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Peroxiredoxin 6 as an Antioxidant Enzyme: Protection of Lung Alveolar Epithelial Type II Cells From H_2O_2-Induced Oxidative Stress

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Abstract We evaluated the antioxidant role of peroxiredoxin 6 (Prdx6) in primary lung alveolar epithelial type II cells (AEC II) that were isolated from wild type (WT), Prdx6−/−, or Prdx6 transgenic (Tg) overexpressing mice and exposed to H_2O_2 at 50–500 μM for 1–24 h. Expression of Prdx6 in Tg AEC II was sevenfold greater than WT. Prdx6 null AEC II exposed to H_2O_2 showed concentration-dependent cytotoxicity indicated by decreased ''live/dead'' cell ratio, increased propidium iodide (PI) staining, increased annexin V binding, increased DNA fragmentation by TUNEL assay, and increased lipid peroxidation by diphenylpyrenylphosphine (DPPP) fluorescence. Compared to Prdx6 null cells, oxidant-mediated damage was significantly less in WT AEC II and was least in Prdx6 Tg cells. Thus, Prdx6 functions as an antioxidant enzyme in mouse AEC II. Prdx6 has been shown previously to reduce phospholipid hydroperoxides and we postulate that this activity is a major mechanism for the effectiveness of Prdx6 as an antioxidant enzyme. J. Cell. Biochem. 104: 1274–1285, 2008.

Key words: cell viability; apoptosis; lipid peroxidation; diphenylpyrenylphosphine; TUNEL; annexin V

Oxidative stress is widely recognized as a major pathophysiologic mechanism in various injuries that lead to either acute or chronic organ failure. This stress is characterized by the increased production of reactive oxygen species (ROS) such as superoxide radical, H_2O_2, and hydroxyl radical, which can result in oxidation of macromolecules including nucleic acids, lipids, and proteins [Fisher et al., 1984; Lang et al., 2002; Chow et al., 2003]. The lung is particularly susceptible to oxidative damage caused either by direct contact with oxidants in ambient air or by blood-borne oxidants delivered through its extensive capillary perfusion [Fisher et al., 1984; Lang et al., 2002]. A broad spectrum of disease states has been attributed to oxidative stress in the lung including sepsis-induced acute lung injury (ALI) and chronic obstructive pulmonary disease (COPD) associated with smoking of cigarettes [Lang et al., 2002; Rahman and Adcock, 2006].

To protect against the harmful consequences of oxidative stress, cells express a number of antioxidant enzymes including superoxide dismutases (SOD), glutathione (GSH) peroxidases (GPx), catalase, and peroxiredoxins (Prdxs), as well as non-enzymatic antioxidants such as GSH [Forman and Fisher, 1981]. The Prdxs, a recent addition to the list of antioxidant enzymes, use cysteine as the catalytic center rather than the selenocysteine that characterizes the GPx enzymes [Wood et al., 2003; Rhee et al., 2005]. These enzymes have been classified as a 1-cys or 2-cys according to the number of conserved cysteine residues that function in catalysis [Wood et al., 2003; Rhee et al., 2005]. The 2-cys Prdxs generally use thioredoxin as a co-factor to reduce H_2O_2 and organic hydroperoxides [Wood et al., 2003; Rhee et al., 2005]. Peroxiredoxin 6 (Prdx6) is a 1-cys enzyme that utilizes GSH as the physiological reductant to

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reduce H_2O_2 and organic hydroperoxides. Prdx6 is the only one of the six mammalian Prdxs reported so far with the ability to reduce phospholipid hydroperoxides (PLOOH) at a significant rate [Fisher et al., 1999; Manevich et al., 2004], although this oxidation product can also be reduced by phospholipid hydroperoxide GPx (also called GPx4) [Ursini et al., 1995].

The role of Prdx6 and other antioxidant enzymes in protecting the lung against oxidant injury has been evaluated in cell and animal models by manipulation of their expression levels. These studies include gain and loss of function for SOD 1 and 2 [Danel et al., 1998; Ho et al., 1998; Folz et al., 1999; Asikainen et al., 2002; Ho, 2002], GPx1 [Ho et al., 1998; Ho, 2002], and catalase [Danel et al., 1998]. These studies have given ambiguous results for their role in antioxidant defense. Similar studies have been carried out more recently with Prdx6 [Manevich et al., 2002; Pak et al., 2002; Wang et al., 2003; Wang et al., 2004a,b; Wang et al., 2006a,b]. Overexpression of Prdx6 protein in human lung-derived NCI-H441 cells attenuated membrane phospholipid peroxidation and apoptosis when cells were subjected to ·OH stress by Cu^{2+}/ascorbate treatment [Manevich et al., 2002], while antisense-mediated decrease in expression of Prdx6 in L2 cells, a rat lung epithelial cell line, resulted in increased oxidant sensitivity and cell death [Pak et al., 2002]. Overexpression of Prdx6 in mouse lungs mediated by adenoviral transfection or by gene transfer techniques decreased oxidative stress and increased survival in hyperoxia [Wang et al., 2004b; Wang et al., 2006b], while loss of Prdx6 by gene inactivation resulted in increased lung injury and mouse mortality associated with hyperoxia or paraquat exposure [Wang et al., 2003; Wang et al., 2004a; Wang et al., 2006a]. These results indicate that Prdx6 plays an important role in defense of the lung against oxidative stress.

