

Phospholipase A₂ of peroxiredoxin 6 has a critical role in tumor necrosis factor-induced apoptosis

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Peroxiredoxin 6 (Prdx6) is a bifunctional enzyme with peroxidase and phospholipase A₂ (PLA₂) activities. Although the cellular function of the peroxidase of Prdx6 has been well elucidated, the function of the PLA₂ of Prdx6 is largely unknown. Here, we report a novel function for the PLA₂ in regulating TNF-induced apoptosis through arachidonic acid (AA) release and interleukin-1 β (IL-1 β) production. Prdx6 knockdown (Prdx6^{KD}) in human bronchial epithelial cells (BEAS2B) shows severe decreases of peroxidase and PLA₂ activities. Surprisingly, Prdx6^{KD} cells are markedly resistant to apoptosis induced by TNF- α in the presence of cycloheximide, but are highly sensitive to hydrogen peroxide-induced apoptosis. Furthermore, the release of AA and the production of IL-1 β induced by proinflammatory stimuli, such as TNF- α , LPS, and poly I/C, are severely decreased in Prdx6^{KD} cells. More interestingly, the restoration of Prdx6 expression with wild-type Prdx6, but not PLA₂-mutant Prdx6 (S32A), in Prdx6^{KD} cells dramatically induces the recovery of TNF-induced apoptosis, AA release, and IL-1 β production, indicating specific roles for the PLA₂ activity of Prdx6. Our results provide new insights into the distinct roles of bifunctional Prdx6 with peroxidase and PLA₂ activities in oxidative stress-induced and TNF-induced apoptosis, respectively.

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Introduction

Peroxiredoxins (Prdxs) are a family of peroxidases that reduce hydrogen peroxide (H₂O₂) and alkyl hydroperoxides to water and alcohol, respectively.^{1,2} Prdxs are classified as either 1-Cys or 2-Cys Prdxs, based on whether the protein contains one or two conserved cysteine residues, respectively. In mammals, six members of the Prdx family have been described. Five of these (Prdx1, Prdx2, Prdx3, Prdx4, and Prdx5) are 2-Cys enzymes that use thioredoxin as the electron donor in their catalytic cycle.^{3,4} In contrast, Prdx6, the sole mammalian 1-Cys Prdx, does not use thioredoxin as a reductant. In addition to peroxidase activity, Prdx6 has Ca²⁺-independent phospholipase A₂ (iPLA₂) activity.⁵ The peroxidase activity of Prdx6 has been widely studied in cells and animal models for its antioxidant properties, which provides protection against the harmful consequences of oxidative stress.^{6–8} However, the PLA₂ activity of Prdx6 has not been studied as widely as the peroxidase activity.

In the classification based upon the Ca²⁺ requirement for their enzymatic activities, PLA₂s can be divided into three categories: secretory PLA₂s that require millimolar concentrations of Ca²⁺; cytosolic PLA₂s (cPLA₂s) that require micromolar concentrations of Ca²⁺; and iPLA₂s that do not require Ca²⁺ for their activity.^{9,10} The cellular functions of iPLA₂ are known to be responsible for phospholipid remodeling as a housekeeping function and for generation of arachidonic acid (AA) and lysophospholipid, which are closely

related to multicellular functions, such as cell proliferation, apoptosis, and inflammatory events.^{11–16} Although a recent report showed that Prdx6 interferes with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced death-inducing signaling complex formation by binding to the death effector domain of caspase,¹⁷ the inhibitory effect seemed to be independent of its enzymatic activity, that is, its peroxidase and iPLA₂ activity. Considering many functions of cellular iPLA₂, the iPLA₂ activity of Prdx6 may be also implicated in controlling cell fate in response to a variety of stimuli.

Prdx6 is expressed in all major mammalian organs, with the greatest protein expression levels in the lung.^{18,19} Within the lung, especially high expression is observed in type 2 alveolar epithelial cells and bronchiolar Clara cells, indicating preferential roles for Prdx6 in lung-resident cells. Based on this observation, we investigated the cellular functions of the PLA₂ of Prdx6 in human bronchial epithelial cell (BEAS-2B) by using cell-based loss-of-function and gain-of-function assays of Prdx6. We report distinct roles of bifunctional Prdx6 with peroxidase and PLA₂ activities in H₂O₂-induced oxidative stress and TNF-induced apoptosis, respectively.

Results

The Prdx6 knockdown BEAS-2B cells exhibit decreased peroxidase and iPLA₂ activities. To investigate the cellular functions of bifunctional Prdx6, we utilized short hairpin RNA

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Abbreviations: PLA₂, phospholipase A₂; iPLA₂, Ca²⁺-independent phospholipase A₂; Prdx6, peroxiredoxin 6; Prdx6^{KD}, Prdx6 knockdown; CHX, cycloheximide; AA, arachidonic acid; H₂O₂, hydrogen peroxide

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(shRNA) technology. A specific shRNA to Prdx6 was cloned into the pSUPER.retro.puro vector as described in Materials and Methods, and confirmed by sequencing (Supplementary Figure 1a). When the vector containing Prdx6shRNA was transfected into the BEAS-2B cells, the endogenous expression of Prdx6 was effectively suppressed in a dose-dependent manner (Figure 1a). To generate stable knockdown cells, retroviruses containing Prdx6shRNA were prepared and used to infect BEAS-2B cells. The cells were then selected in puromycin-containing medium. The Prdx6 knockdown (Prdx6^{KD}) cells revealed a decrease of Prdx6 expression of up to 70% compared with that of wild-type BEAS-2B (wt BEAS-2B; Figure 1b). In terms of morphology, the Prdx6^{KD} cells had fibroblast-like shape compared with wt BEAS-2B (Supplementary Figure 1b). The growth rate in Prdx6^{KD} cells was significantly lower than in wt BEAS-2B cells (Figure 1c). This seemed to be related to decreases in cell-cycle progression of G1/S and S/G2M transitions in Prdx6^{KD} cells (Supplementary Figure 1c). Microarray analysis comparing wt BEAS-2B with Prdx6^{KD} cells revealed downregulation of cell-cycle regulatory genes related to G1 progression, G1/S transition, or cell-cycle regulation (Table 1). This finding was partly consistent with previous observations that Prdx6 affects cell proliferation.^{11,15} We next evaluated peroxidase and iPLA₂ activities in Prdx6^{KD} cells. When the cells were exposed to different concentrations of H₂O₂, the H₂O₂-eliminating efficacy was significantly lower in Prdx6^{KD} cells compared

with that of wt BEAS-2B cells (Figure 1d), indicating an important role for the peroxidase of Prdx6 in the response to eliminate H₂O₂. Furthermore, the iPLA₂ activity was significantly decreased in Prdx6^{KD} cells compared with that in wt BEAS-2B (Figure 1e). The suppression of cellular PLA₂ activity resulted in significant downregulation of genes related to phospholipid metabolism in the Prdx6^{KD} cells (Supplementary Table 1). These results suggest that the stable knockdown of Prdx6 occurred successfully, and critically affected its bifunctional activity with peroxidase and PLA₂.

