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Peroxiredoxin 6 as an antioxidant enzyme: protection of lung alveolar epithelial type II cells from H₂O₂-induced oxidative stress

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Abstract

We evaluated the anti-oxidant role of peroxiredoxin 6 (Prdx6) in primary lung alveolar epithelial type II cells (AEC II) that were isolated from wild-type, Prdx6^{-/-}, or Prdx6 transgenic overexpressing (Tg) mice and exposed to H₂O₂ at 50μM - 500μM for 1-24 hours. Expression of Prdx6 in Tg AEC II was 7-fold greater than wild type. Prdx6 null AEC II exposed to H₂O₂ showed concentration-dependent cytotoxicity indicated by decreased “live/dead” cell ratio, increased propidium iodide 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 wild type AEC II and was least in Prdx6 Tg cells. Thus, Prdx6 functions as an anti-oxidant 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 anti-oxidant enzyme.

Keywords

cell viability; apoptosis; lipid peroxidation; diphenylpyrenylphosphine; TUNEL; Annexin V

Introduction

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, H₂O₂, and hydroxyl radical, which can result in oxidation of macromolecules including nucleic acids, lipids, and proteins [Chow et al., 2003; Fisher et al., 1984; Lang et al., 2002]. 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, as well as non-enzymatic antioxidants such as GSH [Forman and Fisher, 1981]. The peroxiredoxins (Prdx), a recent addition to the list of antioxidant enzymes, use cysteine as the catalytic center rather than the selenocysteine that characterizes the GPx enzymes [Rhee et al., 2005; Wood et al., 2003]. These enzymes have been classified as a 1-cys or 2-cys according to the number of conserved cysteine residues that function in catalysis [Rhee et al., 2005; Wood et al., 2003]. The 2-cys Prdxs generally use thioredoxin as a co-factor to reduce H_2O_2 and organic hydroperoxides [Rhee et al., 2005; Wood et al., 2003]. Prdx6 is a 1-cys enzyme that utilizes GSH as the physiological reductant to reduce H_2O_2 and organic hydroperoxides. Prdx6 is the only one of the six mammalian peroxiredoxins 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 also can be reduced by phospholipid hydroperoxide GPx (also called GPx4) [Ursini et al., 1995].

The role of Prdx 6 and other anti-oxidant 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 [Asikainen et al., 2002; Danel et al., 1998; Folz et al., 1999; Ho, 2002; Ho et al., 1998], GPx1 [Ho, 2002; Ho et al., 1998], and catalase [Danel et al., 1998]. These studies have given ambiguous results for their role in anti-oxidant 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; Wang et al., 2006a; Wang et al., 2004b; Wang et al., 2006b]. Overexpression of Prdx6 protein in human lung derived NCI-H441 cells attenuated membrane phospholipid peroxidation and apoptosis when cells were subjected to $\cdot OH$ 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.

Prdx 6 is enriched in lung compared to other organs and is expressed at the highest levels in alveolar epithelial type II cells, bronchiolar Clara cells, and alveolar macrophages [Kim et al., 1998; Mo et al., 2003]. Alveolar type I and type II epithelial cells (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 iodine staining [Hagen et al., 1986; Piotrowski et al., 2004; Yin et al., 2005]. In the present study, we investigate the role of Prdx6 in anti-oxidant defense using primary isolates of AEC II from lungs of wild type 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 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 TgPrdx6 mice was a genomic clone containing the entire Prdx6 gene including 6644 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 24-28g 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- μ m 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 μ g/ml insulin, 5 μ g/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 [Bates et al., 2002; Bortnick et al., 2003; Dobbs, 1990]. 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 ~495nm/~515nm) obtained by enzymatic conversion of non-fluorescent calcein AM to calcein. Dead cells were indicated by red fluorescence (ex/em ~495nm/~635nm) of ethidium homodimer (EthD-1) which can enter cells through damaged membranes and binds to nucleic acids. AEC II were incubated with 0.5 μ M calcein AM, plus 2 μ M EthD-1 for 20 mins 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) [Aubry et al., 1999; Martin et al., 1995]. Cells that bind annexin V show green staining of the plasma membrane; the red fluorescence of propidium iodide (PI) indicates altered cell membrane permeability. AEC II were incubated with 5 μ l Annexin V and 10 μ l PI in 200 μ l 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 ~1000 cells in 5-6 randomly chosen fields per dish.

