LOCALIZATION AND REGULATION OF PEROXIREDOXINS IN HUMAN LUNG AND LUNG DISEASES

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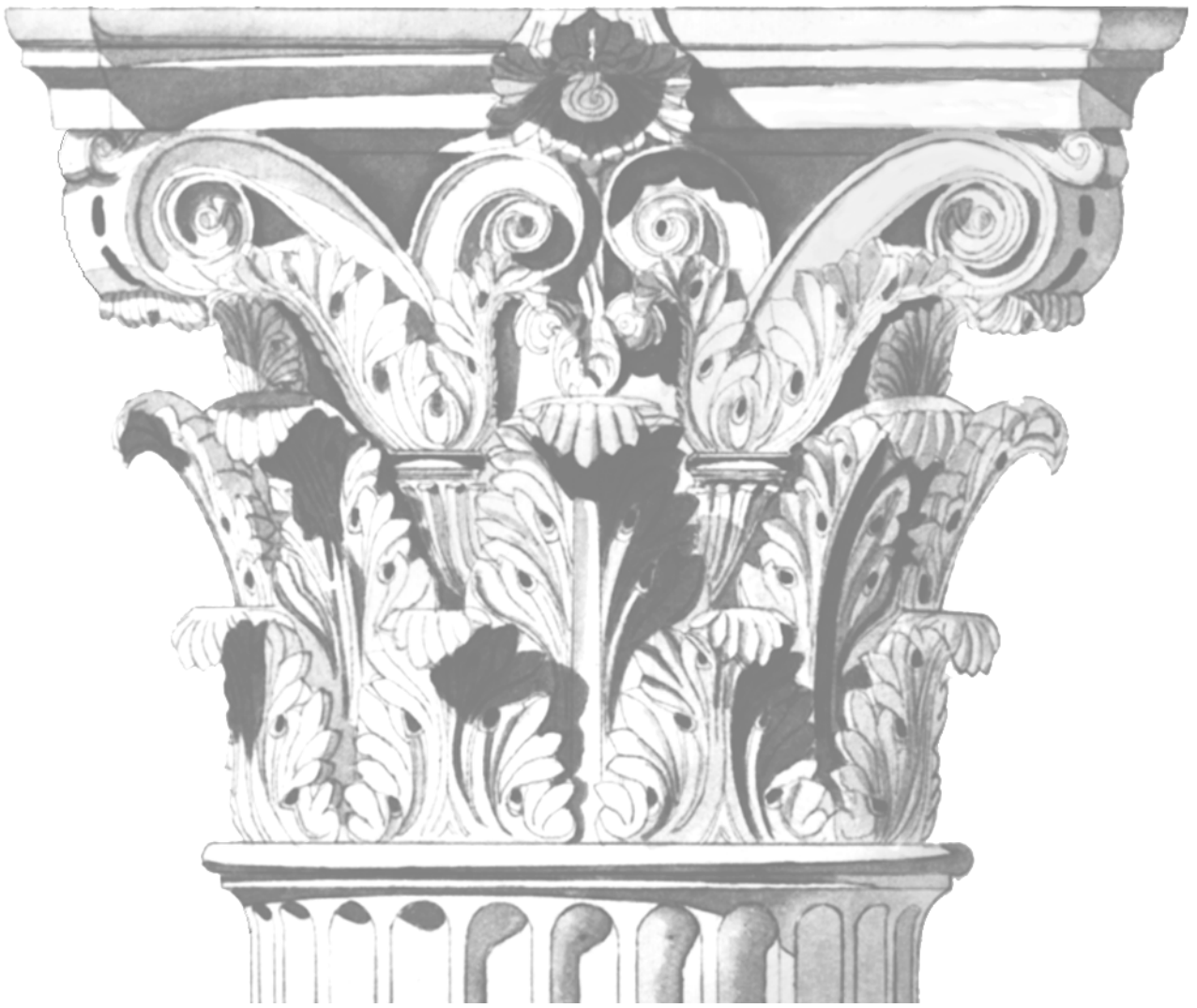

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Abstract

Reactive oxygen species (ROS) can cause severe damage to cells and organs but they are also important mediators of inflammatory responses and cellular signalling. Due to the significant role of ROS, the cells have evolved a broad antioxidative system to regulate the concentration of these species. Peroxiredoxins (Prxs) are enzymes that participate in the regulation of the cellular redox-homeostasis by detoxifying hydrogen peroxide. Prxs are not classified as conventional antioxidant enzymes and their physiological role, whether protective or regulatory, is still unclear.