Prdx6^{KD} cells show strong resistance to TNF- α /cycloheximide-induced apoptosis, but not in H₂O₂-induced apoptosis. Prdx6 as an intracellular peroxidase has an important role in cellular protection in response to oxidative stress, especially stress by increased intracellular H₂O₂.^{6–8} We examined the functional response to H₂O₂-induced apoptosis in wt BEAS-2B and Prdx6^{KD} cells. At low concentrations of H₂O₂ (10–100 μ M), no significant differences in apoptotic cells, as defined by AnnexinV, could be detected in either cell line. At high concentrations of H₂O₂ (> 100 μ M), however, the percent of AnnexinV⁺ cells was markedly increased in Prdx6^{KD} cells compared with that in wt BEAS-2B cells (Figure 2a; open bars, wt BEAS-2B; closed bars, Prdx6^{KD}). To confirm whether the effect is specifically dependent on the peroxidase activity of Prdx6, wt Prdx6 or peroxidase-mutant (C47A) Prdx6 vector was

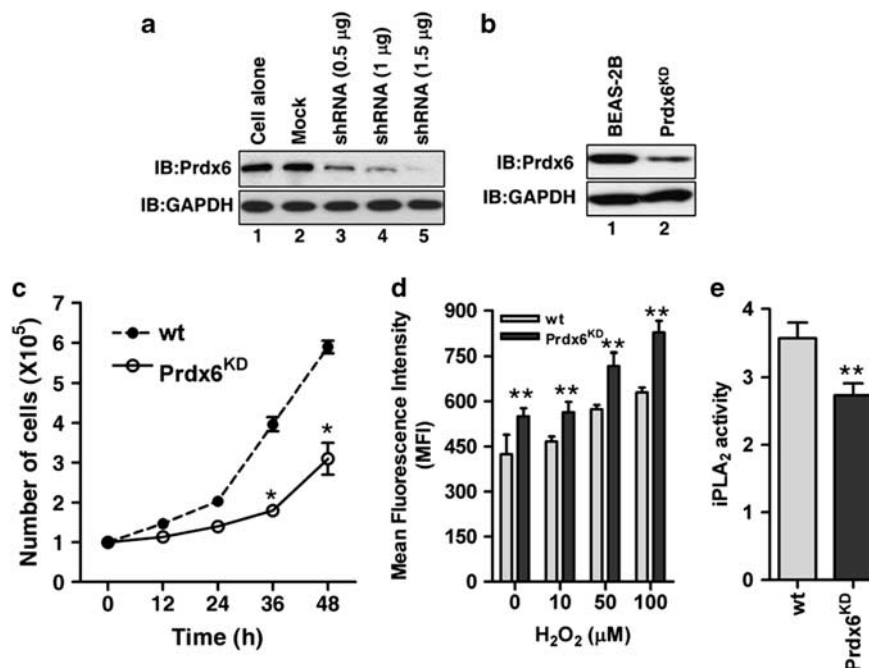


Figure 1 Prdx6^{KD} BEAS-2B cells exhibit decreases of peroxidase and PLA₂ activities. (a) Mock or pSuper.retro vector containing an shRNA specific for Prdx6 was transfected into BEAS-2B cells at different concentrations. The endogenous Prdx6 expression was evaluated. (b) Retroviruses containing an shRNA specific for Prdx6 were produced, as described in Materials and Methods. The cells were infected with the viruses and selected in puromycin-containing media for 30 days. The Prdx6-knockdown efficacy was examined. (c) In all, 1 × 10⁵ cells were plated into six wells and incubated for various times as indicated. At each different time, the cells were harvested and counted under trypan blue. (d) The wt BEAS-2B and Prdx6^{KD} cells were exposed to different concentrations of H₂O₂, as indicated, for 2 h. The intracellular level of H₂O₂ was analyzed by DCF stain, as described in Materials and Methods. (e) The iPLA₂ activities in wt BEAS-2B and Prdx6^{KD} cells were measured according to the manufacturer's recommendations, as described in Materials and Methods. Bars represent mean ± S.D. from at least three independent experiments. *P*-values were calculated using *t*-test versus wt BEAS-2B or Prdx6^{KD} cells (**P* < 0.001 and ***P* < 0.05)

Table 1 Deregulated genes related to cell cycle in wt BEAS-2B and Prdx6^{KD} cells

Target ID	Transcript ID	Symbol	Function in cell cycle	Fold change ^a
<i>Cell cycle-related genes</i>				
ILMN_1770085	NM_006763.2	BTG2	G1/S transition	-3.52
ILMN_1667081	NM_001759.2	CCND2	G1/S transition	-14.38
ILMN_2067656	NM_001759.2	CCND2	G1/S transition	-16.04
ILMN_2374425	NM_001238.1	CCNE1	G1/S transition	-2.25
ILMN_2412384	NM_024779.3	CCNE2	G1/S transition	-2.49
ILMN_1733396	NM_001789.2	CDC25A	G1/S transition	-2.24
ILMN_1802615	NM_001259.5	CDK6	G1 and G1/S transition	-3.12
ILMN_1784602	NM_078467.1	CDKN1A	G1 progression	-11.53
ILMN_1717294	NM_002829.2	PDPN3	Cell-cycle regulator	-2.73

Three independent illumina expression chip analyses were performed in wild-type BEAS2B and Prdx6^{KD} cells

^aFold change (Prdx6^{KD}/wt BEAS2B)

