ANTIOXIDANTS & REDOX SIGNALING
Volume 8, Numbers 1 & 2, 2006


# Original Research Communication

# Peroxiredoxin 6 Gene-Targeted Mice Show Increased Lung Injury with Paraquat-Induced Oxidative Stress

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## ABSTRACT

**Mice with knock-out of peroxiredoxin 6 (Prdx6), a recently described antioxidant enzyme, were evaluated for susceptibility to lung injury with paraquat (PQ) administration. With high dose PQ (30 mg/kg i.p.), all Prdx6−/− mice died ($LT_{50}$ 54 ± 2.05 h, mean ± SE) by 4 days, whereas 86% of the wild-type (WT) mice (C57BL/6) survived ($n = 14$). At 2 days after PQ, lung wet/dry weight ratio increased significantly ($p < 0.05$) to 7.57 ± 0.37 in Prdx6−/− mice *vs*. 5.42 ± 0.25 in WT mice. Total protein and nucleated cells in bronchoalveolar lavage fluid and TBARS and protein carbonyls in lung homogenate also showed more marked increases in Prdx6−/− mice. At 2.5 days after PQ, light microscopy of WT lungs showed mild injury while Prdx6−/− lungs showed epithelial cell necrosis, perivascular edema, and inflammatory cells. With low dose PQ (12.5 mg/kg), mortality and lung injury were less marked but were significantly greater with Prdx6−/− compared to WT mice. These results show that Prdx6−/− mice have increased susceptibility to lung injury with PQ administration. Thus, Prdx6 protects lungs against PQ toxicity as shown previously for hyperoxia, indicating that it functions as an important lung antioxidant enzyme.** *Antioxid. Redox Signal.* 8, 229–237.

## INTRODUCTION

Reactive oxygen species (ROS) are produced during the course of normal cellular metabolism and also in response to external agents such as hyperoxia or chemical agents (3, 15, 18). Strong oxidants create oxidative stress within cells by reacting with macromolecules, resulting in derangements such as mutations in DNA, alterations of protein function, and membrane damage due to lipid peroxidation. Cellular defenses that protect against those effects of ROS include the antioxidant enzymes such as superoxide dismutases (SOD), GSH peroxidases (GPx) and catalase, as well as nonenzymatic antioxidants (10, 12, 19, 35). Peroxiredoxins (Prdx) are a recently described family of nonseleno-peroxidases that catalyze the reduction of a broad spectrum of peroxides. Of the six mammalian members of this family, five (Prdx 1–5) contain two conserved catalytic cysteines and utilize thioredoxin as the reductant (33). Their physiological role may include both cellular signaling and antioxidant functions. Prdx6 has a single redox-active cysteine and utilizes GSH to catalyze the reduction of $H_2O_2$ as well as phospholipid hydroperoxides (PLOOH) (17). Prdx6 is highly enriched in lung compared to other organs and is expressed at especially high levels in Clara and alveolar epithelial type II cells (23).

Previous studies of Prdx6 function have provided evidence of a primary antioxidant role (27). Overexpression of Prdx6 protein protected glutamine synthetase in NIH 3T3 cells against $H_2O_2$-mediated inactivation (22) and inhibited membrane phospholipid peroxidation and apoptosis in H441 cells subjected to $^{\bullet}OH$ stress by $Cu^{2+}$/ascorbate treatment (25). Antisense treatment of L2 cells resulted in increased lipid peroxidation and decreased resistance to oxidative stress (31). *In vivo*, adenovirus-mediated overexpression of Prdx6 in mouse lung increased survival and protected against lung injury with hyperoxia (38). With inactivation of the Prdx6 gene, lung injury and mortality with hyperoxia or with paraquat treatment were increased (36, 37). The latter studies used two different strains of Prdx6 null mice in which either exons 1 and 2 (36) or exon 3 (28) had been targeted.

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Paraquat (PQ) is a quaternary nitrogen herbicide widely used for broadleaf weed control (35). The mechanism of PQ toxicity in various species has been investigated and attributed to the generation of superoxide radical ($O_2^{\bullet -}$) through an oxygen- and NADPH-dependent redox cycle with subsequent oxidative injury (2, 8, 14). The major cause of death in PQ toxicity is respiratory failure associated with damage to the alveolar epithelium (3, 11, 29). In the previous study of paraquat toxicity using mice in which exons 1 and 2 of Prdx6 had been targeted, evidence of lung injury was provided by histologic study (36). The present study with paraquat utilized exon 3 targeted mice to directly compare results with the previous study and to provide more definitive evidence for lung injury associated with the paraquat treatment.

