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Critical Role of Peroxiredoxin 6 in the Repair of Peroxidized Cell Membranes Following Oxidative Stress

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Abstract

Phospholipids are a major structural component of all cell membranes; their peroxidation represents a severe threat to cellular integrity and their repair is important to prevent cell death. Peroxiredoxin 6 (Prdx6), a protein with both GSH peroxidase and phospholipase A_2 (PLA_2) activities, plays a critical role in antioxidant defense of the lung and other organs. We investigated the role of Prdx6 in the repair of peroxidized cell membranes in pulmonary microvascular endothelial cells (PMVEC) and isolated mouse lungs treated with *tert*-butylhydroperoxide and lungs from mice exposed to hyperoxia (100% O_2). Lipid peroxidation was evaluated by measurement of thiobarbituric acid reactive substances, oxidation of diphenyl-1-pyrenylphosphine, or the ferrous xylenol orange assay. The exposure dose was varied to give a similar degree of lipid peroxidation at the end of exposure in the different models. Values for lipid peroxidation returned to control levels within 2 h after oxidant removal in wild type PMVEC and perfused lungs but were unchanged in Pxdx6 null preparations. An intermediate degree of repair was observed with PMVEC and lungs that express only C47S or D140A mutant Prdx6; the former mutant does not have peroxidase activity while the latter loses its PLA_2 activity. Prdx6 null mice showed markedly delayed recovery from lipid peroxidation during a 20 h observation following exposure to hyperoxia. Thus, Prdx6 plays a critical role in the repair of peroxidized phospholipids in cell membranes and the recovery of lung cells from peroxidative stress; the peroxidase and PLA_2 activities each contribute to the recovery process.

Graphical abstract

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INTRODUCTION

Phospholipids are a major structural component of all cell membrane and their peroxidation with oxidative stress represents a severe threat to cellular integrity. Peroxidation of cell membrane-associated phospholipids has been implicated in the pathogenesis of various disorders including neurodegenerative diseases, acute lung injury, and lung fibrosis among others (1, 4, 15, 25, 34). Lipid hydroperoxides are the primary products of lipid peroxidation and are the source of highly toxic secondary products such as aldehydes (4). Thus, the ability to reduce phospholipid hydroperoxides (PLOOH), i.e., repair the peroxidized membrane lipids, is of primary importance in the recovery of cells from oxidative stress. Numerous studies have investigated DNA and protein injury and repair, but there are relatively few studies of the repair of oxidized lipids and the mechanisms for repair are poorly understood. This knowledge is of crucial importance for understanding the recovery from sublethal exposure to oxidants.

Previous studies by our group have shown that peroxiredoxin 6 (Prdx6) can bind to peroxidized phospholipids in the cell membrane following oxidative stress and is associated with a decrease (compatible with repair) in the cell membrane content of phospholipid hydroperoxides (20), suggesting a possibly important and unique role of Prdx6 as an anti-oxidant enzyme. Prdx6 is a bifunctional enzyme with peroxidase and phospholipase A_2 (PLA_2) activities and is known to play a critical role in antioxidant defense of lung and other organs (17, 18, 24, 30–33). In addition to reducing a wide range of hydroperoxide substrates, Prdx6 is the only enzyme in the peroxiredoxin family that can reduce PLOOH through its peroxidase activity (12). This peroxidase activity is dependent on the catalytic Cys at position 47 and uses GSH as the physiological reductant (6, 12). A second enzymatic function of Prdx6 is its unique calcium-independent PLA_2 activity (19). This latter activity is dependent on a catalytic triad: Ser32, His26, and Asp140 (19). The PLA_2 activity catalyzes the hydrolysis of the acyl group at the sn-2 position of glycerophospholipids, with special affinity for phosphatidylcholines, to produce free fatty acids and a lysophospholipid (8).

A previous study indicated that both the peroxidase and PLA_2 activities are important in protecting cells against death associated with oxidant stress (17), but the mechanism for this protective effect of Prdx6 was not determined. One possibility for the protective function of Prdx6 is the scavenging of oxidants such as H_2O_2 or other small hydroperoxides, via its peroxidase activity, in competition with catalase and other GSH peroxidases and peroxiredoxins. Based on our previous studies showing a significantly greater protective role for Prdx6 compared to the important anti-oxidant enzyme GSH peroxidase 1 (GPx1), a scavenging role for Prdx6 seems unlikely to be the major mechanism for its ability to protect against cell death (18).

We propose that the more important antioxidant defense role of Prdx6 is based on its ability to contribute to the repair of peroxidized cell membranes and that this repair is through both the direct reduction pathway via its peroxidase activity and phospholipid remodeling through its PLA_2 activity. The present study evaluated the role of Prdx6 and its 2 activities in the "repair" of peroxidized cell membranes in mouse pulmonary microvascular endothelial cells (PMVECs) in primary culture, in isolated mouse lungs and in intact mice.

MATERIALS AND METHODS

Materials

2-Thiobarbituric was purchased from Sigma-Aldrich (St. Louis, MO). DPBS and DAPI were obtained from Life Technologies (Grand Island, NY). Trichloroacetic acid was purchased from Fisher Scientific (Fair Lawn, NJ). *Tert*-butyl hydroperoxide (*t*-BOOH) was purchased as a 70% solution from MP Biomedicals (Newport Beach, CA). Diphenylpyrenyl phosphate (DPPP) and methyl linoleate hydroperoxide (MeLOOH) were purchased from Cayman (Ann Arbor, MI); the latter was solubilized in dimethyl sulfoxide and stored at −80 °C. Ferrous xylenol orange (FOX) was purchased as an assay kit from Northwest Life Science (Vancouver, Wa). 1-Palmitoyl, 2-linoleoyl, sn-3-glycerophosphocholine hydroperoxide (PLPCOOH) as substrate for the peroxidase assay was prepared as described previously (17). Recombinant wild type Prdx6 protein was expressed in PET Blue-1 (Novagen, Madison, WI) and purified by column chromatography as described previously (6, 19, 20).

Animals

The use of animals for these studies was approved by the University of Pennsylvania Animal Care and Use Committee (IACUC). Male C57Bl/6 wild-type null mice (8–10 weeks old) were obtained from The Jackson Laboratory (Bar Harbor, ME). Prdx6 null mice were bred in our animal care facilities. Their generation and genotyping has been described previously (22) and they have been fully back-crossed to the wild type C57BL/6 background as confirmed using microsatellite marker analysis performed by the Jackson Laboratory (18). Two 'knock in' mouse colonies, C47S and D140A, each containing a Prdx6 mutation on the C57BL/6 background were generated. These mutations were selected based on the previous demonstration that mutation of C47 inactivates the peroxidase but not the PLA_2 activity of Prdx6 while the mutation of D140 inactivates the PLA_2 but does not affect the peroxidase activity (6).

Gene targeting—In order to retrieve the part of the Prdx6 gene to be mutated, short homologous arms for the pL253 retrieval vector (Fredericks NCI, recombineering website) were amplified from a *Prdx6* gene containing BAC clone from a C57Bl/6 Library; 300 bp sequences were amplified by PCR to generate linear fragments flanked by either NotI/HindIII or HindIII/SpeI, respectively, and then co- ligated into the pL253 MCS NotI and SpeI sites. The resulting construct was linearized using HindIII and transfected into *E. coli* SW102 containing heat shock inducible lambda Red recombination proteins and the *Prdx6* BAC clone. Following treatment at 42°C for 15 min and ampicillin selection, resistant colonies were screened for homologous recombination at the 300 bp flanking regions.

pL253 derivatives with a ~12 kB fragment containing exons 1–4 were selected. This fragment served as basic backbone for both the C47 and the D140 contructs.

To generate the C47 mutant allele, a mini-vector was first generated by PCR to combine a ~300 bp long genomic *Prdx6* fragment located ~150 nt upstream of exon 2 together with a ~700 bp *Prdx6* fragment containing exon 2 located just downstream of the ~300 bp fragment. The genomic PCR primers were designed to introduce the appropriate restriction enzyme sites at the ends of the fragments for cloning into PL451 (Fredericks NCI, recombineering website). The two PCR fragments were ligated into pL451 by two subsequent ligation and cloning cycles. This pL451 derivative was mutated by site-directed mutagenesis at codon C47 (TGC to TCC) in exon 2 to construct a mutagenic mini-targeting vector. This vector, which also contained a pgk EM7 neo-polyA cassette flanked by FLP recombinase target sites (FRT), was linearized, transfected, and recombined into the above ~12 kB Prxd6 vector backbone by heat induction of the lambda Red recombination enzymes, as above.

To generate the D140 mutant allele, the same strategy as for the C47 allele was used, except that the two PCR fragments for the mini vector contained ~300 nt of P*rdx*6 sequences upstream of exon 4, and exon 4 itself, respectively. The mutation D140 was introduced as above by site directed mutagenesis of the mini-vector which was then integrated into the backbone vector, as above.

Generation of the final targeting vectors with the C47 and D140 mutations included the neomycin resistance cassette; selection utilized kanamycin (50 μg/ml). This final targeting construct was linearized, sequence verified and electroporated into the C57Bl/6N ES cell line (EAP6, Penn Gene Targeting Core and Laboratory) for insertion of the mutant sequences into the mouse genome by homologous recombination. Positive ES clones showed homologous recombination displaying an extra, larger band upon Southern blotting (Figure 1); these results for the D140A targeting vector were confirmed by Southern blotting using the entire region (not shown). The clones were karyotyped, the C47 or D140 mutation verified by genomic sequencing, and finally used for blastocyst injection into CD-1/BALB/c mice.

Chimeric C47S or D140A mice were bred to C47Bl/6N wild type mice. F1-one pups with black coat color were of pure C57Bl/6N background; these mice were genotyped and those carrying the desired mutation were further bred to homozygosity. Genotyping of knock-in mice was performed by allele-specific PCR. DNA was prepared from mouse tail tips (approximately 3 mm in length) by an alkaline lysis method with addition of 0.05 M NaOH (0.3 ml) to each tail tip in a microfuge tube. The tube was heated to 95° C for 30 min, then placed briefly on ice before centrifugation at ~ 15,900 × g in a microfuge for 5 min. Supernatant (10 μl) from each tail tip was then transferred to another tube containing 90 μl of TE (10 mMTris HCl, 1 mM EDTA, pH 7.5 or 8) and mixed well. One μl of this diluted DNA was used in a 10 μl PCR reaction containing 5 μl KAPA2G Fast HotStart Genotyping Mix (2X) (KAPA Biosystems, Wilmington, MA), along with 0.5 μM of each PCR primer. PCR was started by incubating each reaction for 10 min at 95° C, followed by 35 cycles of denaturation (95° C; 30 sec), annealing (15 s), and elongation (72° C, 30 s). The temperature

of annealing was adjusted depending on the sequence of the PCR primers. After the completion of 35 cycles, the PCR was concluded with 10 min at 72° C and then held at 4° C.

The oligonucleotide primers for the forward reaction for C47 wild type was: 5′ CGGGACTTTACCCCAGTGTG 3′ and for the mutant allele, C47S, 5′ CGGGACTTTACCCCAGTGT**C** 3′. The reverse primer for this PCR was: 5′ CCCTAGTCCTGCCTGGTTC 3′; the annealing temperature was 67° C. The forward primer for genotyping the D140A phenotype was: 5′ CTTGGGGGAATCACATGAGGG 3′. The reverse primers were allele specific. The primer for the D140 wild-type was: 5′ GGATAGACAGCTTCAGTTTCTTGG 3′ and for the mutant allele, D140A was: 5′ GGATAGACAGCTTCAGTTTCTTG**T**3′; the annealing temperature was 64° C. The bolded letter in each mutant construct represents the mutation. The PCR products were subjected to electrophoresis on a 1.5% agarose gel.

