Peroxiredoxin VI in human respiratory system

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Abstract Peroxiredoxins (Prxs) constitute a novel family of antioxidant proteins, which specifically prevent enzymes from metal-catalyzed oxidation. The localization of a member of the mono-cystein subfamily of Prxs, Prx VI in human respiratory system and its antioxidant properties were investigated. By immunoblotting, the Prx VI was found to be present in human respiratory epithelium. Immunostaining with rabbit polyclonal antibody raised against the Prx VI revealed that the said protein was present in apical areas and mucus of all respiratory airways from trachea to bronchioles. Immuno-depletion of the Prx VI profoundly decreased the antioxidant activity of the respiratory epithelium extract. © 2002 Elsevier Science Ltd. All rights reserved.

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INTRODUCTION

Respiratory system due to peculiarities of its texture, structure and functions has increased capability for reactive oxygen intermediates (ROI) formation and for consecutive free radicals reaction as lungs are exposed to both endogenous and inhaled free radicals. The oxidative burden of a healthy lung is balanced by the local antioxidant defenses (1). Since hydrogen peroxide (H₂O₂) is a precursor of highly reactive hydroxyl radical (OH·), lung antioxidant strategy is based in many respects on peroxide inactivation system, that includes several enzymes, of which catalase and glutation-redox cycle enzymes are basic ones. In addition to well-documented antioxidant enzymes, a novel family of antioxidant enzymes, designated peroxiredoxins (Prxs), has recently been discovered in both eucaryote and procaryote cells. Prx family members in the presence of some thiols can specifically prevent biopolymers (proteins, nucleic acids, lipids) from damage by reactive oxygen species (2). Mammalian Prxs are classified into six distinct groups, Prx I—Prx VI, based on amino acid sequences and immunological reactivity (3,4). Prx VI was detected in olfactory and respiratory epithelium of rats (EMBL/GenBank, accession number Y17295) (5–7). Based on electron microscopy immunohistochemistry data authors suppose that respiratory epithelium cells produce Prx VI to epithelial lining fluid, thus Prx VI is a secretory protein (8). It is generally known that ELF constitutes the first and most likely basic line lung antioxidant defense (I). A comparison of rats Prx VI sequences with protein sequences in EMBL and GenBank databases revealed human protein (ORF6) with 91% similarity (GenBank, D14662). The aim of this study is elucidation of Prx VI expression pattern and contribution to antioxidant defense of human respiratory airways.

PREPARATION OF WATER-SOLUBLE EXTRACT OF TRACHEA

Trachea specimens for immunohistochemistry and biochemical analysis were derived from six men at autopsy within 10 h after decrease. Autopsy showed absence of any abnormalities in lungs. Lung biopsy specimens for immunohistochemistry from four subjects with normal histopathology undergoing surgery for lung cancer were retrieved from the files of the Department of Pathology, Pulmonology Research Institute, Moscow.

Isolated tissue from human trachea was homogenized in 10 mM sodium phosphate buffer containing 150 mM NaCl and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4. The homogenate was centrifuged at 20 000 g for 1 h, and the supernatant (total protein concentration approximately 1 mg/ml) was used as water-soluble extract.
PRODUCTION OF RABBIT POLYCLONAL ANTIBODY AGAINST RECOMBINANT HUMAN PEROXIREDOXIN VI, AND IMMUNOBLOTTING

Rabbit polyclonal antibody against the recombinant human Prx VI was produced as previously described (8). The protein (300 µg of antigen per immunization) was emulsified with complete Freund’s adjuvant for the first immunization, then by boost in incomplete Freund’s adjuvant at 3 weeks. The antiserum was diluted 1:200 for light microscopy.

SDS-polyacrylamide gel electrophoresis (PAGE) was performed under reducing condition on 10% slab gels in discontinuous buffer system, according to Laemmli (9). Approximately 20 µg of total water-soluble protein was used for SDS-PAGE gel lane.

Immunoblotting was performed as previously described (8).

Protein concentrations were measured according to the method of Bradford (1970), using serum albumin as standard (10).