Prdx6 is enriched in lung compared to other organs and is expressed at the highest levels in alveolar epithelial type II cells (AEC II), bronchiolar Clara cells, and alveolar macrophages [Kim et al., 1998; Mo et al., 2003]. AEC I and II are major constituents among the 40 or more different cell types that comprise the lung. AEC I cover ~95% of the lung alveolar surface area and form the barrier that is important for gas exchange. AEC II account for ~15% of total lung cells and cover ~5% of the alveolar surface area. These latter cells play a critical role in maintaining lung function by production of surfactant, by their ability to proliferate to restore damaged epithelium, and by their contribution to host defense [Dobbs, 1990]. Because AEC II comprise a relatively small percentage of total lung cells, it is difficult to determine their response to oxidant stress through study of the whole lung. The response to oxidant stress of rat AEC II in primary culture has been studied previously by measuring dye exclusion, generation of thiobarbituric acid reactive substances (TBARS), MTT reduction, Cytochrome *c* release, and propidium iodide (PI) staining [Hagen et al., 1986; Piotrowski et al., 2004; Yin et al., 2005]. In the present study, we investigate the role of Prdx6 in antioxidant defense using primary isolates of AEC II from lungs of wild type (WT) mice and those overexpressing and deficient in Prdx6.

MATERIALS AND METHODS

Animals

The use of animals for these studies was approved by the University of Pennsylvania Animal Care and Use Committee (IACUC). Three experimental groups of mice were studied: a C57BL/6 wild type (WT) group (n = 95), a homozygous Prdx6−/− group (n = 80), and a Prdx6 transgenic (Tg) group (n = 82). The generation and genotyping of Prdx6−/− and transgenic (Tg) Prdx6 mice has been described previously [Mo et al., 2003; Phelan et al., 2003; Wang et al., 2006b]; these mice were bred in the animal facility of the University of Pennsylvania and maintained under HEPA filtered air. The transgene for Tg Prdx6 mice was a genomic clone containing the entire Prdx6 gene including 6,644 bp of upstream sequence [Phelan et al., 2003]. WT mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice of either sex at 8–11 weeks of age and weighing 24–28 g were used to isolate AEC II.

Isolation and Culture of AEC II

AEC II were isolated by enzymatic digestion of mouse lungs as described previously [Bortnick et al., 2003]. Briefly, lungs were cleared of blood by perfusion through the pulmonary artery, instilled with 2 ml of dispase (50 U/ml), and incubated at room temperature for 45 min. Lung tissue was separated from large bronchi

with forceps and transferred to a Petri dish containing 10 ml DMEM with 0.01% DNase I for 10 min incubation at 37°C; subsequently, the cells were filtered sequentially through 100-, 35-, and 17-μm nylon mesh. The cell pellet was resuspended in 10 ml DMEM, plated on mouse IgG (0.75 mg/ml)-coated Petri dishes, and incubated at 37°C for 1 h to remove adherent macrophages. The non-adherent cells were centrifuged, resuspended in DMEM with 10% FBS, and seeded on tissue culture plastic dishes (Techno Plastic, Trasadingen, Switzerland) for a 1 h incubation at 37°C to remove adherent fibroblasts. The remaining cells were resuspended in Ham's F-12 culture medium supplemented with 15 mM HEPES, 0.8 mM $CaCl_2$, 0.25% BSA, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, and 4% mouse serum and seeded on 35 mm type I collagen-coated cell culture dishes (BD Biosciences, Franklin Lakes, NJ). After 36 h, cells were washed and medium was replaced with serum-free medium in order to maintain the AEC II phenotype. Dishes at 48 h of culture contained 10^5–10^6 AEC II and were used for experiments.

Identification of AEC II

AEC II were identified by staining with modified Nile red (Nile Blue A Oxazone, Sigma, St. Louis, MO), a lipophilic fluorescent dye that can label lipid-rich areas and is used to identify lamellar bodies, the organelle that is characteristic of these cells [Dobbs, 1990; Bates et al., 2002; Bortnick et al., 2003]. Nile red, dissolved in dimethylsulfoxide (DMSO), was added at 1:40 to unfixed cells in PBS and swirled in the dish for 10 min at room temperature to label the cells; the dishes were washed with medium and examined using the Rhodamine filter of the fluorescence microscope (Nikon Eclipse TE 2000-U, Nikon, El Segundo, CA).

Cell Viability and Apoptosis

Cell viability was detected by the live/dead cell viability and cytotoxicity kit (Molecular Probes, Eugene, OR). 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 ethidium homodimer (EthD-1) which can enter cells through damaged membranes and binds to nucleic acids. AEC II were incubated with 0.5 μM calcein AM, plus 2 μM EthD-1 for 20 min at 37°C and examined by fluorescence microscopy [Muro et al., 2003].

Apoptosis was evaluated with the ApoAlert Apo 2.7/Annexin V-EGFP kit (Clontech, Mountain View, CA) [Martin et al., 1995; Aubry et al., 1999] Cells that bind annexin V show green staining of the plasma membrane; the red fluorescence of PI indicates altered cell membrane permeability. AEC II were incubated with 5 μl annexin V and 10 μl PI in 200 μl binding buffer with gentle shaking at room temperature for 10 min in the dark and images were obtained by fluorescence microscopy. For each experiment, the percentage of cells positive for annexin or PI staining was counted for ~1,000 cells in 5–6 randomly chosen fields per dish.

