



Published in final edited form as:

Arch Biochem Biophys. 2017 March 01; 617: 68–83. doi:10.1016/j.abb.2016.12.003.

Peroxiredoxin 6 in the repair of peroxidized cell membranes and cell signaling

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Abstract

Peroxiredoxin 6 represents a widely distributed group of peroxiredoxins that contain a single conserved cysteine in the protein monomer (1-cys Prdx). The cys when oxidized to the sulfenic form is reduced with glutathione (GSH) catalyzed by the π isoform of GSH-S-transferase. Three enzymatic activities of the protein have been described: 1) peroxidase with H_2O_2 , short chain hydroperoxides, and phospholipid hydroperoxides as substrates; 2) phospholipase A_2 (PLA $_2$); and 3) lysophosphatidylcholine acyl transferase (LPCAT). These activities have important physiological roles in antioxidant defense, turnover of cellular phospholipids, and the generation of superoxide anion via initiation of the signaling cascade for activation of NADPH oxidase (type 2). The ability of Prdx6 to reduce peroxidized cell membrane phospholipids (peroxidase activity) and also to replace the oxidized *sn*-2 fatty acyl group through hydrolysis/reacylation (PLA $_2$ and LPCAT activities) provides a complete system for the repair of peroxidized cell membranes.

Keywords

Phospholipid hydroperoxide glutathione; peroxidase; Phospholipase A_2 ; Lysophospholipid acyl transferase; NADPH oxidase; Phospholipid remodeling; Anti-oxidant defense

1. Introduction

Peroxiredoxin 6 (Prdx6) was the 6th and final mammalian protein to be recognized as a member of the peroxiredoxin (Prdx) family of non-seleno peroxidases. Because of prior use of the abbreviation *Prx* to identify the *paired-related* homeobox gene family [1], I have used *Prdx*, instead of *Prx*, as a unique identifier for the peroxiredoxin family of proteins. The protein was originally isolated from the ciliary body of the bovine eye and characterized as unique by its N-terminal amino acid sequence [2]. The protein was subsequently isolated from rat and bovine lung and rat olfactory mucosa [3–6]. Around the same time, search of the GenBank and EMBL databases identified the human Prdx6 gene [7,8] that had been generated by random cloning [9] and subsequently the rat and mouse genes were described [6,10,11]. Several names had been attached to the identical protein prior to agreement to designate the family as peroxiredoxins [12]; these former names include non-selenium GSH peroxidase [2,13,14], acidic (or lysosomal) Ca^{2+} -independent PLA $_2$ (aiPLA $_2$) [4,6,8], antioxidant protein 2 (AOP2) [10,15], Clara cell protein 26 (CC26) [16], and p29 [17]. The corresponding gene has been called ORF6 [9], LTW4 [18], and keratinocyte growth factor (KGF)-regulated gene 1 [14].

Prdx6 is a 25 kDa protein formed by 224 amino acids (Fig. 1). Proteins that are homologous to mammalian Prdx6 are widely distributed throughout all kingdoms and have been described in archaea, bacteria, yeast, plants, insects, mollusks, amphibians, and birds [7,19–22]. Prdx6 is sometimes called 1-cys Prdx, reflecting the catalytic mechanism that is based on a single conserved cysteine residue in the Prdx monomer as contrasted with 2-cys Prdx enzymes that function with 2 conserved cys moieties per monomer. However, this nomenclature has anomalies, primarily related to more primitive organisms, since a 1-cys catalytic mechanism occurs in some non-Prdx6 members of the Prdx family while a 2-cys mechanism can be seen in members that are structurally similar to Prdx6 [23–25]. Based on protein structural features, a more recent classification of peroxiredoxins from all phyla identifies 6 subfamilies: Prx1 (Prdx1-4 in the other nomenclature); Prx5 (Prdx5); Prx6 (Prdx6); and 3 subfamilies present only in bacteria and other lower forms (AhpE, PrxQ, and Tpx) [25]. This review of Prdx6 will focus on mammalian enzymes using the older 1-cys and 2-cys terminology. Several reviews of Prdx6 have been published previously [15,19,26–28].

2. Enzymatic activities of prdx6

2.1. Peroxidase activity

H₂O₂, short chain hydroperoxides such as *t*-butyl and cumene hydroperoxides, and peroxyxynitrite (ONOO⁻) are substrates for all of the Prdx enzymes including Prdx6 [22,29,30]. The catalytic cycle for reduction of hydroperoxides by peroxiredoxins involves 3 main chemical steps: peroxidation, resolution, and recycling [31]. The first step (peroxidation) reduces the hydroperoxide substrate resulting in oxidation of the reactive cys moiety to a sulfenic acid; the resolution step results in reduction of the sulfenic cys; and the recycling step leads to regeneration of the reduced cys sulfhydryl, i.e., the active enzyme. While the initial peroxidation step appears to be similar for all peroxiredoxins, the subsequent resolution and recycling steps vary between the 2-cys and 1-cys peroxiredoxins.

2.1.1. 2-cys vs 1-cys enzymes—As described above, the Prdx family members have been classified as either 2-cys (Prdx1-5) or 1-cys peroxiredoxins (Prdx6) based on the number of conserved cys per monomer. Prdx1-4 function as dimers in solution and use the N-terminal cys (peroxidatic cys) of one monomer to reduce hydroperoxides; the resultant oxidized (sulfenic, -SOH) cys interacts with the C-terminal cys of the other monomer (the resolving cys) to generate a protein di-sulfide. Prdx5 is classified as an atypical 2-cys Prdx with both the peroxidatic and the resolving cys on the same monomer forming an internal disulfide during the reaction cycle. In either case (typical or atypical), the resultant disulfide is reduced by thioredoxin [22].

2.1.2. Prdx6 peroxidatic mechanism—Similar to the mechanism for the 2-cys Prdx enzymes, reduction of hydroperoxides by 1-cys Prdx occurs through oxidation of its single conserved cys (located at amino acid 47 in Prdx6) [32]. The measured rates for reduction of H₂O₂ by Prdx6 are ~6 μmol/min/mg protein with K_m 180 μM and rate constant (k₁) ~3 × 10⁷ M⁻¹s⁻¹ [2,30,33,34]. The rate constant for reduction of peroxyxynitrite is about 2 orders of magnitude lower [34]. The reaction of thiol with hydroperoxide is rapid so that the

resolution phase rather than the reduction reaction is rate limiting for the peroxidatic function of the enzyme. The pKa of the reactive thiol in Prdx6 has been measured as 5.2 [31,34], similar to values for the 2-cys enzymes, so that the major fraction of Prdx6 in the cytoplasm (pH ~7) would be in the thiolate form ($-S^-$) that is more reactive than the thiol ($-SH$).

2.1.3. Resolution—Resolution of the oxidized 2-cys enzymes is accomplished through formation of a disulfide by a relatively rapid reaction of the sulfenic with the resolving cys of the Prdx dimer. Unlike the 2-cys Prdx enzymes, Prdx6 does not have a second (resolving) cys, so the resolution of the sulfenic state requires interaction of the protein with an extraneous reductant. The physiological reductant that has been described for the mammalian enzyme is glutathione (GSH). GSH forms a mixed disulfide with oxidized Prdx6 (glutathionylation of Prdx6), analogous to the disulfide formed by interaction of the catalytic and resolving cys residues in the 2-cys enzymes [35–37]. (Protein glutathionylation also is seen in some 2-cys Prdx enzymes, but its role there is not related to their peroxidase activity [38]). Although Prdx6 functionally is a GSH peroxidase, it is classified as a peroxiredoxin based on the role of cys instead of selenocysteine as the redox-active group and its structural homology with other members of the peroxiredoxin family.

While the role of GSH in the reduction of oxidized 1-cys Prdx6 is now accepted, early studies were inconsistent and there were several negative reports [5,32,39]. It was subsequently found that the formation of the disulfide through reaction of the sulfenic 1-cys Prdx with GSH could be catalyzed by the π isoform of the enzyme GSH S-transferase (π GST) [36,37]. Thus, sulfenic Prdx6 can heterodimerize with GSH-loaded π GST followed by glutathionylation of the oxidized Prdx6 monomer [37,40]. This interaction is non-covalent. Prolonged interaction of Prdx6 with π GST can result in formation of a covalent disulfide bond between monomers [37] but, based on kinetic considerations, that is unlikely to be part of the normal reaction cycle. Confirmation of a role for π GST in the reduction of oxidized Prdx6 in intact cells was evaluated with the malignant MCF7 cell line that does not express π GST (or Prdx6). (Note that other studies have demonstrated the presence of some Prdx6 in MCF7 cells [41]; this expression may be strain-dependent). Intracellular delivery of π GST plus Prdx6 resulted in peroxidase activity while delivery of Prdx6 alone was ineffective [36]. The reversible heterodimerization of oxidized Prdx6 with π GST has been demonstrated *in vivo* by proximity ligation assay with fluorescence imaging of intact endothelial cells [42].

Although it is now clear that π GST can catalyse the reaction of GSH with oxidized Prdx6, several early studies demonstrated Prdx6 peroxidase activity in the presence of GSH alone. Those studies showing reduction by GSH that used native Prdx6 after isolation from various organs [2,4,5] may have inadvertently included π GST as a co-isolate with Prdx6, as shown with extraction of bovine lung [36]. However, our initial studies using *recombinant* Prdx6 from a single preparation showed effective resolution of the oxidized state by GSH in the absence of added π GST with activities in the range of 2–5.5 $\mu\text{mol}/\text{min}/\text{mg}$ protein and a rate constant (k_2) $>10^6 \text{ M}^{-1}\text{s}^{-1}$ [13,30], not very different from subsequent assays with GSH plus π GST. The catalytic cys is contained within a narrow pocket that presumably does not permit access of GSH (in the absence of π GST) as needed for Prdx6 reduction [43]. The

ability of GSH alone to reduce the oxidized cys in this one [6] preparation of Prdx6 [13,30] may have reflected a tertiary structural conformation that allowed access of GSH to the catalytic “pocket”.