Cell isolation and culture

PMVEC were isolated from the lungs of WT and Prdx6-null mice as previously described (23, 24). Briefly, freshly harvested mouse lungs were treated with collagenase, followed by isolation of cells through incubation with monoclonal antibody that recognizes platelet endothelial cell adhesion molecule (PECAM) (BD Biosciences Pharmingen, Palo Alto, CA) and Dynabeads (Dynal, Oslo, Norway) coated with sheep anti-rat IgG (25). PMVEC were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, nonessential amino acids, penicillin/streptomycin, and Plasmocin (InvitroGen, San Diego, CA). The endothelial phenotype of the preparation was routinely confirmed by evaluation of several markers including fluorescence microscopy to determine cellular uptake of acetylated low-density lipoprotein labeled with 1, 1′ –dioctadecyl–3,3,3′,3′ –tetramethylindocarbocyanine perchlorate (DiI-AcLDL) and by immunostaining using mouse monoclonal antibodies for PECAM-1 (Santa Cruz Biotech, Santa Cruz, CA) and vascular endothelial cadherin (VE-cadherin) (Abcam, Cambridge, MA) (5). Cells were washed and incubated with secondary antibody, goat anti-mouse IgG conjugated to Alexa 488 (green) for PECAM or Texas Red (red) for VE-cadherin. Cells between passages 8–13 were used for the studies.

Plasmid constructs and lentiviral infection

HMD lentiviral transfer plasmid, packaging plasmid pCMV-dR8.2, and envelope plasmid pCMV-VSVG were a generous gift from the laboratory of Dr. Mitchell Weiss, The Children's Hospital of Philadelphia. The WT and mutant D140A or C47S human Prdx6 plasmids were generated by overlap PCR with hPrdx6 PET21b as a template and inserted into HMD lentivirus transfer plasmid containing EGFP using the Xho1 and EcoR1 restriction sites. The specific primers for generating the D140A mutant HMD lentiviral transfer plasmid were:

5′ GTGTTTGTTTTTGGTCCT***GCC***AAGAAGCTGAAGCTGTC 3′ (forward) and

5′ GACAGCTTCAGCTTCTT***GGC***AGGACCAAAAACAAACAC 3′ (reverse).

The specific primers for making the C47S HMD lentiviral transfer plasmid were:

5′ GGGACTTTACCCCAGTG***AGC***ACCACAGAGCTTGGCAG 3′ (forward) and

5′ CTGCCAAGCTCTGTGGT***GCT***CACTGGGGTAAAGTCCC 3′ (reverse).

Letters in bold italics represent mutated codons.

The specific primers were used with the following general primers in overlap PCR:

5′ ACAC**CTCGAG**GCCACCATGCCCGGAGGTCTGCTTCTCGG 3′ (forward; used with specific reverse primers above) and

5′ ACAC**GAATTC**TTAAGGCTGGGGTGTGTAGCGGAGG 3′ (reverse; used with specific forward primers above).

Letters in bold represent restriction enzyme cleavage sites (forward, XhoI; reverse EcoRI) that were used for cloning.

HMD transfer plasmids containing WT or mutant (D140A, C47S) Prdx6 were packaged with second generation packaging plasmid pCMV-dR8.2 and envelope plasmid pCMV-VSVG in 293T cells using Lipofectamine 2000. The medium containing the virus was collected at 48 and 72 h after transfection. Prdx6 null PMVEC were infected with virus containing medium plus added polybrene (~ 8 μg/ml). EGFP expression in the cells was maximal at 72–96 h after infection and cells were studied during that interval post-transfection. Empty HMD lentiviral transfer plasmid was used as a negative control.

Exposure of cells to oxidants

For oxidant exposure of PMVEC, *t*-BOOH was added to the incubation medium at a final concentration of 0.1–0.3 mM; incubation was continued for 0–8 h. At the end of exposure, the medium was removed, cells were washed with fresh medium, and incubation with oxidant-free medium was continued for an additional 2–6 h following which PMVEC were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% NP-40.

Isolated perfused mouse lung

Isolated lung perfusion was carried out as described previously (11, 13, 18). Mice were anesthetized with intraperitoneal pentobarbital (50 mg/kg) and lungs were cleared of blood by perfusion through the pulmonary artery and placed in the perfusion chamber. Lungs were ventilated continuously at 0.3 ml tidal volume with 5% CO_2 in air and perfused at 2 ml/min with perfusate containing 3% fatty-acid-free bovine serum albumin and 10 mM glucose in Krebs Ringer bicarbonate buffer (pH 7.4) with or without 5 mM *t*-BOOH. If DPPP assay was planned, lungs were pre-perfused with the fluorophore (50 μM) for 30 min and then subjected to oxidative stress. At the end of 1 h perfusion, the pulmonary vasculature was flushed with PBS followed by *en bloc* removal of the heart and lungs. The heart and large airways were dissected away and the cleared lung was rapidly frozen in liquid nitrogen and stored at − 80° C. For subsequent assay, an aliquot of frozen lung was homogenized under N_2 in PBS (1:10) containing 0.01% butylated hydroxytoluene.

Exposure of mice to hyperoxia

Wild-type, Prdx6 null, C47S mutant, and D140A mutant mice were exposed to 100% oxygen in an exposure chamber as described previously (18, 32). After exposure to O_2 for 60 h, the chamber was opened to room air and mice were sacrificed immediately (0 time) or after 4, 12, or 20 h of observation. Mice were anesthetized and lungs were cleared of blood, excised, and homogenized as described above for study of the isolated perfused lung. Lung homogenates were assayed for lipid peroxidation.

Assays for lipid peroxidation

Lipid peroxidation was assayed by 3 different methods (TBARS, DPPP, and FOX). TBARS is a widely used assay that is useful as a screening method; it measures malondialdehyde (MDA) and other aldehydes that are predominantly generated from lipid hydroperoxides by the hydrolytic conditions of the reaction, i.e., high temperature (90–100 °C) and acidic medium; this assay, therefore, does not directly measure lipid peroxidation products (14). In terms of this study, the generation of MDA in the intact cell is not reversible so measurement of MDA *per se* to evaluate repair processes is not appropriate. However, since the MDA and other products are formed during the assay, we reasoned that a decrease in TBARS would reflect a decrease in the lipid hydroperoxides formed *in situ* that give rise to products (MDA, etc) detected *in vitro* by the reaction. DPPP and FOX reagents interact directly with lipid hydroperoxides and, therefore, do reflect the repair process. Our previous studies with oxidant stress indicate that all 3 assays give comparable results for the direction and magnitude of change in lipid peroxidation of lung tissue although absolute values differ with the assay method (18). Although the TBARS measurement can reflect oxidation of biomolecules other than lipids, the qualitatively similar results to the other two assay methods suggests that the results with this assay largely reflect formation of lipid hydroperoxides. Of note, the formation of F2-isoprostanes also is not reversible so that measurement of these products would not be appropriate to assess reversal of lipid peroxidation.

For cell extracts, TBARS were measured fluorometrically at an excitation wavelength of 530 nm and an emission wavelength of 550 nm by using a FLX800 microplate Fluorescence Reader (BioTEK, Winooski, VT), and quantified by comparison to a standard curve obtained by measuring TBARS formation using MDA (0.1–10 mM) to react with thiobarbituric acid under the same conditions. For determination of TBARS in lung homogenates, the absorbance was measured at 535 nm using a Varian Cary® 50 UV-Vis spectrophotometer (Agilent Tech, Santa clara, CA) (18).

Diphenyl-1-pyrenylphosphine (DPPP) is non-fluorescent but fluoresces when oxidized. It reacts with lipid hydroperoxides stoichiometrically to yield the fluorescent product DPPP oxide (DPPP-Ox) and has been used as a fluorescent probe for lipid peroxidation in cells and tissues (18, 21, 23, 31). Cell and lung homogenates were incubated with DPPP (10 μM) in the dark at 4° C for 30 min. For cells, fluorescence was determined with the FLX800 microplate reader at 352 nm excitation and 380 nm emission wavelengths; DPPP-Ox concentration was calculated from a standard curve obtained by reacting DPPP (10 μM) with methyl linoleate hydroperoxide (MeLOOH) under the same conditions. For lung

homogenates, fluorescence was measured at 380 nm (350 nm excitation) using the PTI spectrofluorometer and values are reported as arbitrary fluorescence units (AFU) as described previously (18, 21).

The FOX assay was used as described previously to analyze the lung homogenates from mice exposed to hyperoxia (18). Absorbance of the Fe^{3+}-xylenol orange complex was measured at 560 nm using the Varian spectrophotmeter and lipid hydroperoxides were calculated as described by the supplier.

Peroxidase enzymatic activity assay

Peroxidase activity in lysed cells or homogenized lung was measured as previously described (12) with minor modifications. Activity was assayed using PLPCOOH as substrate by measuring the consumption of NADPH in the presence of GSH and GSH reductase. The PLPCOOH substrate was dispersed in 0.1% Triton X-100. The reaction buffer (3 ml) was 50 mM Tris–HC1 (pH 8.0) containing 2 mM NaN_3, 0.1 mM EDTA, 0.025 mM NADPH, 0.66 mM GSH, and 0.23 units/ml GSH reductase. The cell lysate in buffer was pre-incubated for 5 min with continuous stirring. Fluorescence was continuously recorded at 460 nm (340 nm excitation) using a PTI spectrofluorometer (Photon Technology International, Edison, NJ) equipped with a single photon counting system. After a steady baseline had been achieved, the reaction was started by the addition of 50 μM PCOOH, and the change in fluorescence was recorded for 5–10 min. The rate of reaction was calculated from the linear rate of NADPH oxidation and was corrected for the relatively small baseline nonenzymatic oxidation of NADPH. Enzymatic activity was expressed as nmol NADPH oxidized/min/mg protein.

PLA_2 activity assay

To measure PLA_2 activity, lysed PMVEC were incubated with radiolabeled liposomes as previously described (9, 11). Unilamellar liposomes (~100 nm in diameter) consisting of 1-palmitoyl, 2-[9, 10 ^{3}H]-palmitoyl, *sn*-glycero-3 phosphocholine ([^{3}H]-DPPC), egg phosphatidylcholine, phosphatidylglycerol, and cholesterol (0.5, 0.25, 0.1, 0.15 mol fraction, respectively) were prepared by extrusion through a membrane under pressure. Lysates of cells or lung homogenates were incubated in calcium-free buffer (40 mM sodium acetate, 5 mM ethylene glycol tetraacetic acid) at pH 4 for 1 h. In additional studies to compare activity with reduced vs oxidized phospholipid substrate, recombinant Prdx6 or lung homogenate was evaluated for PLA_2 activity at pH 4 or 7 using ^{3}H-labelled liposomes before or after oxidation of lipids. To oxidize lipids, liposomal substrate was incubated with 10 μM $CuSO_4$ in the presence of 0.20 mM ascorbate for 1 hr followed by dialysis for 2 hr against PBS (pH 7.4) using a 10 kDa molecular mass cutoff membrane (20). For assay at pH 7, Tris (50 mM) was substituted for Na acetate in the reaction buffer. For all incubations, the radiolabeled free fatty acid product was extracted with hexane-ether 1:1 (v/v), resolved on Whatman LK5 silica gel 105A thin-layer chromatography plates using a hexane-ether-acetic acid solvent system, and analyzed by scintillation counting (9, 10).

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 ANOVA followed by the Student t-test as appropriate. Differences between mean values were considered statistically significant at $P < 0.05$.

RESULTS

Lipid peroxidation induced by t-BOOH and recovery in PMVEC

Isolated PMVEC showed the absence of the 2 enzymatic activities of Prdx6 in Prdx6 null lungs/cells as compared to wild type (Table 1), consistent with previous reports (17). To generate lipid peroxidation in cells, PMVEC from wild-type (WT) or Prdx6 null mice were incubated with 50–300 μM *t*-BOOH for 2–8 h. Lipid peroxidation as evaluated by TBARS assay was increased in a dose and time dependent manner in both WT and Prdx6 null cells; however, Prdx6 null PMVEC were more sensitive to the peroxidative stress with higher levels of lipid peroxidation compared to WT cells at each concentration of *t*-BOOH or time point (Fig. 2). We selected 300 μM *t*-BOOH for WT cells and 200 μM for mutant cells as the agent for generating lipid peroxidation for subsequent study of cellular repair. The rationale for using different concentrations of oxidant was to generate an equivalent degree of lipid peroxidation in the WT and mutant cells at the end of the exposure period, i.e., the start of the repair process.