Apoptosis was also detected and quantified by fluorescence using the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN) for Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) [Labat-Moleur et al., 1998; Pak et al., 2002]. ATII 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 after TUNEL staining with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI, 1 ul/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 ATII cells were incubated with recombinant DNase I (2 U/ml) for 10 min to induce DNA strand breaks. For TUNEL negative control staining, the processed ATII cells were incubated with the labeling solution of the TUNEL reaction mixture minus the enzyme.

Prdx6 Protein Expression and Enzymatic Activity

To measure Prdx6 protein content and enzymatic activity, AEC II were treated with lysis buffer (50 mM Tris, 0.1% SDS or 1% NP-40), shaken for 10 min at 4°C in the presence of Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Indianapolis, IN), and centrifuged at 12,000*g* for 5 min. The supernatant

was analyzed for protein concentration by the binding of Coomassie blue (Bio-Rad, Richmond, CA) using bovine gamma globulin as the standard. Samples (20 μg protein per lane) were subjected to 12% SDS–PAGE using a Bio-Rad electrophoresis apparatus (Bio-Rad) and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA). Membranes were incubated for 1 h with Odyssey Blocking Buffer Q diluted 1:1 in PBS, and then probed with polyclonal antibodies to Prdx6 peptide and β-actin followed by fluorescently labeled (IRDye TM800) affinity purified anti-rabbit IgG secondary antibody (Rockland, Gilbertsville, PA) (1:5,000). The membrane was scanned with an Odyssey™ two-color scanner (LI-COR Biosciences, Lincoln, Nebraska) and protein band density was measured using Odyssey software.

Peroxidase activity was measured in cell sonicates by the decrease in NADPH fluorescence in the presence of aminotriazole (20 mM, to inhibit catalase), reduced GSH, an NADPH regenerating system, and 0.66 mM H_2O_2 or 0.1 mM phospholipid hydroperoxide substrate as previously described; the phospholipid hydroperoxide substrate was prepared by incubation of 1-palmitoyl, 2-linoleoyl *sn*-phosphorylcholine (PLPC) with lipoxygenase to yield the oxidized product (PLPCOOH) [Fisher et al., 1999].

Diphenylpyrenylphosphine (DPPP) Detection of Cell Membrane Lipid Peroxidation

DPPP is a fluorescent probe for the detection of cell membrane lipid peroxidation [Okimoto et al., 2000; Matot et al., 2003]. DPPP is non-fluorescent, but becomes fluorescent when oxidized. DPPP (Dojindo, Gaithersburg, MD) was solubilized in DMSO and stored at −20°C. Storage and all subsequent procedures with DPPP were carried out in the dark. AEC were labeled by incubation at 37°C for 10 min with a micellar suspension of DPPP (400 nmol/ml) and washed twice with PBS. DPPP-labeled AEC II from WT, Prdx6−/−, or Tg Prdx6 mice then were treated with 500 μM H_2O_2 for various times as indicated and imaged by fluorescence microscopy (351 nm excitation, 380 nm emission).

DPPP fluorescence of cells also was determined with a PTI spectrofluorometer (Photon Technology Int., Bricktown, NJ) equipped with a single photon counting system. The DPPP-labeled cells were detached from the culture dish with trypsin-EDTA, resuspended in phosphate-buffered saline by gentle swirling in a standard quartz cuvette, and kept on ice until assayed. After recording the initial emission spectrum, the cell suspension was excited at 351 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 [Manevich et al., 2002].

Statistical Analysis

Data are expressed as mean ± SEM for three or more independent experiments. Statistical significance was assessed with SigmaStat software 9.0/3.1 (Jandel Scientific, San Jose, CA). Group differences were evaluated by one-way ANOVA followed by Bonferroni's test or by Student's *t*-test as appropriate. Differences between mean values were considered statistically significant at $P < 0.05$.

RESULTS

Identification of AEC II and Prdx6 Protein Expression

AEC II isolated from WT, Prdx6−/−, and Tg Prdx6 mice showed similar morphology and a similar purity by Nile red staining of ∼95% (Fig. 1A). We have shown previously that Prdx6−/− mice as expected do not express Prdx6 message or protein [Mo et al., 2003; Wang et al., 2004a] and this was confirmed for protein expression with AEC II from Prdx6−/− mice (Fig. 1B). For AEC II from Tg Prdx6 mice, Prdx6 protein expression by Western blot was ∼sevenfold greater as compared to WT mice (Fig. 1B and C). Peroxidase activity in sonicated cells when measured with H_2O_2 substrate showed no significant differences among AEC II from WT and Prdx6 knock-out or overexpressing mice (Fig. 1D). This indicates that other enzymes (e.g., GPx, as catalase was inhibited by the presence of aminotriazole) are largely responsible for the degradation of H_2O_2 in these cells. Peroxidase activity with the phospholipid hydroperoxide substrate (PLPCOOH) was only 10% of the corresponding activity with H_2O_2 in WT cells and this activity was abolished by knock-out of Prdx6 (Fig. 1D). This result is

