Variable overoxidation of peroxiredoxins in human lung cells in severe oxidative stress

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Lehtonen, Siri T., Piia M. H. Markkanen, Mirva Peltoniemi, Sang Won Kang, and Vuokko L. Kinnula. Variable overoxidation of peroxiredoxins in human lung cells in severe oxidative stress. Am J Physiol Lung Cell Mol Physiol 288: L997–L1001, 2005. First published December 30, 2004; doi:10.1152/ajplung.00432.2004.—Peroxiredoxins (Prxs) are a group of thiol containing proteins that participate both in signal transduction and in the breakdown of hydrogen peroxide (H₂O₂) during oxidative stress. Six distinct Prxs have been characterized in human cells (Prxs I–VI). Prx I–IV form dimers held together by disulfide bonds, Prx V forms intramolecular bond, but the mechanism of Prx VI, so-called 1-Cys Prx, is still unclear. Here we describe the regulation of all six Prxs in cultured human lung A549 and BEAS-2B cells. The cells were exposed to variable concentrations of H₂O₂, menadione, tumor necrosis factor-α or transforming growth factor-β. To evoke glutathione depletion, the cells were furthermore treated with buthionine sulfoximine. Only high concentrations (300 μM) of H₂O₂ caused a minor increase (28%, 4 h) in the expression of Prxs I, IV, and VI. Severe oxidant stress (250–500 μM H₂O₂) caused a significant increase in the proportion of the monomeric forms of Prxs I–IV; this was reversible at lower H₂O₂ concentrations (≤ 250 μM). This recovery of Prx overoxidation differed among the various Prxs; Prx I was recovered within 24 h, but recovery required 48 h for Prx III. Overall, Prxs are not significantly modulated by mild oxidant stress or cytokines, but there is variable, though reversible, overoxidation in these proteins during severe oxidant exposure.

peroxiredoxin; lung; oxidant; antioxidant; hydrogen peroxide

LUNG REPRESENTS A UNIQUE TISSUE as it is directly exposed to high oxygen tension and oxidants. Reactive oxygen species (ROS) that are also produced as byproducts of cellular metabolism, are toxic, but at low concentrations they are known to function as messengers in signal transduction processes. Due to ROS toxicity, cells and tissues have developed several enzymatic systems that regulate the concentration of these species inside and outside of the cells. The major ROS decomposing enzymes generally sensitizes cells to oxidant-mediated cell injury and that overexpression of these enzymes confers significant protection (11, 27, 34–36). The regulation of the major antioxidant enzymes in human lung cells is well documented, MnSOD representing an enzyme with highest inducibility (20). In contrast, the significance and regulation of the Prx family of proteins in lung cells are unknown.

Human Prxs comprise a family of six enzymes (Prxs I–VI) that degrade hydrogen peroxide (H₂O₂) using their thiol groups of cysteines (Cys) as catalytical centers (3, 4, 14). Prxs I–IV are called typical 2-Cys Prxs. Disulfide bonds formed in their reactions can be reduced by Trx, which in turn receives its reducing power from NADPH by Trx reductase. Prx V is considered as an atypical 2-Cys Prx, as it forms an intramolecular disulfide bridge, which is also reduced by Trx. Prx VI belongs to a group of 1-Cys Prxs and its physiological reducing agent is unknown. In animal models and/or cultured cells, overexpression of Prxs has been shown to provide significant protection against hyperoxia and the deficiency of these enzymes can enhance lipid peroxidation and apoptosis (1, 29, 30, 37, 38, 42, 43).

Several proteins of the Prx family are highly overexpressed in human lung malignancies (15, 22, 25). Prx I has been found to be somewhat increased in ovalbumin-induced allergic inflammation, whereas no major changes in Prxs I–VI were detected in the inflammatory/granulomatous states of human parenchyma (10, 21). In rat and baboon lungs, Prxs I, II, and/or VI have been found to be differentially regulated during lung development and hyperoxia such that the levels of Prx I and Prx VI mRNA increase after birth and are elevated by hyperoxia (9, 17, 19). Prxs can be inactivated by overoxidation on the catalytical cysteine caused by H₂O₂ (7, 32, 40) or by cell cycle dependent phosphorylation (5). Oxidized Prx VI has also been found to interact with other compounds containing thiol groups, for example with glutathione (28), which is evidence of the complex interactions between Prxs with other enzymes regulating cellular redox state and defense in oxidant exposure.