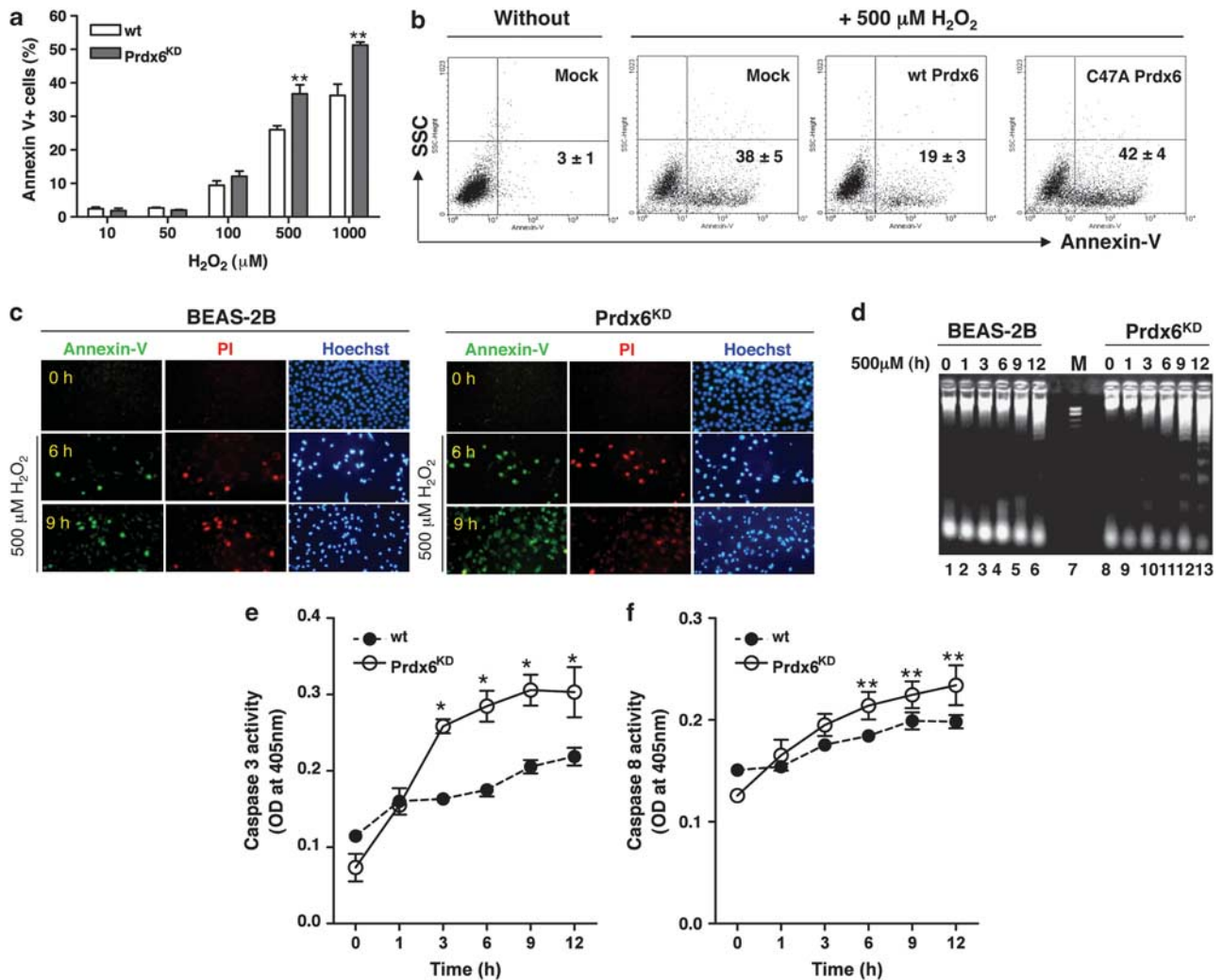


Figure 2 Prdx6^{KD} cells are highly susceptible to oxidative stress-induced apoptosis. (a) The wt BEAS-2B and Prdx6^{KD} cells were exposed to different concentrations of H₂O₂ for 24 h. The cells were stained for AnnexinV. The percentage of AnnexinV-positive cells was analyzed with the FACSCalibur system and determined with the CellQuest software. The results are expressed as mean ± S.D. for triplicate assays. (b) Prdx6^{KD} cells were transfected with mock, wt Prdx6, and peroxidase-mutant Prdx6 (C47A) cells. The cells were exposed to 500 μM H₂O₂ for 24 h, and then AnnexinV⁺ cells were analyzed by flow cytometry. Bars represent mean ± S.D. from at least three independent experiments. (c) The wt BEAS-2B and Prdx6^{KD} cells were exposed to 500 μM H₂O₂ for 24 h. The cells were stained with AnnexinV/PI/Hoechst 33342, as described in Materials and Methods, and visualized with a fluorescence microscope. (d) The wt BEAS-2B and Prdx6^{KD} cells were exposed to 500 μM H₂O₂ for various times as indicated. Cells were homogenized in 1 ml of lysis buffer. The genomic DNA extracts were prepared, as described in Materials and Methods, run on 1.8% agarose gels and visualized under UV illumination. (e and f) The wt BEAS-2B and Prdx6^{KD} cells were exposed to 500 μM H₂O₂ for different times as indicated. Caspase-3 (e) and caspase-8 (f) activities were measured using the CaspACE kit according to the manufacturer's instructions. The results are expressed as mean ± S.D. for triplicate assays. *P*-values were calculated using *t*-test versus wt BEAS-2B cells (**P* < 0.001 and ***P* < 0.05)

transfected into the Prdx6^{KD} cells. The cells were exposed to 500 μ M H₂O₂ and then analyzed for apoptosis. The H₂O₂-induced apoptosis was significantly reduced in wt Prdx6-overexpressing Prdx6^{KD} cells compared with that in peroxidase-mutant C47A Prdx6-overexpressing Prdx6^{KD} cells (Figure 2b: 19 \pm 3 versus 42 \pm 4%, respectively), indicating a specific role for the peroxidase of Prdx6 in response to H₂O₂. Moreover, similar results were confirmed in both immunofluorescence microscopy assay and the DNA-laddering assay (Figures 2c and d, respectively). We next assessed whether the apoptotic event is related to the caspase-induced pathway. When the cells were exposed to 500 μ M H₂O₂, enzymatic activation of caspase-3 and -8 was significantly increased in wt BEAS-2B cells in a time-dependent manner (Figures 2e and f: closed circles). Moreover, the activities were higher in Prdx6^{KD} than in wt BEAS-2B cells (Figures 2e and f: open circles). These results suggest that the peroxidase activity of Prdx6 is important for the regulation of intracellular H₂O₂ against H₂O₂-induced apoptosis.

We next addressed the cellular functions of the PLA₂ activity of Prdx6. Recent studies have shown that, among the 10 groups of PLA₂, the calcium-independent-type VIA cPLA₂, iPLA₂, has an important role in lipid remodeling.^{8–10} Activation of endogenous iPLA₂ during apoptosis contributes to exposure of the phospholipid antigen, lyso-PC, on the cell surface.¹³ Furthermore, a recent report has shown that Prdx6 modulates TRAIL signaling.¹⁷ These results suggest that PLA₂ activity of Prdx6 is related to TNFR-mediated apoptosis. To test the possibility, wt BEAS-2B and Prdx6^{KD} cells were treated with TNF- α /cycloheximide (CHX) for various times, and then apoptotic cells were assessed by flow cytometry after staining with AnnexinV. In wt BEAS-2B cells, AnnexinV⁺ apoptotic cells were greatly increased in a time-dependent manner (Figure 3a: open bars). Interestingly, the apoptotic cells in TNF- α /CHX-treated Prdx6^{KD} were markedly decreased compared with that in wt BEAS-2B cells (Figure 3a: closed bars). These results were confirmed in immunofluorescence microscopy assays and DNA-laddering assays (Figures 3b and c, respectively). In contrast to H₂O₂-induced apoptosis as shown in Figures 2e and f, enzymatic activation of caspase-3 and -8 was markedly decreased in TNF- α /CHX-treated Prdx6^{KD} cells in a time-dependent manner (Figures 3d and e: open circles) compared with that in TNF- α /CHX-treated wt BEAS-2B cells (Figures 3d and e: closed circles). In western blotting, significant results were consistently observed (Supplementary Figure 2). These results suggest that the peroxidase activity of Prdx6 may modulate TNF- α /CHX-induced apoptosis as a positive regulator.

PLA₂ activity of Prdx6 has a pivotal role in TNF- α /CHX-induced apoptosis. To gain more direct evidence of whether PLA₂ activity of Prdx6 is involved in TNF- α /CHX-induced apoptosis, we performed the rescue experiment in Prdx6^{KD} cells. The wt Prdx6 vector was transfected into the Prdx6^{KD} cells (Figure 4a). The wt BEAS-2B and Prdx6^{KD} cells transfected with Prdx6 wt vector were treated with TNF- α /CHX for various times. We found that the proteolytic processing of caspase-3 and degradation of caspase-8 were significant in both BEAS-2B and Prdx6^{KD} cells transfected

with Prdx6 wt (Figure 4b). When we measured their activities, enzymatic activation of caspase-3 and -8 was markedly increased in both cell lines (Figures 4c and d). In apoptotic analysis by AnnexinV staining, AnnexinV⁺ apoptotic cells were greatly increased in a time-dependent manner (Figure 4e: open bars, BEAS-2B + mock; closed bars, Prdx6^{KD} + Prdx6 wt). These results suggest that Prdx6 is involved in TNF- α /CHX-induced apoptosis as a positive regulator. We next explored whether the PLA₂ activity of Prdx6 is critical in apoptosis. The Prdx6^{KD} cells were transfected with mock, wt Prdx6, and PLA₂-mutant Prdx6 (S32A), and then apoptotic cells were analyzed after treatment with TNF- α /CHX for different times. The percent of apoptotic cells was significantly decreased in Prdx6^{KD} cells compared with that in wt BEAS-2B (Figure 5a: wt BEAS-2B and Figure 5b: Prdx6^{KD}). This finding was consistent with Figure 3. However, the reintroduction of wt Prdx6 to Prdx6^{KD} significantly increased apoptotic cells to a similar degree as in wt BEAS-2B (Figures 5a and c). More interestingly, no significant increases were detected in Prdx6^{KD} cells transfected with PLA₂-mutant Prdx6 (S32A; Figure 5d). These results strongly suggest that PLA₂ activity of Prdx6 has a pivotal role in TNF- α /CHX-induced apoptosis.