After 4 h of *t*-BOOH exposure, the lipoxidation level was increased by approximately 4-fold in both WT and null cells (zero time in Fig. 3A, B). The medium containing *t*-BOOH was then replaced with fresh medium and cells were harvested for measurement of lipoxidation at 2, 4, and 6 h. In WT cells, the TBARS value had returned to its control levels by the 2 h time point (Fig. 3A). The control level is shown in Table 2. By contrast, TBARS in Pxdx6 null PMVEC remained unchanged during the 6 h observation period (Fig. 3A). Similar results were obtained for measurement of cellular lipid hydroperoxides based on the oxidation of DPPP (Fig. 3B). These results indicate that the presence of enzymatically active Prdx6 plays a critical role in the repair of peroxidized cell membranes of PMVEC subjected to peroxidative stress.

Role of peroxidase and PLA_2 activities of Prdx6 in the repair of peroxidized cell membrane phospholipids

We next evaluated the relative roles of the 2 activities of Prdx6 in the repair of peroxidized cell membrane phospholipids. Prdx6 null PMVEC were infected with lentivirus to express mutant Prdx6 with only peroxidase activity, only PLA_2 activity, or both activities (i.e., comparable to WT Prdx6); control cells were infected with lentivirus vector alone and did not express either activity of Prdx6 (control cells). Evaluation of GFP fluorescence showed an infection efficiency of approximately 90% of cells and was similar for all 4 constructs (vector alone, WT, C47S, D140A) (Fig. 4A). Assay for the Prdx6-related activities of infected cells showed the expected absence of both activities in control cells, loss of PLA_2 activity in D140A infected cells and a marked decrease of peroxidase activity (PLPCOOH substrate) in C47S infected cells (Table 1). The values for peroxidase activity in the WT and

D140A infected cells were similar to peroxidase activity levels previously reported for WT PMVEC while the PLA_2 activity in WT and C47S infected cells was about 1/3 decreased as compared to the previously reported WT values (17). The Prdx6 null PMVEC (non-infected as well as infected) show a spindle-shaped morphology (Fig. 4B) unlike the polygonal and cobblestone morphology that is characteristic of WT endothelial cells, including those isolated in our laboratory (not shown). However, these spindly cells stained positively for PECAM and VE-cadherin (Fig. 4B), protein markers that define endothelial cells. Further, additional passages of the spindly cells sometimes resulted in their reversion to cobblestone morphology. Spindle-shaped morphology has been reported previously for placental microvascular endothelial cells (16). Therefore, we conclude that these Prdx6 null cells even with the aberrant morphology are PMVEC.

The infected Prdx6 null cells were exposed to *t*-BOOH and the subsequent recovery from this oxidative stress was determined. Prdx6 null PMVEC expressing WT Prdx6 were treated with 300–μM *t*-BOOH and Prdx6 null PMVEC expressing mutant Prdx6 or vector alone were treated with 200 μM *t*-BOOH for 4 h; cells then were evaluated at 2 h intervals for up to 6 h after removal of the oxidant. Values for lipid peroxidation returned to control levels (see Table 2 for control values) in 2 h in WT cells (i.e., Prdx6 null PMVEC infected with the WT Prdx6 construct) (Fig. 5). Indices of lipoperoxidation were unchanged at 6 h after oxidant removal in Prdx6 null PMVEC, i.e., Prdx6 null PMVEC infected with lentivirus vector alone (Fig. 5). Thus, the infected cells (WT and null, Fig. 5) had the same response as the corresponding non-infected cells (Fig. 4), showing that infection with lentivirus *per se* had no effect on the cellular response to oxidative stress. A slower rate of repair compared to WT was observed with Prdx6 null PMVEC infected with C47S or D140A mutant Prdx6 and cells required 4–6 h to return to the baseline value (Fig. 5). The cells without peroxidase activity were affected to a slightly greater degree that those without PLA_2 activity. These results confirm that Prdx6 plays a critical role in the repair of cell membrane lipid peroxidation in PMVECs and that both the peroxidase and PLA_2 activities of Prdx6 contribute to the repair process.

Isolated perfused mouse lung

As previously reported (11, 21), lungs of Prdx6 null mice do not express either enzymatic activity of Prdx6 (Table 1). The “knock-in” mice show the expected loss of peroxidase activity with the C47S mutation and loss of PLA_2 activity with the D140A mutation (Table 1). The enzymatic activities in the knock –in models were similar to the respective activities measured in WT lungs, i.e., C47S had similar PLA_2 activity to WT and D140A had similar peroxidase activity to WT (Table 1). *t*-BOOH was used to induce oxidant injury in isolated perfused lungs and parameters of phospholipid peroxidation subsequently were measured in homogenates of these lungs. WT lungs were perfused with 25 mM *t*-BOOH and Prdx6 null and mutant lungs were perfused with 15 mM *t*-BOOH in order to generate similar degrees of peroxidative stress (Fig. 6). After 60 min of oxidant exposure, the oxidant was removed and recovery was evaluated over the subsequent 2 h. WT lungs recovered fully during the 2-h post-exposure observation period, while Prdx6 null lungs showed no recovery (Fig. 6). C47S Prdx6 lungs recovered by about 30% while D140A Prdx6 lungs recovered by about 50% from the lipid peroxidation at 2 h after removal of the oxidant (Fig. 6). Thus, similar to the

results obtained with PMVEC, the presence of Prdx6 was crucial for the repair of peroxidized phospholipids in lung. Both peroxidase and PLA_2 activities of Prdx6 contributed to the repair process although the repair was slightly less efficient with the loss of peroxidase activity.

Mice exposed to hyperoxia

Wild-type, Prdx6 null, and C47S and D140A mutant mice were exposed to 100% oxygen, and evaluated for oxidation of lung lipids by using TBARS, DPPP fluorescence, and FOX assays. Exposure to oxygen resulted in significantly increased levels of lipid peroxidation in all 4 mouse types as indicated by all 3 assays (Fig. 7 A, B, C). The degree of lipid peroxidation at 60 h of hyperoxia was greater in the Prdx6 null mice compared to WT, consistent with our previous observations (18, 32). The extent of lipid peroxidation at end-exposure in the Prdx6 null and mutant (C47S, D140A) mice was not significantly different.

After the 60 h exposure period, mice were allowed to recover in room air. Lipid peroxidation in lungs of WT mice as determined by all 3 assays showed gradual return of the parameters to normal pre-exposure levels (as shown in Table 1) by 20 h in room air, but no significant recovery was observed in Prdx6 mice (Fig. 7). Lungs of C47S Prdx6 mutant mice expressing only PLA_2 activity recovered by about 30% to 40% during the 20 h post-exposure period in room air (Fig. 7); lungs of D140A Prdx6 mutant mice expressing only peroxidase activity recovered by about 50% (Fig. 7). Thus, as with the cell and isolated lung experiments, Prdx6 had a critical role in protecting lung from hyperoxic injury by participating in the repair of peroxidized phospholipids. Also similar to the cell and isolated lung studies, the D140A mutant (expressing peroxidase but not PLA_2 activity) was slightly more efficient in the repair of lipid peroxidation than the C47S mutant (expressing PLA_2 but not peroxidase activity).

PLA_2 activity of recombinant Prdx6

Assay of lysed PMVEC and homogenized lungs demonstrated the presence of both peroxidase and PLA_2 activities of Prdx6 (Table 1). However, peroxidase activity was assayed at pH 7 (cytosolic pH) while PLA_2 activity was assayed at pH 4, as there is relatively little PLA_2 activity if assayed at cytosolic pH with non-oxidized phospholipid substrate and non-phosphorylated enzyme (20, 35). For its potential physiological role in "repair" of oxidized phospholipid in cells, PLA_2 activity would be required at pH 7 since the reaction presumably occurs at the interface of membrane and cytosol. Our previous studies have shown a strong correlation between binding of Prdx6 to lipids and subsequent enzymatic activity, i.e. Prdx6 binds to phospholipids at pH 4, and activity is present, but binds poorly at pH 7 and demonstrates relatively little PLA_2 activity (20). On the other hand, Prdx6 binds to oxidized lipids equally well at both pH 4 and 7 (20). Thus, PLA_2 activity at pH 7 was studied using liposomes that had been treated with oxidants, i.e. oxidized phospholipid, as the substrate. With this substrate, PLA_2 activity of Prdx6 at pH 7 was significantly increased, representing a 200-fold (recombinant protein) or 45-fold (lung) increase over the activity with the reduced substrate (Table 3).

DISCUSSION

Oxidative stress plays an important role in many disease processes so that understanding the mechanisms by which cells guard against oxidant injury and how they repair its effects is of crucial importance to the development of therapeutic strategies. Peroxidation of phospholipids, especially those with an unsaturated fatty acid in the *sn*-2 position, by reactive oxygen species is a major threat to cellular integrity. Thus, the repair of the peroxidized membrane phospholipids is essential to cell survival. Although many studies have evaluated oxidant stress in the lungs, relatively few have evaluated the mechanisms for cellular repair. The chain reaction of lipid peroxidation via free radical mechanisms can be quenched by radical based anti-oxidants such as the α-tocopherols, but this will not repair the lipid peroxides that have been generated prior to radical quenching. In this study, we have specifically investigated the role of Prdx6 in the repair process.

Peroxiredoxins are a ubiquitously distributed family of peroxidases that function in cellular redox reactions (8, 26). Prdx6, the only member of the peroxiredoxin family that contains a single conserved Cys, uniquely possesses PLA_2 activity in addition to its peroxidase activity (6, 8). Also uniquely, Prdx6 is able to reduce peroxidized phospholipids (12, 20). Previous studies by our group have shown that cytoplasmic Prdx6 can bind to peroxidized phospholipids in the cell membrane following oxidative stress and its activity catalyzes their reduction (20). This suggests an important protective function of Prdx6 in antioxidant defense of lung cells related to its ability to repair peroxidized cell membranes. Of note, glutathione peroxidase 4 (GPx4) also can reduce phospholipid hydroperoxides (PLOOH), and MCF-7 cells stably transfected with this protein and exposed to singlet O_2 showed removal of PLOOH that was proportional to the GPx4 expression level (29). However, this enzyme has little expression in the lung and its content in other tissues is variable (3).

In the present study, we utilized 3 distinct models of oxidant exposure and evaluated recovery following removal of the oxidant from the incubation medium (cells), perfusate (isolated lungs), or inspired gas (mice). These experimental models were: i) PMVECs from WT and Prdx6 null mice with expression of mutant Prdx6 in the null cells via lentivirus infection; ii) isolated perfused lungs from WT, Prdx null, and Prdx6 mutant ("knock in") mice; and iii) lungs isolated from intact WT, Prdx6 null, and "knock-in" mice following oxidant exposure and recovery *in situ*. The oxidants that were used were *t*-BOOH for the cells and isolated lungs and hyperoxia for the intact mice. As a first step, we evaluated the effect of *t*-BOOH on lipid peroxidation in PMVEC. The incubation of *t*-BOOH with PMVECs resulted in increased lipid peroxidation in a time and dose dependent manner in both WT and Prdx6 null cells with a significantly higher level of lipid peroxidation in the null cells (Fig. 1), compatible with our previous results indicating an anti-oxidant role for Prdx6 (18, 31–33). In order to compensate for the potential effect on the starting point for recovery, we used a higher concentration of *t*-BOOH for exposure of WT vs mutant cells. With removal of *t*-BOOH from the incubation medium, lipoxidation in Prdx6 null cells remained unchanged without any significant recovery over an 8 h period; however, the degree of lipid peroxidation in WT PMVEC had returned to control levels in about 2 h (Fig. 3). Similar results were obtained with isolated mouse lungs from WT and Prdx6 null mice exposed to *t*-BOOH with full recovery of lungs from WT mice in 2 h but essentially no

recovery in lungs from Prdx6 null mice (Fig. 6). The lungs from intact mice exposed to hyperoxia also showed a similar response although a much longer period (20 h instead of 2 h) was required for full recovery by the WT lungs (Fig. 7). We do not know the reason for the longer time to recovery with the hyperoxia model, but it could be related to on-going lung inflammation after removal from hyperoxia or the significantly longer duration of exposure required to generate lipid peroxidation. Nevertheless, results from the 3 different experimental oxidant stress models show a remarkably similar pattern of response, despite the differing scales for both degree of injury and time for recovery. The major finding is that there is insignificant recovery from lipid peroxidation in the absence of Prdx6. Previous results have shown that knock-out of Prdx6 has no significant effect on the expression of anti-oxidant enzymes other than Prdx6 (30, 32), providing support to the conclusion that the observed effects in these models are due to loss of Prdx6.