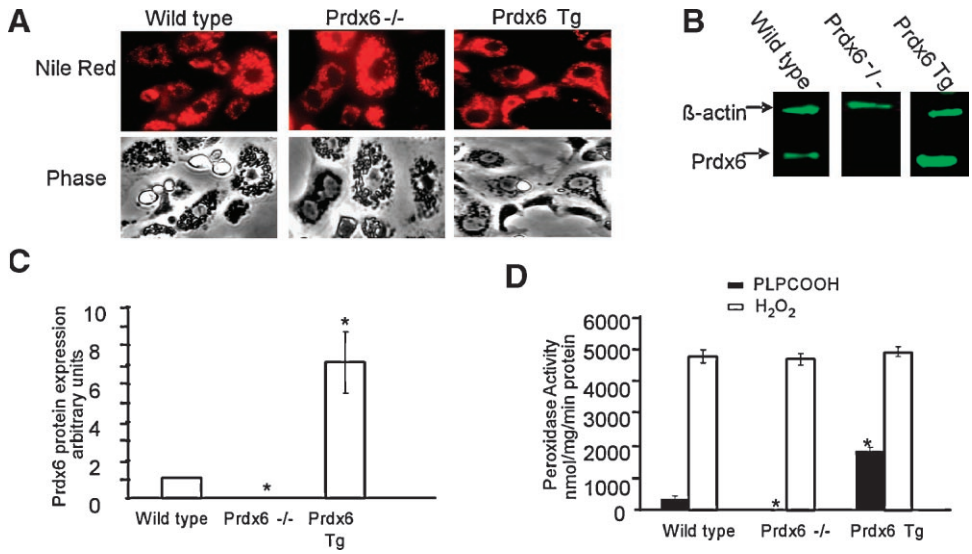

Fig. 1. Identification of lung AEC II. AEC II were isolated from WT, Prdx6 null, and Prdx6 Tg mice and maintained in culture for 36–48 h. **A**: Fluorescence and phase images after treatment of AEC II with Nile red, a dye that localizes in the lamellar bodies. **B**: Western blot following SDS–PAGE (20 mg AEC II protein per lane) using polyclonal antibody to Prdx6 followed by fluorescently labeled secondary antibody. β-actin was used as a loading control. **C**: Quantitation of Western blots for Prdx6 protein expression under basal conditions showing mean ± SE for n = 3. Results were normalized to β-actin. Values were zero for Prdx6−/−. **D**: Prdx6 peroxidase activity of sonicated AEC II cells with 660 μM H_2O_2 or 100 μM 1-palmitoyl-2-linoleoyl phosphatidylcholine hydroperoxide (PLPCOOH) as substrate. Aminotriazole (20 mM) was added for assay with H_2O_2. The value for Prdx6−/− cells with PLPCOOH substrate was zero. Values are means ± SE for n = 3. *$P < 0.05$ compared to WT for the same substrate.

consistent with our previous observation that Prdx6 is the only peroxidase in the lung with the capacity to significantly degrade PLPCOOH [Wang et al., 2006a]. Peroxidase activity with PLPCOOH substrate in Tg AECII was increased fivefold compared to WT.

The change in cellular Prdx6 protein expression after 24 h of treatment with H_2O_2 at increasing concentrations (50–500 μM) was determined by Western blotting (Fig. 2A). This oxidant stress induced a dose-dependent increase in Prdx6 expression in both WT and Prdx6 Tg AECII with an increase in expression levels by 55–85% with 500 μM H_2O_2 treatment (Fig. 2B). As Prdx6−/− AEC did not express Prdx6, they were not evaluated for the effect of H_2O_2.

Viability and Apoptosis of AEC II With H_2O_2 Treatment

Viability of AEC II was studied with calcein and ethidium staining (Fig. 3A). This allowed calculation of the percentage of "live" (viable) cells, or conversely the percentage of "dead" cells. The percentage of "dead" cells with treatment for 24 h varied with the concentration of H_2O_2 (Fig. 3B). The percentage of viable AEC II (100 minus percentage of "dead" cells) after treatment with 50 μM H_2O_2 for 24 h was 67% for Prdx6−/− cells, 85% for WT cells, and 95% for Prdx6 Tg overexpressing cells. With 500 μM H_2O_2, viability decreased to ~50% for Prdx6−/− cells but was essentially unchanged from control for the Prdx6 overexpressing AEC II (Fig. 3B).

Staining of WT AEC II for annexin V under control conditions showed <10% positive and this was not significantly changed with exposure to 50–250 μM H_2O_2 for 24 h (Fig. 4A). However, the percent of annexin positive Prdx6−/−AEC II was twofold greater than control at 50 μM H_2O_2 and increased progressively with increasing H_2O_2 concentration (Fig. 4A). This result is compatible with the early stages of apoptosis due to oxidant stress. Prdx6 Tg cells were <5% annexin positive under control conditions and increased only slightly with H_2O_2 exposure (Fig. 4A). At 500 μM H_2O_2, PI positive cells were ~45% in Prdx6−/−, 23% in WT, and 6% in Prdx6 Tg (Fig. 4B). Uptake of PI reflects cell membrane damage indicating late apoptosis or necrotic cell death. These results