This study was undertaken to obtain an accurate insight into the regulation of all six Prxs in human lung cells. The regulation of Prxs was also compared with that of MnSOD, an enzyme known to be highly inducible by cytokines such as tumor necrosis factor (TNF)-α and downregulated by growth factors such as transforming growth factor (TGF)-β (31, 33). The regulation of the six Prxs was investigated by using two studies have shown that the lack of the major antioxidant enzymes generally sensitizes cells to oxidant-mediated cell injury and that overexpression of these enzymes confers significant protection (11, 27, 34–36). The regulation of the major antioxidant enzymes in human lung cells is well documented, MnSOD representing an enzyme with highest inducibility (20). In contrast, the significance and regulation of the Prx family of proteins in lung cells are unknown.

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well-characterized human lung cell lines, A549 cells and BEAS-2B cells. One special feature of the A549 cells is their very low level of Prx II (21). We assessed alterations in the Prxs at the transcriptional, translational, and posttranslational levels after exposing cells to H₂O₂, menadione, TNF-α, TGF-β₁, or to L-buthionine-[S,R]-sulfoximine (BSO), a compound that is known to increase oxidant stress by depleting intracellular glutathione by γGCL inhibition. In addition to being the major cytokines in human lung disorders, TNF-α and TGF-β₁ are also known to evoke ROS production inside the cells.

**MATERIALS AND METHODS**

**Cell cultures and treatments.** Two well-characterized human airway epithelial cell lines were used, A549 and BEAS-2B cells (American Type Culture Collection, Manassas, VA). A549 cells are malignant cells originating from human alveolar type II pneumocytes and BEAS-2B cells are SV40 transformed human nonmalignant airway epithelial cells. A549 cells were cultured in Ham’s F-12 medium (GIBCO Invitrogen, Carlsbad, CA) supplemented with fetal bovine serum, and BEAS-2B cells were cultured in bronchial epithelial cell growth medium (Cytotech ApS, Hellebæk, Denmark). Cells were exposed to 5–500 μM H₂O₂, 10 ng/ml TNF-α, 2 ng/ml TGF-β₁, 10 μM menadione, or 0.5 mM BSO (all from Sigma-Aldrich Chemie). For posttranslational state analysis a 30-min pulse of H₂O₂ was given after which the medium was replaced with fresh medium. During the other exposures, the medium was not replaced. Each set of exposures was performed independently at least twice. The cells were counted after the exposures.

**RT-PCR.** Total RNA was isolated from cultured A549 cells by RNeasy Mini Kit (Qiagen, Westburg, Netherlands), or messenger RNA was isolated by Quick Prep Micro mRNA Purification Kit (Amersham Pharmacia Biotech Europe, Freiburg, Germany). RT-PCR was performed as described earlier (25). The primers used and the expected sizes of PCR products are shown in Table 1.

**Quantitative RT-PCR.** The preparation of the controls for Prx IV and VI quantitative RT-PCR has been described earlier (25). Controls for Prx I–III and V were prepared in a similar manner, based on modified PCR amplification according to Jin and coworkers (12). Shortened PCR products were subcloned into a pGEM3Zf(+) vector (Promega, Madison, WI), and RNA was produced in vitro from purified plasmids by Riboprobes System-T7 (Promega). Competitive controls contained a deletion of 60–125 bp but did contain intact binding sites for the PCR primers used. Sizes of the shortened PCR products are shown in Table 1.

### Table 1. RT-PCR primers and sizes of the PCR products for peroxiredoxins

<table>
<thead>
<tr>
<th>cDNA</th>
<th>AS or S</th>
<th>Primer</th>
<th>RT-PCR Product, bp</th>
<th>Competitor Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prx I</td>
<td>AS</td>
<td>CAGCCCGACGCTGCTATTGCC</td>
<td>295</td>
<td>214</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>CAGACCGGAGACGCGGATTTGC</td>
<td>373</td>
<td>316</td>
</tr>
<tr>
<td>Prx II</td>
<td>AS</td>
<td>CTGCCGAAGAACCTTTGCATC</td>
<td>352</td>
<td>227</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>GGCACAAGGTTGTTTGATGCGG</td>
<td>328</td>
<td>253</td>
</tr>
<tr>
<td>Prx III</td>
<td>AS</td>
<td>CTGCCGTATTATATACGGCAACGATGGGG</td>
<td>296</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>GCCGGACGTGCCGCCGTTATCCG</td>
<td>329</td>
<td>259</td>
</tr>
</tbody>
</table>

Peroxiredoxin; AS, antisense; S, sense.