2.1.4. Recycling—The final step (recycling) in the reaction pathway is to regenerate the active protein. For this, glutathionylated Prdx6 reacts with GSH to return to the fully reduced state (Figs. 2 and 3) [36,37]. This step appears to utilize free GSH as the reductant [36], although a possible role for π GST (or glutaredoxin) in mediating the interaction of the glutathionylated intermediate with GSH has not been excluded. Both oxidized GSH (GSSG) formed by reduction of 1-cys Prdx and oxidized thioredoxin formed by reduction of 2-cys Prdx are themselves reduced by NADPH-dependent enzymes (GSH and thioredoxin reductases, respectively). Thus, the reaction cycles for both the 1- and 2-cys Prdx enzymes comprise oxidation of the cys thiol to the sulfenic form followed by disulfide formation and then reduction to the “active” sulfhydryl with reducing equivalents ultimately derived from NADPH.

2.1.5. Structure-function relationships—The motif surrounding the peroxidatic C47 in human, mouse, rat, and bovine Prdx6, ⁴³TPVCTTE⁵⁰ [28,30], and the corresponding peroxidase motif in 2-cys Prdxs, TFVCPTE [22], are largely homologous. Based on the tertiary structure of oxidized Prdx6, it was proposed that the adjacent H39 and R132 stabilize the C47 thiolate in its active form [43] but further study has shown a more complex relationship between the active site and adjacent amino acids. Peroxidatic activity for the 2-cysPrdx enzymes requires a triad (Pro,Thr,Arg) surrounding the catalytic cys [31]. The catalytic ⁴⁷C in Prdx6 is surrounded by an identical triad (⁴⁰P, ⁴⁴T, ¹³²R); the Pro, Thr, and peroxidatic cys (C_p) are found in a contiguous sequence (PXXXTXXC_p) in Prdx6 as in the 2-cys enzymes [31]. The triad appears to play an important role in the activation of the peroxide substrate. Specific functions for the individual amino acids have been postulated: 1) the Pro shields the thiolate from unwanted reactions and positions the Thr and Arg for hydrogen bonding; 2) the Arg positions and activates the thiolate; and 3) the Thr and Arg position and activate one oxygen atom (the electrophilic center) of the peroxide substrate [31]. The relationship of the peroxidase motif to other important motifs in the Prdx6 amino acid sequence is shown in Fig. 1.

2.1.6. Prdx6 as a dimer—Although the catalytic cycle for Prdx6 does not involve an interaction of two monomers as with the typical 2-cys enzymes, the native (reduced) protein nonetheless is dimeric in crystal structure as well as in solution with the monomers arranged in an antiparallel state [35,44,45]. Dimerization is through hydrogen bonding involving the hydrophobic interface between the seven β -strands of each monomer, resulting in an extended 10-stranded β -sheet; the hydrophobic residues Leu145, Ile147, Leu148, and Pro150 help to stabilize the interface [35,43,44]. This configuration has been designated as a B-type dimer [25]. The crystal structure of Prdx6 shows the “thioredoxin fold” characteristic of peroxiredoxins and an extended C-terminus relative to most of the other Prdxs [43,46]. The formation of higher oligomers (e.g., decamers) of Prdx6 has been suggested [23], but the required experimental conditions and their possible physiological significance are unclear. The dimer affinity of Prdx6 ($K_d < 20$ nM) decreases upon its oxidation, providing

oxidized monomer for heterodimerization with GSH-loaded π GST [35–37]. The heterodimer of Prdx6 and π GST forms only in the presence of GSH and removal of the thiol results in dissociation of the complex [37]. Interestingly, while the oxidized protein is largely monomeric in solution [35], it crystallized as a dimer rather than a monomer [43], possibly reflecting the use of conditions, such as high concentrations of protein, that favor the dimeric form.

2.1.7. π GST:GSH and other reductants—Reduction of oxidized Prdx6 as studied with recombinant enzyme is through glutathionylation mediated by π GST. Like Prdx6, π GST also exists in solution as a mixture of homodimers and monomers in equilibrium allowing heterodimerization of its monomer with an oxidized Prdx6 monomer [40]. Heterodimerization of oxidized Prdx6 with π GST utilizes the same hydrophobic interface as homodimerization of reduced Prdx6; this interface is centered on ¹⁴⁵L-P¹⁵⁰ of Prdx6 (Fig. 1), so that hetero-dimerization essentially represents a “swap” of a Prdx6 monomer for a π GST monomer [35,37]. The rate of heterodimerization has not been measured but presumably is rapid based on the overall k of $>10^6 \text{ M}^{-1}\text{s}^{-1}$ cat for the coupled assay (see below). Polymorphisms associated with the π GST gene have been shown to influence the affinity of the protein for interaction with Prdx6 and presumably the rate constant for binding; π GST1-1A and C subtypes ($K_d \sim 50 \text{ nM}$) have greater affinity for binding to Prdx6 as compared to π GST1-1B and D ($K_d \sim 100 \text{ nM}$) [41].

GSTs are a large family of proteins and 13 different cytosolic as well as additional microsomal and mitochondrial isoforms have been identified in vertebrates [47]. Additional classes of GSTs have been described in non-vertebrates and plants, although documentation is still incomplete [48]. π GST is a widely distributed isoform of the GST family that is expressed in most mammalian organs and at especially high levels in lungs, heart, and brain [49]. The distribution of GSTs within organs can vary with cell type; for example, in the liver, hepatocytes express predominantly α GST while biliary tract cells express predominantly the π isoform [49]. Thus, hepatocytes that express Prdx6 at a relatively high level [50] have little to no expression of π GST. The mechanism for resolution of oxidized Prdx6 in the absence of π GST in these cells has not been adequately evaluated. Although preliminary studies from our laboratory have shown that the μ isoform of GST may catalyze the glutathionylation of Prdx6 (unpublished results), the possible role of this and other GST isoforms in resolution of oxidized 1-cys Prdx in cells that do not express π GST will require further study.

Other potential physiological reductants for Prdx6 have been proposed. Ascorbic acid was shown to reduce yeast mitochondrial 1-cys Prdx [51]; however, subsequent studies with the same protein have shown glutathionylation of the yeast enzyme (in the absence of π GST) rather than ascorbate-mediated reduction as the primary mechanism for resolution of the oxidized state [52,53]. Evaluation of this latter mechanism could provide insights into alternate means for glutathionylation of Prdx6 in mammalian systems that do not express π GST. Oxidized mammalian Prdx6 also can be reduced by ascorbate *in vitro* [51]; however, the rate constant for this reaction appears to be at least an order of magnitude less than that for GSH: π GST, suggesting that ascorbate is not the primary physiological reductant in mammals. Nonetheless, ascorbate-mediated reduction could be a pathway for the reduction

of oxidized Prdx6 in the absence of GST or under special circumstances, e.g., depletion of GSH, and may be the primary reductant for 1-cys Prdx enzymes in plants [54]. Finally, cyclophilin A has been shown to bind to and to reduce Prdx6 *in vitro* [55] but this reaction is not specific and its possible physiological role as a reductant for oxidized Prdx6 has not been demonstrated.

2.1.8. Phospholipid hydroperoxide GSH peroxidase activity—Uniquely among the Prdxs, Prdx6 also is able to reduce phospholipid hydroperoxides (PLOOH); the activity responsible for PLOOH reduction has been called phospholipid hydroperoxide GSH peroxidase (PHGPx). Phosphatidylcholine hydroperoxide (PCOOH) has been the primary substrate used to measure PHGPx activity of Prdx6. The rate of reduction of PCOOH by recombinant Prdx6 is $\sim 6 \mu\text{mol}/\text{min}/\text{mg}$ protein with K_m 120–150 μM ; the rate constant for reduction (k_1) is $\sim 10^7 \text{ M}^{-1} \text{ s}^{-1}$ with k_2 (k_{cat}) $2\text{--}5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ [13,36,37]. The reaction rate was similar for triton-dispersed PCOOH and oxidized phospholipids in liposomes (mol fraction: 0.5 dipalmitoyl phosphatidylcholine; 0.25 palmitoyl, linoleoyl phosphatidylcholine; 0.15 cholesterol; 0.10 phosphatidylglycerol) [33]. These reaction rates and kinetic constants for PCOOH are similar to those for H_2O_2 (see above).

The only protein besides Prdx6 for which significant PHGPx activity has been demonstrated is GSH peroxidase 4 (GPx4) [56,57] and none of the 2-cys Prdx enzymes have been shown to express this activity. (In this review, I have used PHGPx to describe the activity potentially shared by other enzymes and GPx4 as the name of a specific protein.) The reported activity for GPx4 in the reduction of peroxidized phospholipids is 20–30 $\mu\text{mol}/\text{min}/\text{mg}$ protein with a rate constant (k_2, k_{cat}) $\sim 1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ [57–59]; these values are only slightly greater than the corresponding values for Prdx6 indicated above. Although GPx4 may play an important role in the reduction of oxidized membrane lipids in some organs, its expression varies considerably among tissues [60–63]. For example, it is expressed at a relatively low level in lungs, an organ susceptible to oxidant stress based on its organismal location and physiological function. “Knock-out” of Prdx6 (Prdx6 null) diminished the PHGPx activity of lungs by 97% (Fig. 4), suggesting that the reduction of oxidized phospholipids in this organ largely reflects Prdx6 activity [64]. Homogenates from several organs obtained from heterozygotic mice that express only one allele for wild type GPx4 and the other allele for inactive GPx4 showed relatively little difference from control mice in their phospholipid hydroperoxide GSH peroxidase activity [63]. This result is compatible with, although not definitive for, a major role for Prdx6 with minimal input from GPx4 in the reduction of peroxidized phospholipids in these organs (heart, liver, kidney, spleen, lung). The structural basis for the activity of Prdx6 toward oxidized phospholipids is discussed below.