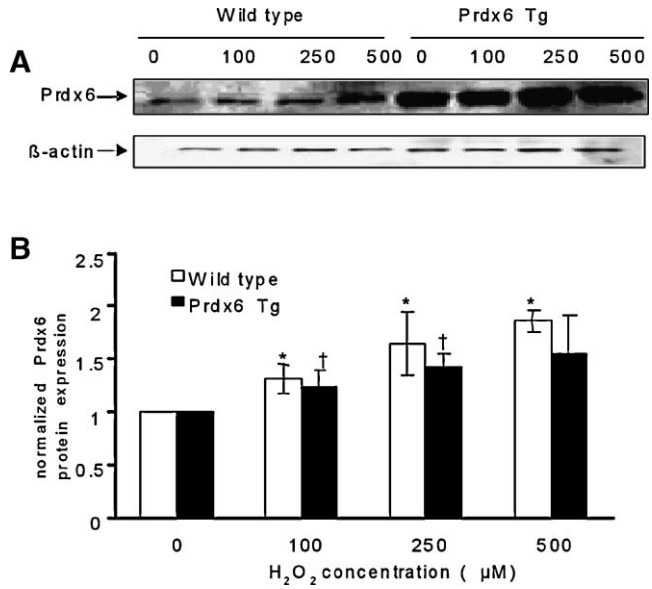

Fig. 2. Prdx6 expression in WT and Prdx6 Tg AEC II cells following treatment with H_2O_2. **A**: Cells were treated for 24 h with 0–500 μM H_2O_2 and evaluated by Western blot using β-actin as a loading control. **B**: Quantitation of Western blots for Prdx6 protein expression in AEC II cells. The ratio of Prdx6 to β-actin for each H_2O_2 concentration was normalized to the respective WT or Tg control (no H_2O_2 treatment). Results represent the mean ± SE for three experiments. $^*P < 0.05$ compared to zero point in the same group; $^\dagger P < 0.05$ compared to WT at the same H_2O_2 concentration.

for PI uptake (Fig. 4B) correlate well with the results for ethidium uptake (Fig. 3) as both reflect increased cell membrane permeability.

TUNEL staining was used to determine the effect of H_2O_2 exposure on DNA cleavage in ATII cells as another index of apoptosis. Under control conditions, the percentage of TUNEL positive cells was ~3% in WT, 12% in Prdx6−/− and <1% in Prdx6 Tg AEC II (Fig. 4C). At 24 h exposure to H_2O_2, there was a concentration-dependent increase in TUNEL positive cells in the WT and Prdx6−/− groups, while the Tg cells showed relatively small changes (Fig. 4C). At 500 μM H_2O_2, ~40% ATII cells in Prdx6−/− group were TUNEL positive compared to 20% in the WT and about 5% in Tg cells.

Lipid Peroxidation With H_2O_2 Treatment

DPPP was used as a fluorescent probe to monitor lipid peroxidation in cellular membranes as a more direct index of oxidative stress. AEC II isolated from WT, Prdx6−/−, and Tg Prdx6 mice were treated with 500 μM H_2O_2 for 1 or 2 h (Fig. 5). DPPP fluorescence was evaluated both by microscopy and by measurement in a fluorometer. Control cellular fluorescence after incubation with DPPP in WT cells was relatively low but increased progressively with time during the H_2O_2 exposure (Fig. 5A). AEC II from Prdx6−/− mice showed significantly greater DPPP fluorescence compared to WT at both time points. The greater change in DPPP fluorescence with the Prdx6 null cells was confirmed by measurements in the spectrofluorometer (Fig. 5B). The fluorescence signal from Tg and WT cells appeared similar by microscopy (Fig. 5A), although DPPP fluorescence by fluorometry indicated less lipid peroxidation in Prdx6 overexpressing cells (Fig. 5B).

DISCUSSION

It is now recognized that ROS, including superoxide radicals and H_2O_2, are produced biologically by many different types of cells and can serve as signaling molecules [Thannickal and Fanburg, 2000]. However, ROS when generated at greater than normal concentrations are considered to be agents of cell injury [Forman and Fisher, 1981; Fisher et al., 1984; Lang et al., 2002; Chow et al., 2003] H_2O_2 exposure has been widely used as a model of cellular oxidant stress as its interactions can generate more potent oxidizing radicals. Enzymes that directly scavenge H_2O_2 include the GPxs, catalase, and Prdxs [Forman and Fisher, 1981; Fisher et al., 1999; Rhee et al.,