PLA₂ activity of Prdx6 regulates the release of AA for the synthesis of leukotriene in response to TNF- α stimulation and the production of interleukin-1 β (IL-1 β) in response to proinflammatory stimulation. Based on Figures 4 and 5, we asked how the PLA₂ activity of Prdx6 is associated with TNF- α /CHX-induced apoptosis. Previous reports have shown that the endogenous calcium-independent iPLA₂ is involved in apoptotic pathway mediated by AA that regulates the ceramide pathway or/and regulation of membrane integrity, such as the hydrolysis of plasma membrane phospholipids.^{20–23} Therefore, we assessed whether the PLA₂ activity of Prdx6 can regulate the release of AA for the synthesis of leukotriene in response to TNF- α stimulation. In response to stimulation with TNF- α , the production of AA was markedly increased in wt BEAS-2B cells, whereas a significant decrease was detected in Prdx6^{KD} cells (Figure 6a). The synthesis of leukotriene in response to TNF- α stimulation was also markedly attenuated in Prdx6^{KD} cells (Figure 6b). More interestingly, the level of leukotriene was dramatically elevated in both wt Prdx6-expressing BEAS-2B and Prdx6^{KD} cells (Figure 6c: closed bars). In contrast, no significant change was detected in both mutant Prdx6 (S32A)-expressing BEAS-2B and Prdx6^{KD} cells (Figure 6d: closed black bars). These results demonstrate that the PLA₂ activity of Prdx6 can regulate the release of AA for the synthesis of leukotriene in response to TNF- α stimulation, and may thereby modulate TNF/CHX-induced apoptosis by AA and inflammation by leukotriene derived from AA as depicted in Figure 8.

Cellular iPLA₂ activity is also involved in proinflammatory response regulating the production of IL-1 β via the modulation of caspase-1 activity.²⁴ In addition, it has been reported that the activation of the IL-1 β -converting enzyme family is likely to be one of the critical events of TNF-induced cytotoxicity.²⁵ We next examined the role of the PLA₂ activity in the production of IL-1 β in response to proinflammatory stimuli. When we measured the levels of IL-1 β and IL-6 in wt BEAS-2B and

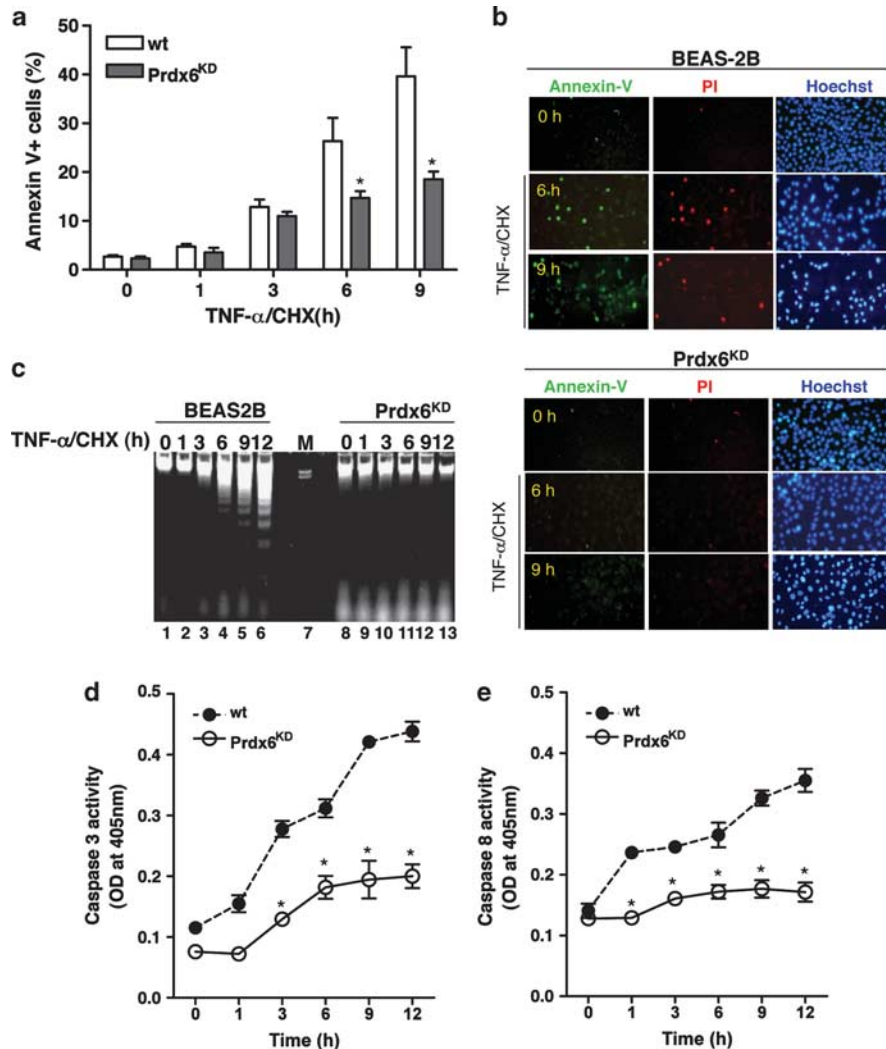


Figure 3 Prdx6^{KD} cells show strong resistance to TNF- α /CHX-induced apoptosis. (a) The wt BEAS-2B and Prdx6^{KD} cells were treated with 100 ng/ml TNF- α /3 μ M CHX for various times as indicated. The cells were stained for AnnexinV. The percentage of AnnexinV-positive cells was analyzed with the FACSCalibur system and determined with the CellQuest software. The results are expressed as mean \pm S.D. for triplicate assays. (b) The wt BEAS-2B and Prdx6^{KD} cells were treated with TNF- α /CHX for 9 h. The cells were stained with AnnexinV/PI/Hoechst 33342, as described in Materials and Methods, and visualized with a fluorescence microscope. (c) The wt BEAS-2B and Prdx6^{KD} cells were treated with TNF- α /CHX for various times as indicated. Cells were homogenized in 1 ml of lysis buffer. The genomic DNA extracts were prepared, as described in Materials and Methods, run on 1.8% agarose gels, and visualized under UV illumination. (d and e) The wt BEAS-2B and Prdx6^{KD} cells were treated with TNF- α /CHX for different times as indicated. Caspase-3 (d) and caspase-8 (e) activities were measured using the CaspACE kit according to the manufacturer's instructions. The results are expressed as mean \pm S.D. for triplicate assays. *P*-values were calculated using *t*-test versus wt BEAS-2B (**P* < 0.05)

Prdx6^{KD} cells after stimulation with TNF- α , LPS, poly I/C, and IL-1 β , the production of IL- β was severely attenuated in Prdx6^{KD} cells but not in wt BEAS-2B cells (Figure 7a). But no significant changes could be detected in the production of IL-6 in both cells (Figure 7b), indicating that Prdx6 may be not involved in the IL-6 production by proinflammatory stimuli. Furthermore, microarray analysis comparing wt BEAS-2B and Prdx6^{KD} cells revealed significant downregulation of cytokine-related genes, such as *CCL5*, *IL-13R α 2*, *IL-1 α* , and *IL-1 β* (Supplementary Table 1). These results indicate that PLA₂ activity of Prdx6 may be specifically involved in the production of IL-1 β . To validate the specificity of its PLA₂ activity, wt BEAS-2B and Prdx6^{KD} cells were transfected with wt Prdx6- or mutant Prdx6 (S32A)-expressing vector, and then the production of IL-1 β was measured after stimulation

with TNF- α , LPS, and poly I/C. The level of IL- β was significantly elevated in the wt Prdx6-overexpressing wt BEAS-2B and Prdx6^{KD} cells (Figures 7c, d, and e: dim bars), whereas no significant increases could be detected in mutant Prdx6 (S32A)-overexpressing wt BEAS-2B and Prdx6^{KD} cells (Figures 7c, d, and e: black bars). Taken together, these results suggest that the PLA₂ activity of Prdx6 specifically regulates the release of AA for the synthesis of leukotriene in response to TNF- α stimulation and the production of IL-1 β in response to proinflammatory stimulation.