There are 2 major pathways that have been proposed for the repair of oxidized phospholipids (27). The first is the direct enzymatic reduction of the oxidized fatty acyl carbonyl group (equation 1). As demonstrated with Prdx6 null mice (18), Prdx6 is the only enzyme in lungs that has significant activity to reduce PLOOH, the major product of cell membrane lipoperoxidation. Prdx6 uses GSH as the physiological reductant to reduce PLOOH:

$$PLOOH + 2GSH \xrightarrow{\mathrm{Prdx6}} PLOH + GSSG + H_2O \quad (1)$$

PLOH is then further reduced to phospholipid. Because of its relatively slow reaction kinetics with hydroperoxides, GSH functions mainly as a co-factor rather than a direct reductant in the enzymatic reduction of H_2O_2 and other peroxides (7). The role for Prdx6 in this direct reduction pathway was evaluated in the present study using cells and lungs that express mutant D140A Prdx6; this mutant expresses only the peroxidase and not the PLA_2 activity of the protein. D140A Prdx6 restored about 60–70% of the “repair” function that was absent in Prdx6 null PMVEC and lungs. Thus, the direct reduction of oxidized phospholipids by Prdx6 peroxidase activity represents a major mechanism for cellular membrane repair following oxidative stress.

The second pathway proposed for cell membrane repair is the remodeling pathway, analogous to the remodeling pathway for phospholipid synthesis (28). Prdx6 possesses PLA_2 activity in addition to its peroxidase activity and, as shown in Table 3, this activity is present with oxidized substrate at cytosolic pH. The remodeling pathway requires the combined activity of PLA_2 to liberate the peroxidized fatty acid (FAOOH) (equation 2) followed by reacylation of the lysophospholipid (lysoPL) with a fatty acid by a fatty acyl-CoA transferase to regenerate the reduced phospholipid (PL) (equation 3):

$$PLOOH \xrightarrow{PLA_2} lysoPL + FAOOH \quad (2)$$

$$lysoPL + FA - CoA \xrightarrow{acyltransferase} PL \quad (3)$$

The released FAOOH can be reduced to free fatty acid or metabolized via fatty acid oxidation pathways.

To study the role of the PLA_2 activity of Prdx6 in membrane repair, mutant cells and mice were generated to express C47S Prdx6; this mutant expresses the PLA_2 but not the peroxidase activity of Prdx6. PMVECs and lungs expressing C47S Prdx6 regained their function of repairing lipid peroxidation although at a rate slightly less than that seen with the D140A mutant. Our previous studies have demonstrated that Prdx6 does not bind to membranes at cytosolic pH and the PLA_2 activity of Prdx6 is quiescent, so that normal cell membranes are not at risk of attack. With oxidized membranes, however, Prdx6 does bind (20) and shows greatly increased PLA_2 activity at cytosolic pH (Table 3) as required for it to function as a membrane repair enzyme. Thus, the present results indicate that both the peroxidase and PLA_2 activities of Prdx6 can play a critical role in the repair of peroxidized phospholipids in cell membranes and the recovery of lung cells from peroxidative stress.

In summary, this study has evaluated the pathways that lung cells utilize for repair of cell membranes following oxidative stress. Repair in PMVEC and lungs depends on the presence and activity of Prdx6. Thus, Prdx6 is key to the cell membrane repair process; activity of this bi-functional enzyme can result in repair of peroxidized membranes through both the direct reduction pathway via its peroxidase activity and the remodeling pathway though its PLA_2 activity. A direct reduction pathway has been estimated to be several orders of magnitude faster than a remodeling pathway in a model system (2), but this has not yet been evaluated experimentally. In the present study, direct reduction appeared to be slightly more efficient than a remodeling pathway in the repair of peroxidized phospholipids although either pathway was partially effective in the absence of the other. The role for each pathway under physiological conditions likely will depend on the relative kinetics for the 2 pathways in any particular cell.

Acknowledgments

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ABBREVIATIONS

***t*-BOOH**	*tert*-butyl hydroperoxide
BHT	butylated hydroxytoluene
DPPP	diphenyl-1-pyrenylphosphine
DPPP-Ox	DPPP oxide
FRT	FLP recombinase target sites
GPx4	Glutathione peroxidase 4
lysoPL	lysophospholipid
MeLOOH	methyl linoleate hydroperoxides

Prdx6	peroxiredoxin 6
PLA_2	Phospholipase A_2
PLOOH	phospholipid hydroperoxides such as PLPCOOH (palmitoyl, linoleoyl phosphatidylcholine hydroperoxide)
PMVECs	pulmonary microvascular endothelial cells
TBARS	thiobarbituric acid reactive substances

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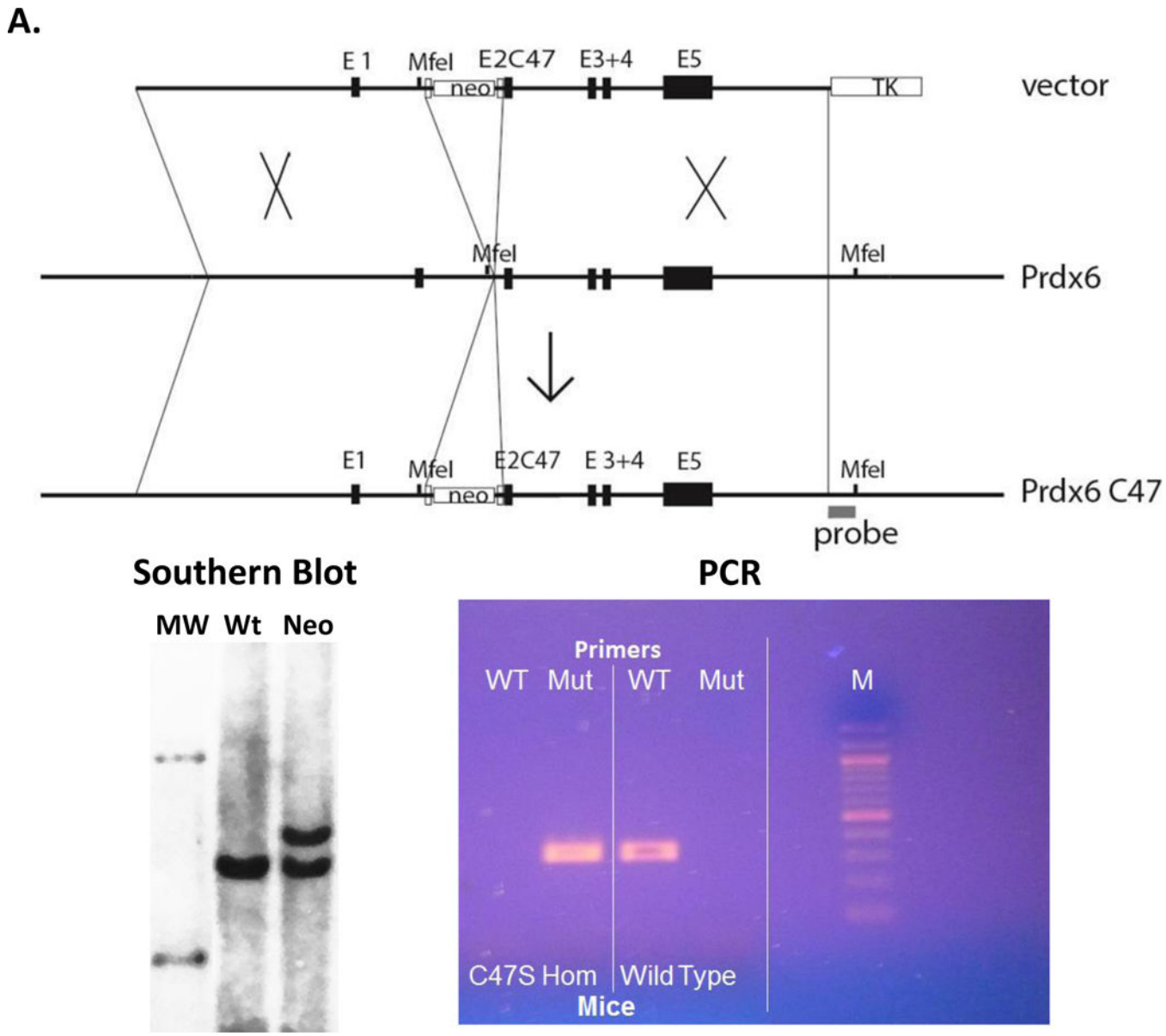
HIGHLIGHTS

- Peroxiredoxin 6 expresses both glutathione peroxidase and phospholipase A_2 activities.
- Deletion of peroxiredoxin 6 in pulmonary microvascular endothelial cells results in increased lipid peroxidation associated with peroxidative stress.
- Lipid peroxidation resolves gradually in wild type endothelial cells and lungs subjected to oxidative stress but is markedly delayed with deletion of peroxiredoxin 6.
- Both peroxidase and phospholipase activities of peroxiredoxin 6 participate in the resolution of lung lipid peroxidation.