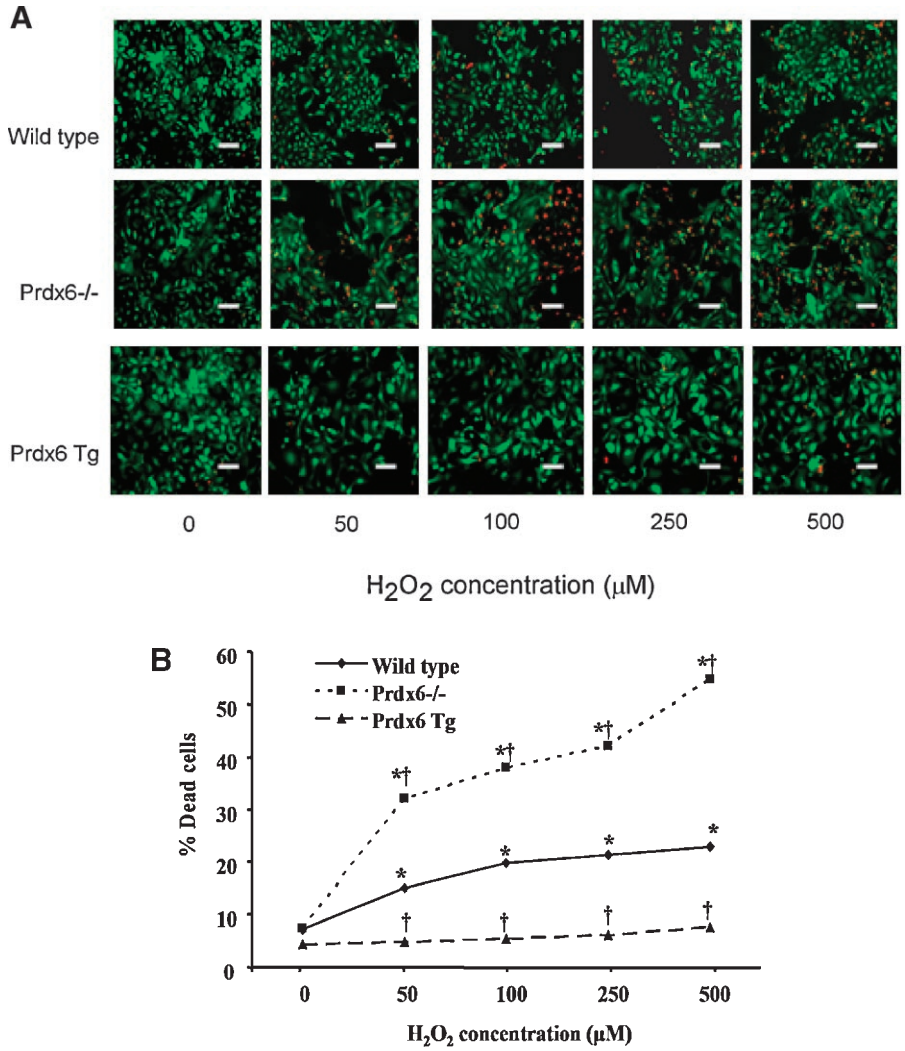

Fig. 3. Effect of H_2O_2 treatment on AEC II viability. AEC II were treated with 0–500 μM H_2O_2 for 24 h. **A**: Fluorescence images with calcein AM to detect ''live'' cells (green) and ethidium homodimer to detect ''dead'' cells (red). The calcein stain is diffuse in the cytoplasm while the ethidium stain is concentrated in the nucleus. The scale bar is 100 μm. Original magnification100×. **B**: Percentage of ''dead'' cells from experiments shown in A determined from approximately 1,000 total cells in 15 microscopic fields. Values are means ± SE for n = 3 separate experiments. $^{*}P < 0.05$ versus the corresponding zero time value. $^{\dagger}P < 0.05$ versus WT at the same concentration of H_2O_2.

1999]. We have extensively studied the antioxidant role of Prdx6, one of the six mammalian Prdxs [Manevich et al., 2002; Pak et al., 2002; Wang et al., 2004a, b; Wang et al., 2006a, b]. This enzyme differs from the other mammalian Prdxs in that it uses GSH following glutathionylation mediated by GSH S-transferase pi (πGST) as the physiological co-factor reductant [Manevich et al., 2004; Ralat et al., 2006]. Further, Prdx6 is able to directly reduce phospholipid hydroperoxides (PLOOH) to the less toxic alcohol [Fisher et al., 1999], a pathway that has been estimated as 10^4 times more efficient than the alternate pathway of PLA_2-mediated hydrolysis followed by reduction of the fatty acyl hydroperoxide [Zhao et al., 2003]. Both isolated cell and intact mouse models with Prdx6 overexpression or deletion have demonstrated that this enzyme has a major role in antioxidant defense [Manevich et al., 2002; Pak et al., 2002; Wang et al., 2003; Wang et al., 2004a,b; Wang et al., 2006a, b].

AEC II are important for lung homeostasis as they are the cells primarily responsible for lung surfactant production and for repair of the alveolar epithelial surface associated with lung