Apoptosis also was 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 label 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 μ l/ml). Cells were evaluated by fluorescence microscopy (ex/em~495nm/~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 mins at 4°C in the presence of Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Indianapolis, IN), and centrifuged at 12,000 x 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 μ g 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) (1:5000). 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 glutathione (GSH), an NADPH regenerating system, and 0.66 mM H₂O₂ or 0.1 mM phospholipid hydroperoxide substrate as previously described; the phospholipid hydroperoxide substrate was prepared by incubation of 1-palmitoyl, 2-linolenoyl *sn*-phosphorylcholine (PLPC) with lipoxygenase to yield the oxidized product (PLPCOOH) [Fisher et al., 1999].

DPPP detection of cell membrane lipid peroxidation

Diphenyl-1- pyrenylphosphine (DPPP) is a fluorescent probe for detection of cell membrane lipid peroxidation [Matot et al., 2003; Okimoto et al., 2000]. DPPP is nonfluorescent, 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° 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 H₂O₂ 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 (3nm slit) over the subsequent 60 sec. 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 sec and normalized to the total number of cells [Manevich et al., 2002].

Statistical Analysis

Data are expressed as mean $\pm$ SEM for 3 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, Prdx 6 protein expression by western blot was ~7-fold greater as compared to WT mice (Fig 1B, C). Peroxidase activity in sonicated cells when measured with H₂O₂ substrate showed no significant differences among AEC II from wild type and Prdx6 knock-out or overexpressing mice (Fig. 1D). This indicates that other enzymes (for example, GSH peroxidases, as catalase was inhibited by the presence of aminotriazole) are largely responsible for the degradation of H₂O₂ in these cells. Peroxidase activity with the phospholipid hydroperoxide substrate (PLPCOOH) was only 10% of the corresponding activity with H₂O₂ in wild type cells and this activity was abolished by knock-out of Prdx6 (Fig. 1D). This result is 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 5-fold compared to wild type.

The change in cellular Prdx6 protein expression after 24 h of treatment with H₂O₂ at increasing concentrations (50-500 μ M) was determined by western blotting (Fig 2A). This oxidant stress induced a dose-dependent increase in Prdx6 expression in both wild type and Prdx6 Tg AECII with an increase in expression levels by 75-100% with 500 μ M H₂O₂ treatment (Fig 2B). As Prdx6^{-/-} AEC did not express Prdx6, they were not evaluated for the effect of H₂O₂.

Viability and apoptosis of AEC II with H₂O₂ treatment

Viability of AEC II was studied with calcein and ethidium staining (Fig. 3A). This allowed calculation of the % of “live” (viable) cells, or conversely the % of “dead” cells. The % of “dead” cells with treatment for 24 h varied with the concentration of H₂O₂ (Fig 3B). The percentage of viable AEC II (100 minus % of “dead” cells) after treatment with 50 μ M H₂O₂ for 24 h was 67% for Prdx6^{-/-} cells, 85% for WT cells, and 95% for Prdx6 Tg overexpressing cells. With 500 μ M H₂O₂, viability decreased to ~50% for Prdx6^{-/-} cells but was essentially unchanged from control for the Prdx6 overexpressing AEC II (Fig. 3B).

