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Forum News & Views

Does the Oxidative Stress in Chronic Obstructive Pulmonary Disease Cause Thioredoxin/Peroxiredoxin Oxidation?

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ABSTRACT

The thioredoxin/peroxiredoxin system comprises a redox-regulated antioxidant family in human lung; its significance, regulation, or oxidation has not been evaluated in smoking-related lung diseases. Here, we present the expression of the thioredoxin/peroxiredoxin system in lung biopsies from normal lung ($n = 14$), smokers ($n = 21$), and patients with chronic obstructive pulmonary disease (COPD, $n = 38$), and assess the possible inactivation/oxidation of this system by nonreducing Western blotting, two-dimensional gel electrophoresis, and mass spectrometry. Our study shows that the thiol status of the Trx/Prx-system can be modulated *in vitro*, but it appears to have high resistance against the oxidative stress in COPD. *Antioxid. Redox Signal.* 10, 813–819.

INTRODUCTION

THE MOST IMPORTANT RISK FACTOR for chronic obstructive pulmonary disease (COPD) is cigarette smoke, which contains not only free radicals but also evokes the accumulation of reactive oxygen species (ROS) by activating inflammatory cells such as neutrophils and macrophages in the lung (4, 23). The major contributor to the development of the damage is, however, the balance between increased oxidant generation and antioxidant defense (13) that may also explain why only a proportion of smokers develop COPD.

Primary attention for the importance of ROS detoxifying enzymes in human lung and lung diseases has been focused on glutathione metabolism and on the major antioxidant enzymes, such as superoxide dismutases and gamma-glutamylcysteine synthetase (glutamate cysteine ligase) (13, 24). The levels of these enzymes have been shown to be unchanged, increased, or declined in smoker's lung, also depending on COPD severity (8, 9, 24). Recent studies have, however, shown that human lung also possesses several other enzymes such as thioredoxins (Trxs) and peroxiredoxins (Prxs) (14, 25) that can offer potential protection against exogenous oxidants and participate in the redox-regulation *in vivo*. These thiol enzymes have been suggested to have an essential role in the transition of neonatal lung to the oxygen-rich environment after birth, protect against hyperoxia and oxidant generating drugs in experimental animal models, and be involved both in nonmalignant and malignant diseases of human lung.

Thioredoxin system participates in cell proliferation and protects cells and tissues against oxidant stress (7). Trxs have a broad reducing capacity mediated by Trx reductase (TrxR). There are at least two different forms of Trxs and TrxRs in human cells, one cytosolic and one mitochondrial. Cytosolic Trx participates in signaling, for example, in the regulation of transcription factors and kinase activities (10, 20). One target of Trx is the family of Prxs, which decomposes hydrogen peroxide (H_2O_2) and organic peroxides into water and the corresponding alcohols. Prxs are reduced by Trx or other reductants. Like Trxs, Prxs represent powerful defense against oxidative stress, participate in signal transduction and cell proliferation,

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and protect cells against apoptosis (11). Six different Prxs have been detected in human cells with different ultrastructural compartmentalization. With the exception of recent microarray studies on the bronchial brushings of healthy smokers and emphysematous lung (6,8), these enzymes have not been investigated in smokers, and no studies have assessed them in smoking-related nonmalignant lung diseases. The thiol groups of the dimeric 2-Cys Prxs have been shown to be oxidized to disulfide monomers in cell cultures *in vitro*, leading to a potential loss of their functional integrity (16, 22, 28, 29), but whether this overoxidation occurs *in vivo*, in lung diseases associated with increased oxidative stress, is unclear.

In this study, the expression of Trx and all Prxs was first investigated in lung homogenates from nonsmokers, smokers with normal lung function, and smokers with COPD. Subsequently, the cell-specific expression of Trx, TrxR, and selected Prxs was analyzed by immunohistochemistry and their possible inactivation by nonreducing Western blotting, two-dimensional gel electrophoresis (2-DE), and mass spectrometry, with lung samples obtained from controls and patients with COPD.