## MATERIALS AND METHODS

### *Animals*

The use of animals for these studies was approved by the University of Pennsylvania Animal Care and Use Committee (IACUC). Two experimental groups of mice were studied: homozygous Prdx6 null mice and C57BL/6 wild-type control mice ($n$ = 35 for each group). All experimental mice were male weighing 24–28 g (age 8–11 weeks) at the time of study. C57BL/6 wild type mice, free of specific pathogens, were obtained from the Jackson Laboratory (Bar Harbor, ME); Prdx6 null mice were bred in our animal facilities. The generation of Prdx6−/− mice by targeting of exon 3 in 129/SvJ cells and injection into C57BL/6 blastocysts has been described previously (28). The lungs of Prdx6−/− contained no Prdx6 mRNA by real time PCR assay and no Prdx6 protein by immunoblot assay (28). The Prdx6−/− mice appear grossly normal and show normal growth and ability to reproduce (28). Analysis of the Prdx6−/− genotype by genome scanning (Jackson Laboratory) showed approximately equal distribution of the genetic background from C57BL/6 and 129/SvJ mice as expected (data not shown). When exposed to hyperoxia, C57BL/6 wild type showed greater sensitivity to oxidant stress compared to 129/SvJ mice (21, 37); thus, we used the more sensitive strain (C57BL/6) as control for the current study. In a limited number of experiments, Prdx6 +/+ and Prdx6−/− littermates resulting from Prdx6 +/− mating were compared.

### *PQ-induced oxidative stress in mice*

Mice were injected intraperitoneally with PQ (Sigma, St. Louis, MO) at 12.5 (low dose) or 30 (high dose) mg/kg in 0.2 ml phosphate-buffered saline (PBS). The low and high doses of PQ were chosen to correspond to previous reports from other laboratories (10, 12, 36). Mice after injection were observed at approximately 2 h intervals (except overnight) for up to 14 d. Mice were allowed food and water *ad libitum* and maintained on a 12 h dark-light cycle. Cages were opened daily for change of water, food, and bedding, and removal of dead mice. The time to death for 50% of exposed animals ($LT_{50}$) was determined. To evaluate lung injury, additional mice were injected with high dose PQ and sacrificed after 24 or 48 h (i.e., 1 or 2 d) or low dose PQ and sacrificed after 96 h (4 d).

### *Harvesting and preparation of lung tissue*

After induction of anesthesia (pentobarbital, 50 mg/kg intraperitoneally) at the end of exposure, a midline laparotomy/thoracotomy was performed. The trachea was cannulated for continuous ventilation and mice were exsanguinated by transection of the left renal artery and vein. In some experiments, the whole lung was processed for evaluation of tissue histology. In most experiments, 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 thrice by instillation and aspiration of 0.5 ml PBS containing 0.5 m*M* EDTA, pH 8.0. The bronchoalveolar lavage fluid (BALF) was placed on ice for subsequent cell counting and analysis of protein concentration. The pulmonary vasculature then was flushed with PBS by cannulation of the pulmonary artery via the right ventricle followed by *en bloc* removal of the heart and lungs. The heart and large airways were dissected away from the lungs and discarded. The perfused lung was then rapidly frozen in liquid nitrogen and stored at −80°C for subsequent measurement of thiobarbituric acid-reactive substances (TBARS) and protein carbonyls.

### *Wet-to-dry weight ratio and BALF analyses*

Wet-to-dry lung weight ratio and BALF analyses were performed immediately after sample collection. Lungs were lightly blotted, weighed immediately, and then placed in a heated vacuum chamber (Scientific Glass Apparatus, Bloomfield, NJ) until repeated weighing demonstrated no change in weight (about 3 d). The volume of the BALF was measured and total nucleated cells in an aliquot were counted with a Coulter Counter (Coulter Electronics, Luton, England). The remaining BALF was centrifuged for 20 min at 1000 rpm and 4°C for determination of protein concentration in the supernatant.

### *Prdx6 immunoblot analysis*

Frozen lung tissue was thawed in 0.05% Tween 20-TBS buffer containing 10 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, and homogenized using a Potter-Elvehjem homogenizer at 4°C in the presence of Complete Protease Inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN). Tissue extracts were sonicated, centrifuged at 10,000 g for 20 min, and protein concentration in supernatant was determined. Protein samples (10 μg) were subjected to 12% SDS-PAGE gel electrophoresis (BioRad, Richmond, CA) and then were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA). Membranes were incubated in blocking solution (0.1% Tween 20-TBS buffer containing 10% nonfat dry milk) for 1 h and then were probed with a polyclonal antibody to Prdx6 (1:2000 dilution) followed by peroxidase-conjugated anti-rabbit secondary antibody (1:2000 dilution) as described previously (25, 38). The blot was detected by enhanced chemiluminescence (ECL, NEN Life Science,

Boston, MA) and quantitated by densitometric scanning of x-ray film using a Fluor-S multi-imager (BioRad, Hercules, CA).