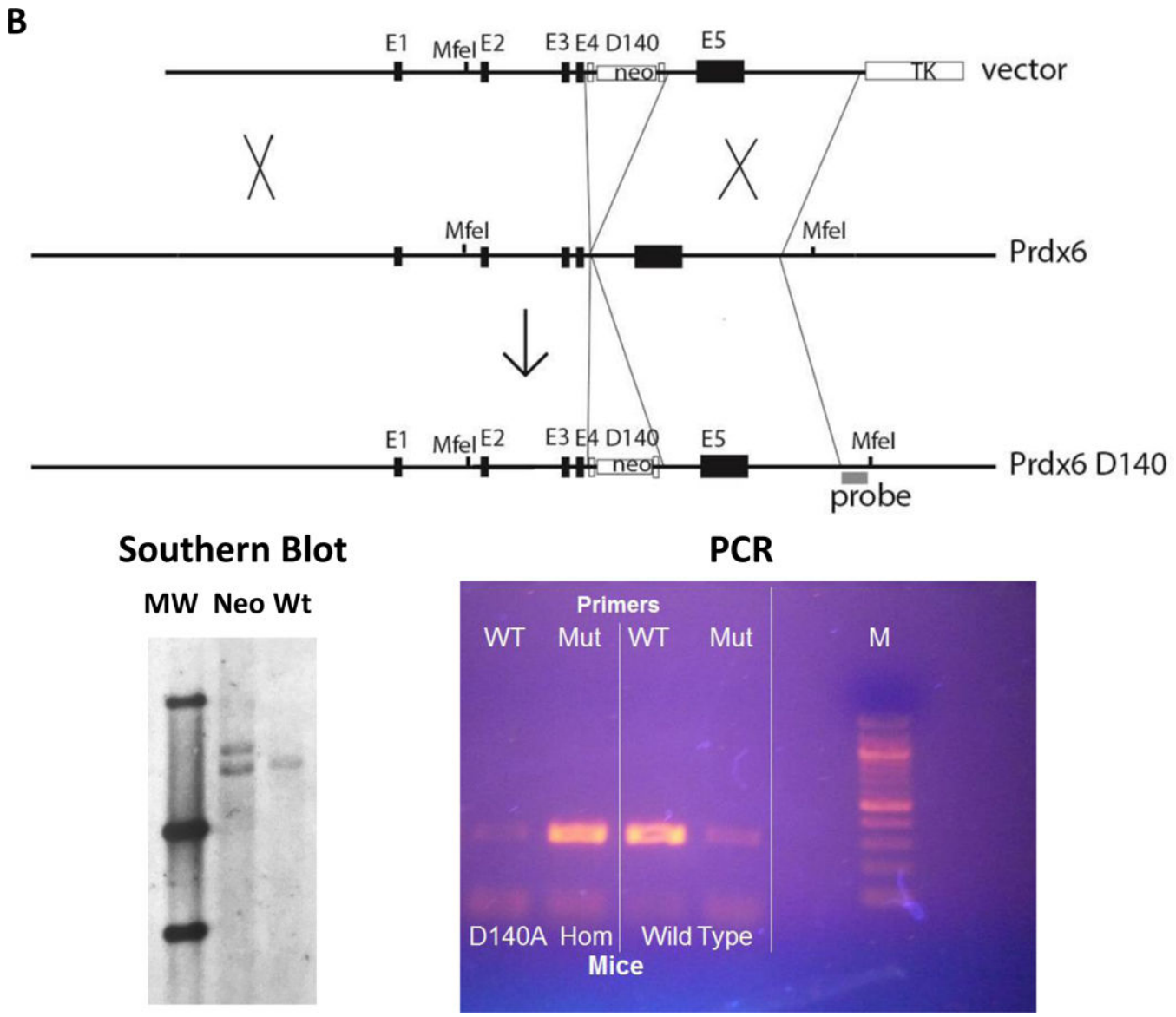
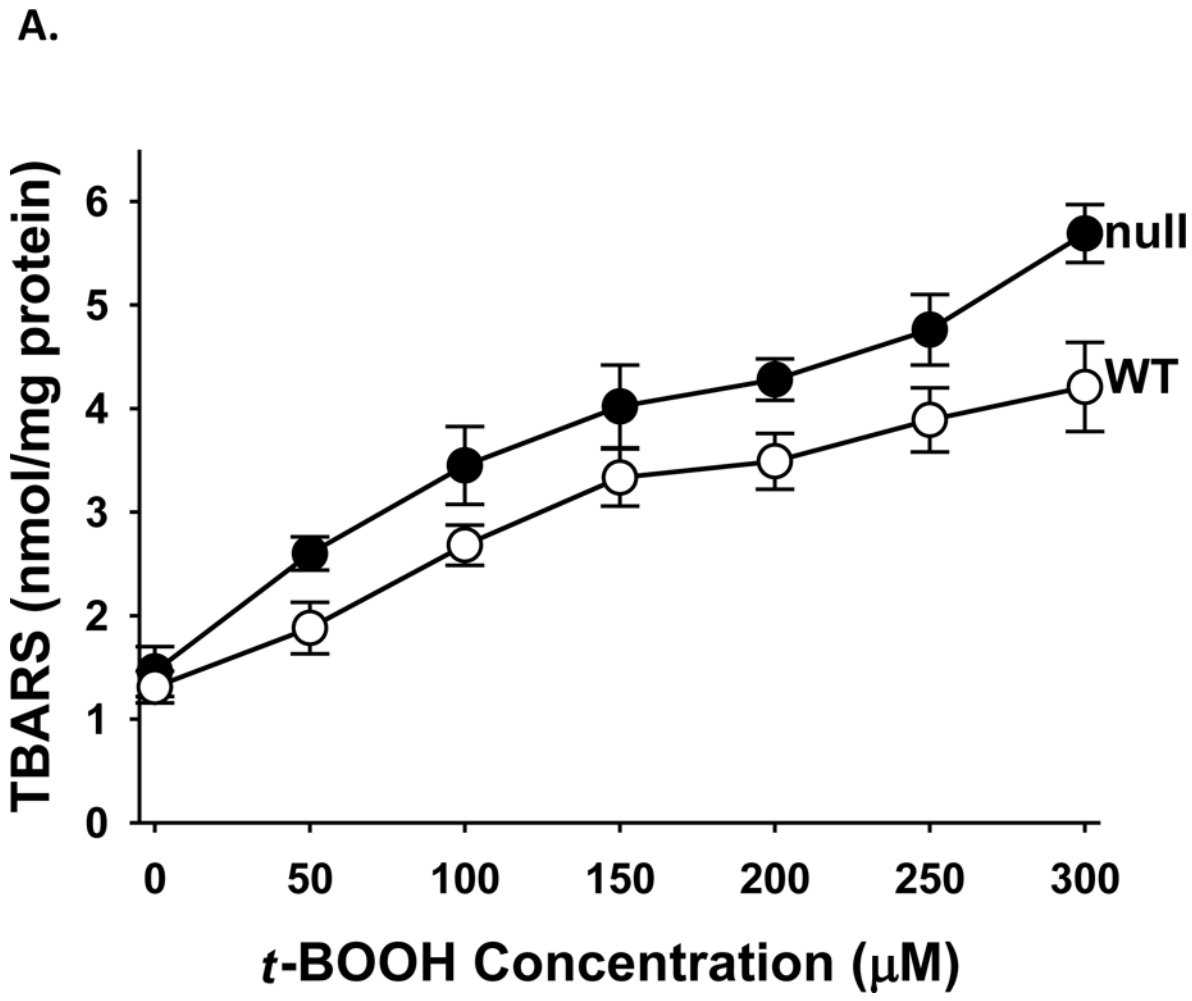


Fig. 1. Targeting constructs for generation of Prdx6 mutant mice

A). Prdx6 C47 mice: A targeting vector was constructed with an FRT flanked neo-cassette inserted upstream of exon 2 and electroporated into C57Bl/6 ES cells. Neo-positive ES clones were screened by Southern blotting using a probe located just downstream of the 3′ end of the vector. The probe was used for Southern blotting of genomic DNA cut with MfeI; positive ES clones yielded the expected 13.0 kB mutant band due to insertion of the neo-cassette. Mice were genotyped by using allele-specific PCR. The presence of a band with the specific primer indicates the presence of that allele. B). Prdx6 D140 mice: The targeting vector had the FRT neo-cassette from A) inserted just downstream of exon 4, but all other characteristics of the targeting scheme including the Southern strategy and PCR for genotyping of mice were essentially identical to A). Black boxes, exons; unlabeled white boxes, FRT sites; M, mol. mass marker; WT, wild type primer; Mut, mutant primer; Hom, homozygous.



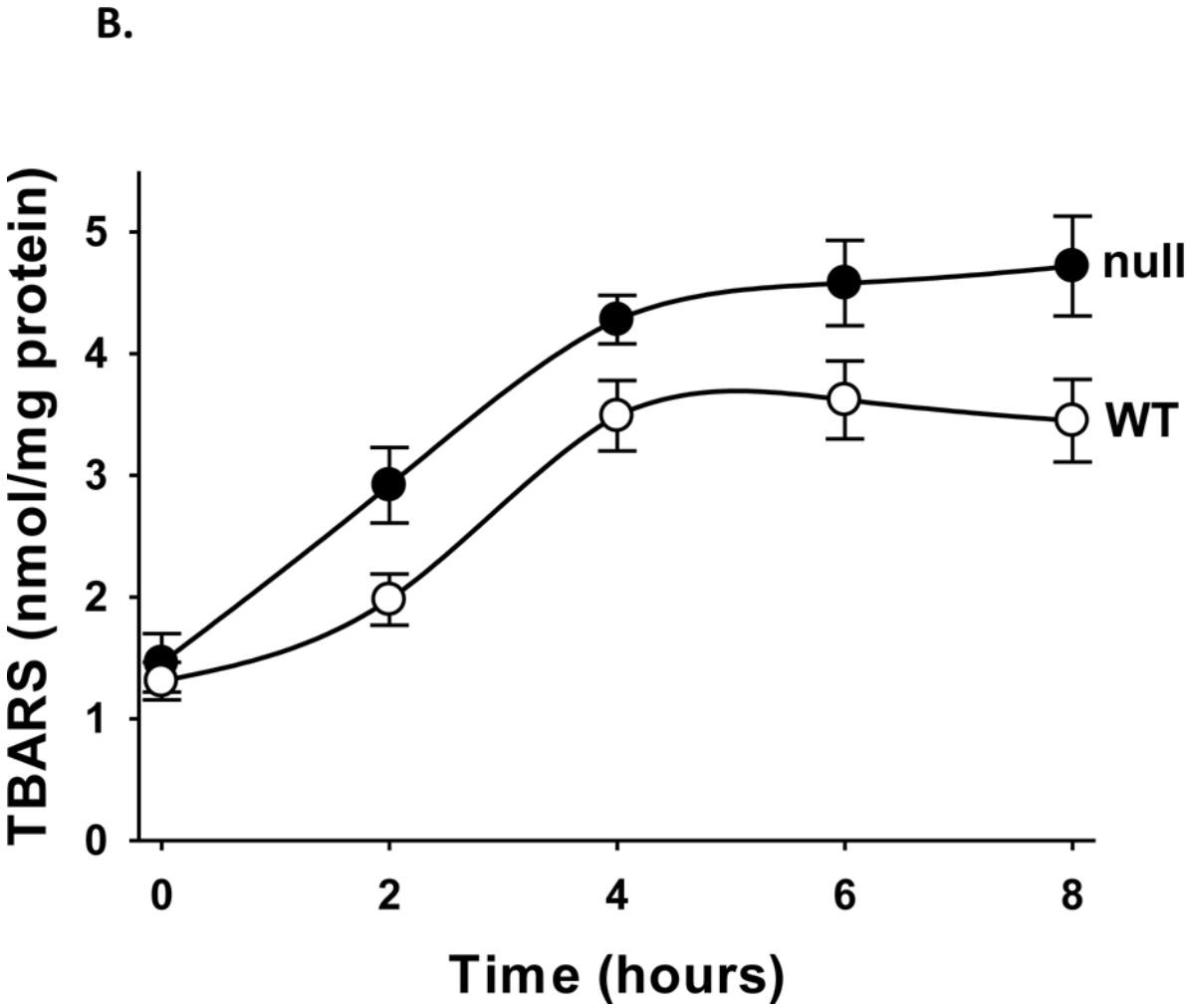
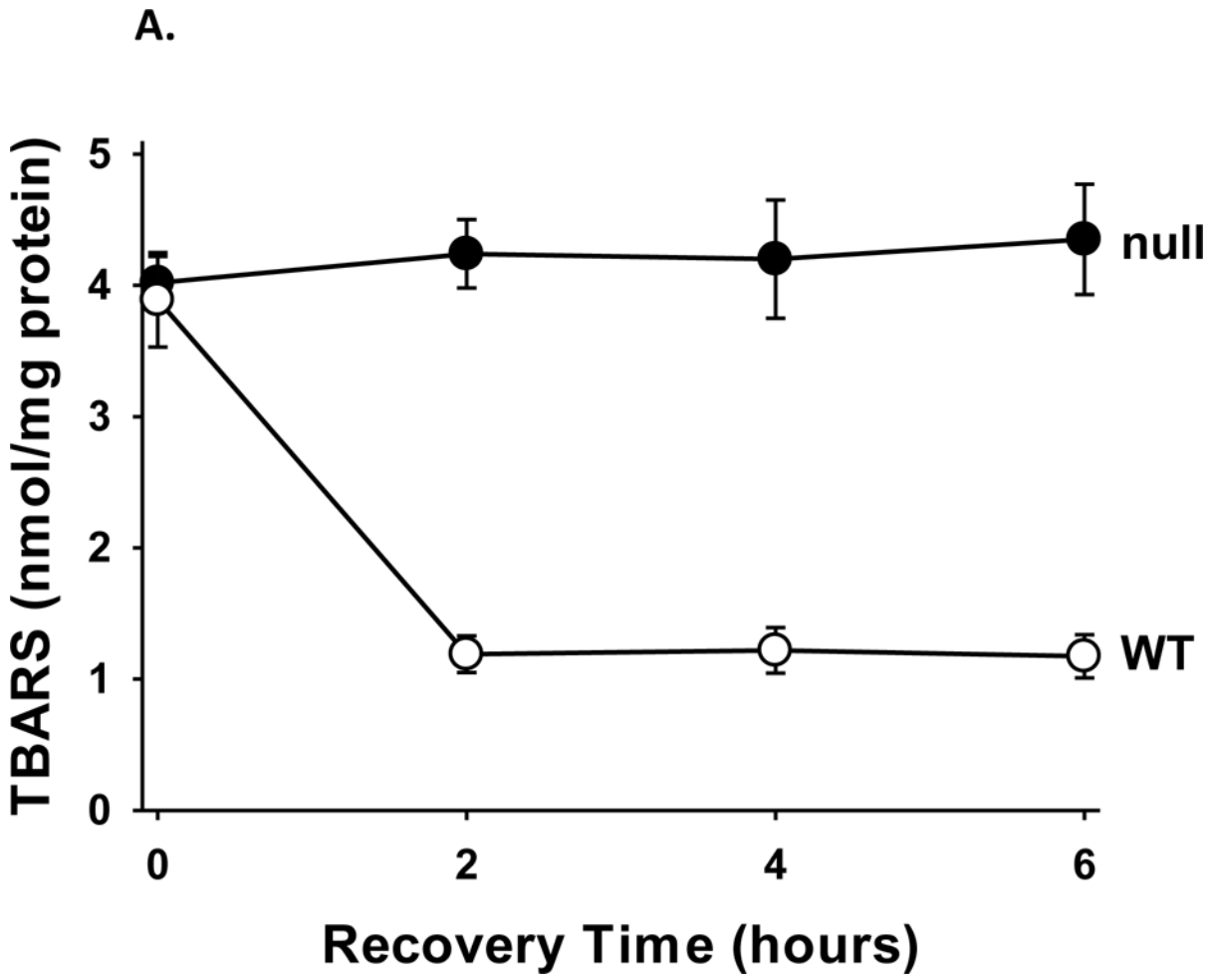


Fig. 2. Lipid oxidation with *t*-BOOH evaluated by TBARS measurement in wild type and Prdx6 null PMVEC

TBARS measurements were done immediately after exposure of cells to 0–300 μM *t*-BOOH for 4 h (A) or exposure to 200 μM *t*-BOOH for 0–8 h (B). Data are expressed as means ± SE (n=3). P<0.05 for all values for Prdx6 null vs WT except for 0 μM and 0 h.