THIOREDOXIN SYSTEM

Studies on the Western analysis of the lung homogenates represent total immunoreactivity of the enzyme protein in the whole lung, including the cells and matrix. There was extensive heterogeneity in the Trx immunoreactivity, but there was also a marginal increase in the Trx expression in COPD ($p = 0.05$) (Fig. 1A and B). Trx was expressed especially in the bronchial epithelium and alveolar macrophages, with occasional nuclear reactivity (Fig. 1C and D). The localization of Trx in macrophages was confirmed by the double staining methodology. Trx and TrxR were expressed in all macrophages analyzed. TrxR was found especially in the alveolar macrophages of smokers and COPD patients, and in bronchial epithelium (Fig. 1E and F). TrxR intensity was higher in the macrophages of the patients with smoking history (healthy smokers and patients with COPD) than in nonsmokers ($p = 0.009$).

These results are in agreement with a previous microarray analysis that showed significant increase of the TrxR mRNA and a minor increase of Trx expression in the bronchial brushings of smoker's lung (8). The results are also in line with the regulation of Trx in other inflammatory states of human lung, one example being pulmonary sarcoidosis, and also with the inducibility of Trx by cytokines and oxidants *in vitro* (25). It is highly likely that there is induction of the Trx-system in COPD that represents attempts to improve the pulmonary defense against the increased inflammation/oxidative stress.

PEROXIREDOXINS

Studies on the total lung homogenates by Western blotting analysis confirmed the expression of all Prxs in the lung of the controls and COPD patients (Fig. 2A), the expression of Prxs V ($p = 0.045$) and VI ($p = 0.048$) being increased in COPD, and Prx I in Stage IV COPD ($p = 0.032$) (Fig. 2B and C). Previous and present studies of our laboratory and others have also shown high Prx V and Prx VI expression in the airways/alveoli, and Prx V also to be a peroxynitrite reductase (5, 12, 14, 15, 17, 19, 27). For these reasons, these two proteins were selected for more detailed analysis in 49 lung specimens. Prx V was expressed especially in the airway epithelium and alveolar macrophages of nonsmokers, smokers, and COPD with occasional nuclear staining (Fig. 2D and F). Cytoplasmic staining was partly granular, as expected from its ultrastructural (*i.e.* mitochondrial) location. Altogether 62% of the macrophages in nonsmokers were positive, the corresponding numbers for smokers and COPD patients being 47% and 70% ($p = 0.025$ healthy smokers *versus* COPD patients). Prx VI was localized in the alveolar macrophages, airway epithelium, and occasionally in alveolar epithelium. The expression in macrophages was ensured by double staining (Fig. 2F). Again as with Prx V, the number of Prx VI positive macrophages was increased in COPD ($p = 0.001$, 46% positive for nonsmokers, 51% for smokers, 82% for COPD patients).

The family of Prxs has a cell-specific expression in human lung, Prx V and Prx VI being expressed mainly in the airway epithelium and alveolar macrophages with very minor, if any, induction (14, 15, 17). Our findings are in line with these previous observations and in agreement with a microarray study that showed no major changes of Prx mRNA expression in the bronchial brushings of smoker's lung (8). Interestingly, very high mRNA and protein levels of Prxs have been detected in malignant lung/pleural diseases (15, 17). Both Prxs V and VI are prominently expressed in the critical areas of the lung. Prx V also functions as a peroxynitrite reductase with a high rate constant (5), and thereby may have special importance in lung diseases associated with increased oxidative/nitrosative stress. In contrast to many other antioxidant enzymes, Prxs are expressed at high levels in healthy lung with no signs of their decline during COPD progression.

The significance of the marked expression of Trx/Prx-system in the alveolar macrophages in smoker's lung and COPD is difficult to estimate. Both Trx and Prxs contribute to cell growth and proliferation, and they also may prolong the survival of the inflammatory cells. Macrophages not only protect the lung, but they also may have a pivotal role in the pathophysiology of COPD. There is a significant correlation of macrophage numbers in the airways and the severity of COPD. Furthermore, macrophages are localized to sites of alveolar wall destruction in emphysema. It has also been suggested that alveolar macrophages may have prolonged survival in the lungs of COPD (1). It is therefore likely that the relatively high expression of several antioxidant enzymes, including Trx and Prxs, may also contribute to the proliferation, survival, and persistence of macrophages in smoker's lung.