Discussion

Tumor necrosis factor receptor-1 (TNFR1) can stimulate gene expression through the activation of transcription factors,

such as AP-1 and NF- κ B, that are involved in regulating the expression of inflammatory mediators and antiapoptotic proteins. In contrast, TNFR1-mediated signal can also induce apoptosis through the activation of caspase-8 and -3.^{26–28} Although many of the molecular details of the signaling pathway engaged by TNFR1 family molecules have been investigated, it is still unclear how TNF-induced activation of

caspases is linked to the activation of second messengers, which eventually lead to the wide variety of TNF-induced effects. At the molecular level, the recruitment of receptor-interacting protein and TNF receptor-associated factor into the death domain (DD) of TNFR1 is critical for the regulation of the expression of inflammatory mediators and antiapoptotic proteins via the activation of AP-1 and NF- κ B.^{27,28} On the other hand, associations of TRADD and FADD with DD of TNFR1 are involved in TNF- α -induced apoptosis.^{27,28} Additionally, it cannot be ruled out that other cellular proteins or mediators modulate the apoptotic cell death. One such protein is cellular PLA₂ enzyme. Previous reports have shown that TNFR1-induced apoptosis partly depends on the cellular activity of PLA₂ enzyme, which catalyzes the release of AA from the sn-2 position of membrane phospholipids, thereby inducing apoptotic cell death in a caspase-dependent manner.^{14,29,30} As an important mediator of endotoxin-induced vascular collapse and other inflammatory reactions, TNF- α can induce the activation of cellular PLA₂.^{30,31} PLA₂ activation is closely linked to the generation of AA, which is eventually involved in a signal transduction pathway resulting in cell death.^{29–31}

Our present study demonstrates for the first time that the PLA₂ activity of Prdx6 regulates the release of AA for the synthesis of leukotriene in response to TNF- α stimulation, and thereby is involved in TNF- α /CHX-induced apoptosis as a positive regulator in human bronchial epithelial cells, BEAS-2B. Among six Prdxs, Prdx6 is the sole mammalian 1-Cys Prdx and has a key role in the elimination of H₂O₂ in oxidative stress. In addition, Prdx6 has an iPLA₂ activity.⁵ The peroxidase activity of Prdx6 has been widely studied in cells and animal models for its antioxidant properties, which provides protection against the harmful consequences of oxidative stress.^{6–8} However, the iPLA₂ activity of Prdx6 remains poorly understood. We recently reported that Prdx6 is involved in H₂O₂-induced cellular toxicity through the hyperoxidation and upregulation of the iPLA₂ activity of Prdx6.⁶ Our current work suggests that Prdx6 has dual functions in apoptotic cell death that are dependent on its peroxidase and PLA₂ activities. The knockdown of Prdx6 rendered cells highly sensitive to H₂O₂-induced apoptosis but resistant to TNF- α /CHX-induced apoptosis. The latter event was dependent on the PLA₂ activity of Prdx6 when examined with the S32A PLA₂-mutant construct in Prdx6^{KD} cells. Moreover, the release of AA for the synthesis of leukotriene in response to TNF- α stimulation was severely defective in Prdx6^{KD}, whereas the release was markedly recovered in a gain-of-function study, indicating that the PLA₂ activity of Prdx6 may be closely linked to the AA pathway. Based on the results of the current study, we propose a possible model of cellular signaling and apoptotic cell death regulation by bifunctional Prdx6 (Figure 8). The peroxidase of Prdx6 exerts a protective effect on cellular toxicity against increased cellular H₂O₂

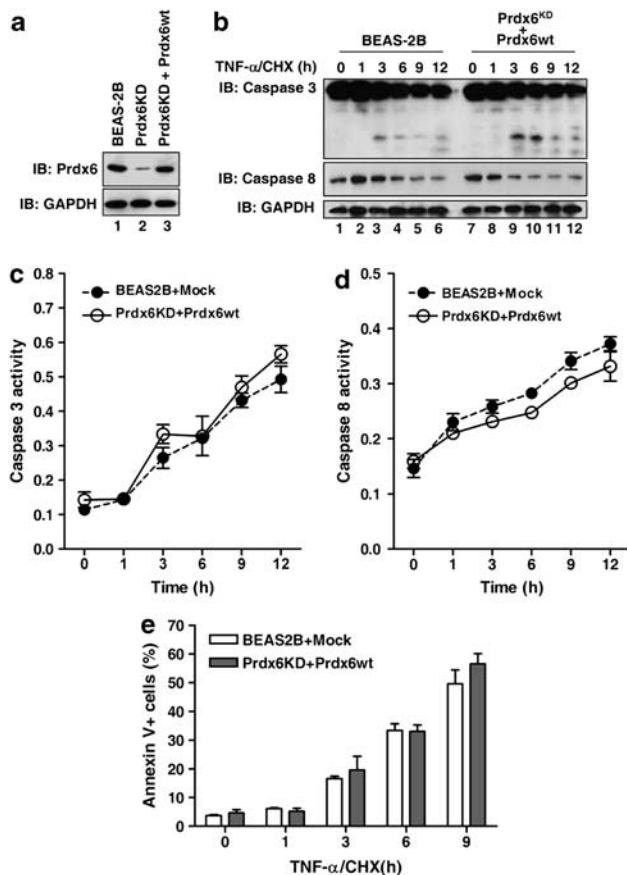
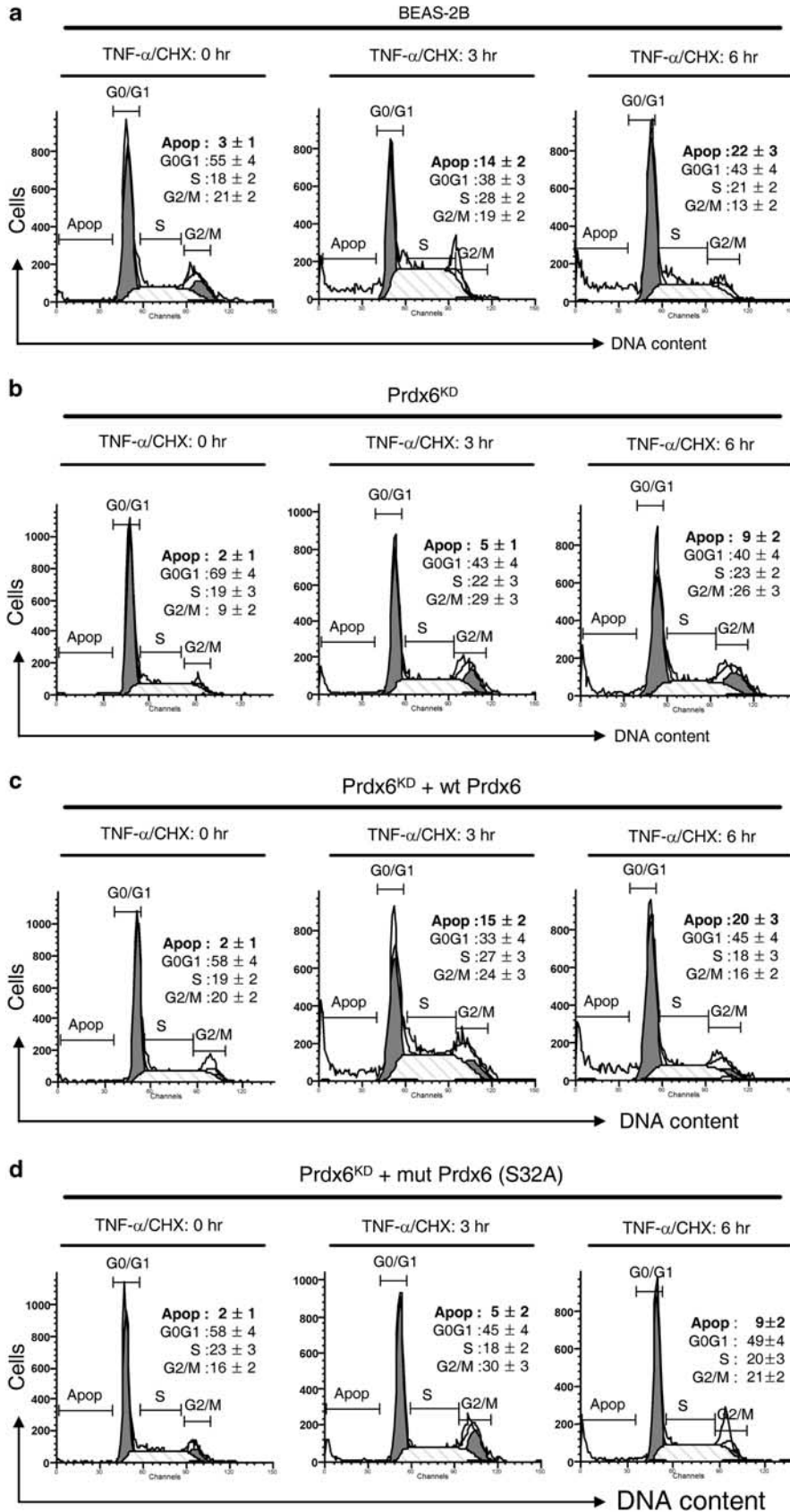