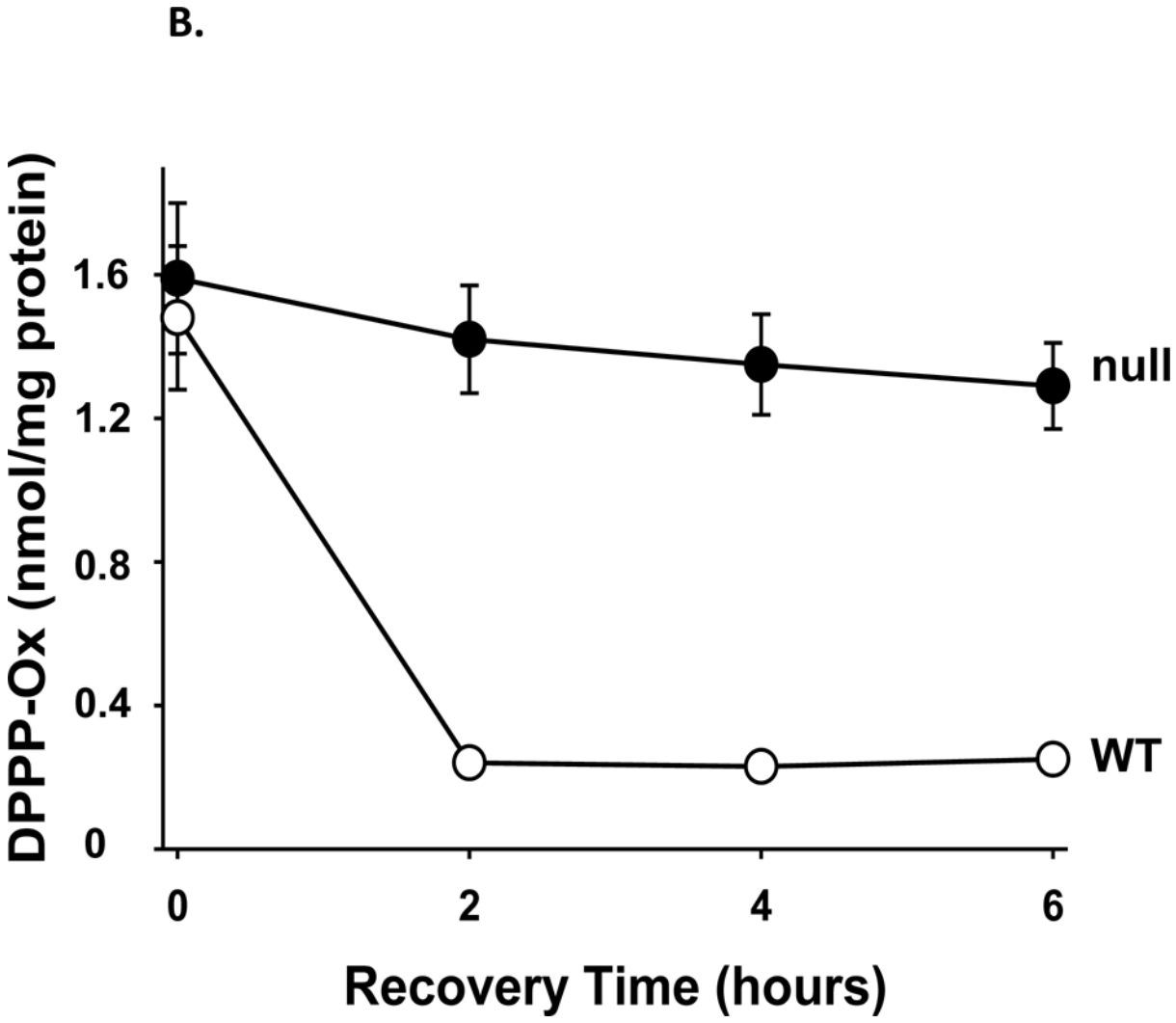


Fig. 3. Time dependence for recovery of WT and Prdx6 null PMVEC from lipid peroxidation as evaluated by TBARS (A) or DPPP-Ox (B) measurement

Cells were treated with 300 μM (WT) or 200 μM (null) *t*-BOOH for 4 h (shown as 0 time) and evaluated at intervals for recovery in medium free of *t*-BOOH. Different concentrations of t-BOOH were used for exposure in order to achieve approximately equal end-exposure lipid peroxidation values in the 2 types of cells. Data are expressed as means ± SE (n=3). $P<0.05$ for all values for Prdx6 vs WT except for 0 h.

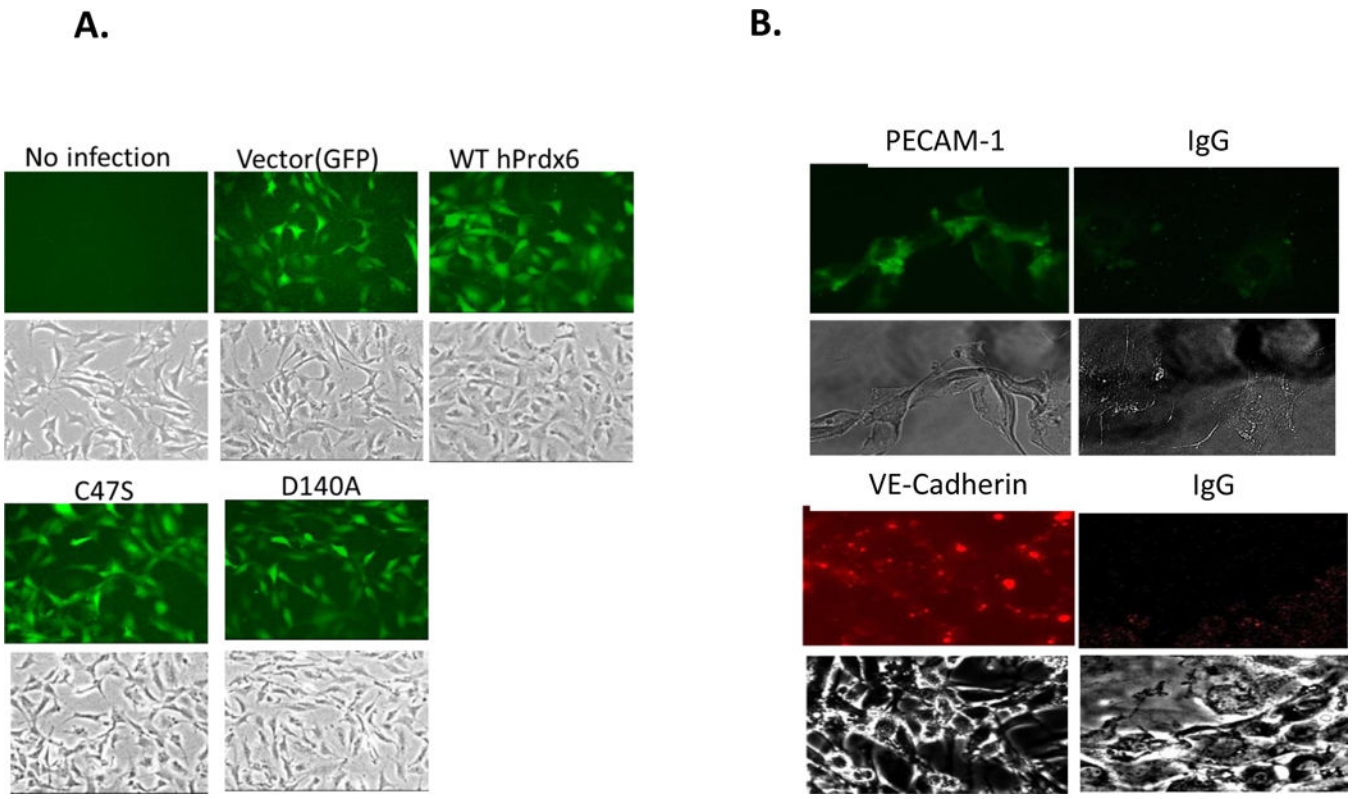


Fig. 4. Infection of Prdx6 null PMVEC with lentiviral vector constructs

A). The upper (green panels) show GFP fluorescence; the lower panels are the corresponding phase pictures. B. PMVEC: upper images, immunofluorescence using monoclonal antibody or non-immune IgG; lower images, phase contrast. PMVEC stain positively for the endothelial markers, PECAM-1 (green) and VE-cadherin (red).