RESISTANCE OF THE THIOREDOXIN AND PEROXIREDOXIN SYSTEM TO OXIDATIVE STRESS

Oxidative damage in COPD (23), was confirmed by 4-hydroxynoneal detection using Western blotting analysis (Fig. 3A, $p = 0.014$). Typical 2-Cys Prxs can be oxidized to their monomeric forms by oxidants, this alteration was confirmed in cell cultures of alveolar epithelial cells exposed to 50 and 250 μM H_2O_2 and investigated by the nonreducing SDS-PAGE

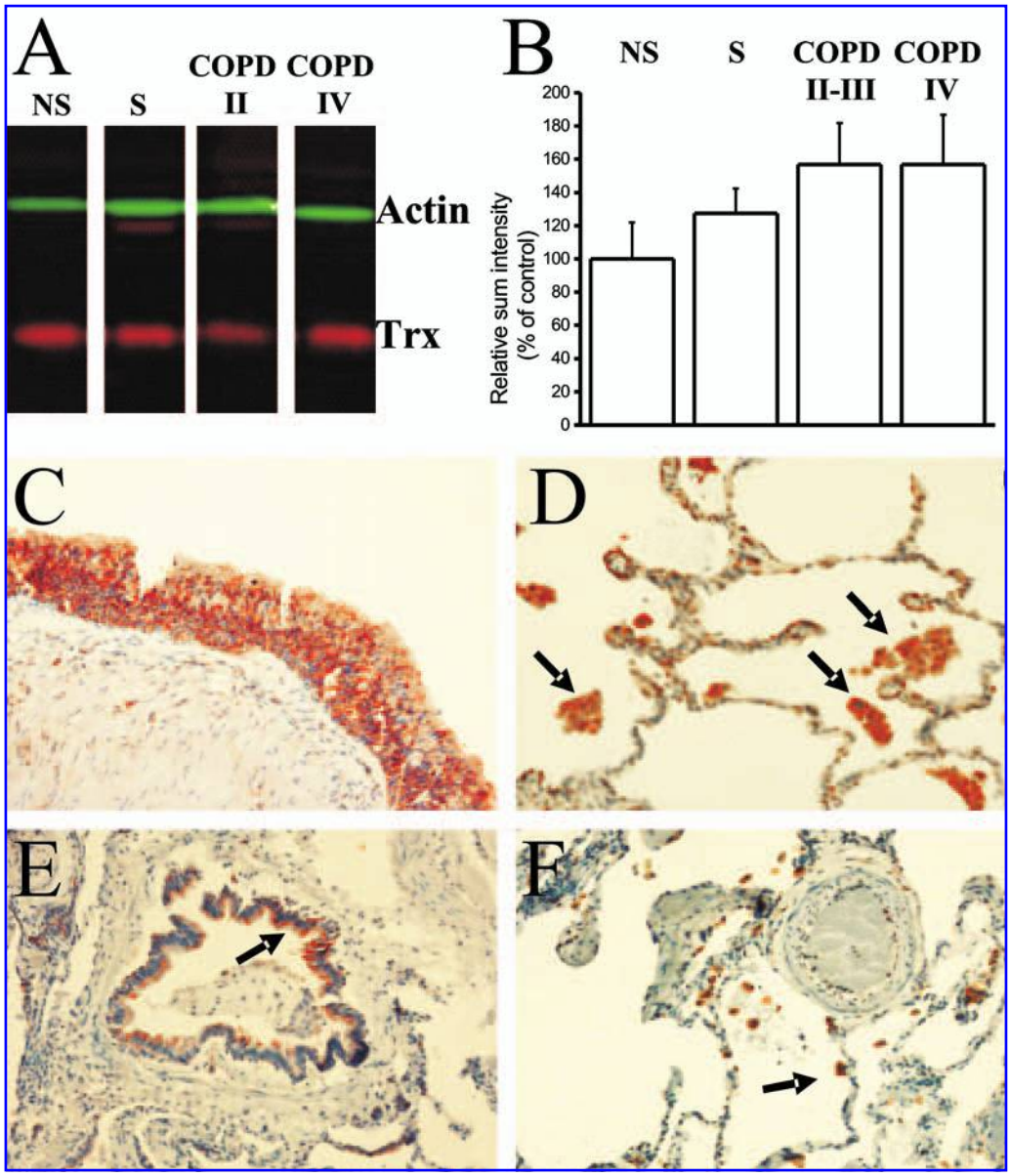

FIG. 1. Representative Western analysis of Trx and b-actin in the lung homogenates from nonsmoker (NS), smoker (S), COPD Stage II, and COPD Stage IV (A). Quantitated results of Trx Western analysis were normalized against β-actin expression (**B**). Moderate/strong staining of Trx is seen in bronchial epithelium of smoker with normal lung function (**C**), alveolar macrophages and in some epithelial cells of smoker with normal lung function (**D**). TrxR positivity can be detected in bronchiolar epithelium of smoker with normal lung function (**E**) and in alveolar macrophages of patient with COPD stage II (**F**). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