Staining of wild type AEC II for annexin V under control conditions showed <10% positive and this was not significantly changed with exposure to 50-250 μ M H₂O₂ for 24 h (Fig 4A). However, the percent of annexin positive Prdx6^{-/-} AEC II was 2-fold greater than control at 50 μ M H₂O₂ and increased progressively with increasing H₂O₂ 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 H₂O₂ exposure (Fig. 4A). At 500 μ M H₂O₂, 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 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 H₂O₂ exposure on DNA cleavage in AECII cells as another index of apoptosis. Under control conditions, the percentage of TUNEL positive cells was ~3% in wild type, 12% in Prdx6 ^{-/-} and <1% in Prdx6 Tg AECII (Fig. 4C). At 24 h exposure to H₂O₂, there was a concentration dependent increase of TUNEL positive cells in the wild type and Prdx6 ^{-/-} groups, while the Tg cells showed relatively small changes (Fig. 4C). At 500 μM H₂O₂, ~40% AECII cells in Prdx6 ^{-/-} group were TUNEL positive compared to 20% in the wild type and about 5% in Tg cells.

Lipid peroxidation with H₂O₂ 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 wild type, Prdx6 ^{-/-} and Tg Prdx6 mice were treated with 500 μM H₂O₂ 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 wild type cells was relatively low but increased progressively with time during the H₂O₂ exposure (Fig. 5A). AEC II from Prdx6 ^{-/-} mice showed significantly greater DPPP fluorescence compared to wild type 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 wild type 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 H₂O₂, 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 [Chow et al., 2003; Fisher et al., 1984; Forman and Fisher, 1981; Lang et al., 2002] H₂O₂ 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 H₂O₂ include GPxs, catalase, and the Prdxs [Fisher et al., 1999; Forman and Fisher, 1981; Rhee et al., 1999]. We have extensively studied the anti-oxidant role of Prdx6, one of the six mammalian Prdxs [Manevich et al., 2002; Pak et al., 2002; Wang et al., 2004a; Wang et al., 2006a; Wang et al., 2004b; Wang et al., 2006b]. 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 10⁴ times more efficient than the alternate pathway of PLA₂-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 anti-oxidant defense [Manevich et

al., 2002; Pak et al., 2002; Wang et al., 2003; Wang et al., 2004a; Wang et al., 2006a; Wang et al., 2004b; Wang et al., 2006b].

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 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 H₂O₂ as the oxidant stress revealed that AEC II from Prdx6 null mice are significantly more sensitive to injury compared to cells from wild type mice while AEC II from Tg Prdx6 overexpressing mice are relatively resistant. The increased resistance to H₂O₂ shown by Tg AEC II cells indicates that the overexpressed protein is targeted to the relevant compartment(s) for anti-oxidant protection. The induction of Prdx6 in Tg cells with H₂O₂ exposure (Fig. 2) is compatible with presence of the endogenous promoter in the >6000 base pairs of upstream sequence used to generate the overexpressing construct [Phelan et al., 2003]. Finally, these results suggest availability of sufficient π GST and GSH, necessary co-factor for the Prdx6 catalytic cycle [Manevich et al., 2004; Ralat et al., 2006].

Evidence for toxicity of H₂O₂ in the present studies was the decreased cell viability indicated by increased uptake of ethidium homodimer (Fig. 3) and propidium iodide (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 propidium iodide 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 [Aubry et al., 1999; Martin et al., 1995]; 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 (4-HNE) as influencing the signaling pathway for apoptosis and promoting cell death by attacking nucleophilic amino acids such as Cys, His, and Lys [Castillo et al., 2007; Esterbauer et al., 1991; Pettus et al., 2002; Ryter et al., 2007]. For each of the parameters that were evaluated, the response to H₂O₂ was greater with the Prdx6 null cells and was lessened with Prdx6 overexpression compared to wild type.