2.1.9. Sulfenylamide formation—In contrast to the 2-cys Prdxs that can be reduced from the sulfenic (–SOOH) state by sulfiredoxin, hyperoxidation of Prdx6 is irreversible [65]. Thus, preventing further oxidation of the sulfenic is essential for Prdx6 function. The π GST-catalyzed reaction of the sulfenic group with GSH to form a disulfide can stabilize the protein (pending its reduction) and prevent its hyperoxidation *in vitro* [66]. However, it is not clear that the π GST required for hetero-dimerization would be readily available

intracellularly to prevent hyperoxidation of the protein under physiological conditions. Our mass spectroscopic evaluation of the recombinant protein following storage (that presumably was accompanied by auto-oxidation of the protein) indicated a molecular mass that was 2 Da less than the predicted mass of the reduced protein [35]. Based on this finding, we have proposed that a sulfenylamide forms through dehydration of oxidized Prdx6 (reduced protein +16 for “O” –18 for H₂O = –2) [35]; an alternative possibility for the measured mass is a thioether, but that is extremely rare in nature [67]. As noted in the review by Forman in this volume, sulfenylamide formation of a protein *in vivo* requires the reactivity of its sulfhydryl group toward peroxides that is in the range demonstrated by Prdx6. Thus, sulfenylamide formation could represent a mechanism for stabilization of the oxidized thiol (Fig. 3) and this reaction could account for the surprising stability reported for oxidized (sulfenic) bovine Prdx6 [38,39]. The possible partner with C47 for sulfenylamide formation remains to be determined, but includes the adjacent V46 or T48 or, in the tertiary structure, H39 or R132. Although sulfenylamide formation was demonstrated for this protein *in vitro*, its formation and possible role for stabilization of Prdx6 has not yet been demonstrated under physiological conditions.

2.2. Phospholipase A₂ activity

2.2.1. Mechanism for activity—Prdx6 also expresses a second major activity related to phospholipid metabolism as it is able to hydrolyze glycerophospholipids at the *sn*-2 position; thus, Prdx6 exhibits phospholipase A₂ (PLA₂) activity [4,6,8,30]. Properties of the enzyme have been studied with Prdx6 protein isolated from rat and bovine lungs [4,8] and recombinant rat and human protein [68–70]. Under optimal conditions, PLA₂ activity of recombinant Prdx6 is ~100 nmol/min/mg protein with K_m ~350 μM using radiolabeled dipalmitoyl phosphatidylcholine (PC) in mixed unilamellar liposomes (mol fraction: 0.5 dipalmitoyl PC, 0.25 egg PC, 0.15 cholesterol, 0.10 phosphatidylglycerol) as substrate; a rate constant for this reaction has not been reported. For comparison, the activity of secreted (type II) human platelet PLA₂ is ~300 nmol/min/mg protein while Ca²⁺-independent PLA₂ from a macrophage cell line is ~5 nmol/min/mg protein [71,72]. However, these comparisons have little meaning because of the varied substrate specificity and the complex conditions necessary for demonstration of *in vitro* activity. Prdx6 PLA₂ has a decided preference for phosphatidylcholine as a component of liposomes as substrate with ~40% less activity toward phosphatidylethanolamine and even lower activity towards phospholipids with other head groups; there is no preference for the fatty acyl group in the *sn*-2 position although an alkyl substituent results in a decrease in activity by ~50% [4,6]. The active sites for PLA₂ activity and peroxidase activity are distinct, making this a true “moonlighting” protein [30].

The PLA₂ activity of recombinant Prdx6 as well as Prdx6 isolated from lungs is greatest under acidic conditions (~pH 4) and, unlike many other PLA₂ enzymes, does not require the presence of Ca²⁺ [73–75]; thus, this activity initially was called either acidic calcium-independent PLA₂ (aiPLA₂) [4,6,73,75] or lysosomal PLA₂ [8,30,74,76]. (Not to be confused with a 45 kDa protein that was identified subsequently and also called lysosomal PLA₂ [77]). While the PLA₂ activity of Prdx6 with the usual phospholipid substrates is much lower at cytosolic pH, activity at pH 7 towards phosphatidylcholines with an oxidized

fatty acid in the *sn*-2 position, i.e., a phosphatidylcholine hydroperoxide, is similar to the level seen with this substrate under acidic conditions [78]. Unlike the case for peroxidase activity, PLA₂ activity is present in the Prdx6 monomer and dimerization is not required [35].

aiPLA₂ activity shows a distinctive response to chemical PLA₂ inhibitors; it is insensitive to most of the inhibitors of other PLA₂ enzymes but is inhibited competitively by 1-hexadecyl-3-trifluoroethylglycero-*sn*-2-phosphomethanol (MJ33), a tetrahedral mimic of the transition state [4,6,8,73,76,79]. (It should be noted that many purported PLA₂ inhibitors function by making the substrate inaccessible to the enzyme, and in that sense are not classical enzyme inhibitors [80]). Inhibition by MJ33 is > 80% at a ratio of 1:100 inhibitor to substrate concentrations (mol:mol).

The motif in Prdx6 that is responsible for phospholipid hydrolysis is ³²S-¹⁴⁰D-²⁶H [78], a catalytic triad that is commonly found in lipases and proteases [81,82]. This triad is present in all mammalian Prdx6 proteins for which the amino acid sequence has been determined and those examined have all shown PLA₂ activity [28]. The ³²S amino acid residue as well as the other members of the catalytic triad also are conserved in many non-mammalian 1-cys Prdx enzymes including those from avian, fish, amphibian, and *Drosophila* species [68], although whether that translates into PLA₂ activity has not yet been demonstrated. A homologous triad is not present in the 2-cys Prdxs and none of these latter proteins has been reported with PLA₂ activity.

2.2.2. Phosphorylation of Prdx6—The post-translational modification with the greatest effect on PLA₂ activity is its phosphorylation at the T177 position. Activity of the phosphorylated enzyme is ~1.3 μmol/min/mg protein, a >10-fold increase compared to the non-phosphorylated protein; further, activity towards reduced phospholipid substrate is similar under acidic and neutral pH conditions [69]. Prdx6 is phosphorylated by MAP kinases (p38, ERK) as shown *in vitro* with recombinant protein and in cells by the use of MAP kinase inhibitors [69,83]. *In silico* analysis of Prdx6 based on the crystal structure shows accessibility of MAPK docking sites on the protein surface [69].

The site for phosphorylation, T177, in the crystal structure of Prdx6 is buried in the homodimer interface and would not be solvent accessible for phosphorylation *in vitro* [43]. However, analysis of the reduced protein in solution by zero length chemical crosslinking and homology modeling demonstrated considerable divergence from the crystal structure of the oxidized (sulfenic) protein, particularly in the C-terminal region [45]. Although these latter measurements indicate solvent accessibility to T177, that site is nonetheless far removed from the active site for PLA₂ activity requiring a conformational change to influence substrate binding and/or enzymatic activity as described below.

2.3. Lysophospholipid acyl transferase activity

Prdx6 also demonstrates a third distinct activity, namely the ability to acylate lysophosphatidylcholine with a free fatty acid (CoA derivative); this activity is called lysoPC-acyl CoA transferase (LPCAT) [84]. Prdx6 exhibits one of the motifs, ²⁶HxxxxD³¹, that characterize LPCAT activity [85,86] (Fig. 1). LPCAT activity is abolished by mutation

of D31 but, surprisingly, was unaffected by mutation of H26 [84]. The activity expressed by Prdx6 has a marked preference for choline (lysoPC) as compared to other lysophospholipid head groups and also a preference for palmitoyl CoA as the *sn*-2 substituent fatty acid. Kinetic studies demonstrated that hydrolysis by Prdx6 (PLA₂ activity) and reacylation (LPCAT activity) represent a continuous process without release of the lysoPC intermediate that is generated by the PLA₂ reaction [84]. Thus, in the presence of palmitoyl CoA, the combined PLA₂ and LPCAT activities essentially result in the generation of PC with palmitate in the *sn*-2 position. Prdx6 is one of 5 mammalian enzymes that have been described with LPCAT activity [85–87] but it is the only one to date that shows PLA₂ and LPCAT activities in the same protein and also the only one with a predominantly cytosolic localization (see below).

2.4. Phospholipid binding

The three reactions described above that involve phospholipids, namely, the reduction of PLOOH (PHGPx activity), the hydrolysis of PC (PLA₂ activity), and the acylation of lysoPC (LPCAT activity), all require the binding of Prdx6 to a phospholipid substrate. Binding of Prdx6 to liposomes as a model for cell membranes ($K_d \sim 230$ nM) has been demonstrated using circular dichroism, microfiltration analysis, fluorescence spectrofluorometry, time-resolved fluorescence spectroscopy, isothermal titration calorimetry, and other techniques [33,78]. Binding is greatest with liposomes containing an anionic phospholipid and decreases by ~50% with non-charged (neutral) liposomes [78]. This means that the negative charge (e.g., associated with the presence of phosphatidylglycerol) facilitates binding of Prdx6 to the liposome although the subsequent hydrolytic activity is greatest towards phosphatidylcholine. Prdx6 binds to liposomal phospholipids at pH 4 but binds less well at pH 7, consistent with assays of its PLA₂ activity. Also consistent with its PLA₂ activity, phosphorylated Prdx6 binds avidly at both acidic and neutral pH while Prdx6 binds equally at both pHs to liposomes containing an oxidized phospholipid [33,69]. Thus, substrate binding appears to be a key element for understanding the lipid-related enzymatic activities of Prdx6.

Evaluation of Prdx6 by the biophysical techniques mentioned above provide evidence that the mechanism for the effect of phosphorylation on PLA₂ activity is through increased binding associated with conformational change in the protein structure [88]. This latter study demonstrated that phosphorylation induces Prdx6 to assume a molten globule state that shows maximal enzymatic (PLA₂) activity. This state is the configuration associated with activity for many proteins, allowing greater structural flexibility and a greater range of conformations for binding to substrates as compared with the fully folded native state [89–91]. The greater binding of Prdx6 to phospholipids at acidic pH and the increased binding to oxidized substrate also may be a consequence of altered protein conformation subsequent to thiol protonation (–SH) or oxidation (–SOH), although those possibilities have not yet been evaluated.

The amino acid residues ³⁰G-G³⁴ in Prdx6 (GDSWG) constitute what has been called a lipase domain (GxSxG) (Fig. 1); this motif is commonly observed in proteins that show lipolytic activity [92,93]. The amino acids of the lipase domain with extension to H26 are

the key site for binding of Prdx6 to phospholipid substrates [33,78]. Based on analysis of the crystal structure [43], we have proposed that upon binding of phosphatidylcholine to the Prdx6 surface, the *sn*-2 fatty acyl group is positioned into a surface invagination (catalytic pocket) where it is susceptible to: a) reduction by the peroxidatic site centered on C47 that is located at the base of the pocket, or b) hydrolysis by the lipolytic site centered on S32 at the entrance to the pocket (Fig. 5). These structure-function relationships of Prdx6 are ultimately responsible for its unique enzymatic activities.