(Fig. 3B). All lung specimens (controls and COPD) showed almost exclusively only the dimeric forms of Prxs, even though individual variability in the level of monomeric form could be detected (Fig. 3C and D). The oxidation stage of Trx and Prxs *in vivo* (four controls and four COPD Stage IV) was further evaluated using more specific 2-DE analysis. This analysis was focused on the cytoplasmic Trx and Prx II and mitochondrial Prx III (Fig. 4). The detected position of Prx III indicates the presence of the maturated protein upon import into the mitochondria, which might also explain the lower intensity of this spot within the total cell extracts. Cysteine oxidation of Prxs caused by oxidative stress is reflected by a spot shift within the 2-D gel and has been shown to be reversible (3, 22, 26). Here, no corresponding spot shift for Trx, Prx II, or Prx III between 2-D gels in any control or COPD could be detected, indicating no differences in the oxidation status of the catalytic cysteines (Fig. 4). Mass spectrometry analyses confirmed with peptides containing nonoxidized cysteins at position 51 (3058.5422) for Prx II, position 127 (3450.6283) for Prx III, and position 32 and 35 (1738.8291) for Trx that the nonoxidized cysteines were present. In addition, Prx II and Prx III spots matched to the nonoxidized forms of an earlier study (26).

Diminished functional activity of Prxs (*i.e.*, their inactivation/oxidation) may have multiple consequences in the diseased lung. Prxs can become inactivated by oxidation/overoxidation in severe oxidative stress, at least in cultured cells *in vitro* (16,