To evaluate the effects of PQ, fresh whole lungs were excised from PQ treated and control mice, washed repeatedly with ice cold PBS, and inflation fixed for 30 min at 4°C by instilling 1 ml 0.2% glutaraldehyde plus 2% formaldehyde in PBS. The fixed tissues were dehydrated in graded ethanol followed by xylene and embedded in paraffin. Longitudinal sections were cut at a thickness of 6 μm and stained with hematoxylin and eosin for examination by light microscopy (37).

### *Biochemical measurements*

For analysis of tissue TBARS, an aliquot of frozen lung (1:10) was homogenized under $N_2$ in PBS containing 0.01% butylated hydroxytoluene and the homogenate was reacted with thiobarbituric acid and assayed at 535 nm as described previously (5, 38). For analysis of tissue protein carbonyls, frozen lung was homogenized under $N_2$ in buffer containing Complete Protease Inhibitor cocktail (Boehringer Mannheim) and centrifuged at 100,000 g for 15 min; the supernatant was reacted with dinitrophenylhydrazine and assayed at 360 nm as previously described (5, 38). Protein content in BALF and lung homogenate was measured by Coomassie blue dye binding with bovine gamma globulin as the standard (BioRad).

### *Statistical analysis*

Data are expressed as mean ± SE. Statistical significance was assessed with SigmaStat software (Jandel Scientific, San Jose, CA). Group differences were evaluated by one way ANOVA or by Student's *t* test as appropriate. Differences between mean values were considered statistically significant at $p < 0.05$.

## RESULTS

### *General response and mortality in PQ-induced oxidative stress*

As previously described (28), Prdx6−/− mice grew and reproduced normally with no obvious phenotypic difference from the wild type. Wild-type mice injected with high dose PQ showed a significant increase in the lung Prdx6 content at 1 d and a further increase at 2 d to a value approximately double the control; lung Prdx6 had returned to control levels at 8 d after PQ treatment (Fig. 1). Wild-type mice appeared lethargic and responded poorly to stimulation during the initial 3 d period after high dose PQ injection, but 86% then recovered (Fig. 2). By contrast, Prdx6−/− mice showed a more marked response that culminated in death. At 4 d following PQ injection, all Prdx6−/− mice had died compared with only 14% of the wild-type control mice (Fig. 2A). The time to 50% lethality ($LT_{50}$) for Prdx6−/− mice treated with high dose PQ was 54 ± 2.0 h. With low dose PQ, there was no obvious effect on behavior of wild-type mice and all survived the 14 d observation period (Fig. 2B). In Prdx6−/− mice, low dose PQ resulted in death of 25% of the animals between 3.5 and 4.5 d; the remaining 75% survived the 14 d observation period (Fig. 2B).

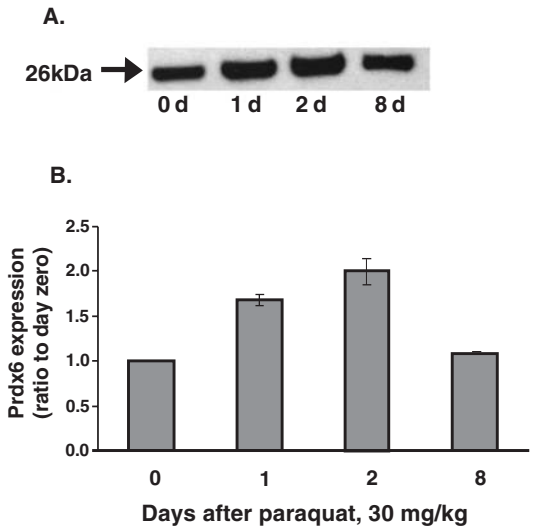


**FIG. 1. Prdx6 protein expression in the lungs of wild-type mice with paraquat (PQ) injection.** (**A**) Representative immunoblots using a polyclonal Prdx6 antibody indicating migration at 26 kDa. Mouse lungs were evaluated before (day 0) and at days 1, 2, and 8 following injection of PQ, 30 mg/kg i.p. (**B**) Western blots for Prdx6 protein were quantified for Prdx6 expression with FluorS MultiImager and Quantity One software; density of bands was expressed relative to the value at day 0.