3. Expression of Prdx6 protein and activities

3.1. In vitro expression

The multiple states of Prdx6 and its susceptibility to auto-oxidation have led to difficulty in isolation and storage of enzymatically active protein. We have developed conditions for generating active Prdx6 in relatively high yield and in the reduced state (<5% oxidized) using codon optimized *E. coli* for expression of the protein and ion-exchange chromatography for purification [45].

3.2. Cellular and subcellular localization

Prdx6 is widely distributed throughout all organs with especially high concentrations in the lung, brain, liver, kidney, and testis [6,27,94]. At present, no normal mammalian cells are known that lack Prdx6. The subcellular localization of Prdx6 is predominantly cytosolic. However, as demonstrated through subcellular fractionation, immunofluorescence, and immunogold electron microscopy, Prdx6 in lung alveolar type 2 epithelial cells is present in lysosomes and lamellar bodies (LB) as well as cytosol [52,70,95–97]. LB are lysosomal-related organelles (LROs) that, like lysosomes, maintain an acidic internal pH [98] and are the intracellular site for storage and metabolism of the lung surfactant [99–101]. Prdx6 plays an important role in the intra-LB metabolism of phospholipids (see below). The possible presence of Prdx6 in other LROs (e.g., melanosomes) has not been reported. Prdx6 was not detected in the lysosomal fraction isolated from human A431 epidermoid carcinoma cells [32].

The transport of Prdx6 from its site of synthesis in the endoplasmic reticulum to its localization in the luminal compartment of the LB of the type 2 alveolar epithelial cell proceeds via the vesicular pathway as shown by its inhibition with brefeldin A [96]. The amino acid sequence in Prdx6 between positions 31–40 (DSWGILFSHP) is required for LB targeting of the protein [96]. Within this sequence, the amino acids S32 and G34 appear to be critical while mutation of S38 has no effect on targeting; the importance of the other individual amino acids in the sequence has not been evaluated. Binding of Prdx6 to lipid is not required for targeting of the protein [96]. The cytosolic chaperone protein 14-3-3 ϵ , after its activation by MAP kinase activity, binds to Prdx6 and is required for organellar targeting [95] although the precise mechanism for determining the distribution between cytosolic vs. organellar localization for the protein is not known. Mutation of S32 to threonine had no effect on lipid binding or enzymatic activities of Prdx6 but did abolish its binding to 14-3-3 ϵ and its targeting to LB [68].

3.3. Regulation of expression

Prdx6 in rat lungs is expressed at relatively low levels in the fetus but is markedly increased immediately after birth and reaches adult levels at ~1–2 weeks of age [102]. Prdx6 expression postnatally is induced by oxidative stress as shown by treatment of lungs or cell lines with H₂O₂, hyperoxia, or paraquat [103–105]. Transcriptional activity for Prdx6 synthesis also is stimulated by KGF and by a glucocorticoid analogue (dexamethasone) independently of oxidants [14,102,105,106]. Both oxidant stress and KGF induce transcription through activation of the transcription factor Nrf2 that translocates to the nucleus and binds to an anti-oxidant (electrophilic) response element (ARE or ERE) located between 357 and 349 nucleotides upstream of the Prdx6 translational start site [104,106]. Dexamethasone activates Prdx6 transcription by binding to a glucocorticoid response element (GRF) located ~750 nucleotides upstream of the Prdx6 translational start site [106]. The effects of KGF and dexamethasone on Prdx6 protein expression are synergistic, consistent with their regulatory effect via separate but interactive elements in the Prdx6 DNA promoter [106]. Prdx6 expression also is regulated by the transcription factors CCAAT/enhancer protein β (C/EBP β) and cAMP response element binding protein (CREB) [107].

3.4. Regulation of activity

Several potential physiological modulators of the PLA₂ activity of Prdx6 have been shown, but no endogenous regulators of its peroxidase activity are known. As described above, phosphorylation of Prdx6 at position T177 mediated by MAP kinase leads to an increase in PLA₂ activity of >10-fold [69]. Phosphorylation under physiological conditions has been shown with intact endothelial cells that were treated with angiotensin II [83]. With phosphorylation, cytosolic Prdx6 can bind to cell membranes, attaining substrate for its PLA₂ activity [69].

Two specific cellular proteins have been shown to inhibit PLA₂ activity. The first is surfactant protein A (SP-A), a protein that is present in the type 2 alveolar epithelial cells and the extracellular airspaces of lungs [97]. SP-A (monomeric mol wt ~26 kDa) binds to Prdx6 through hydrophobic interaction and H⁺-binding; the stoichiometry of binding is one Prdx6 monomer per SP-A trimer [70]. Inhibition of PLA₂ activity is non-competitive with Ki~10 μ g/ml (~130 nM for the SP-A trimer). The sites for interaction are ¹⁹⁵E-K²⁰⁴ in Prdx6 and ⁸³D-L⁹⁹ in SP-A [108]; the role of specific amino acid residues in these sequences has not been investigated. Prdx6 PLA₂ activity was significantly increased in lung homogenates from SP-A null mice [109] while LB that had been isolated from rat lungs showed a significant increase in PLA₂ activity when treated with a chemical inhibitor of SP-A binding to Prdx6 [97]. These findings are compatible with a physiological role for SP-A in the regulation of Prdx6 PLA₂ activity. Since Prdx6 and SP-A co-exist only in lung epithelial cells, lung LB, and the lung airway extracellular spaces, the regulatory role of SP-A on Prdx6 function likely would be limited to those compartments.

A second endogenous regulator of Prdx6 PLA₂ activity is the cytosolic protein p67^{phox} that is associated with the NADPH oxidase type 2 (NOX2) complex [17]. p67^{phox} binds strongly to phosphorylated Prdx6 (Ki 65 nM) and inhibits its PLA₂ activity while binding to non-

phosphorylated Prdx6 is considerably weaker [110]. The NOX2 activation pathway requires phosphorylation of p67^{phox} but this abolishes its binding to Prdx6 [110]. Prdx6 and p67^{phox} co-exist in the cytoplasm of polymorphonuclear leukocytes, macrophages, endothelium, and possibly other cell types and their interaction in those cells may play a regulatory role related to NOX2 activation as discussed below.

4. Physiological roles of Prdx6

4.1. Prdx6 null mice

Prdx6 null mice have been generated by two different molecular strategies [111,112]. In both cases, mice appear to develop and reproduce normally with no overt phenotype. However, closer evaluation shows a characteristic phenotype including: 1) increased susceptibility to oxidant-mediated injury with altered repair of peroxidized cell membranes, 2) disturbances of sperm mobility with consequent effects on fertility associated with aging, 3) abnormalities related to lung lipid metabolism with age-related phospholipidosis and altered lung surfactant composition, and 4) greatly decreased activation of NADPH oxidase (type 2) with decreased O₂⁻ generation and altered cell signaling. These abnormalities are now discussed with relevance to the physiological roles of Prdx6.

4.2. Antioxidant defense

4.2.1. Response to oxidative stress—There is much evidence that Prdx6 can protect against cell injury associated with oxidative stress. Studies have evaluated the effects of both over- and under-expression of Prdx6 in isolated cells, isolated organs, and whole animals. Cells in culture over-expressing Prdx6 have been obtained by their isolation from transgenic Prdx6 over-expressing mice, by generation of stable cell lines, and by use of TAT peptide as a protein delivery agent. Cells over-expressing Prdx6 have shown an increase in survival compared to control when oxidatively challenged with hydroperoxides, paraquat, UVB radiation, or OH generated by Cu²⁺/ascorbate [113–116]. Prdx6 over-expression through adenoviral-mediated intratracheal delivery of a Prdx6 expression vector or in transgenic mice protected lungs from hyperoxic injury [117,118].

Prdx6 null mice showed increased lung or liver injury with exposure to hyperoxia, lipopolysaccharide (LPS), or paraquat [64,112,119,120]. Lung alveolar type 2 epithelial cells, lung pulmonary microvascular endothelial cells, peritoneal macrophages, and sperm that were isolated from Prdx6 null mice were more sensitive (increased injury) with exposure to paraquat or various hydroperoxides [64,121–123]. Prdx6 null mice as compared to wild type demonstrated increased age-related oxidative damage to their sperm chromatin [124] as discussed further below. Treatment of a lung epithelial cell line with a Prdx6 antisense oligonucleotide depressed Prdx6 expression and resulted in oxidative stress during routine cell culture with increased cytotoxicity on exposure to an •OH-generating system [125].

4.3. Mechanism for antioxidant protection

Based on the studies described above, there is a distinct correlation between the level of Prdx6 expression and susceptibility to a variety of agents that produce their effects through

oxidant stress. However, the precise mechanism(s) for protection against oxidant injury by Prdx6 is not clear. One possibility would be the direct scavenging of H₂O₂ (or other low molecular weight hydroperoxides) by Prdx6. For this function, Prdx6 would compete with various cellular enzymes such as the GPx enzymes, other Prdxs, and catalase. A second possibility for the effect of Prdx6 is related to its ability to reduce PLOOH.

To gain an understanding of the relative roles for scavenging of H₂O₂ vs reduction of PLOOH in antioxidant protection by Prdx6, we compared the response of Prdx6 null and glutathione peroxidase 1 (GPx1) null mice to oxidant stress [64]. GPx1 is considered to be a major scavenger of cellularly-generated H₂O₂ and simple hydro-peroxides. Mice were exposed to hyperoxia, isolated lungs were exposed to *t*-BOOH or paraquat, and primarily isolated pulmonary microvascular endothelial cells also were exposed to *t*-BOOH. Lungs from the two types of mice showed similar levels for expression of mRNA or protein for antioxidant enzymes, except for the targeted protein. For all parameters investigated in each experimental model, there was relatively little injury in the GPx1 null mice while injury in the Prdx6 null was significantly greater [64]. The results of these comparative studies [64,121] suggest (with caveats, of course) that in these models of oxidant injury, the ability to reduce H₂O₂ has relatively minor importance while the ability of Prdx6 to either reduce or hydrolyze phospholipid hydroperoxides is crucial to its protective role.