Figure 4 TNF- α /CHX-induced apoptosis was dramatic in Prdx6-overexpressing Prdx6^{KD} cells. (a) Prdx6 was transfected into Prdx6^{KD} cells. The expression of Prdx6 was compared in wt BEAS-2B, Prdx6^{KD}, and Prdx6-overexpressing Prdx6^{KD} cells with an antibody specific for Prdx6. (b) The wt BEAS-2B and Prdx6-overexpressing Prdx6^{KD} cells were treated with TNF- α /CHX for various times as indicated. The lysates were examined by western blotting using anti-caspase-3, anti-caspase-8, and anti-GAPDH. (c and d) The wt BEAS-2B and Prdx6-overexpressed Prdx6^{KD} cells were treated with TNF- α /CHX for different times as indicated. Caspase-3 (c) and caspase-8 (d) activities were measured using the CaspACE kit according to the manufacturer's instructions. The results are expressed as mean \pm S.D. for triplicate assays. (e) The wt BEAS-2B and Prdx6-overexpressed Prdx6^{KD} cells were treated with TNF- α /CHX for various times as indicated. The cells were stained for AnnexinV. The percentage of AnnexinV-positive cells was analyzed with the FACSCalibur system and determined with the CellQuest software. The results are expressed as mean \pm S.D. for triplicate assays

Figure 5 The PLA₂ activity of Prdx6 is essential for TNF- α /CHX-induced apoptosis. The wt Prdx6 and mutant Prdx6 (S32A) were expressed in Prdx6^{KD} cells. The wt BEAS-2B (a), Prdx6 (b), wt Prdx6-overexpressing Prdx6^{KD} (c), and mutant Prdx6 (S32A)-overexpressing Prdx6^{KD} cells (d) were treated with TNF- α /CHX for different times as indicated. Cells were washed with HBSS, stained with PI, as described in Materials and Methods, and analyzed with the FACSCalibur system. The cell-cycle distributions were determined with the Modfit LT 3.0 software. The results are expressed as mean \pm S.D. for triplicate assays



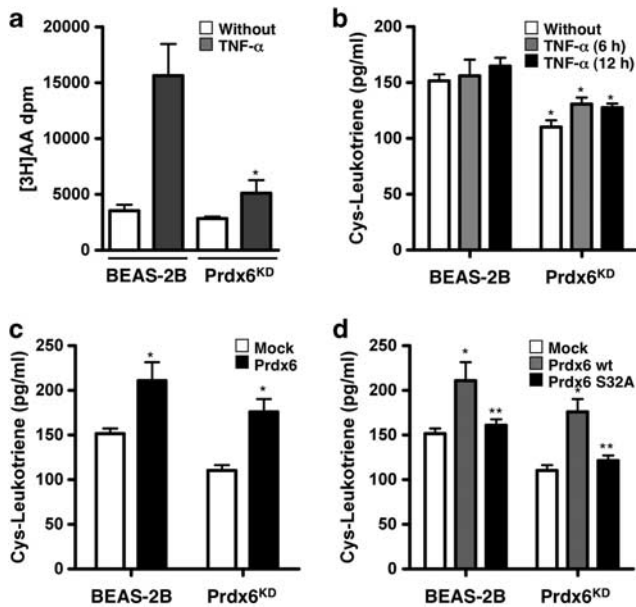


Figure 6 The PLA₂ activity of Prdx6 regulates the release of AA for the synthesis of leukotriene in response to TNF- α stimulation. (a) The wt BEAS-2B and Prdx6^{KD} cells were incorporated with [³H] AA and stimulated with TNF- α , as described in Materials and Methods. The supernatants were collected from three separate wells. The samples were chromatographed by HPLC and the elutions were measured for radioactivity. The results are expressed as mean \pm S.D. for triplicate assays. (b) The wt BEAS-2B and Prdx6^{KD} cells were stimulated with TNF- α for various times as indicated. The supernatants were collected, and then levels of cysteinyl leukotriene were measured with the Cysteinyl leukotriene Express EIA Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol. The results are expressed as mean \pm S.D. for triplicate assays (c) Mock or wt Prdx6 vector was transfected into wt BEAS-2B and Prdx6^{KD} cells. After 48 h, supernatants were collected, and then levels of cysteinyl leukotriene were measured. The results are expressed as mean \pm S.D. for triplicate assays. (d) Mock, wt Prdx6, and Prdx6 S32A-mutant vectors were transfected into wt BEAS-2B and Prdx6^{KD} cells. After 48 h, supernatants were collected, and then levels of cysteinyl leukotriene were measured. The results are expressed as mean \pm S.D. for triplicate assays. *P*-values were calculated using *t*-test versus wt BEAS-2B cells (**P* < 0.001 and ***P* < 0.05)

induced by oxidative stress. On the other hand, the PLA₂ activity of Prdx6 can induce the release of AA. The increased AA may be involved in either H₂O₂-mediated cellular signals through the regulation of NADPH oxidase activity or TNFR1-mediated apoptosis through the AA-induced apoptotic pathway.^{26–28,32–34}

The present results also indicated that PLA₂ activity of Prdx6 is involved in the proinflammatory response through the regulation of IL-1 β production. IL-1 β is a proinflammatory cytokine produced by macrophages in response to microbes or danger signals.^{24,35} IL-1 β acts synergistically with other cytokines in the orchestration of the host response and contributes to the development of inflammatory disease, fever, and septic shock. Although the production of IL-1 β is under tight and complex regulation, for simplicity it can be divided into two separate steps: induction of pro-IL-1 β and activation of caspase-1. Previous reports have shown that iPLA₂ has an important role in the activation of caspase-1 induced by proinflammatory stimuli.^{36,37} These results strongly suggest that the PLA₂ activity of Prdx6 may be involved in the proinflammatory response through the regulation