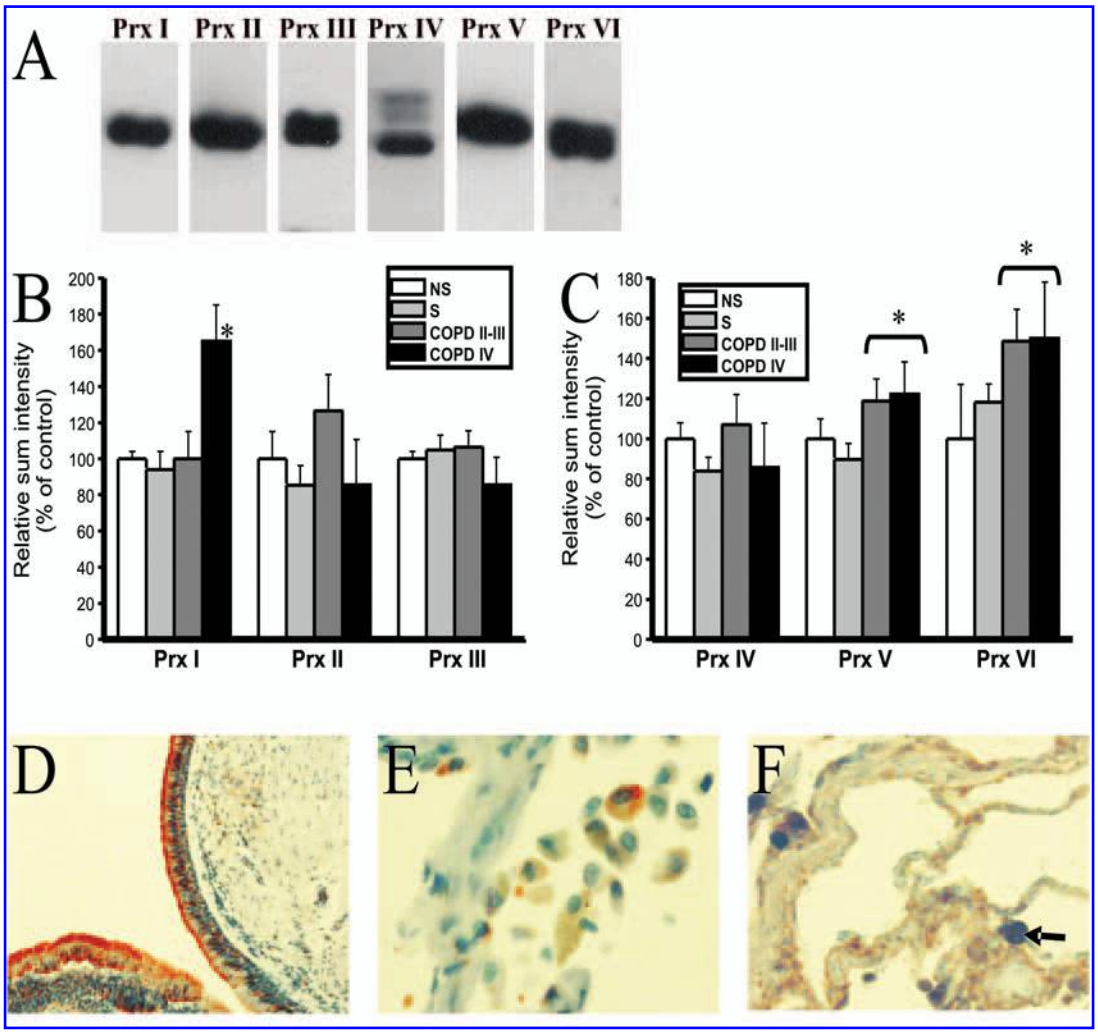

FIG. 2. Expression of various Prxs in human lung. Representative Western analyses of all Prxs are shown in (**A**), and the quantitation of each protein in (**B**) and (**C**). Prx V immunoreactivity in bronchial epithelium and in some macrophages of healthy smoker are shown in (**D**) and (**E**). Double staining of CD68 and Prx VI shows Prx VI positivity in alveolar macrophages; weak staining can also be seen in the alveolar epithelium of patient with COPD Stage II (**F**). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

22, 26, 28, 29), and this oxidation can be detected by nonreducing Western blotting, 2-DE, and mass spectroscopy. Reversible oxidation of Prxs has also been detected during the normal catalytic cycle, but even their overoxidized cysteine sulfinic acid forms have been found to be reversible *in vitro* (2, 3, 22, 26, 28, 29). In the present study, altered Prx oxidation could be seen in cultured A549 cells after H_2O_2 exposure, indicating transient inactivation of Prxs under oxidative stress. A549 cells have unusual intracellular signaling and high glutathione level, but similar oxidation of Prxs has been detected also in other cell lines

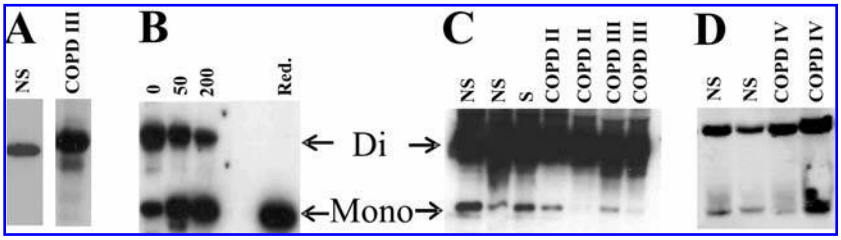

FIG. 3. Alteration in the oxidation state of Prxs can be detected *in vitro* but generally not *in vivo*. Representative Western analysis of 4′-hydoxynoneal in the lung homogenates of nonsmoker (NS) and smoker with COPD Stage III to ensure the oxidative stress (**A**). Nonreducing Western analysis was performed for Prx I in cultured human lung cells (**B**) and lung tissue homogenates of nonsmokers (NS), smokers without COPD (S), and COPD patients with different Stages of COPD (**C**) and (**D**). Cultured cells were exposed to 0, 50, or 200 μM H_2O_2 for 1 h, one lane was analyzed under reducing conditions (*red*). Sites of monomeric (*Mono*) and dimeric (*Di*) forms are indicated by *arrows.*