TABLE 1. LUNG WET/DRY RATIO AFTER PARAQUAT (PQ) INJECTION

| *Days after PQ* | *Dose (mg/kg)* | *Wild type* | *Prdx6−/−* |
|---|---|---|---|
| 0 | 30 | 3.96 ± 0.16 | 4.00 ± 0.16 |
| 1 | 30 | 4.69 ± 0.1† | 4.85 ± 0.19† |
| 2 | 30 | 5.42 ± 0.25† | 7.57 ± 0.37*† |
| 4 | 12.5 | 4.12 ± 0.22 | 4.96 ± 0.14*† |

Values are means ± SE for $n = 4$ in each group.
*$p < 0.05$ vs. wild type at the same time and dose of exposure.
†$p < 0.05$ vs. the corresponding zero time control.

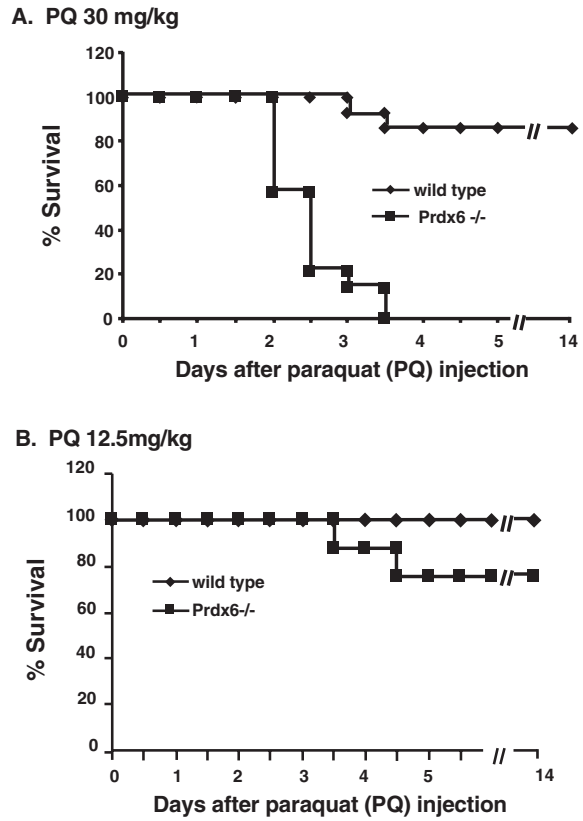


**FIG. 2. Survival of wild type and Prdx6 null mice with PQ-induced oxidative stress.** PQ at high (**A**) or low (**B**) dose was injected intraperitoneally in age-matched (10 weeks) wild type (C57BL/6) and Prdx6−/− mice from several litters and the survival times were measured ($n = 14$ for each group). Mice were observed for 14 d after injection.

### *Lung wet-dry weight ratio and lung morphology*

The lung wet-dry weight ratio increased slightly at 1 d after high dose PQ exposure in both wild type and Prdx6−/− mice (Table 1). At 2 d, the ratio was only slightly greater than the 1 d value in the wild-type mice but had increased to a value compatible with marked edema in the Prdx6−/− mice (Table 1). Lung dry-wet ratio was measured at 4 d after low dose PQ and showed small increases that were similar to 1 d after high dose PQ. At both doses, the greater increase in the Prdx6−/− mice compared to wild type was statistically significant (Table 1).

Lung structure and morphology was similar in untreated Prdx6−/− and wild type mice (Fig. 3). At 2.5 d (60 h) after high dose PQ treatment, the lungs of wild-type mice showed relatively mild inflammatory changes while lungs from Prdx6−/− mice showed areas of more severe injury with peribronchial edema and infiltration of PMN and mononuclear cells into the alveolar space. Representative areas of injury are shown in Figure 3. Lung histology after low dose PQ was examined at 5 d and showed essentially no abnormalities in the wild type and only mild cellular infiltration in the knock-out mice (Fig. 3).

### *BALF analysis*

Both the protein content and nucleated cell counts in BALF were unchanged or slightly increased from control at 1 d in wild-type mice and increased modestly at 2 d following high dose PQ treatment (Fig. 4). The change in BALF protein was significantly greater in Prdx6−/− mice. At 2 d after high dose PQ, BALF protein concentration and nucleated cells counts in Prdx6−/− mice were increased nearly six-fold and three-fold, respectively, as compared to control, or approximately double the effect seen in wild-type mice (Fig. 4). With low dose PQ, both BALF parameters were increased in Prdx6−\− mice at 4 d after injection while they were unchanged from control in wild-type mice (Fig. 4).