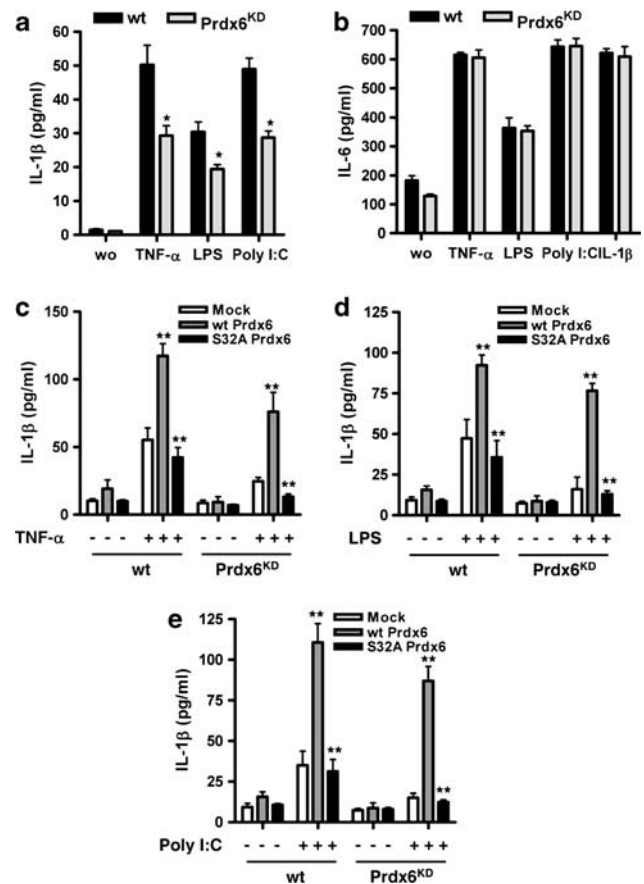


Figure 7 The PLA₂ activity of Prdx6 regulates the production of IL-1 β in response to proinflammatory stimulation. (a and b) The wt BEAS-2B and Prdx6^{KD} cells were stimulated with TNF- α , LPS, poly I/C, and IL-1 β for 12 h. Supernatants were collected, and then levels of IL-1 β (a) and IL-6 (b) were measured, as described in Materials and Methods. The results are expressed as mean \pm S.D. for triplicate assays. (c) Mock, wt Prdx6, and S32A Prdx6-mutant vectors were transfected into wt BEAS-2B and Prdx6^{KD} cells. The cells were stimulated with TNF- α , and then the level of IL-1 β was measured, as described in Materials and Methods. The results are expressed as mean \pm S.D. for triplicate assays. (d) Mock, wt Prdx6, and S32A Prdx6-mutant vectors were transfected into wt BEAS-2B and Prdx6^{KD} cells. The cells were stimulated with LPS, and then the level of IL-1 β was measured, as described in Materials and Methods. The results are expressed as mean \pm S.D. for triplicate assays. (e) Mock, wt Prdx6, and S32A Prdx6-mutant vectors were transfected into wt BEAS-2B and Prdx6^{KD} cells. The cells were stimulated with poly I/C, and then the level of IL-1 β was measured, as described in Materials and Methods. The results are expressed as mean \pm S.D. for triplicate assays. *P*-values were calculated using *t*-test versus wt BEAS-2B cells, mock-transfected, or wt Prdx6-transfected cells (**P* < 0.05 and ***P* < 0.001)

of IL-1 β production via the activation of caspase-1.²⁵ In our results, we found that the production of IL-1 β in response to TNF- α , LPS, and poly I/C was markedly attenuated in Prdx6^{KD} cells, whereas no significant changes were detected in the production of IL-6. Loss-of-function and gain-of-function studies using Prdx6^{KD} revealed that the production of IL-1 β was dependent on the PLA₂ activity of Prdx6: the IL-1 β production was dramatically recovered in wt Prdx6-expressing Prdx6^{KD} cells, but not in PLA₂-mutant (S32A) Prdx6-expressing Prdx6^{KD} cells.

The findings of this study are summarized in Figure 8. The bifunctional Prdx6 has an important role in the cellular

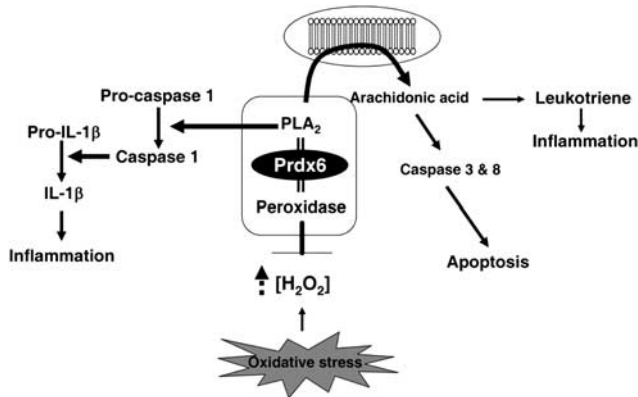


Figure 8 A possible model of cellular functions of bifunctional Prdx6 with peroxidase and PLA₂ activities. The peroxidase of Prdx6 acts to eliminate the increased intracellular H₂O₂ induced by a variety of stimuli and thereby has a key role in the protection against cellular damages in oxidative stress, as shown in the current work and many previous reports. Other cellular functions of Prdx6 may be involved in the TNF-induced apoptosis and the proinflammatory response. PLA₂ induces the release of AA, which is involved in the inflammatory response by its metabolic mediator or in apoptosis linked with a caspase-dependent pathway. In addition, PLA₂ induces the activation of IL-1 β -converting enzyme and caspase-1, and in turn regulates the production of IL-1 β . IL-1 β as a potential proinflammatory cytokine is involved in the inflammatory response

protection against oxidative stress. Under oxidative stress induced by a variety of cellular stimuli, the peroxidase of Prdx6 actively acts in stressful conditions to eliminate harmful H₂O₂. In addition, the iPLA₂ of Prdx6 can regulate cellular responses for TNF- α /CHX-induced apoptosis via regulation of AA release and IL-1 β production. However, the biological relevance of these putative cellular functions of the PLA₂ activity of Prdx6 is unclear at the moment, as the other dual functions regulating AA release and IL-1 β production are also complicated by proinflammatory responses as well as TNF-induced apoptosis. Although these related functions remain to be addressed, understanding the cellular functions of bifunctional Prdx6 with peroxidase and iPLA₂ activities may suggest strategies to manipulate the apoptotic pathway induced by either oxidative stress or TNFR1 and the inflammatory response for therapeutic purposes in diseases, such as cancer and inflammatory diseases.

Materials and Methods

Cell culture. BEAS-2B bronchial epithelial cells (derived from adenovirus 12-SV40-transformed normal human bronchial epithelium) were purchased from the American Type Culture Collection and maintained in 100-cm² tissue culture plates coated with LHC-9 medium containing fibronectin (Calbiochem, San Diego, CA, USA), vitrogen (Cohesion Technologies, Palo Alto, CA, USA), and 0.1% BSA (Biosource, Rockville, MD, USA). Cells were maintained in LHC-9 serum-free medium (Biosource) supplemented with penicillin–streptomycin (50 U/ml) in a 5% CO₂ incubator at 37 °C. Cells were utilized between passages 1 and 15 for all experiments. Prdx6-knockdown BEAS-2B cells (Prdx6^{KD}) were also maintained in the same medium containing 5 μ g/ml puromycin.