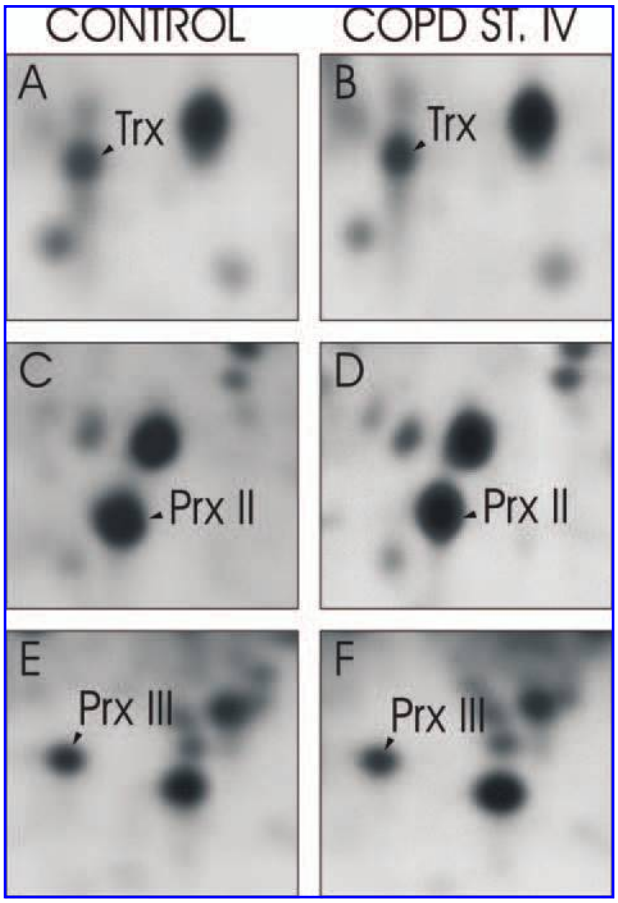

FIG. 4. Two-dimensional electrophoresis (2-DE) reveals no difference between healthy control (A, C, and E) and very severe COPD (Stage IV) (B, D, and F) lung in Trx and Prx oxidation. Lung homogenates were separated by 2-DE (pH 4–7) and gel parts presenting their expression are shown here. Trx (**A** and **B**), Prx II (**C** and **D**), and Prx III (**E** and **F**) were identified by mass spectrometry. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

(16, 22, 28, 29). However, despite the presence of oxidant burden in COPD, Trx or Prx oxidation was not associated with COPD or with COPD severity, suggesting that the functional integrity of the Prx/Trx system is not seriously disturbed in COPD. In agreement, the results obtained by 2-DE analyses revealed that Trx, Prx II, and Prx III are not irreversibly oxidized in COPD. The patients with severe COPD were on corticosteroid therapy, which in theory could affect the result obtained. However, the patients with COPD Stage II–III without corticosteroid therapy had no signs of Prx oxidation, as analyzed by nonreducing Western. Since earlier studies showed that the regeneration from the oxidized to the nonoxidized state was faster for Prx I than for Prx III, and a similar recovery rate was observed for Prx III and Prx VI (3), we suppose that also other Prxs, such as Prx I and Prx VI, retain their activity in COPD. This indicates that a functional Trx/Prx system, with the ability to regenerate, is also present in COPD. It is even possible that enzymes participating in Prx catalytical cycle may be enhanced during chronic oxidative stress and thus help to maintain the Prx activity.

Overall, these results suggest a modest enhancement of the Trx/Prx system in COPD with no signs of its irreversible oxidation /inactivation.

APPENDIX

Notes

1. Lung tissue specimens. Lung tissue specimens were obtained from patients operated for lung tumor (either malignant or nonmalignant, such as hamartoma) and were retrieved from the files of the Department of Pathology, Oulu University Hospital and Department of Pathology, Helsinki University Hospital. COPD was defined according to GOLD criteria (FEV1 $<$ 80% of reference, FEV1/FVC $<$ 70%, and bronchodilatation effect $<$ 12%) (21). Tissue specimens from tumor-free peripheral lung tissue were selected. The patients were not under corticosteroid therapy (neither inhaled nor systemic) with the exception of lung transplantation cases, who were on inhaled corticosteroids, and did not suffer from asbestos-related disease. The ethical committees of the University of Oulu and Northern Ostrobothnia Hospital District, and Helsinki University Hospital accepted this study.

The tissues for immunohistochemistry were fixed in 10% buffered formalin and embedded in paraffin. The material contained 9 life-long nonsmokers, 15 smokers with normal lung function, 17 smokers with Stage I–III COPD and 8 ex-smokers with Stage IV (very severe) COPD without α-1 antitrypsin deficiency undergoing lung transplantation (Table 1).