**Western analysis.** Collected cells were sonicated, and protein was measured by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) after clearing of cell lysate by centrifugation. Proper amount of protein was analyzed by standard or nonreducing SDS-PAGE and blotted onto membranes, which were stained by anti-Prx antibodies as described previously (21, 22, 25). Antibody against β-actin (Sigma-Aldrich Chemie) was used to assure equal loading of proteins. Anti-MnSOD (generous gift from Professor J. D. Crapo, National Jewish Medical and Research Center, Denver, CO) was used as a positive control for TNF-α induction.

**RESULTS**

All six Prxs were clearly expressed in both BEAS-2B and A549 cells, but Prx II expression was very weak in A549 cells. Exposure of BEAS-2B cells to 300 μM H₂O₂ increased the expressions of Prx I, Prx IV, and Prx VI (27, 28, and 19% in 4 h, respectively) at the protein level, but no effect was found at lower H₂O₂ concentrations (Fig. 1). The analysis at the transcriptional level showed no induction of any of these Prxs in A549 cells exposed to 100 μM H₂O₂ (<5%, Fig. 2). Exposure to TNF-α, TGF-β₁, BSO, or to a sublethal menadione concentration did not affect Prx expression at the protein level.
level in either cell line (Fig. 3), nor did these exposures alter the mRNA level in A549 cells (data not shown). The expressions of all Prxs in both cell lines were analyzed at four to six time points from 1 to 72 h. Figure 3 shows a representative analysis of BEAS-2B cells after the 24-h exposures. MnSOD, of all antioxidant enzymes, is unique as it is highly inducible by cytokines, but its levels remain relatively constant during oxidant exposure of lung cells in vitro (20, 23). In agreement, MnSOD expression was increased by TNF-α (4.8-fold in 24 h, Fig. 3) but was not significantly changed by direct oxidant exposure in vitro.

The Prx oxidation state was assessed by nonreducing SDS-PAGE, which leaves disulfide bridges intact. Under normal conditions, the bands for dimeric and monomeric forms of Prxs I, III, and IV and two bands for Prx VI could be detected (Fig. 4). Exposure to H2O2 caused a clearly visible and immediate increase in the monomeric forms of Prxs I, III, and IV. This overoxidation could be seen only at high, toxic (>250 μM) concentrations. As expected, this phenomenon could not be observed for Prx VI, which is 1-Cys Prx. The oxidation of Prx II could not be assessed in A549 cells due to low expression. When the cells were exposed to lower, but still severe oxidant stress (250 μM H2O2), the oxidation of the Prxs was reversible. Prx I recovered during the first 24 h but 48 h was required for recovery of Prx III (Fig. 5). Low, sublethal (5–100 μM) concentration of H2O2 did not cause any detectable overoxidation (not shown).

The effect of each exposure on cell growth and survival was estimated by counting the cells at different time points in each experiment (Fig. 6). These studies confirmed the toxicity of oxidant exposure of lung cells in vitro (20, 23). In agreement, MnSOD expression was increased by TNF-α (4.8-fold in 24 h, Fig. 3) but was not significantly changed by direct oxidant exposure in vitro.
those higher H$_2$O$_2$ concentrations that were associated with Prx oxidation.

DISCUSSION

This study shows that low, sublethal levels of H$_2$O$_2$, menadione, TNF-α, TGF-β, or BSO did not cause significant induction or downregulation of any of the Prxs in human lung cells. The response did not differ in two lung cell lines investigated. In contrast, severe oxidant stress caused remarkable, but transient, overoxidation of Prxs with variable recovery.