LIGHT-MICROSCOPIC IMMUNOHISTOCHEMISTRY

Paraffin sections 4–5 µm thick were cut on microtome and mounted on gelatin-coated microscope slides. The sections were deparaffinized in xylene and rehydrated though a series of graded alcohols from absolute to water. Sections were treated with 0.3% hydrogen peroxide for 5 min to inactivate the endogenous peroxidase activity and blocked for 30 min with 2% bovine fetal serum in 10 mM phosphate-buffered saline, pH 7.5. Sections were washed with 10 mM phosphate-buffered saline, pH 7.5, and incubated with a primary rabbit antiserum against Prx VI at a dilution 1:200 for 1 h. Antibody binding was detected using horseradish peroxidase-coupled goat anti-rabbit secondary antibody. The complex was visualized in 10 mM sodium phosphate buffer, pH 7.5, containing 0.15 mM NaCl, 0.5 mg/ml 3′, 3-diaminobenzidine tetrahydrochloride, and 0.015% hydrogen peroxide. To evaluate nonspecific staining, controls were incubated with preimmune serum. The stained sections were examined under a camera-equipped Zeiss microscope. All procedures were carried out at room temperature.

IMMUNODEPLETION

Anti-Prx VI IgG was coupled to CNBr-activated sepharose (Pharmacia, Sweden) according to the manufacturer’s instructions. For immunodepletion of the Prx VI, 10 ml of water-soluble extract of human trachea containing 10 mg of total water-soluble protein was passed twice through an antibody-affinity column (0.5 × 3 cm)². The degree of the Prx VI immunodepletion in the trachea extract was evaluated by immunoblotting.

INACTIVATION OF GLUTAMINE SYNTHETASE BY THE Fe³⁺/DITHIOTHREITOL/O₂ SYSTEM

Escherichia coli glutamine synthetase was inactivated by the Fe³⁺/dithiothreitol/O₂ system as described previously (II). The inactivation reaction was performed in 60-µl reaction mixture containing 5 µg of glutamine synthetase, 3 mM dithiothreitol (DTT), 3 µM FeCl₃, and 50 mM HEPES buffer, pH 7.3. Protein sample (20 µl of untreated or depleted extract of trachea) was also added. After incubation at 25°C for 10 min, the remaining activity of glutamine synthetase was measured as described (II).

RESULTS

By immunoblotting, using rabbit polyclonal antibody raised against the human recombinant Prx VI, we showed here that Prx VI was present in water-soluble extract of human trachea (Fig.1). In the case when antibodies against rat 28-kDa peroxiredoxin were used for immunoblotting, a similar picture was revealed (figure missing). Thus Prx VI was identified in human trachea.

We performed light-microscopic immunohistochemistry examination with the purpose of clarifying Prx VI localization in human trachea and lungs. The most intensive immunostaining in trachea was observed in the apical area and mucus (Fig. 2).

The immunoreactivity in lung was observed in bronchi and bronchioles of all generations including terminal bronchioles in a pattern similar to that in the trachea (Fig. 3). No immunoreactivity was apparent in other areas of lung, such as alveolus and lung blood vessels.

To elucidate the role of the Prx VI in the endogenous antioxidant system, we tested the effect of immunodepletion of the Prx VI on the antioxidant property of the trachea extract. For this study, we used the human trachea epithelium depleted for Prx VI. For immunodepletion, we used chromatography of the extract of the trachea epithelium on anti-Prx VI antibody-affinity column, and monitored the efficiency of the Prx VI depletion by immunoblotting. As shown in Fig. 4, two-step chromatography completely removed the Prx VI from the trachea tissue extract.

For testing the ability of the antioxidant inherent in trachea tissue extract to prevent damages caused by AOS, we used Fe³⁺/DTT/O₂ system as its components serve as electron donors for Prxs and can simultaneously generate free radicals. As a component sensitive to
oxidation by Fe$^{3+}$/DTT/O$_2$ system, we employed glutamine synthetase, the enzyme commonly used for investigating the properties of the antioxidants.

As shown in Fig. 5, under the given experimental conditions the levels of the antioxidant activity depleted extracts were not sufficient to protect glutamine synthetase from inhibition by Fe$^{3+}$/DTT/O$_2$ system. The low antioxidant activity exerted by the depleted isotonic extracts can be attributed to catalase, which has previously been shown to protect glutamine synthetase from inactivation by Fe$^{3+}$/ascorbate/O$_2$ system in the absence of any electron-donor compounds (12).