Tissue specimens for the Western blotting and two-dimensional gel electrophoresis (2-DE) were gathered from corresponding areas of the lung and quickly frozen to liquid nitrogen and stored at -80°C. The analysis for the Western included 11 subjects with normal lung function and 13 cases of COPD with variable severity (Table 2) and for the 2-DE four cases with normal lung from nonsmokers, also confirmed

Table 1. Clinical Information of the Patients Analyzed by Immunohistochemistry

	Nonsmokers Mean (SD)	*Smokers Mean (SD)*	*Smokers with COPD st II-III Mean (SD)*	*Ex-smokers with COPD st IV Mean (SD)*	*ANOVA or Fisher's exact test* (p)
Total (*n*)	9	15	17	8	
Male/Female	2/7	12/3	14/3	5/3	
Age years	64 (15)	62 (7)	64 (5)	54 (8)	
Pack years	0	48 (20)*‡	40 (11)†	29 (15)	<0.0001
FEV1 (I/s)	2.94 (1,07)	3.01 (0.72)‡	2.09 (0.40)†	0.6 (0.3)	<0.0001
FEV1, % of ref.	99.1 (12,4)	90.5 (9.6)‡	63.8 (10.1)†	19.9 (7.8)	<0.0001
FEV1/FVC, %	85.9 (10,7)	82.7 (11.5)‡	61.1 (7.0)†	33.8 (8.9)	<0.0001

*Comparison between nonsmokers and smokers without COPD significant, †comparison between nonsmokers and smokers with COPD significant, ‡comparison between smokers without COPD and with COPD significant.

TABLE 2. CLINICAL INFORMATION OF THE PATIENTS ANALYZED BY WESTERN BLOTTING AND/OR 2DE

	Nonsmokers Mean (SD)	*Smokers Mean (SD)*	*Smokers with COPD st II-III Mean (SD)*	*Ex-smokers with COPD st IV Mean (SD)*	*ANOVA or Fisher's exact test (p)*
Total (*n*)	5	6	7	6	
Male/Female	2/3	4/2	4/3	5/1	
Age years	64 (15)	60 (7)	64 (5)	53 (6)	
Pack years	0	42 (20)*‡	40 (11)†	32 (22)	<0.0001
FEV1 (I/s)	2.94 (1,07)	3.01 (0.72)‡	2.04 (0.40)†	0.52 (0.19)	<0.0001
FEV1, % of ref.	99.1 (12,4)	90.5 (9.6)‡	63.8 (10.1)†	16.1 (7.2)	<0.0001
FEV1/FVC, %	85.9 (10,7)	82.7 (11.5)‡	61.1 (7.0)†	35.1 (12.2)	<0.0001

*Comparison between nonsmokers and smokers without COPD significant, †comparison between nonsmokers and smokers with COPD significant, ‡comparison between smokers without COPD and with COPD significant.

by histopathology, and four nonsmokers with Stage IV COPD without α-1 antitrypsin deficiency.

2. Cell cultures. Human alveolar epithelial A549 cells (American Type Culture Collection, Manassas, VA) were cultured in Ham's F12 medium (Gibco Invitrogen Corporation, Carlsbad, CA). Cells were exposed to 50 or 200 μM H_2O_2 for 60 min and used as positive controls for the investigation of the oxidation state of Prxs.

3. Western analysis. Tissue biopsies or cultured cells were homogenized in phosphate buffered saline (PBS), and 50 μg of protein was electrophoresed either by reducing or nonreducing SDS-PAGE and blotted into membranes, which were stained with 0.2% Ponceaus S (Sigma–Aldrich Co., St. Louis, MO) in 1% acetic acid to ensure equal loading of proteins. Membranes were probed as described earlier with all six rabbit polyclonal Prx antibodies (14). Dilutions of 1:5,000–1:1,000 were used for Trx (Sigma–Aldrich Co.), TrxR (Sigma–Aldrich Co.), 4-hydroxynonenal (Calbiochem-Novabiochem Corp., San Diego, CA) or β-actin (Sigma-Aldrich Co.) antibodies, followed by anti-rabbit or anti-mouse treatment. Enhanced chemiluminesence system (ECL, Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) or Odyssey Infrared Imaging System (LiCor Biotechology, Lincoln, NE) was used for detection and for quantification of the band sum intensity.