### *Lung TBARS and protein carbonyls*

The content of TBARS and protein carbonyls in the lungs of control mice was similar to previously reported values for normal rat lungs (5, 6, 16, 20). These values for TBARS are lower than in our previous report of mouse lungs (37, 38) because of a calculation error in the latter. The lung content of protein carbonyls was unchanged in wild-type lungs at 2 d after high dose PQ while TBARS was increased slightly (Fig. 5). With high dose PQ exposure in Prdx6−/− mice, the lung content of both TBARS and protein carbonyls increased progressively at 1 and 2 d and was significantly higher at both time points compared to wild-type mice (Fig. 5). At 2 d, the level of TBARS and protein carbonyls in lung homogenate of Prdx6−/− mice was increased 110% and 139%, respectively, above control as compared to increases of 70% and 74% in wild-type mice (Fig. 5). Low dose PQ, studied at 4 d after injection, resulted in changes of lung TBARS and protein carbonyls that were significantly greater in the Prdx6−/− compared to wild-type mice (Fig. 5). Values for these parameters at 4 d after low dose PQ were unchanged from control for wild-type mice and were approximately the same as the 2 d values with high dose PQ for Prdx6−/− mice (Fig. 5).

## DISCUSSION

PQ, an herbicide which is highly toxic to mammals as well, causes necrotic damage to the lung and other organs (3, 8, 9, 11, 13, 30). The lungs are preferentially targeted because of the rapid uptake and accumulation of PQ in lung cells, especially alveolar epithelium, through polyamine transporters (32). A major mechanism for PQ toxicity is redox cycling which generates ROS and can initiate lipid peroxidation (1, 30, 34, 39). The oxidative changes in lung tissue result in increased membrane permeability and loss of membrane integrity resulting in alveolar edema and cell death (13, 30, 35). Depletion of cellular NADPH may contribute to the oxidative stress and promote cellular dysfunction (2).

Prdx6 is expressed at a relatively high level in the lung where it is localized primarily to cytosol, although it also is present in lamellar bodies of granular pneumocytes and lysosomes (23, 27, 33). The enzyme utilizes GSH to catalyze the reduction of $H_2O_2$ and phospholipid hydroperoxides (PLOOH) (17, 26). Several lines of evidence using models of both over and under expression of Prdx6 in lung cells and