4.3.1. Repair of peroxidized cell membranes—Lipid peroxidation, primarily affecting cell membranes, is recognized as one of the major mechanisms for cell injury associated with oxidant stress [126]. Lipid peroxidation occurs through proton abstraction, commonly from a phospholipid with an unsaturated fatty acid in the *sn*-2 position, resulting in a lipid radical that can initiate a chain reaction. Quenching of the chain reaction (for example, by α -tocopherol) can prevent further oxidation of lipids but does not alter the lipid hydroperoxides that are already formed; these can break down gradually to products including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) that have signaling or toxic effects and are commonly assayed to detect lipid peroxidation [126]. Peroxidation of cell membranes generally leads to cell death, primarily by apoptosis or by the recently described pathway of ferroptosis, although extensive injury can result in cellular necrosis as well [126,127]. Thus, the repair of peroxidized cell membranes is crucial for the survival of cells with oxidatively damaged cell membranes.

Two major reaction pathways have been proposed for the reduction of oxidized cell membrane phospholipids. The first is the direct reduction of the phospholipid hydroperoxides through PHGPx activity [128,129]. Prdx6 and GPx4 are the only two enzymes that have been demonstrated to reduce phospholipid hydroperoxides at a significant rate; both proteins are present in cytoplasm and use GSH as the physiological reductant. GPx4 also can reduce cholesterol hydroperoxides that may be present in oxidized cell membranes [59]; the possible reduction of that substrate by Prdx6 has not yet been evaluated. Loss of GPx4 (GPx4 null mice) results in fetal lethality and in cell death by ferroptosis, although whether those effects reflect primarily the loss of PHGPx activity is unclear [127,130]. Since the relative expression of Prdx6 and GPx4 may vary in different organs, their relative roles in cell membrane repair also may vary with cell type.

The other mechanism for “repair” of peroxidized phospholipids in cell membranes is the deacylation/reacylation pathway, also called a remodeling or the Lands pathway [129,131]. In this pathway, the peroxidized phospholipid is hydrolyzed to remove the oxidized *sn*-2 fatty acyl chain, generating a lysolipid that then is reacylated with a non-oxidized fatty acid; reacylation restores the normally reduced state of membrane lipids. The PLA₂ activity of Prdx6 could catalyze the first step in this pathway while the LPCAT activity of Prdx6 could catalyze the second step of the deacylation/reacylation pathway. Thus, Prdx6 expresses all 3 activities that are known to be involved in the repair of peroxidized cell membranes (Fig. 6). In essence, the PHGPx plus the PLA₂/LPCAT activities of Prdx6 can repair peroxidized cell membranes by either direct reduction, deacylation/reacylation, or both pathways acting in concert. Prdx6 thus represents a complete protein for the enzymatic repair of cell membrane lipid peroxidation.

We have studied the role of Prdx6 in the repair of lipid peroxidation in lungs from mice exposed to hyperoxia and in isolated mouse lungs and isolated pulmonary microvascular endothelial cells exposed to *tert*-butyl hydroperoxide (*t*-BOOH). The content of lipid peroxidation products in wild type lungs and cells from these models of injury showed a gradual decrease over several hours following removal of the oxidant stress; however, there was essentially no repair in the absence of Prdx6 during the time frame studied [132] (Fig. 7). Restoration of either the PLA₂ activity or the peroxidase activity of Prdx6 through transfection of the null cells with relevant mutants demonstrated that either activity was partially effective in reducing the degree of membrane phospholipid peroxidation and improved cell survival; transfection with Prdx6 constructs leading to expression of both activities restored the wild type phenotype [121,132]. Mutants that specifically lack LPCAT expression have not yet been evaluated. The results obtained using transfection with mutant proteins provide evidence that Prdx6 does indeed have an important role in the repair of peroxidized cell membranes, at least in lungs, and that both its PHGPx and PLA₂ activities play important roles. Of note, GPx4 appeared to play no role in the repair of the peroxidized lung cell membranes in these models.

Prdx6 is present in the cytoplasm of cells but is not a cell membrane protein. So, how does it act to reduce oxidized membrane phospholipids? As described above, Prdx6 in cytosol (pH 7) does not normally bind to phospholipids; however, it does bind with high affinity at cytosolic pH to oxidized phospholipids, thus becoming a membrane associated protein [33]. In the membrane-bound state, there is access to phospholipid hydroperoxide substrate for PHGPx activity of Prdx6 and also activation of its PLA₂ activity. This oxidation-mediated translocation to the membranes allows Prdx6 to be inactive as a cytosolic protein (PLA₂ activity under resting conditions generally would be undesirable) but to activate following oxidative stress as necessary for cell membrane repair (Fig. 8). Oxidative stress-induced translocation to the plasma membrane has been shown with A549 cells in culture [33]; oxidative stress to liver resulted in translocation of Prdx6 to mitochondria, possibly reflecting its binding intracellularly to oxidized mitochondrial membranes [133].

4.3.2. Reproductive efficacy—A role for Prdx6 in reproduction was indicated by finding that the total number of litters, the litter size, and the total number of pups were significantly lower per Prdx6 null male as compared to WT controls [124]. Examination of sperm from

2-, 8-, and 20 month old male Prdx6 null mice showed age-dependent impairment of motility and maturation, increased DNA oxidation and fragmentation, and decreased DNA compaction and protamination [124]. These results suggest a protective role of Prdx6 in the age-associated decline in mouse sperm quality and fertility. This protective effect is presumably related to the anti-oxidant and the cell membrane repair roles of Prdx6 [123]. Interestingly, infertile men with low sperm motility and evidence of DNA damage have lower levels of Prdx6 in sperm as compared to control men [134].

4.4. Phospholipid synthesis and catabolism

Prdx6 is important in the metabolism of lung surfactant, a secreted product of lung epithelium that is essential for maintaining normal lung function. Lung surfactant comprises specific proteins (~10% of mass), phospholipids (~75%), the major fraction of which is dipalmitoyl phosphatidylcholine (DPPC), and neutral lipids (~15%). Surfactant components are synthesized in the endoplasmic reticulum, transported to the LB, and secreted by exocytosis into the alveolar airspaces [99]. After a short extracellular residence (1/2 time ~10 h), phospholipids (and proteins) are endocytosed by a receptor-mediated pathway and delivered to LB for either degradation or remodeling and resecretion [99,135,136]. The physiological role of this recycling pathway is not clear, although both quality as well as quantity control have been considered as possible “reasons”.

Prdx6 participates in both the degradation and remodeling pathways for phospholipid metabolism in the LB [73–76,79,136] (Fig. 9). The processes of degradation and remodeling both start with PLA₂ activity to generate lysoPC. As the LB pH is ~5 [98], Prdx6 (non-phosphorylated) can bind to and hydrolyze native phospholipids. The degradative pathways result in liberation of free fatty acids and choline phosphate that can be reutilized for *de novo* phosphatidylcholine synthesis by the Kennedy pathway [131] or otherwise metabolized. The remodeling pathway is essentially the same as described for repair of peroxidized membrane phospholipids, although the phospholipid substrate in this case generally is not oxidized.

Lung surfactant requires dipalmitoylphosphatidyl choline (DPPC) for its surface-active properties and this product can be provided by either *de novo* synthesis or by remodeling of phosphatidylcholines that do not contain palmitate in the *sn*-2 position [99,135]. The generation of DPPC is crucial to the proper function of the lung surfactant. The interaction of Prdx6 with SP-A, an endogenous regulator of the PLA₂ activity of Prdx6 (see above), presumably plays an important role in the regulation of PC degradation and remodeling in lung LB, although the precise details of this regulation have not been investigated.

Lungs from Prdx6 null mice show a markedly decreased turnover rate of lung surfactant phospholipids with progressive accumulation (normalized to body wt) of phospholipids in the whole lung and alveolar air spaces as the mice age [74]. By contrast, the normalized phospholipid content of wild type lungs is stable following the neonatal period. Isolated lungs from Prdx6 null mice or with chemical (MJ33) inhibition of Prdx6 PLA₂ activity in wild type lungs also show decreased lung surfactant phospholipid turnover [74,79]. The inverse, i.e., increased degradation of phospholipids, decreased phospholipid content and increased activity of the reacylation pathway of phospholipid metabolism is seen with lungs

from transgenic mice overexpressing Prdx6 [137]. A pattern of altered lung surfactant phospholipid metabolism similar to Prdx6 null is seen in mice that express normal total lung Prdx6 but lack Prdx6 in the lamellar bodies due to a failure of its targeting [68,138]. This latter observation highlights the important role of the lamellar body and its Prdx6 content in lung surfactant metabolism.

4.5. Cell signaling

4.5.1. Activation of NOX2—NOX2, a member of the family of seven NOX/DuOX enzymes that generate $O_2^{\cdot-}$ or H_2O_2 as their primary product, has major roles in bacterial killing and cell signaling. NOX2 is normally inactive in the cell and is activated as required. PLA₂ activity has long been suspected to have a role in the activation of NOX2, initially considered to be related to the generation of arachidonic acid as a substrate for the generation of eicosanoids [139]. However, an understanding of which one or more of the numerous PLA₂ proteins that is involved in the activation process was elusive. Consistent with its known high specificity for the release of arachidonic acid, cytosolic PLA₂ (cPLA₂) was investigated as the enzyme responsible for NOX2 activation [140]. However, a careful study of the cPLA₂ null mouse demonstrated that this enzyme has minimal effect on activation of NOX2 in response to agonists [141]. Further, the role of arachidonate in NOX2 activation proved to be primarily an *in vitro* phenomenon, although arachidonic acid (as well as other unsaturated fatty acids) may bind to components of the NOX2 complex *in vivo* to stabilize their expression [142]. Subsequent attention focused on lysophospholipid as the product of PLA₂ activity that is required for NOX2 activation.

A possible role for Prdx6 in the activation of NOX2 was first suggested through co-immunoprecipitation of Prdx6 and p67^{phox} from the cytosol of non-stimulated polymorphonuclear leukocytes (PMN) and increased $O_2^{\cdot-}$ generation by addition of Prdx6 to a reconstituted system containing PMN cell membranes [17]. The role of specific Prdx6 enzymatic activities in the activation process was not determined. Subsequent studies using chemical and molecular inhibition showed that the PLA₂ activity of Prdx6 is absolutely required for NOX2 activation in a variety of cell types (endothelium, macrophages, PMN) as well as in several cell lines [83,143,144]. Activation of NOX2 is greatest with MAP kinase-mediated phosphorylation of Prdx6 in response to an appropriate agonist [83] (Fig. 10). Experimental agonists for activation of NOX2 include angiotensin II or phorbol ester as well as some disease models such as LPS administration or the response to ischemia; a requirement for Prdx6 PLA₂ activity as a mediator in the activation process has been demonstrated for these specific agonists and interventions [83,144,145]. As noted above, phosphorylation of Prdx6 results in its binding to cell membranes at cytosolic pH and a marked increase in PLA₂ activity [69]. PLA₂ activity results in the generation of lysoPC plus a free fatty acid as its two products. LysoPC is then hydrolyzed to lysophosphatidic acid (LPA) that signals through its receptor leading to activation of *rac* [146], one of the co-factors (in addition to phosphorylated p47^{phox} and p67^{phox}) that are necessary for NOX2 activation [147] (Fig. 10). The proteins downstream from Prdx6 that are involved in the *rac* activation cascade are autotaxin (lysophospholipase D) and LPA receptor 1 (LPAR1), although a possible role for other proteins has not been excluded [146].