Construction of plasmid, mutagenesis, and shRNA. The human Prdx6 cDNA (GenBank accession number, NM_004905) was cloned from a human cervical cancer cell line, HeLa, into the pCDNA3.0 expression vector. Mutations were made using the MORPH plasmid DNA mutagenesis kit supplied by 5' \rightarrow 3' Inc. (Boulder, CO, USA). Cysteine at position 47 was replaced by alanine (C47A) and/or serine at position 32 was substituted by alanine (S32A). Mutagenesis experiments

were performed as described previously⁵. All mutants were verified by automated DNA sequencing. shRNA against human Prdx6 (5'-GCTGGTGCTGTGAGCCA GA-3') was produced from chemically synthesized DNA oligonucleotides that were cloned into the pSUPER.retro vector according to the manufacturer's instruction (OligoEngine, Seattle, WA, USA). DNA transfections were performed using FuGENE6 (Roche, Mannheim, Germany) or MP-100 micro-Porator (Digital Bio, Seoul, Korea) according to the respective manufacturer's instructions.

Generation of Prdx6-knockdown BEAS-2B cells. To generate retroviruses, packaging cells were plated at 2 \times 10⁶ cells per 10 cm plate at 24 h before FuGENE6 transfection with pSUPER.retro vector containing Prdx6shRNA. The medium was changed 24 h after transfection; at 48 h after transfection, the viral supernatant was collected, supplemented with polybrene (8 μ g/ml), and added to BEAS-2B cultures. At 48 h after addition of viral supernatant, BEAS-2B cells were resuspended in fresh medium containing puromycin at 1.5 μ g/ml and selected for approximately 3–4 weeks. Puromycin-resistant BEAS-2B cells were maintained in media containing 1.5 μ g/ml puromycin.

Immunoblotting. Cell lysates were resolved on SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. After blocking with 5% skim milk in TBS-T (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20), the membranes were probed with antibodies. The antibody–antigen complexes were detected using the ECL detection system (Amersham, Buckinghamshire, UK). An antibody to Prdx6 was purchased from Lab Frontier. Antibodies to GAPDH (Cell Signaling Technology, Beverly, MA, USA), caspase-3 (Cell Signaling Technology), and caspase 8 (Cell Signaling Technology) were used for western blot analysis.

Measurement of iPLA₂ activity and intracellular 2',7'-dichlorodihydrofluorescein staining. iPLA₂ activities were measured as described,⁶ according to the manufacturer's recommendations (Cayman Chemicals, Ann Arbor, MI, USA). The 2',7'-dichlorodihydrofluorescein (DCFH) staining was performed as described.³⁴ Briefly, cells were treated with different concentrations of H₂O₂ for 60 min. DCFH was added at a final concentration of 20 μ M and incubated for 30 min at 37 °C. The cells were washed once in phosphate-buffered saline (PBS) and maintained in 1 ml of medium. Cellular fluorescence was determined by flow cytometry (FACSCalibur, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

AnnexinV/propidium iodide/Hoechst 33342 staining. The immunostaining was performed with an Annexin-V-FLUOS staining kit (Roche Applied Sciences) according to the manufacturer's instructions. Briefly, cells were washed with PBS and incubated with AnnexinV/propidium iodide (PI)/Hoechst 33342 for 15 min at room temperature. Cells were visualized with a fluorescence microscope.

DNA fragmentation analysis. Cells were homogenized in 1 ml of lysis buffer (20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.5% SDS, and 0.5 mg/ml proteinase K) and incubated for 15 h at 42 °C under constant agitation. Proteins were then precipitated with 6 M NaCl and centrifuged at 2500 \times g at 4 °C for 15 min. Supernatants containing genomic DNA were then treated with RNase A at 37 °C for 30 min. The genomic DNA was precipitated for 3 h at –70 °C with 2.5 volumes of 100% ethanol and 0.2 volume of 3 M sodium acetate. Samples were then centrifuged at 20 800 \times g at 4 °C for 30 min. The resulting pellets were washed with 70% ethanol and resuspended in 40 μ l of nuclease-free water. Genomic DNA extracts (10–20 μ l) were run on 1.8% agarose gels and visualized under UV illumination.

Caspase-3 and -8 activity measurement. Caspase-3 and -8 activities were measured using the CaspACE kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, cells were collected by trypsinization, followed by centrifugation at 470 \times g at 4 °C for 10 min. The cell pellet was washed with cold PBS, centrifuged at 470 \times g at 4 °C for 10 min, and then resuspended in 40 μ l of lysis buffer. After three rapid freeze–thaw cycles, the lysate was incubated on ice for 15 min and then centrifuged at 15 000 \times g at 4 °C for 20 min. The protein concentration in the supernatant was determined by the Bradford assay, and 75 μ g of proteins was incubated with the caspase-3 substrate at 37 °C for 4 h. The absorbance of the reaction was then spectrophotometrically measured at 414 nm.

Cell cycle and apoptosis analysis. Cells were treated with TNF- α /CHX for various times and washed with HBSS. Cells were harvested by trypsinization,

washed twice with PBS, resuspended in a fluorochrome-staining solution (3.8 mM sodium citrate, 0.05 mg/ml PI, 0.1% Triton X-100, and 7 Kunitz units/ml RNase B), and incubated on ice for 3 h or kept at 4 °C for up to 2 weeks before flow cytometric analysis. The cell cycle was analyzed with the FACSCalibur system and determined with the CellQuest software and Modfit LT 3.0 software (Becton Dickinson, San Jose, CA, USA).

[³H] Arachidonic release assay. The [³H] arachidonic release assay was performed as described.^{38–40} Briefly, BEAS-2B and Prdx6-knockdown BEAS-2B cells (Prdx6^{KD}) cells were cultured in LHC-9 medium containing fibronectin. The [³H] AA (final concentration 1 μCi/ml, American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) was added to medium and incubated with cells (18 h, 37 °C). After repeated washing with media, fresh media were added to each plate. Subsequently, the cells were stimulated with or without 20 ng/ml TNF-α for 12 h. Following incubation with TNF-α, the supernatants collected from three separate plates were prepared for subsequent high-pressure liquid chromatography (HPLC) analysis. The samples were extracted by octadecylsilane C18 cartridges (Sep-Pak C18; Waters Associates, Milford, MA, USA) and chromatographed by reversed-phase HPLC as previously described.^{38–40} The AA fraction of HPLC elution was collected and measured for radioactivity.

Measurement of cysteinyl leukotriene levels. The samples were immediately centrifuged at 10 000 g for 10 min to remove cellular debris. The supernatant was removed and stored at –70 °C until analysis. The cysteinyl leukotriene concentration was measured by ACE Enzyme Immunoassay Kit according to the manufacturer's protocol (Cayman Chemical, Ann Arbor, MI, USA).

Measurement of cytokines. Levels of IL-1β and IL-6 were measured in the supernatants derived from wt BEAS-2B, Prdx6^{KD} wt Prdx6-overexpressing BEAS-2B, wt Prdx6-overexpressing Prdx6^{KD} S32A-mutant Prdx6-overexpressing BEAS-2B, S32A-mutant Prdx6-overexpressing Prdx6^{KD}, and cells stimulated with TNF-α, LPS, poly I/C, and IL-1β according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA).

Microarray analysis. Experimental procedures for microarray were performed according to the Marogen Illumina BeadStation 500 × manual. Briefly, biotinylated cRNA were made by Illumina Amplification Kit (Ambion Inc., San Diego, CA, USA) and prepared by RNeasy kit (Qiagen, Valencia, CA, USA). After hybridization into Sentrix HumanRef-8 Expression BeadChip (Illumina Inc., San Diego, CA, USA), the chip was washed according to the manual. The data were analyzed by a BeadStudio program provided by Illumina Inc. (detection *P*-value, < 0.05).

Statistical analysis. Data are expressed as mean ± S.D. Statistical comparisons between groups were performed using one-way analysis of variance by the Student's *t*-test. Probabilities of *P* < 0.05 or *P* < 0.001 were considered statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)