The aim of this project was to study the localization and regulation of Prxs in normal human lung and also their role in selected lung disorders (pulmonary sarcoidosis, pleural mesothelioma, lung carcinomas and chronic obstructive disorder, COPD). Additionally the expression of thioredoxin (Trx) and thioredoxin reductase (TrxR) was analysed in the lung of smokers and COPD patients. These enzymes are important reductants in cell and Prxs are one of their targets. Lung is an important organ in the field of ROS and antioxidant research since it is especially vulnerable to exogenous oxidative stress caused by pollutants, cigarette smoke and also by high oxygen pressure.

The results showed that all six human Prxs were expressed in healthy human lung but in a cell-specific manner. The most prominent expression was detected in the epithelium and in macrophages, the cells most prone to oxidative stress. There were also differences in subcellular locations of Prxs.

The expression of Prxs in non-malignant lung diseases (pulmonary sarcoidosis and COPD) and in smoker's lung was very similar with that in normal lung. Higher expression of Prx V and VI was detected in a subpopulation of macrophages sampled from COPD patients' lung. In contrast, Trx expression was induced in the bronchial epithelium of smoker's lung.

Differences in the expression compared to normal lung were seen in lung malignancies (pleural mesothelioma and lung carcinomas). Interestingly, different Prxs were highly expressed in different types of carcinomas. In pleural mesothelioma, all Prxs except Prx IV were highly expressed when compared to normal pleura, in adenocarcinoma Prxs I, II, VI and especially IV, and in squamous cell carcinoma Prxs I, II and IV were upregulated.

Tests performed on cultured cells *in vitro* revealed only a minor increase in the Prx expression after severe oxidant stress in malignant lung cell line originating from alveolar type II pneumocytes (A549) or non-malignant cell line derived from bronchial epithelium. None of the tested growth factors or cytokines affected Prx expression or oxidation state, but severe oxidant stress influenced remarkably the oxidation state of the Prxs.

Keywords: antioxidant enzyme, chronic obstructive pulmonary disease, lung neoplasms, mesothelioma, oxidants, peroxiredoxin, sarcoidosis

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Abbreviations

Ap1	Activator protein 1
Ap2	Activator protein 2
ATP	Adenosine triphosphate
$RO^{\bullet}$	Alkoxyl radical
AOE	Antioxidant enzyme
ARE	Antioxidant response element
bp	Base pair
BAL	Bronchoalveolar lavage
BSO	Buthionine sulfoximine
COPD	Chronic obstructive pulmonary disorder
CoA	Coenzyme A
cDNA	Complementary deoxyribonucleic acid
CuZnSOD	Copper zinc superoxide dismutase
Cys	Cysteine
CYP	Cytochrome P450 oxidase
Da	Dalton
DNA	Deoxyribonucleic acid
EM	Electron microscopy
EGF	Epidermal growth factor
ECSOD	Extracellular superoxide dismutase
GCL	Glutamate cysteine ligase
GPx	Glutathione peroxidase
GSH	Glutathione
GTP	Guanosine triphosphate
HBP	Heme binding protein
H_2O_2	Hydrogen peroxide
$HOO^{\bullet}$	Hydroperoxyl radical
$OH^{\bullet}$	Hydroxyl radical
Hif1	Hypoxia inducible factor 1
MSP	Macrophage stress protein
MnSOD	Manganese superoxide dismutase

mRNA	Messenger RNA
Met	Methionine
NKEF	Natural killer enhancing factor
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
Nox	NADPH oxidase
$NO^{\bullet}$	Nitric oxide radical
$NO_2^{\bullet}$	Nitric dioxide radical
NFκB	Nuclear factor kappa B
Nfr2	Nuclear factor erythroid 2 related factor 2
ORF	Open reading frame
OSF	Osteoblast specific factor
O_3	Ozone
Prx	Peroxiredoxin
PMP	Peroxisomal membrane protein
$ROO^{\bullet}$	Peroxyl radical
$OONO^-$	Peroxynitrite
PAG	Proliferation associated gene
PKC	Protein kinase C
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase – polymerase chain reaction
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
$O_2^{\bullet-}$	Superoxide anion radical
SOD	Superoxide dismutase
Sp1	Specificity protein 1
Trx	Thioredoxin
TDPX, TPx	Thioredoxin peroxidase
TrxR	Thioredoxin reductase
TAD	Transactivation domain
TGFβ	Transforming growth factor β
TNFα	Tumor necrosis factor α
XO	Xanthine oxidase

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals. In addition some unpublished data is presented.