Based on its binding to phosphorylated Prdx6 with inhibition of its PLA₂ activity (see above), we have postulated that p67^{phox} (but not the phosphorylated protein) can terminate the NOX2 activation process [110]; however, the possible physiological role for this latter interaction needs further study. Prdx6 also has been suggested as required for activation of NOX1, possibly related to its PLA₂ activity, but those studies are relatively preliminary and the mechanism for the effect has not been determined [148].

4.5.2. Prdx6 in endothelial cell mechanotransduction—Endothelium is constantly exposed to varying mechanical strain associated with shear stress; an increase or decrease in shear outside of the normal range results in a signaling response termed mechanotransduction. We have investigated this response to reduced shear (that is, simulated ischemia) using an isolated lung preparation and isolated endothelial cells that have been flow adapted *in vitro* [149,150]. The loss of shear is sensed by a cell membrane complex (mechanosome) that is localized to caveolae; the mechanosome consists of platelet endothelial cell adhesion molecule (PECAM), vascular endothelial growth factor receptor 2 (VEGFR2), vascular endothelial (VE)-cadherin, and possibly other elements [149,151]. The initial response to loss of shear is the inactivation of the inwardly-rectifying K_{ATP} channels that results in cell membrane depolarization [149,152]; these channels are normally maintained in the open configuration by shear stress. Cell membrane depolarization is the initiating signal for activation of kinases leading to phosphorylation of Prdx6 with initiation of the cascade that leads to activation of NOX2 (see above) and generation of O₂^{·-} [153,154]. O₂^{·-} (or, more likely, H₂O₂ formed by dismutation of O₂^{·-}) is the mediator that signals for the liberation of vascular endothelial growth factor (VEGF) to promote neovascularization, important for repair of the vascular lesion [155]. Cell membrane depolarization also results in activation of voltage gated Ca²⁺ channels allowing Ca²⁺ influx into the cell, thereby activating eNOS to generate NO, a vasodilator [156]. These responses (vasodilation and neovascularization) represent homeostatic responses to the absence of shear (i.e., tissue ischemia) and conceptually can restore the impeded blood flow. This paradigm demonstrates a role for Prdx6-linked generation of H₂O₂ in an important cellular signaling function (Fig. 11). O₂^{·-} generated by NOX2 also plays an important role in cell motility and a variety of other cell biological processes including hypertrophy, proliferation and migration, and activation of various transcription factors [157], but the role of Prdx6 in the regulation of these activities, while probable, has not yet been studied.

4.5.3. Prdx6 as a therapeutic target—In its normal physiological role, activation of NOX2 and the subsequent generation of O₂^{·-}/H₂O₂ is important for mediating various important homeostatic functions [158–160]. On the other hand, generation of O₂^{·-}/H₂O₂ as a major component of inflammation can be responsible for promoting cell injury in a variety of diseases [161]. One such condition is the syndrome of Acute Lung Injury (ALI) that is associated with sepsis and many other etiologies and has a mortality rate of ~40%. Based on its role in production of H₂O₂, inhibition of Prdx6 PLA₂ activity has been proposed as a mechanism to prevent NOX2 activation in conditions characterized by inflammation. Treatment with an inhibitor of Prdx6 PLA₂ activity (MJ33) protected against experimental ALI in mice subjected to intratracheal administration of lipopolysaccharide (LPS) or to a model for lung ischemia-reperfusion [144,145]. These relatively preliminary studies suggest

a potential therapeutic role for the inhibition of Prdx6 PLA₂ activity to prevent injury from O₂^{·-}/H₂O₂ generation associated with NOX2 activation. However, the antioxidant vs pro-oxidant roles of Prdx6 need to be considered when designing therapeutic strategies. An example of these opposing roles is the finding that inhibition of Prdx6 PLA₂ activity protects against lung lipid peroxidation with hyperoxia [162] while the protein is required for the repair of peroxidized cell membranes [132]. In essence, the protein (Prdx6) is available to “clean up” its own mess generated by over-exuberant oxidant production by NOX2. Obviously, if lipid peroxidation is prevented, repair will not be necessary, but additional studies with Prdx6 PLA₂ inhibitors will be required to sort out these issues.

Acknowledgments

I thank Dr. Mahendra Jain for helping to initiate the studies related to PLA₂ activity and his important insights into the catalytic mechanisms for this enzyme. I also thank Dr. Henry Forman for his very helpful suggestions and Drs. David Speicher, Sheldon Fein-stein, Avinash Chander, Yefim Manevich, Shampa Chatterjee, Elena Sorokina, Chandra Dodia, Sandra Harper, Jose Vazquez-Medina and the many other collaborators who have helped to investigate the complexities of peroxidoxin 6 structure and function. Finally, I thank Dawn Williams for assistance in preparing the manuscript.

Abbreviations

| | |
|--------------------------|---|
| aiPLA₂ | acidic Ca ²⁺ -independent PLA ₂ |
| ALI | acute lung injury |
| ARE | antioxidant response element |
| <i>t</i>-BOOH | <i>tert</i> -butyl hydroperoxide |
| DPPC | dipalmitoyl PC |
| GSH | glutathione |
| GSSG | oxidized GSH |
| GPx | GSH peroxidase |
| GST | GSH S-transferase |
| KGF | keratinocyte growth factor |
| LB | lamellar bodies |
| LPCAT | lysophosphatidylcholine acyl transferase |
| LRO | lysosomal related organelle |
| NOX | NADPH oxidase |
| PC | phosphatidylcholine |
| PCOOH | PC hydroperoxide |
| PHGPx | phospholipid hydroperoxide GSH peroxidase |

| | |
|--------------|------------------------------|
| PLOOH | phospholipid hydroperoxide |
| PMN | polymorphonuclear leukocytes |
| Prdx | peroxiredoxin |
| SP-A | surfactant protein A |

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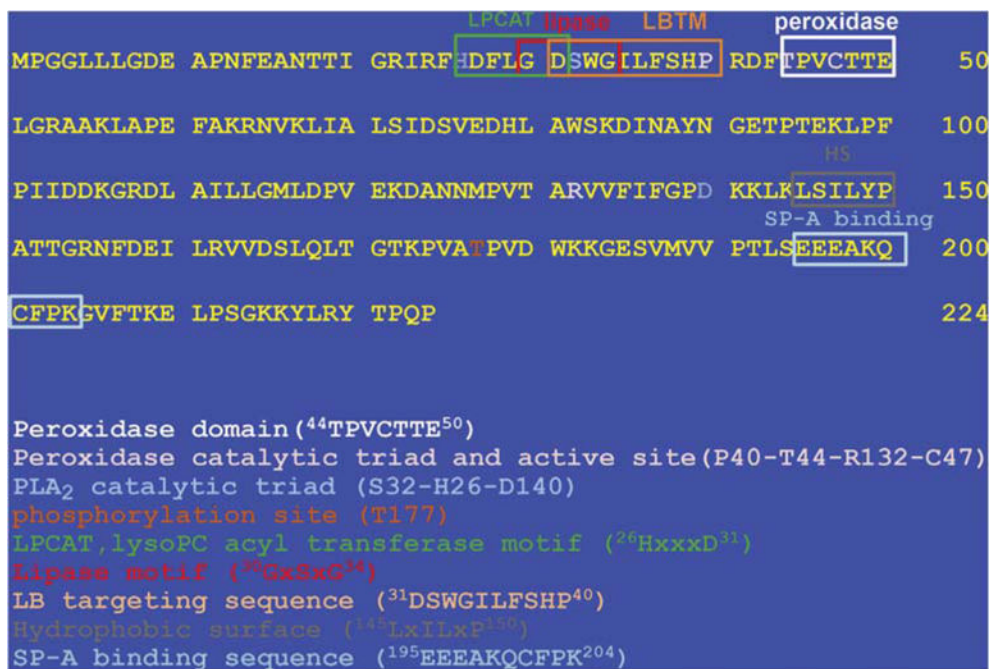


Fig. 1. Amino acid sequence for mouse Prdx6

The full length sequence is shown. The consensus sequences are indicated for peroxidase and LPCAT activities, the PLA₂ and peroxidase catalytic triads, the lipase motif, the motif (LBTM) for targeting of Prdx6 to the lamellar bodies, the hydrophobic surface (HS) for dimerization, the SP-A binding sequence, and the Prdx6 phosphorylation site.