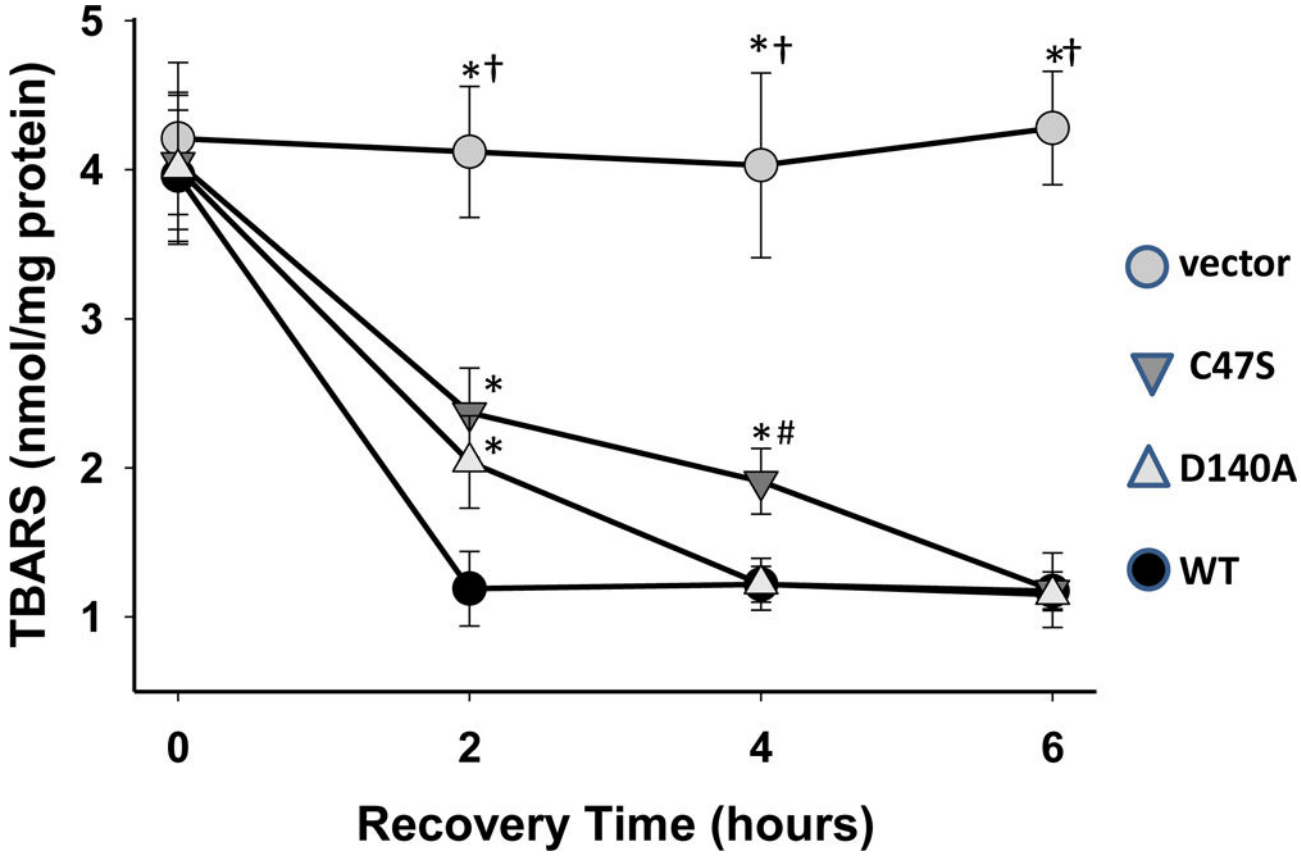
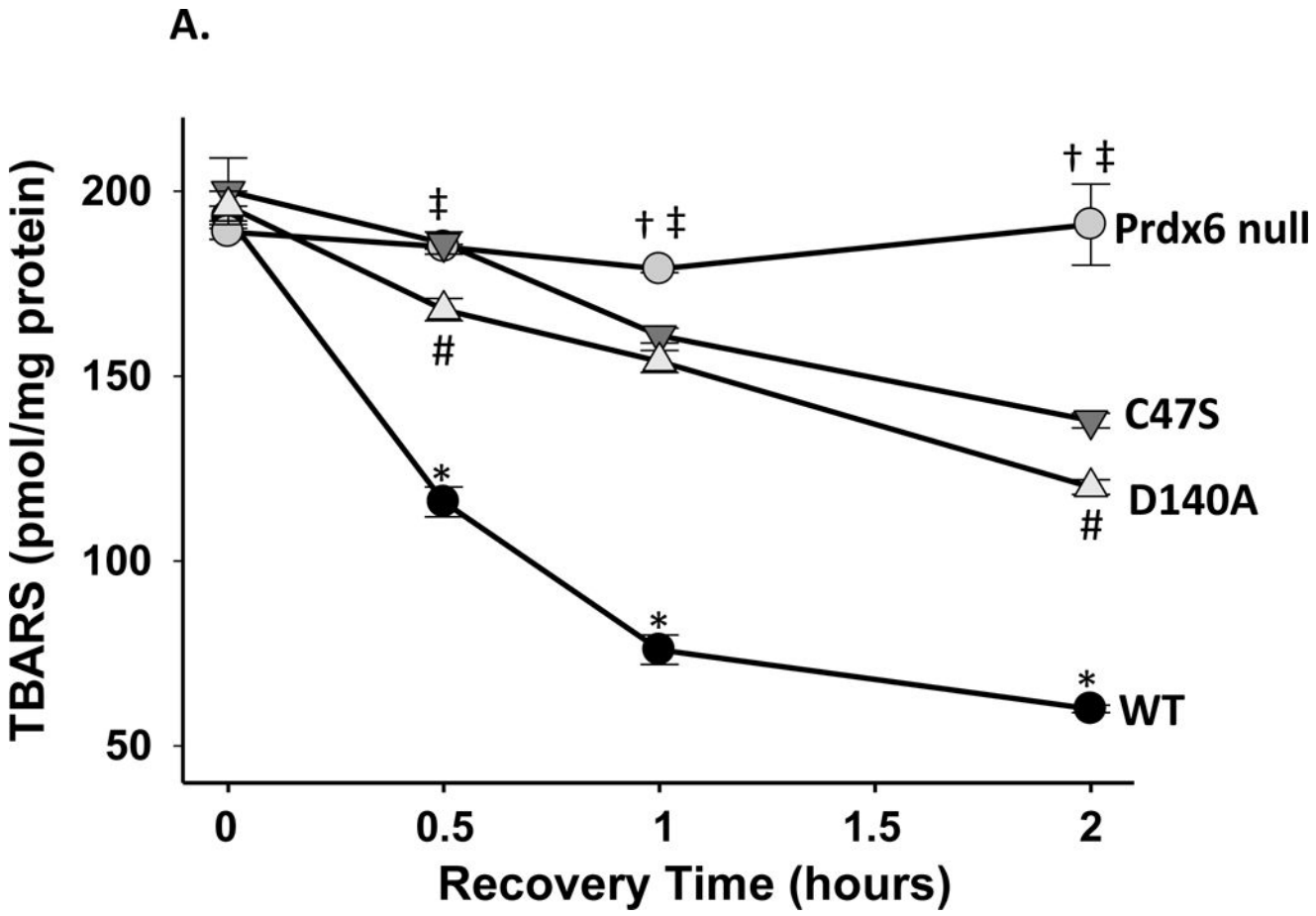


Fig. 5. Relative roles of peroxidase and PLA_2 activities of Prdx6 in the "repair" of peroxidized PMVEC membranes

Prdx6 null cells were infected with lentivirus to express either Prdx6 wild type, C47S Prdx6 (without peroxidase activity), or D140A Prdx6 (without PLA_2 activity). Control was infection with the HMD vector only. The protocol for exposure to t-BOOH and recovery was the same as described in Fig. 3. Lipid peroxides were measured by assay with DPPP. Data are expressed as means ± SE (n=3). *P<0.05 vs WT at the corresponding recovery time; †P<0.05 for null vs C47S or D140A; # P<0.05 for C47A vs D140A.



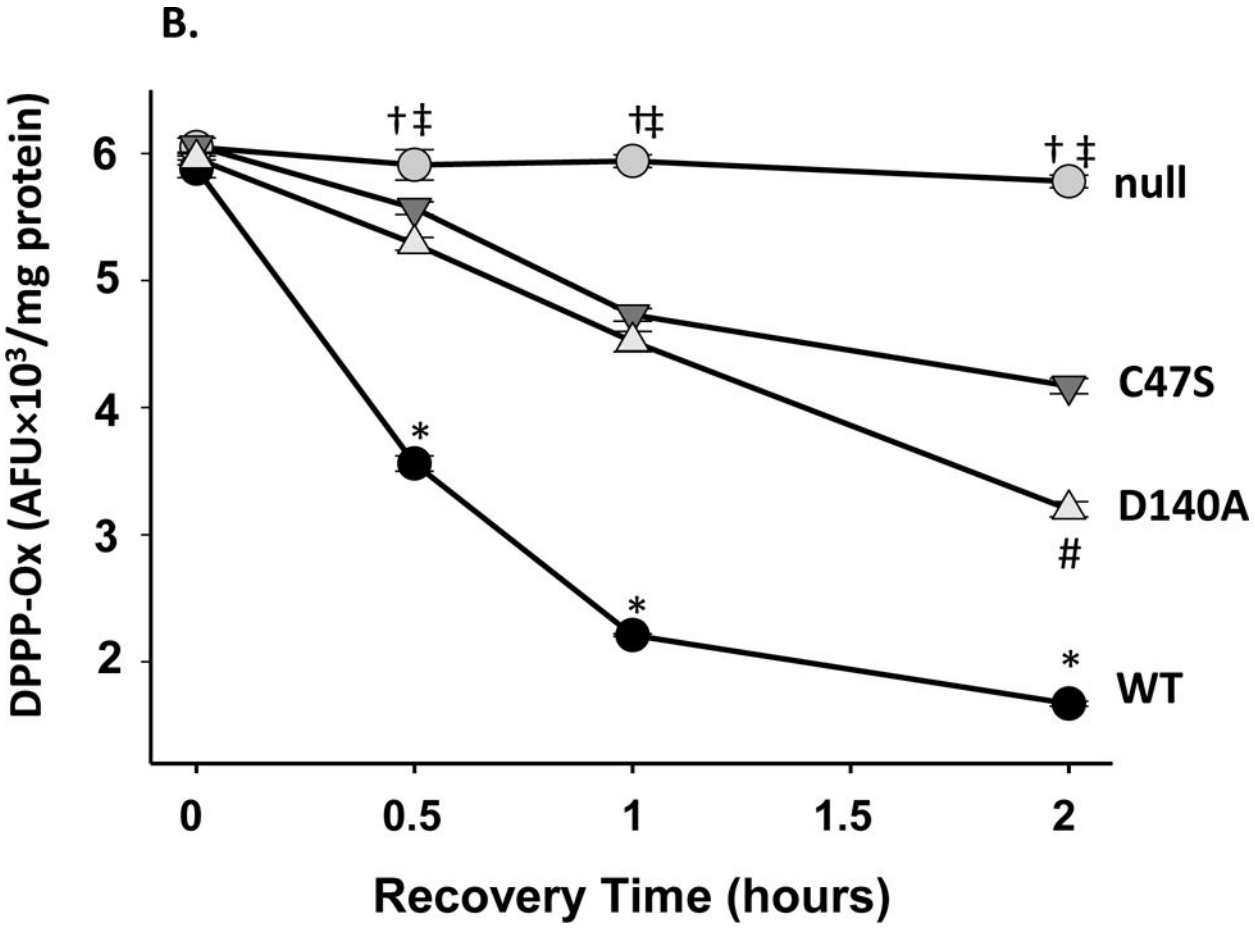
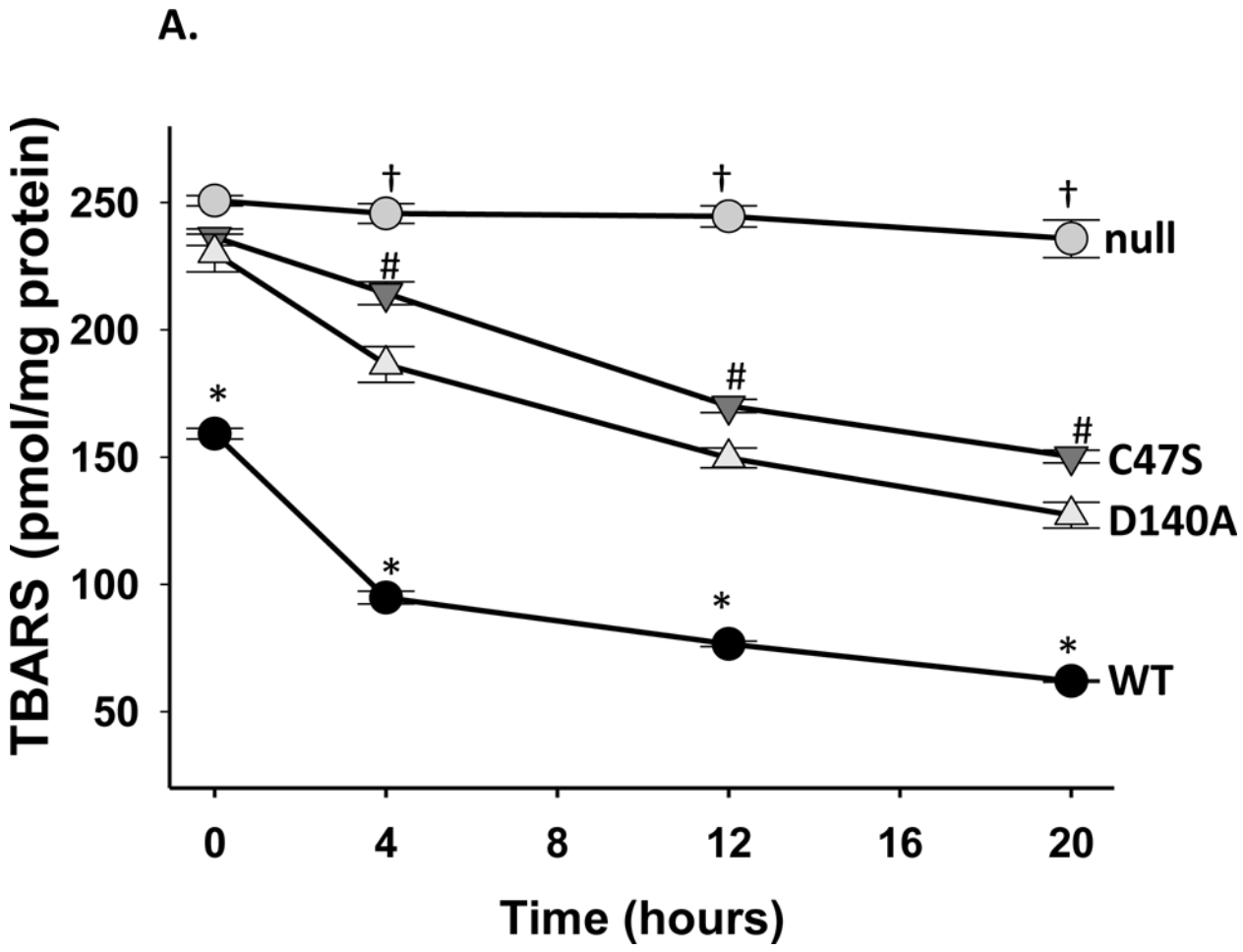
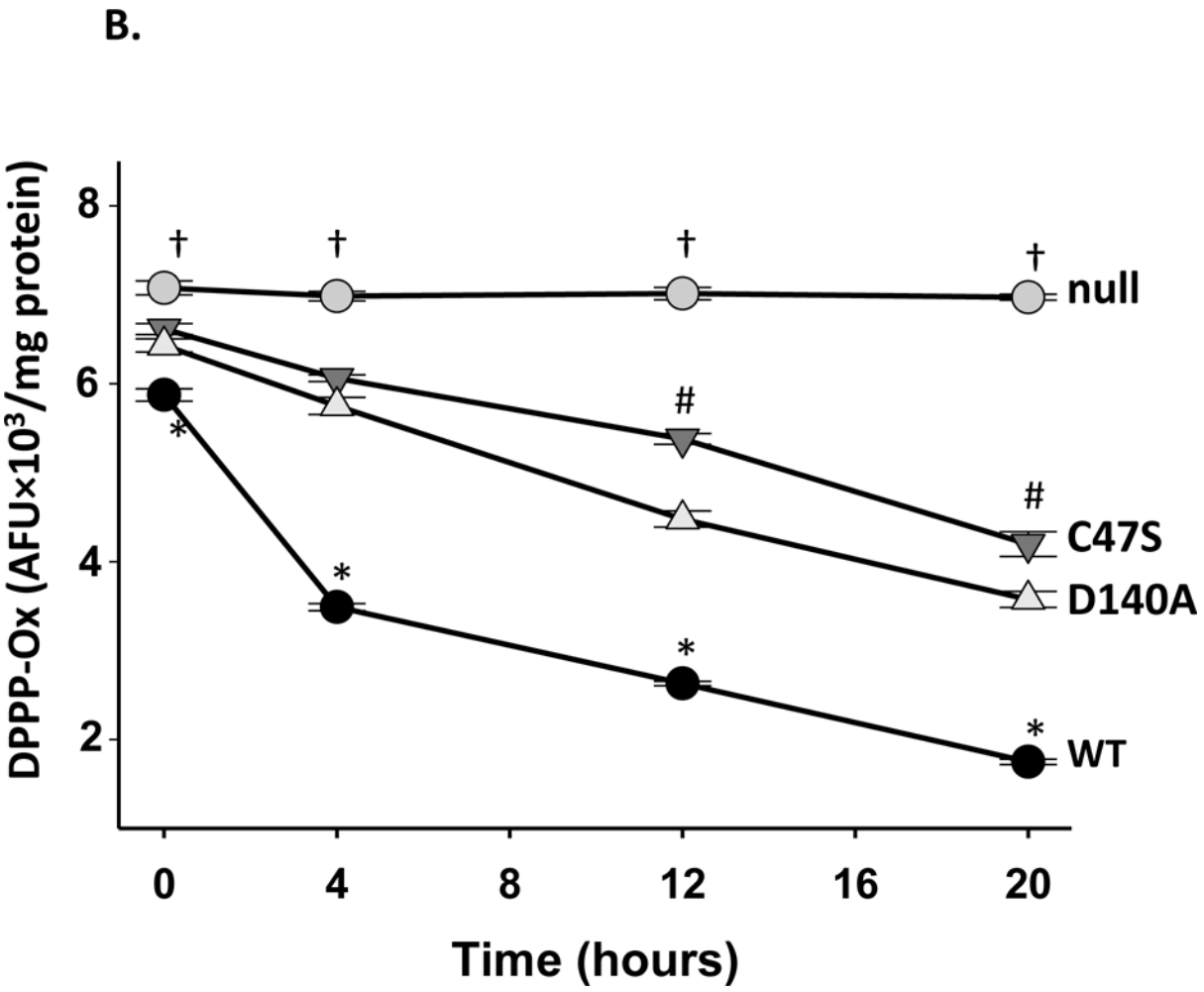