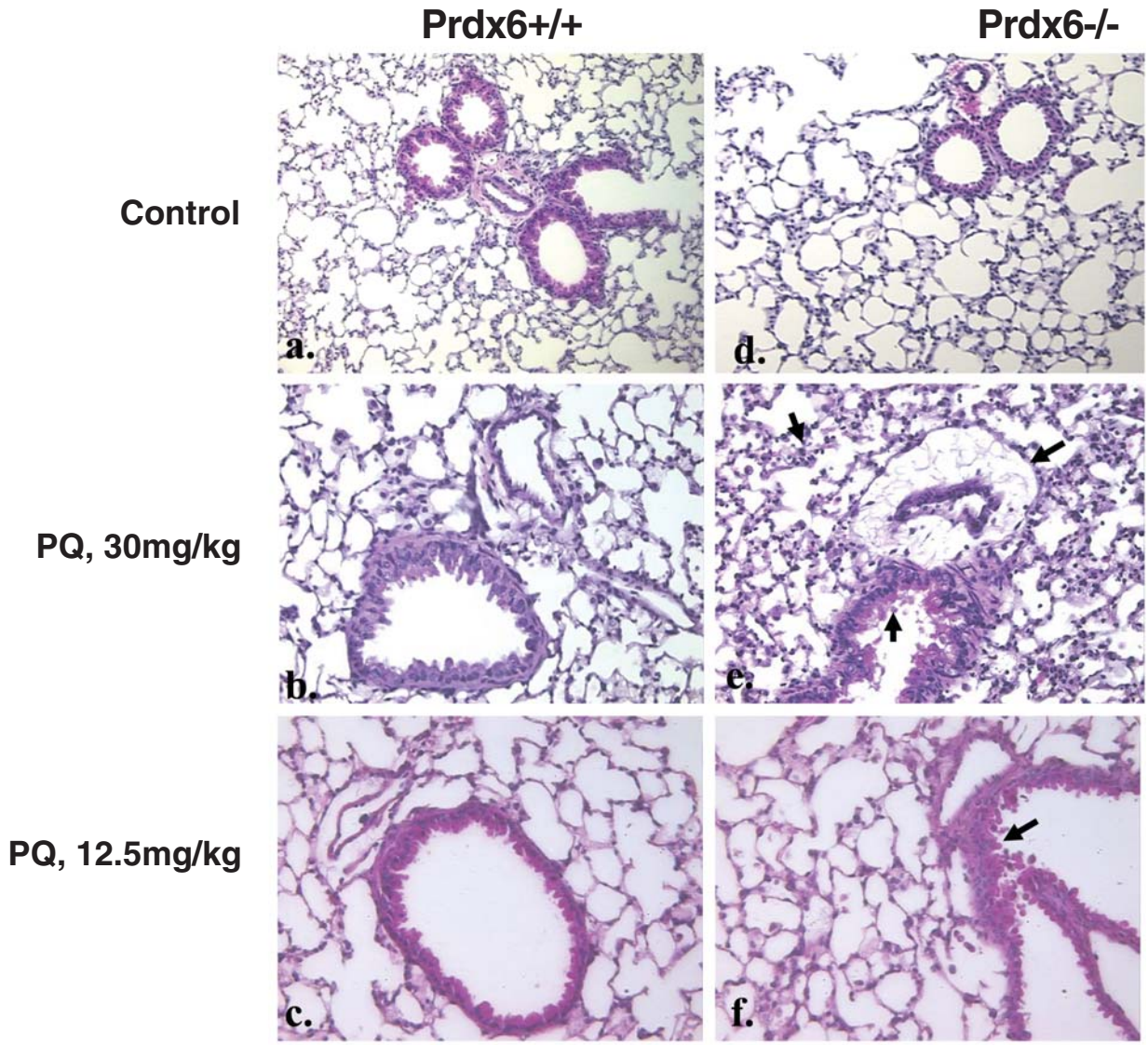


**FIG. 3. Lung histology with PQ exposure.** Lung tissues were prepared from Prdx6+/+ mice and age matched Prdx6−/− littermates and stained with hematoxylin and eosin. Representative sections are shown for control mice (no PQ) (**a**, **d**), at 2.5 d after high dose PQ (30 mg/kg) (**b**, **e**), and at 5 d after low dose PQ (12.5 mg/kg) (**c**, **f**). *Arrows* in **e** and **f** indicate areas of cellular infiltration. Photomicrographs are representative of $n = 3$ in each group. Magnification ×200.

in mouse lung have indicated that Prdx6 functions as an antioxidant enzyme (25, 31, 36–38). These studies have demonstrated an important role for Prdx6 in defense against oxidant stress associated with exposure to hyperoxia (37, 38) and to PQ (36). Consistent with these results, Prdx6−/− mice in the present study showed increased sensitivity to PQ-induced oxidative stress. Although 86% of wild-type mice survived after injection of PQ at 30 mg/kg, all Prdx6−/− mice died within 4 d with 50% lethality ($LT_{50}$) at 54 h. The exon 3 gene targeted mice used in the present study show slightly greater sensitivity compared to previously published results using a Prdx6 exon 1, 2 gene-targeted mouse where the $LT_{50}$ was 72 h and 20% survived >7 d with PQ at 30 mg/kg (36). This latter report showed that expression and response to PQ for other antioxidant enzymes including SOD1, 2 and 3, GPx1, 2, 3 and 4, catalase, thioredoxin 1 and 2, glutaredoxin 1 and 2, and Prdx1, 2, 3, 4 and 5 were similar in wild type (129/SvJ) and Prdx6−/− mice (36). Our laboratory has shown a similar level of expression for SOD1 and 2, catalase, and GPx1 in wild type (C57BL/6) and Prdx6−/− mice, although the response to PQ was not evaluated (37). Thus, the results from two laboratories using knock-out mice of different genotype indicate no change in other antioxidant enzymes and provide strong evidence for the importance of Prdx6 in antioxidant defense with PQ-induced oxidative stress.

Although the cause of death for mice in the present report was not determined, previous studies have indicated that delayed death (days) with PQ is largely due to lung injury,