4. Immunohistochemistry. Normal looking central bronchi and peripheral airways of each patient were selected for analysis, two slides were analyzed from each patient ($n = 49$). Four μm thick sections were treated with rabbit polyclonal Trx, TrxR, Prx V, or Prx VI antibodies (dilutions 1:2,500, 1:1,000, 1:2,000, and 1:2,000, respectively). The immunostaining of the samples and negative controls (PBS or rabbit primary antibody isotype control) was performed by Histostain-Plus Kit (Zymed Laboratories Inc., San Francisco, CA) according to the manufacturer's instructions. All antibodies have shown to be specific and sensitive in detecting these proteins both by Western blotting and immunohistochemistry in human lung (14, 15, 17). The slides were evaluated in a blinded fashion by consensus between two experienced pathologists, problematic cases were also assessed by a third pulmonary pathologist (RK-W, YS, and PP). Two slides from each specimen and at least ten microscopic fields were evaluated. The percentage of Trx and Prx positive macrophages was calculated. The intensity of the immunostaining in alveolar macrophages, airway bronchial, and bronchiolar epithelium was divided in two groups as follows: negative/weak and moderate/strong. Nuclear staining was considered either negative or positive.

In the double labeling, alveolar macrophages were detected by CD68 (KP-1) (Dakopatts, Copenhagen, Denmark) using a dilution of 1:50. The method used was alkaline phosphatase with deep purple as the chromogen by Zymed Histostain DS Broad Spectrum Kit (Zymed Laboratories Inc.). The same sections were then immunostained for Trx, Prx V, and Prx VI, as described above.

5. Two-dimensional gel electrophoresis (2-DE) and protein identification. Lung tissue samples were powdered as frozen and further purified by acetone precipitation. The protein extract was resuspended in urea buffer [7 *M* urea, 2 *M* thiourea, 4% (wt/vol) CHAPS, 0.15% (wt/vol) DTT, 0.5% (vol/vol) carrier ampholytes 3–10, Complete Mini protease inhibitor cocktail (Roche, Mannheim, Germany)] and disrupted for 10 min in an ultrasonic bath. The protein separation for each sample, done in triplicate, was performed as previously (18). In brief, the protein solution was adjusted with urea buffer to a final volume of 350 μl and in-gel rehydration was performed overnight. Isoelectric focusing was carried out in IPG strips (pH 4–7, 18 cm, GE Healthcare, Uppsala, Sweden) with the Multiphor II system (GE Healthcare) under paraffin oil for 55 kVh. SDS-PAGE was done in polyacrylamide gels (12.5% T, 2.6% C) with the Ettan DALT II system (GE Healthcare) at 1–2 W per gel and 12°C. The gels were silver stained, analyzed with the 2-D PAGE image analysis software Melanie 3.0 (GeneBio, Basel, Switzerland) and reproducible changes in spot intensity (at least twofold) were marked in the gel.

For protein identification, excised spots were in gel digested as described (18). Peptide masses were measured with a VOYAGER-DE™ STR (Applied Biosystems, Foster City, CA), and proteins identified with ProFound database version 2005.02.14 (http://prowl.rockefeller.edu/prowl-cgi/profound.exe, last accessed March 16, 2007) and following parameters (20 ppm; 1 missed cut; MH+; +C2H2O2@C [Complete], +O@M [Partial]). Detailed analysis of cysteine oxidation within the detected peptides was done with PeptideMass (http://au.expasy.org/tools/peptide-mass.html, last accessed March 16, 2007).

6. Statistical analysis. Statistical analysis was performed with Statistical Package for Social Studies (SPSS) version 11.5.1 (Chicago, IL). The continuous data was studied by one-way variance analysis, followed by *post hoc* comparisons (two tailed *t*-test) and categorical data by Fisher's exact probability test and nonparametric median test. Probability values $p \leq 0.05$ were considered significant.

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ABBREVIATIONS

COPD, chronic obstructive pulmonary disease; Cys, cysteine; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; H_2O_2, hydrogen peroxide; NS, nonsmoker; Prx, peroxiredoxin; PBS, phosphate-buffered saline; ROS, reactive oxygen species; S, smoker; Trx, thioredoxin; TrxR, thioredoxin reductase; 2-DE, two-dimensional electrophoresis.

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