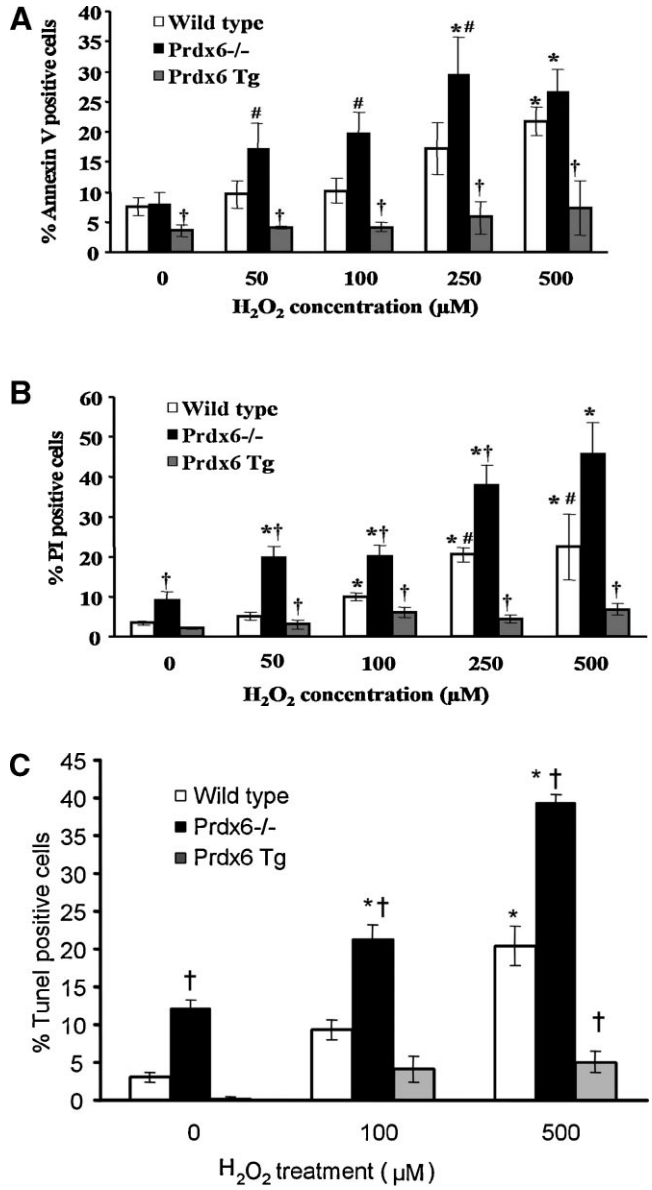

Fig. 4. The effect of Prdx6 expression on AEC II apoptosis with H_2O_2 treatment. AEC II from WT, Prdx6−/−, and Prdx6 Tg mice were treated for 24 h with 0–500 μM H_2O_2 and fluorescence staining for cell membrane expression of annexin V, uptake of PI, and DNA cleavage (TUNEL) were evaluated. Values are means ± SE for n = 3–4 experiments. **A**: Percentage of AEC II positive for annexin V. **B**: Percentage of AEC II positive for PI uptake. **C**: Percentage of AEC II positive for TUNEL. $^{*}P < 0.05$ compared to zero point in the same group; $^{\#}P < 0.05$ compared to WT for the same concentration of H_2O_2; $^{\dagger}P < 0.05$ for Tg versus Prdx−/− for the same concentration of H_2O_2.

injury. Prdx6 is prominently expressed in AEC II cells [Kim et al., 1998]. However, the antioxidant role of Prdx6 in primary AEC II cells, or for that matter, in any primary cell, has not previously been reported. The present study with H_2O_2 as the oxidant stress revealed that AEC II from Prdx6 null mice are significantly more sensitive to injury compared to cells from WT mice while AEC II from Tg Prdx6 overexpressing mice are relatively resistant. The increased resistance to H_2O_2 shown by Tg AEC II cells indicates that the overexpressed protein is targeted to the relevant compartment(s) for antioxidant protection. The induction of Prdx6 in Tg cells with H_2O_2 exposure (Fig. 2) is compatible with presence of the endogenous

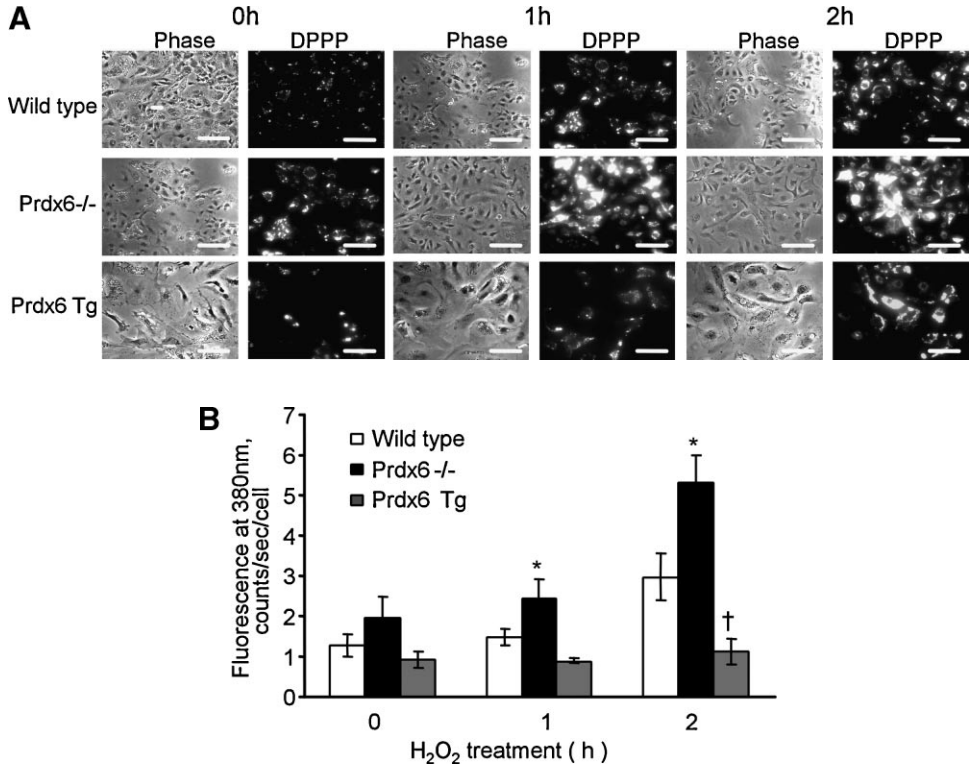

Fig. 5. The effect of Prdx6 on AEC II cellular membrane lipid peroxidation with H_2O_2 treatment as indicated by DPPP fluorescence. DPPP fluorescence was measured by microscopy (**A**) or by a spectrofluorometer (**B**). A: Fluorescence images of DPPP-labeled AEC II from WT, Prdx6−/−, and Prdx6 Tg mice after treatment with 500 μM H_2O_2 for either 1 or 2 h. The scale bar is 50 μm. Original magnification 200×. B: DPPP fluorescence measured by spectrofluorometry in AEC II cells from WT, Prdx6−/−, and Prdx6 Tg mice. Cells were exposed to 500 μM H_2O_2 for 1 or 2 h. Values are means ± SE for n = 3–4 experiments. *$P < 0.05$ compared to control (zero H_2O_2) in the same group; †$P < 0.05$ compared to WT at the same time point.

promoter in the >6,000 base pairs of upstream sequence used to generate the overexpressing construct [Phelan et al., 2003]. Finally, these results suggest availability of sufficient πGST and GSH, necessary co-factors for the Prdx6 catalytic cycle [Manevich et al., 2004; Ralat et al., 2006].