As shown in Fig. 5, the protein concentration of the untreated extract required for the 50% glutamine synthetase protection was approximately 0.2 mg/ml, while depleted extract even with concentration of 1 mg/ml provided only 15% of glutamine synthetase protection. Calculated Prx VI contribution in total antioxidant activity of human airways epithelium comes about 70%.

**DISCUSSION**

Currently, virtually all of the known antioxidants were revealed in ELF (13). However, it should be stressed that the main body of this results was obtained from bronchoalveolar lavage (BAL) studies. BAL studies do not allow to detect the site of antioxidant enzymes synthesis and the capacity of cells to secrete such protein actively. At present, a rather limited number of immunohistochemistry data on cellular localization of antioxidants exists.

However, lung cellular residence of the most widely investigated hydrogen peroxide scavenging mechanisms including catalase and extracellular glutathione peroxidase (eGPx) are well known now. The most intensive catalase immunostaining was seen in type II pneumocytes, a lesser degree immunopositivity for catalase was detected in bronchial epithelium and alveolar macrophages (14). The human airway epithelium and alveolar macrophages express and secrete eGPx into the apical surface lining fluid (15).
Great attention has lately been paid to Prxs — a novel family of antioxidants, though only a few studies of Prxs in the field of respiratory medicine have so far been published. Kinnula and co-authors (16) reported that the bronchial epithelium showed moderate-to-high expression of Prxs I, III, V and VI. The alveolar epithelium expressed mainly Prxs V and VI, alveolar macrophages expressed Prxs I, III, V, and VI. In this study, the immunoreactivity for Prx VI was observed in apical area of trachea and bronchi, including bronchioles. No immunoreactivity was apparent in other areas of lung, such as alveolus and lung blood vessels in all biopsy specimens.

These findings correspond with the facts obtained from studies on rat which showed on immunopositivity for Prx VI in alveolar area (8). It is possible that Prx VI expression in human lung varies to some extent among individuals given the fact that in Kinnula’s research Prx VI showed immunoreactivity in alveolar type II cells only in three of the four cases.

Despite the fact that many aspects of lung antioxidant biology are currently well known, relationship between different peroxide scavengers and contribution each of peroxide scavengers to overall antioxidant activity are still not clarified completely. Usage of a model system in

![Image](a)

**FIG. 3.** Localization of peroxiredoxin VI in human lung. (a) Immunohistochemical staining of the peroxiredoxin VI with anti-peroxiredoxin VI antiserum. Immunoreactivity was visualized using peroxidase reaction with diaminobenzidine as a substrate. (b) Haematoxyline-and-eosin staining. The immunoreactivity is concentrated in mucus and some epithelial cells. Bars 50 mm.

![Image](b)

**FIG. 4.** Immunodepletion of the Prx VI in water-soluble extract of respiratory epithelium. Immunoblot of untreated extract (lane 1), immunoblot of depleted extract (lane 2).

**FIG. 5.** Protection of glutamine synthetase by water-soluble extract of respiratory epithelium against the Fe³⁺ dithiothreitol/O₂ Balck circles represents untreated extract, white circles immunodepleted extract.
which antioxidant of activity of untreated epithelium extract is compared with antioxidant activity of epithelium extract lacking in specific enzyme may constitute one of the possible approaches to the above problem. We applied a similar approach to our research. Difference in ability of untreated extract and of extract depleted by Prx VI to prevent the inactivation of glutamine synthetase by Fe$$^{3+}$$/DTT/O2 comes to about 70%, which enables to suppose that Prx VI plays a leading role in scavenging AOS by tissues. Despite the fact that the operation was conducted in the presence of nonphysiological Prx activator (since physiological activator is not yet identified), this experiment allows to evaluate to certain extent a great Prx VI contribution to total antioxidant activity of human airways. This high activity of Prx VI is consistent to its localization at the epithelium surface of all respiratory airways from trachea to bronchioles implying that Prx VI probably constitutes a powerful antioxidant of first line of defense.

REFERENCES

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