Fig. 6. Repair of lung lipid peroxidation during perfusion of isolated mouse lungs

Lungs were harvested from wild type, C47A mutant, D140A mutant, or Prdx6 null mice. Lungs were perfused for 60 min with medium containing 15 mM (mutants) or 25 mM *t*-BOOH (wild type) followed by 120 min perfusion with perfusate without *t*-BOOH. Lipid oxidation was evaluated by measurement of TBARS (A) or DPPP-Ox (B). Data are expressed as means ± SE (n=3). Recovery was significant ($P<0.05$) by WT, C47S, and D140A for both assays. *$P<0.05$ vs all other conditions at the corresponding time point; †$P<0.05$ for null vs C47S, ≠$P<0.05$ for null vs D140A, # $P<0.05$ for D140A vs. C47S.





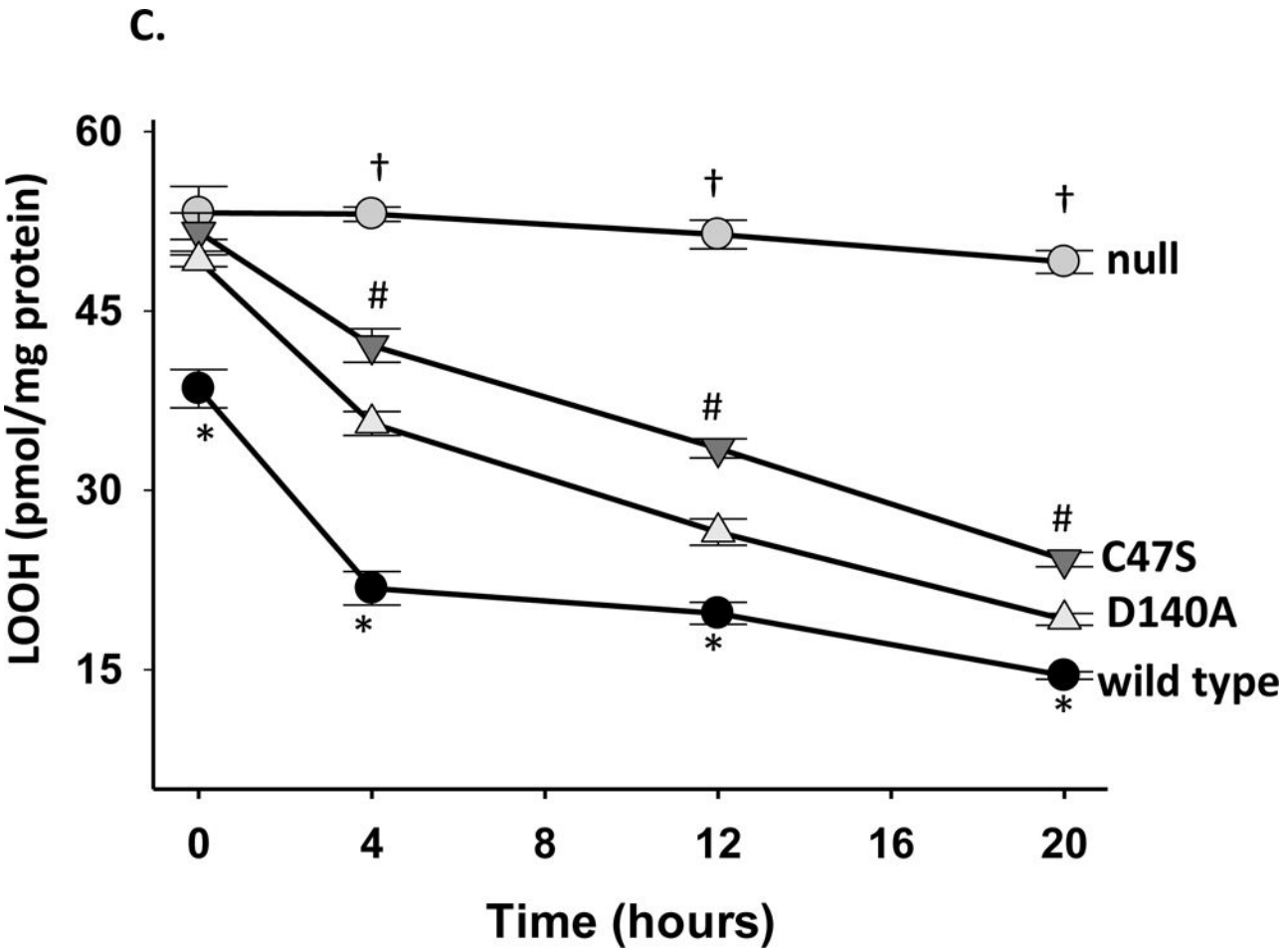


Fig. 7. Lipid peroxidation in lungs of mice exposed to hyperoxia and then returned to room air

Wild type (WT), Prdx6 null, and Prdx6 mutant (C47S, D140A) mice were exposed to 100% O_2 at 1 atmosphere for 60 h (0 time) and then allowed to recover in room air for an additional 20 h. Mice were sacrificed at intervals and lungs were removed and homogenized for lipid hydroperoxide assay. A). TBARS assay; B). DPPP-Ox measurement; C). FOX assay. Data are expressed as means ± SE (n=3). Recovery was significant ($P<0.05$) in WT, C47S, and D140A lungs for all 3 assays. *$P<0.05$ for WT vs all other conditions; † $P<0.05$ for null vs C47S or D140A; # $P<0.05$ for C47S vs D140A.

Table 1

Peroxidase and PLA_2 activities for Prdx6 mutant PMVEC and lungs

PMVEC null cells were infected with lentivirus incorporated with various p-GFP-Prdx6 genes; lungs were harvested from wild type (WT) and mutant mice. Nil, activity <0.5.

	PMVEC		LUNGS	
	PLA_2 nmol/h/mg prot	Peroxidase nmol/min/mg prot	PLA_2 nmol/h/mg prot	Peroxidase nmol/min/mg prot
Null*	nil†	1.0 ± 0.04†	nil†	nil†
Wild type	5.8 ± 0.3	12.2 ± 0.2	8.8±0.7	5.1±0.1
C47S	6.1 ± 0.2	1.1 ± 0.4†	8.8±0.3	nil†
D140A	nil†	10.1 ± 1.3	nil†	4.9±0.45

Values are mean ± SE for n= 3. PLA_2 activity was measured at pH 4 in Ca^{2+} - free buffer. Palmitoyl, linoleoyl-phosphatidylcholine hydroperoxide (PLPCOOH) was substrate for the peroxidase assay.

*Null PMVEC were infected with empty vector.

†P<0.05 vs. corresponding WT.

Table 2

Control values for indices of lipid peroxidation in PMVEC and lungs

Cells and lungs were evaluated under resting conditions without exposure to oxidants.

	PMVEC		LUNGS		
	TBARS	DPPP	TBARS	DPPP	FOX
	nmol/mg		pmol/mg	AFU/mg	pmol/mg
Wild Type	1.3 ± 0.2	1.2 ± 0.2	53 ± 2	1640 ± 14	14.6 ± 0.1
Prdx6 null	1.4 ± 0.2	1.2 ± 0.2	57 ± 2	1720 ± 16	14.4 ± 0.1
C47S Prdx6	1.3 ± 0.2	1.2 ± 0.2	63 ± 3	1750 ± 32	14.4 ± 0.1
D140A Prdx6	1.3 ± 0.2	1.2 ± 0.2	61 ± 3	1680 ± 7	13.9 ± 0.2

Results are expressed per mg cell or lung protein. Values are mean ± SE for n=3.

Table 3

PLA_2 activity of recombinant wild type Prdx6 and lysate of wild type lung homogenate at pH 4 and pH 7 with normal (reduced) and oxidized phospholipid substrate

	PLA_2 activity			
	Protein nmol/min/mg protein		**Lung nmol/h/mg protein**	
	pH 4	**pH 7**	**pH 4**	**pH 7**
Reduced phospholipid	80.4 ± 2.3	0.4 ± 0.03*	8.7 ± 0.1	0.25 ± 0.04*
Oxidized phospholipid	81.1 ± 1.0	80.1 ± 4.4	8.9 ± 0.1	8.8 ± 0.2

Values are mean ± SE for n=4 (Protein) or n=3 (Lung).

* $P<0.05$ vs corresponding value at pH 4.