Evidence for toxicity of H_2O_2 in the present studies was the decreased cell viability indicated by increased uptake of ethidium homodimer (Fig. 3) and PI (Fig. 4B), decreased uptake of calcein (Fig. 3), increased cellular binding of annexin V (Fig. 4A), increased DNA fragmentation indicated by TUNEL staining (Fig. 4C), and increased DPPP fluorescence indicating lipid peroxidation (Fig. 5). Uptake of ethidium and PI indicate increased permeability of the plasma membrane suggesting peroxidation of plasma membrane lipid components [Muro et al., 2003]. The increased annexin V binding indicates increased accessibility of phosphatidyl serine to the extracellular milieu [Martin et al., 1995; Aubry et al., 1999]; this change plus the positive TUNEL assay are compatible with cell death by apoptosis [Labat-Moleur et al., 1998]. Recent evidence implicates the endogenous production of cell membrane-derived lipid metabolites including ceramide or the aldehyde 4-hydroxy-2-nonenal (HNE) as influencing the signaling pathway for apoptosis and promoting cell death by attacking nucleophilic amino acids such as Cys, His, and Lys [Esterbauer et al., 1991; Pettus et al., 2002; Castillo et al., 2007; Ryter et al., 2007]. For each of the parameters that were evaluated, the response to H_2O_2 was greater with the Prdx6 null cells and was lessened with Prdx6 overexpression compared to WT.

The mechanism for cellular toxicity of H_2O_2 is not clear. H_2O_2 itself is a mild oxidant although in the presence of $O_2^{-\cdot}$ and/or transition metals it can generate the very toxic ·OH [Forman and

Fisher, 1981]. The latter is a powerful oxidant that can readily oxidize cellular proteins and lipids. H_2O_2 is also a signaling molecule and its effect on signaling cascades could contribute to its toxicity [Thannickal and Fanburg, 2000]. Thus, removal of excess H_2O_2 would be important for the prevention of cellular damage. However, the present results (Fig. 1) indicate that Prdx6 plays a minor role in H_2O_2 removal in the AEC II and suggest that the ability to reduce H_2O_2 is unlikely to be the mechanism for Prdx6-mediated protection against cell injury. We have suggested that reduction of phospholipid hydroperoxides may be the major role for Prdx6 in antioxidant protection [Manevich et al., 2002; Pak et al., 2002]. Evidence for lipid peroxidation due to H_2O_2 in the present study is the demonstration of increased DPPP fluorescence [Okimoto et al., 2000; Matot et al., 2003]. Although DPPP reacts with oxidized lipids in solution, its fluorescence yield is considerably enhanced by reaction with oxidized membrane lipids [Okimoto et al., 2000]. Also, oxidation of membrane phospholipids is a likely mechanism for the increased cell permeability and increased incidence of apoptosis observed with H_2O_2 treatment in the present study. We have previously obtained similar evidence for lipid peroxidation associated with oxidant stress and protection by Prdx6 in studies of cell lines [Manevich et al., 2002; Pak et al., 2002] and intact lungs [Wang et al., 2004a; Wang et al., 2006a,b]. These results suggest that the ability of Prdx6 to reverse cell membrane lipid peroxidation may be the predominant mechanism for protecting cells from irreversible cell injury related to H_2O_2 treatment and other forms of oxidative stress.

Previous studies of oxidant stress have utilized models for varied expression of the classical antioxidant enzymes such as SODs, catalase, and GPxs [Ho et al., 1997; Danel et al., 1998; Ho et al., 1998; Folz et al., 1999; Asikainen et al., 2002; Ho, 2002]. Unlike the results for altered expression of Prdx6, studies of these other enzymes generally have yielded ambiguous results for their role in defense of the lung against oxidative stress. Thus, Prdx6 appears to be an especially important antioxidant enzyme and may play a critical role in the lung. While SOD can remove $O_2^{-\cdot}$ (thereby generating H_2O_2) and catalase and GPxs can reduce H_2O_2 to H_2O, these enzymes forestall but are unable to reverse ROS-mediated damage. As described above, we have postulated that the basis for this special protective effect of Prdx6 is its ability to reduce PLOOH. Phospholipids are an important component of cell membranes and their oxidation to PLOOH can result in altered transport and other functions of the plasma membrane and/or disruption of cellular organelles. Like Prdx6, GPx4 has the ability to reduce PLOOH [Ursini et al., 1995]. However, we have found that peroxidase activity with PLOOH as substrate is reduced by 96% in Prdx6 null lung homogenate [Wang et al., 2004a] and was undetectable in Prdx6 null AEC II cells (Fig. 1). These results indicate that Prdx6 is mainly responsible for PLOOH reduction activity in lung and that GPx4 has at best a minor role. Thus, we conclude that Prdx6 plays a unique role in protection of AEC II and the lung against oxidant stress.

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