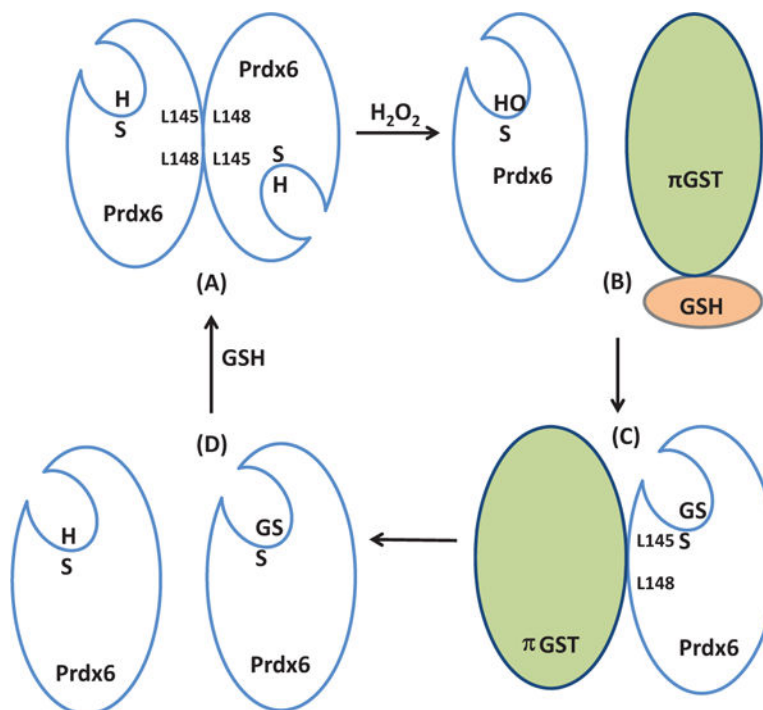


Fig. 2. Mechanism for Prdx6 peroxidase activity and its resolution

Clockwise from upper left. (A) In solution, Prdx6 exists as a homodimer through hydrophobic interactions surrounding the L145-L148 residues; the reduced peroxidatic sulfhydryl (SH) is at the bottom of a pocket. (B) Reduction of H₂O₂ (peroxidase activity) results in oxidation of the Prdx6 sulfhydryl to the sulfenic form (SOH) leading to dissociation of the homodimer. Interaction of the oxidized monomer of Prdx6 with GSH-loaded πGST results in: (C) heterodimerization via the L145-L148 hydrophobic interface of Prdx6 and glutathionylation of Prdx6 (SSG), followed by: (D) dissociation of the glutathionylated Prdx6 and πGST heterodimer. Interaction of the glutathionylated monomer with free GSH restores its normally reduced state allowing reformation of the homodimer.

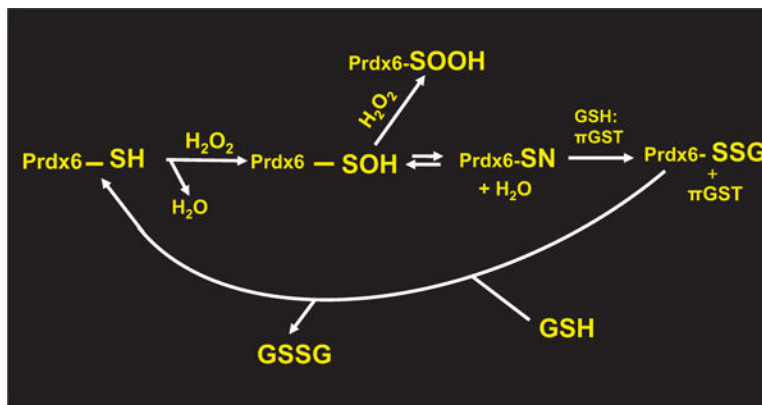


Fig. 3. Reaction scheme for Prdx6 peroxidase activity with *proposed* formation of a sulfenylamide intermediate

Interaction of Prdx6 with H_2O_2 generates the sulfenic state (Prdx6-SOH) of the reactive cysteine. The sulfenic cys is susceptible to further (irreversible) oxidation to the sulfinic form (Prdx6-SOOH). The formation of a reversible sulfenylamide (Prdx6-SN) could stabilize the sulfenic state and protect the protein from irreversible oxidation (see text). Interaction of the Prdx6 sulfenylamide with $\pi\text{GST}:\text{GSH}$ results in Prdx6 glutathionylation (Prdx6-SSG) and subsequent resolution by interaction with GSH as described in Fig. 2. GSSG formed during the cycle is reduced by NADPH mediated by GSH reductase to regenerate 2 GSH (not shown).

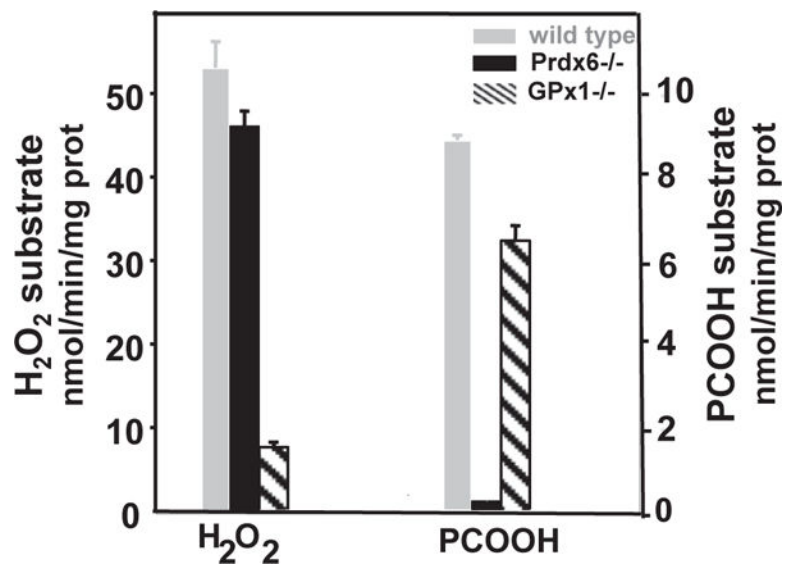


Fig. 4. Effect of Prdx6 “knock-out” on hydroperoxide reduction by mouse lung homogenate
 Knock-out of Prdx6 (Prdx6^{-/-}) has only a minor effect on the GSH-mediated reduction of H₂O₂ (GPx activity, *left y-axis*) by the lung homogenate but results in a marked decrease in the GSH-mediated reduction of phosphatidylcholine hydroperoxide (PHGPx activity, *right y-axis*). Knock-out of GPx1 (GPx1^{-/-}) resulted in the inverse with a large decrease in GPx activity but relatively little effect on PHGPx activity. PCOOH, phosphatidylcholine hydroperoxide. Reprinted from Ref. [64] with permission.

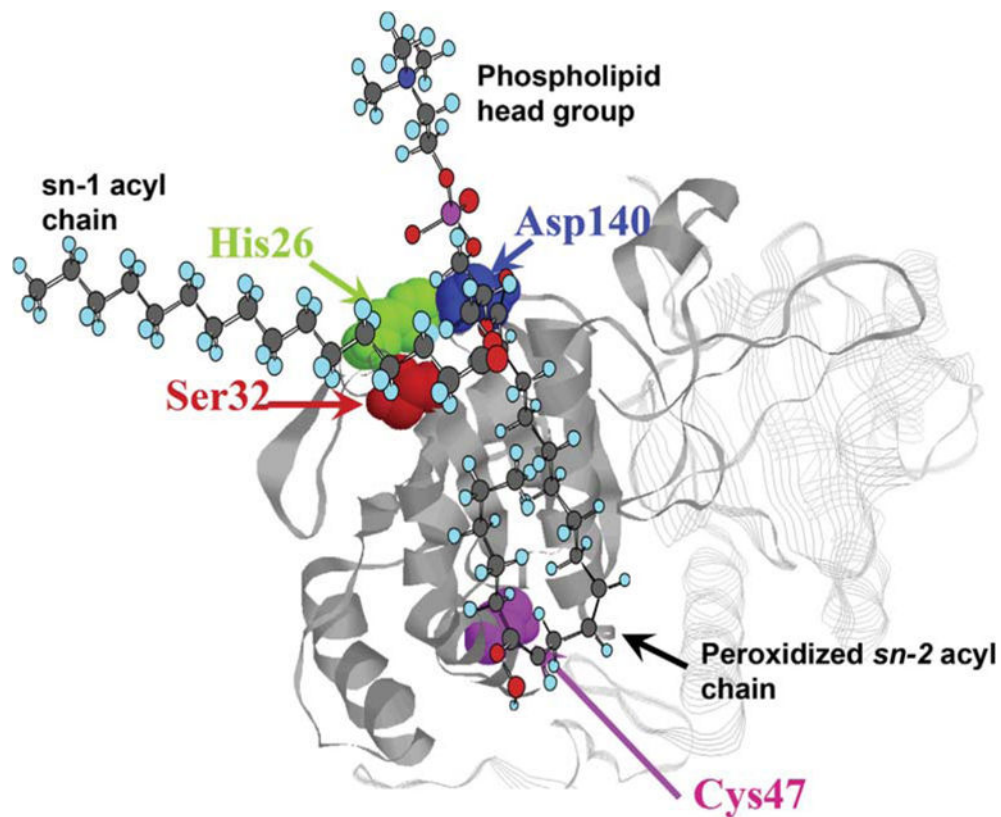


Fig. 5. Model for phospholipid-associated activities of Prdx6

The model is based on the crystal structure of oxidized human Prdx6 [43]. Prdx6 binds to oxidized phosphatidylcholine at the lipid binding region associated with the lipase motif in the protein. We propose that the peroxidized *sn*-2 acyl chain inserts into the pocket containing the peroxidatic cys and is positioned at the surface-expressed PLA₂ catalytic triad (³²S-²⁶H-¹⁴⁰D). The *sn*-1 acyl chain and the phospholipid head group remain outside of the pocket. Thus, Prdx6 can reduce the oxidized acyl moiety (PHGPx activity) or hydrolyze the acyl bond (PLA₂ activity) of the peroxidized phospholipid. This complex could be at the cytoplasmic face of a cell membrane at the left (not shown). The hydrophobic *sn*-1 fatty acyl chain and the hydrophilic head group would be within the membrane while the peroxidized *sn*-2 fatty acyl chain would be at the membrane surface facilitating interaction with Prdx6. Modified and reprinted with permission from Ref. [78].

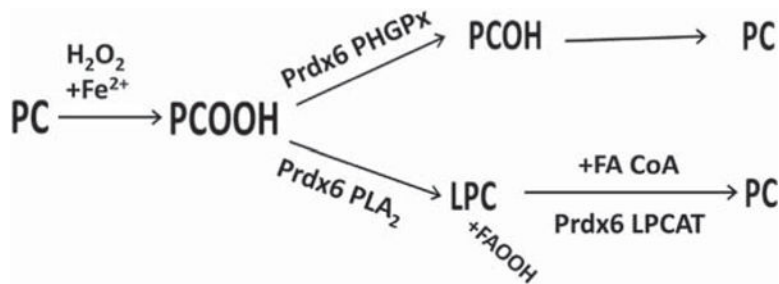


Fig. 6. Role of Prdx6 in the repair of phospholipid hydroperoxides

Phosphatidylcholine (PC) containing an *sn*-2 unsaturated fatty acid can be oxidized by H_2O_2 ($+Fe^{2+}$) to PC hydroperoxide (PCOOH). To restore the normal state, the hydroperoxide can be reduced by the phospholipid hydroperoxide GSH peroxidase (PHGPx) activity of Prdx6 to the PC alcohol that is then reduced to the sulfhydryl. An alternative pathway for removal of PCOOH is hydrolysis at the *sn*-2 position mediated by Prdx6 PLA_2 activity to generate lysoPC plus fatty acyl hydroperoxide; lysoPC can be reacylated with an acyl CoA substrate by the LPCAT activity of Prdx6 to regenerate the reduced phospholipid. Enzymes responsible for reduction of the PC alcohol (PCOH) and fatty acid hydroperoxide (FAOOH) that result from these reactions are not shown. Modified from Ref. [132] and reprinted with permission.