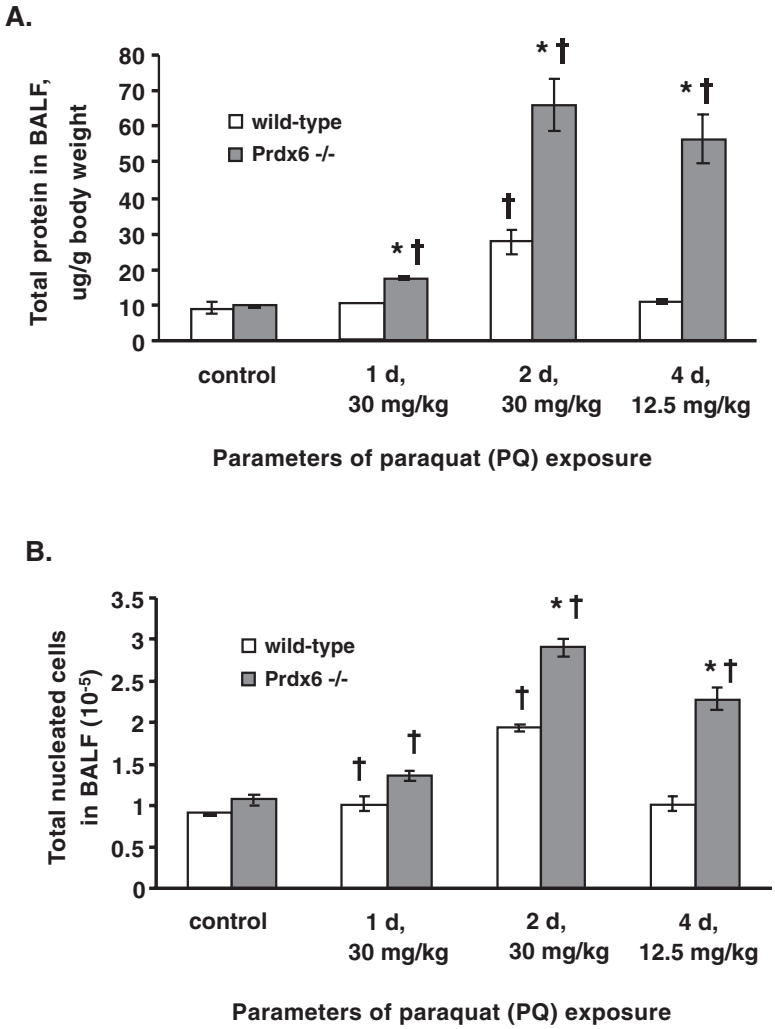


**FIG. 4. Lung bronchoalveolar lavage fluid (BALF) analysis.** BALF was obtained from Prdx6−/− and wild type (C57BL/6) control (unexposed) mice at 1 and 2 d after injection of high dose PQ (30 mg/kg) and at 4 d after low dose PQ (12.5 mg/kg). (**A**) Protein content in BALF. (**B**) Nucleated cells in BALF. Results are mean ± SE ($n = 4$ for each time point). *$p < 0.05$ vs. wild type at the same time of exposure. †$p < 0.05$ vs. the corresponding zero time control.

whereas acute mortality (minutes) appears to have a different mechanism (11). Lung injury in PQ-treated mice in the present study was identified by qualitative histological examination and by evaluation of BALF and lung tissue. There was an increased number of nucleated cells in the BALF indicating inflammation, increased lung wet weight and BALF protein indicating increased alveolar permeability, and increased TBARS and protein carbonyls indicating lipid and protein oxidation in lung tissue. For each of these parameters, the degree of lung injury with PQ was significantly greater in the Prdx6−/− as compared with wild-type mice.

Possible mechanisms for the protection by Prdx6 against PQ-induced lung injury include scavenging of $H_2O_2$ produced by dismutation of $O_2$ or the reduction of PLOOH to the less toxic phospholipid alcohols. Both $H_2O_2$ and PLOOH are efficiently reduced by Prdx6 (17). However, compared to Prdx6, other scavenging enzymes for $H_2O_2$ (catalase, GPx1) are present in higher concentration and show higher activity. For example, both the lung content of GPx1 and its rate constant for $H_2O_2$ reduction are each greater by approximately ten-fold compared to Prdx6. Thus, it would be predicted that GPx1 would be ~100 times more active than Prdx6 in scavenging intracellular $H_2O_2$. This explains why deletion of Prdx6 had no effect on $H_2O_2$ reduction by mouse lung homogenates although reduction of PLOOH was markedly reduced (37). Intracellular enzyme localization also may be important in $H_2O_2$ scavenging but both GPx1 and Prdx6 are primarily cytosolic enzymes. Thus, it seems unlikely that scavenging of intracellular $H_2O_2$ is an important function of Prdx6 in the lung epithelium and it is more likely that the protective effect of Prdx6 in oxidative stress is mediated by its ability to reduce PLOOH. The latter are generated during oxidant stress by peroxidation of cellular lipids, as indicated by increased TBARS in mouse lungs with PQ, and represent