The mechanism for cellular toxicity of H₂O₂ is not clear. H₂O₂ itself is a mild oxidant although in the presence of O₂⁻ 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. H₂O₂ is also a signaling molecule and its effect on signaling cascades could contribute to its toxicity [Thannickal and Fanburg, 2000]. Thus, removal of excess H₂O₂ would be important for the prevention of cellular damage. However, the present results (Fig. 1) indicate that Prdx6 plays a minor role in H₂O₂ removal in the AEC II and suggest that the ability to reduce H₂O₂ 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 anti-oxidant protection [Manevich et al., 2002; Pak et al., 2002]. Evidence for lipid peroxidation due to H₂O₂ in the present study is

the demonstration of increased DPPP fluorescence [Matot et al., 2003; Okimotoa et al., 2000]. Although DPPP reacts with oxidized lipids in solution, its fluorescence yield is considerably enhanced by reaction with oxidized membrane lipids [Okimotoa et al., 2000]. Also, oxidation of membrane phospholipids is a likely mechanism for the increased cell permeability and increased incidence of apoptosis observed with H₂O₂ 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; Wang et al., 2006b]. 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 H₂O₂ 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 [Asikainen et al., 2002; Danel et al., 1998; Folz et al., 1999; Ho, 2002; Ho et al., 1997; Ho et al., 1998]. 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 anti-oxidant enzyme and may play a critical role in the lung. While SOD can remove O₂^{•-} (thereby generating H₂O₂) and catalase and GPxs can reduce H₂O₂ to H₂O, 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 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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Figure 1. Identification of lung alveolar type II alveolar epithelial cells (AEC II)

AEC II were isolated from wild type (WT), Prdx6 null and Prdx6 Tg mice and maintained in culture for 36-48h. 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 H₂O₂ or 100 μM 1-palmitoyl-2-linolenoyl phosphatidylcholine hydroperoxide (PLPCOOH) as substrate. Aminotriazole (20 mM) was added for assay with H₂O₂. The value for Prdx6 ^{-/-} cells with PLPCOOH substrate was zero. Values are means ± SE for n=3. * *p* < 0.05 compared to wild type for the same substrate.

Figure 2. Prdx6 expression in wild type and Prdx6 transgenic (Tg) AEC II cells following treatment with H₂O₂

A. Cells were treated for 24h with 0-500 μM H₂O₂ 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 H₂O₂ concentration was normalized to the respective wild type or Tg control (no H₂O₂ treatment). Results represent the mean ± SE for 3 experiments. *P<0.05 compared to zero point in the same group; †P<0.05 compared to wild type at the same H₂O₂ concentration.

A.

Figure 0003

B.

Figure 0004

Figure 3. Effect of H₂O₂ treatment on AEC II viability

AEC II were treated with 0-500 μM H₂O₂ 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 100μm. Original magnification x 100. 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 vs. the corresponding zero time value. † P<0.05 vs. wild-type at the same concentration of H₂O₂.

Figure 0005

C.

Figure 0006

Figure 4. The effect of Prdx6 expression on AEC II apoptosis with H₂O₂ treatment

AEC II from wild type, Prdx6 ^{-/-} and Prdx6 Tg mice were treated for 24 h with 0-500 μM H₂O₂ and fluorescence staining for cell membrane expression of annexin V, uptake of propidium iodide (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 wild type for the same concentration of H₂O₂; †P<0.05 for Tg vs. Prdx^{-/-} for the same concentration of H₂O₂.

Figure 0007**B.** **Figure 0008****Figure 5.**

The effect of Prdx6 on AEC II cellular membrane lipid peroxidation with H₂O₂ 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 H₂O₂ for either 1 or 2 h. The scale bar is 50 μm. Original magnification x 200. B. DPPP fluorescence measured by spectrofluorometry in AEC II cells from wild type, Prdx6^{-/-}, and Prdx6 Tg mice. Cells were exposed to 500 μM H₂O₂ for 1 or 2 h. Values are means ± SE for n=3-4 experiments. * p<0.05 compared to control (zero H₂O₂) in the same group; † P<0.05 compared to wild type at the same time point.