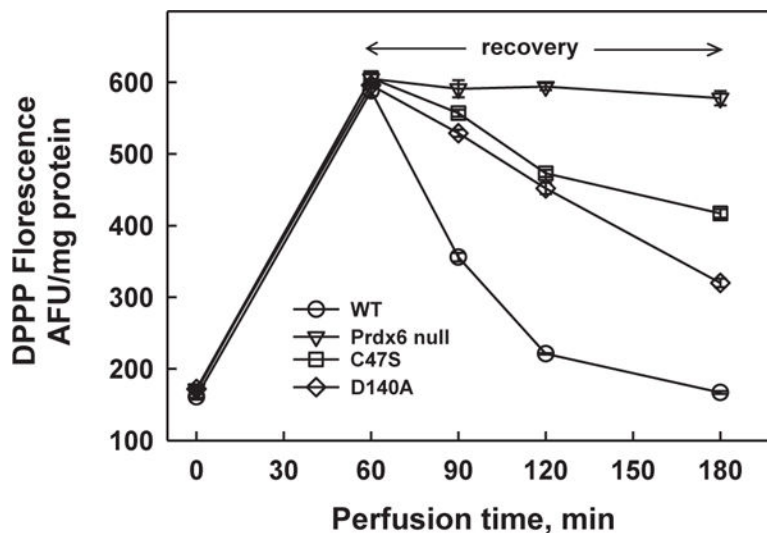


Fig. 7. Recovery of isolated perfused mouse lungs from lipid peroxidation induced by exposure to *t*-BOOH

Lungs were exposed to 25 mM (wild type) or 15 mM (mutants) *t*-BOOH for 1 h followed by 2 h of perfusion with oxidant-free medium. Different concentrations of *t*-BOOH were used for wild type and mutant lungs to produce equal degrees of oxidant stress during the exposure period. Lung homogenate was analyzed for fluorescence of diphenyl-1-pyrenylphosphine (DPPP), an indicator for phospholipid hydroperoxides [122,163]. The increase of DPPP fluorescence indicates lipid peroxidation. Lungs were from wild type (red), Prdx6 null (blue), C47S Prdx6 knock-in (black), and D140A Prdx6 knock-in (green) mice. The C47S mice do not express the peroxidase activity of Prdx6; the D140A mice do not express the PLA₂ activity of Prdx6. Each point is mean for *n* = 3, the small SE is not shown. Modified and reprinted with permission from Ref. [132].

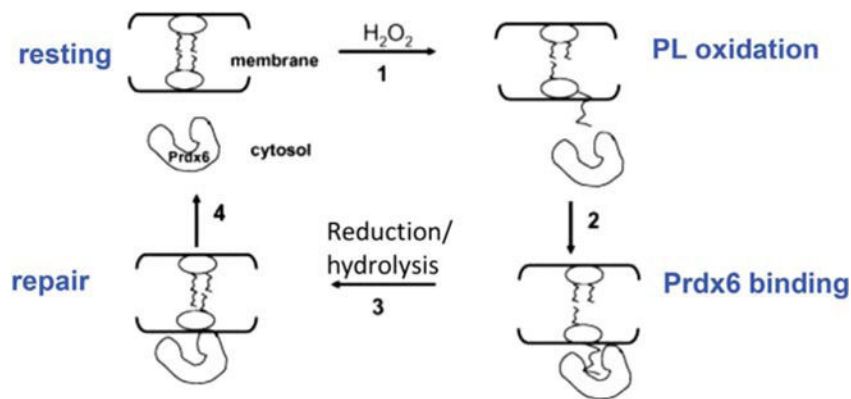


Fig. 8. Mechanism for repair of oxidized cell membranes

Prdx6 is cytosolic under normal resting conditions. Oxidation of a membrane phospholipid fatty acyl residue results in its increased hydrophilicity and its 'flotation' toward the membrane surface; interaction of Prdx6 with the oxidized lipid results in cell membrane association of the protein. Reduction and/or hydrolysis (plus acyl transferase) of the oxidized fatty acyl moiety 'repairs' the cell membrane phospholipid. Prdx6 then dissociates from the membrane to return to the resting state. Prdx6 exists as a dimer in the cytoplasm but only the peroxidatic monomer is shown. Modified from Ref. [33] and reprinted with permission.

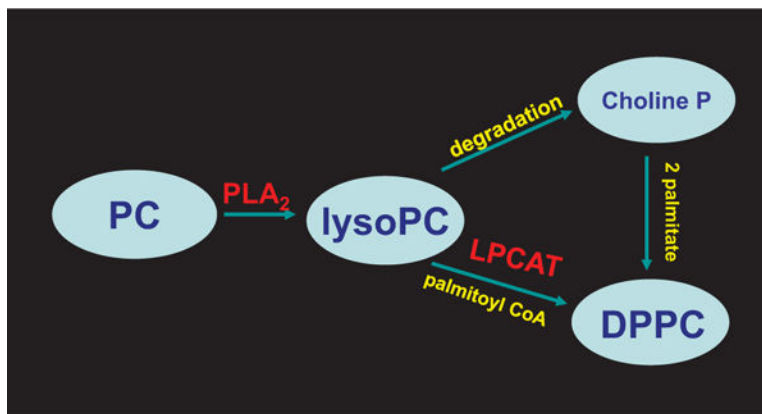


Fig. 9. Pathways for synthesis of dipalmitoyl phosphatidylcholine

These pathways are important for the generation of the major surface active component of the lung surfactant. Lysophosphatidylcholine (lysoPC) is produced by hydrolysis of the *sn*-2 acyl bond of phosphatidylcholine (PC) through the PLA₂ activity of Prdx6. LysoPC can be further degraded by various enzymes; the choline phosphate moiety is conserved and used along with two palmitates for DPPC synthesis by the *de novo* pathway. *sn*-1Palmitoyl lysoPC also can be re-acylated with a palmitoyl CoA by the LPCAT activity of Prdx6 (remodeling pathway) to generate DPPC.

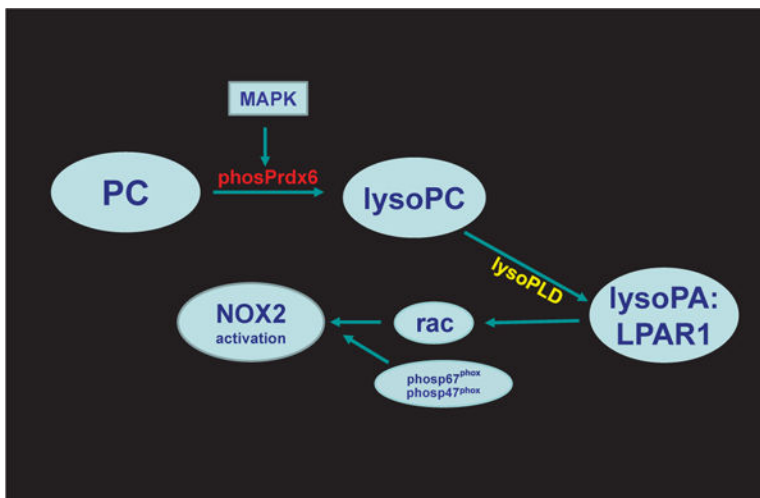


Fig. 10. Pathway for generation of lysophosphatidic acid and the activation of NADPH oxidase type 2 (NOX2)

With an appropriate signal, Prdx6 is phosphorylated via mitogen activated protein kinase (MAPK) activity and binds to phospholipid in the cell membrane. LysoPC that is generated from PC by the PLA₂ activity of Prdx6 can be metabolized via lysophospholipase D (lysoPLD) to lysophosphatidic acid (lysoPA). Interaction of lysoPA with its receptor (LPAR type 1) leads to release of rac (rac1 or 2 depending on cell type), one of the cytosolic protein co-factors required for NOX2 activation [146]. Other important co-factors for activation include phosphorylated p47^{phox} and phosphorylated p67^{phox}.

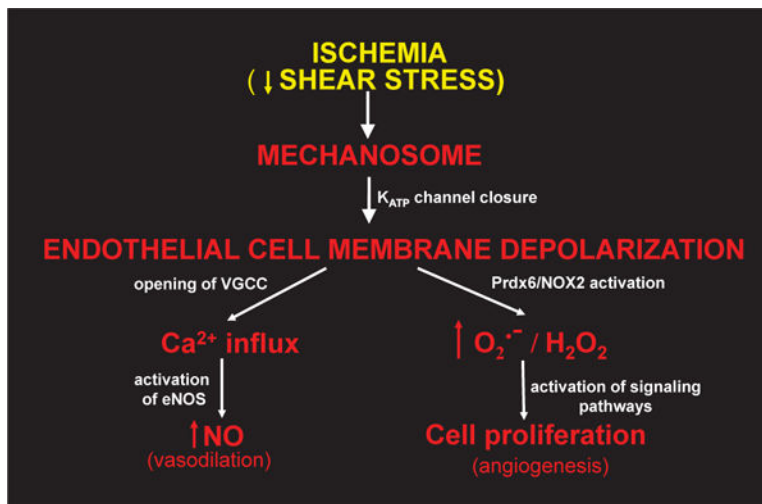


Fig. 11. Scheme for endothelial mechanotransduction

Decreased shear stress associated with stop of blood flow (ischemia) is sensed by the mechanosome leading to closure of inwardly rectifying K_{ATP} channels with resultant cell membrane depolarization, phosphorylation of Prdx6, and activation of NOX2 as shown in Fig. 10. The subsequent generation of O₂^{·-}/H₂O₂ results in release of angiogenic factors resulting in cellular proliferation and neovascularization. Cell membrane depolarization also leads to opening of voltage-gated Ca channels (VGCC) followed by Ca²⁺ influx into the cell and generation of the vasodilating agent NO via endothelial NO synthase (eNOS).