There are several in vivo and in vitro studies on the induction of different Prxs by oxidative stress and cytokines such as TNF-α, but very few experiments have been conducted with human cells. In animal studies, the levels of Prxs I and VI have been shown to increase after birth and/or by hyperoxia (17–19), and a modest induction of Prx VI has been observed in rat lung L2 epithelial cells after severe oxidant stress (18). On the other hand, in vitro studies on mouse Raw264.7 macrophage cells and HeLa cells have shown that oxidative stress evoked by H$_2$O$_2$ does not cause any Prx I–III induction (40, 41). However, some other studies have reported that H$_2$O$_2$ exposure can induce Prx I but not Prx II in FRTL-5 thyroid cells (16), whereas in human gastric carcinoma SNU638 cells Prx II was induced by H$_2$O$_2$ (8). Also in Jurkat T-cell lymphoma cells exposure of 75 μM tert-butyl hydroperoxide caused Prx II but not Prx I, III, or VI induction (32). In our study no major changes in the expression of any Prx could be found after mild to moderate oxidant stress at the level of mRNA or immunoreactive protein. It is likely that Prxs are already highly expressed in cells at ambient oxygen tension, a phenomenon suggested also by studies on the developmental expressions of various Prxs in the lung and their induction after delivery. Minor induction of some Prxs was observed at high H$_2$O$_2$ concentrations, but as described for A549 cells (39), this kind of change may also be related to a selection of certain cell subpopulation under toxic conditions instead of real induction. We used two different human cell lines, BEAS-2B cells, which are SV40 transformed bronchial epithelial cells, and A549 cells, which are malignant alveolar epithelial cells. The extrapolation of these results to the situation in vivo and to human airway cells in primary culture may be difficult. Also human airway cells change significantly within the first days in culture (24). Overall, our results and very minor induction of Prxs in both studied cell lines are in full agreement with the recent study showing minor, if any, change of Prxs in the airways of human inflammatory/granulomatous parenchymal lung disease (sarcoidosis) in vivo (21).

Both TNF-α and TGF-β are known to induce H$_2$O$_2$ production, and it is also known that Prxs participate in H$_2$O$_2$ breakdown (14). We, however, could not detect any effect by TNF-α or TGF-β exposure on Prx expression patterns in either cell line investigated especially compared with the magnitude of MnSOD induction. This does not exclude the possibility that Prxs can break down H$_2$O$_2$ generated by TNF-α and TGF-β, but obviously this occurs without causing significant inactivation by overoxidation. The unchanged levels of Prxs in the inflammatory parenchymal lung disease, sarcoidosis, also is evidence of a minor role of cytokines in evoking Prx induction.

Normal lung cells are well buffered against exogenous oxidants. Prxs constitute a potent defense mechanism in the maintenance of the redox balance both in normal conditions and under oxidant stress. Oxidant stress has already been found to cause Prx overoxidation where the active-site cysteine is selectively oxidized, which then leads to the inactivation of Prx peroxidase activity (7, 40). In line with these concepts, Prx I, III, and IV were oxidized in A549 cells by severe acute oxidant exposure at high H$_2$O$_2$ concentration, which also caused a minor induction of Prx expression. Importantly, there appeared to be a rapid but variable recovery in the oxidation state of Prxs even in severe oxidant stress, with the fastest recovery being observed for Prx I. Recent studies show that this recovery of overoxidation is catalyzed by sulfiredoxin (2, 6). A high H$_2$O$_2$ level also caused an apparent loss of the monomeric form of Prx IV without any clear increase in the other size bands. These high oxygen concentrations may occur locally in the inflammatory states of human lung especially when combined to exogenous oxidant stress (one example being cigarette smoke). Our results suggest that massive overoxidation of Prxs takes place only under very extreme conditions when cell damage is evident.

The importance of Prxs as antioxidants has been a matter of intense debate. According to our studies, these enzymes are not markedly induced by cytokines or oxidants especially if compared with the severalfold increase seen in the level of another antioxidant enzyme, MnSOD, occurring after TNF-α treatment. This study points to a prominent role for Prxs as buffers against oxidative damage of H$_2$O$_2$ as their oxidation state rapidly responded to H$_2$O$_2$ addition. Not only may this be a mechanism that protects other cellular molecules, but it may even be a crucial step in the regulation of cellular signaling cascades, processes like apoptosis and lung injury. The clinical significance of the oxidation of Prxs needs to be investigated in future studies.

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GRANTS

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REFERENCES

6. Chang TS, Jeong W, Woo HA, Lee SM, Park S, and Rhee SG. Characterization of mammalian sulfiredoxin and its reactivation of hyper-