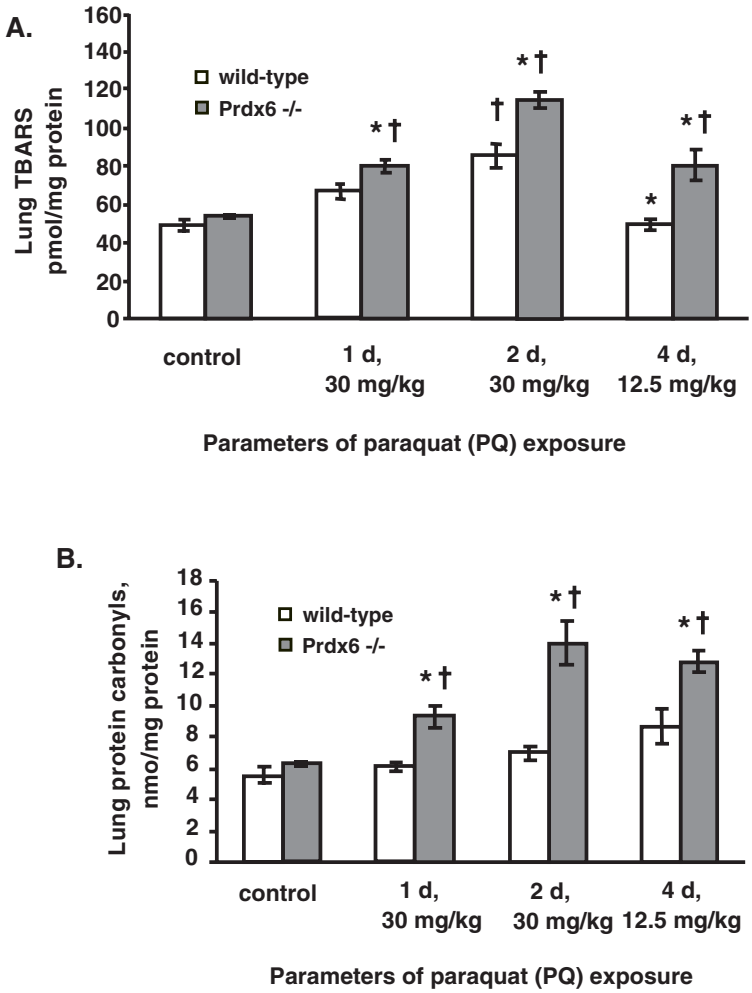


**FIG. 5. Lung lipid and protein oxidation with PQ induced oxidative stress.** TBARS (**A**) and protein carbonyls (**B**) in mouse lung homogenate were measured before treatment (control), at 1 and 2 d after high dose PQ (30 mg/kg), and at 4 d after low dose PQ (12.5 mg/kg). Values are means ± SE ($n = 4$ for each time point). *$p < 0.05$ versus wild type at the same time of exposure. †$p < 0.05$ vs. the corresponding zero time control.

an important mediator of cellular toxicity. Since cytosolic GPx (GPx1) is unable to reduce PLOOH (17, 24), it does not participate in this aspect of antioxidant defense. Whereas phospholipid hydroperoxide glutathione peroxidase (GPx4) does utilize PLOOH as substrate, it is primarily a testicular enzyme and is expressed at significantly lower levels in the lung (40). Thus, Prdx6 may be the primary enzyme in the lung responsible for reduction of PLOOH and restoration of cell membrane integrity following PQ. Several studies have demonstrated that GSH content in the lung, as well as in other tissues, is decreased during oxidative stress and reflects increased oxidant sensitivity (4, 7). GSH has relatively limited ability to directly scavenge ROS but is important as a co-factor in $H_2O_2$ reduction by GPx. We speculate that another major role for GSH in antioxidant defense is to serve as the electron acceptor for Prdx6-mediated reduction of PLOOH.

## ACKNOWLEDGMENTS

The authors thank Dr. Peter Bell for assistant with histology, Dr. Chandra Dodia for assistance with TBARS assay, and Ms. Jennifer Rossi for typing the manuscript. Presented in part at the annual meeting of the American Thoracic Society in San Diego, CA, May 2005. Support was provided by HL-65543 and ES-06639 from the National Institutes of Health and CFFS886 from the Cystic Fibrosis Foundation.

## ABBREVIATIONS

BALF, bronchoalveolar lavage fluid; GPx, glutathione peroxidase; PBS, phosphate buffered saline; PLOOH, phospholipid hydroperoxides; PQ, paraquat; Prdx, peroxiredoxin;

SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

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Received for publication April 6, 2005; accepted August 15, 2005.

**This article has been cited by:**


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