I Kinnula VL, Lehtonen S, Kaarteenaho-Wiik R, Lakari E, Pääkko P, Kang SW, Rhee SG & Soini Y (2002) Cell specific expression of peroxiredoxins in human lung and pulmonary sarcoidosis Thorax. 57: 157-164.

II Kinnula VL, Lehtonen S, Sormunen R, Kaarteenaho-Wiik R, Kang SW, Rhee SG & Soini Y (2002) Overexpression of peroxiredoxins I, II, III, V, and VI in malignant mesothelioma. J Pathol 196: 316-323.

III Lehtonen ST & Svensk A-M, Soini Y, Pääkkö P, Hirvikoski P, Kang SW, Säily M & Kinnula VL (2004) Peroxiredoxins, a novel new protein family in lung cancer. Int J Cancer. 111: 514-521.

IV Lehtonen ST, Harju T, Kaarteenaho-Wiik R, Pääkkö P, Kang SW & Kinnula VL Cell-specific enhancement of Thioredoxin/Peroxiredoxin family in smoker's lung. Submitted.

V Lehtonen ST, Markkanen P, Peltoniemi M, Kang SW & Kinnula VL (2005) Variable over-oxidation of peroxiredoxins in human lung cells in severe oxidative stress. Am J Physiol Lung Cell Mol Physiol. 288: L997-1001.

Contents

1 Introduction

Oxygen (from two Greek words *oxy*=acid and *genes*=forming) was first described and named by Antoine Laurent Lavoisier (1743-1794), who is considered as one of the fathers of modern chemistry. However, this mysterious component of the air and water had puzzled researchers already prior to Lavoisier's innovative experiments. Already during the 18th century, it became obvious that this component was essential for life but later findings also suggested a dual role of oxygen as under certain circumstances it may also be toxic (Hensley & Floyd 2002). Current knowledge has further supported this dual roles i.e. oxygen is both essential and harmful to life. Both these roles are based on the reactivity of oxygen and this property has given rise to the entire concept of oxidation/reduction reactions.

During the last century, researchers noted that oxygen may form even more reactive molecules, termed reactive oxygen species (ROS). These include both radicals and other oxygen derivatives. Henry John Horstman Fenton (1854-1929) discovered that certain metals have the capability to improve the effect of hydrogen peroxide (H_2O_2), one member of the ROS family. Today we know that this phenomenon is based on hydroxyl radical production from H_2O_2 by using metal ion as a catalyst. This was the first important discovery utilising oxygen centred free radicals and even today the so called Fenton reaction is used in detoxifying certain chemicals. However, despite the possibility of utilising ROS in industrial processes and cleaning, it is now evident that ROS can be very harmful for living organisms. These molecules can disrupt the structure of all the critical cellular macromolecules, namely DNA, RNA, lipids, proteins and carbohydrates. Damage to DNA can lead to mutations, which can make cells susceptible to diseases like cancer. However, in processes like signal transduction even our own cells have learned to utilise ROS in controlling cell growth and proliferation and ROS have also a significant role in immune defence (Burdon *et al.* 1995, Thannickal & Fanburg 2000). Therefore their concentration in and outside the cells is strictly controlled by enzymatic systems, which include both ROS-producing and ROS-degrading enzymes, the latter being called antioxidant enzymes (AOEs). Classical AOEs contain superoxide dismutases (SODs, EC 1.15.1.1), catalase (EC 1.11.1.6) and glutathione peroxidases (GPxs, EC 1.11.1.9). Furthermore there are several AOE related proteins i.e. thioredoxins (Trxs), glutaredoxins (EC 1.20.4.1), and peroxiredoxins (Prxs, EC 1.11.1.15).

Prxs are small but abundant thiol-based enzymes that catalyse the degradation of H_2O_2, but they are also capable of decomposing other peroxides, such as certain oxidised lipids. They have been found in a wide variety of organisms from archea and eubacteria to humans. There are six distinct Prxs characterised in human cells, these being called Prxs I-VI, which share structural and functional similarities but have different tissue distributions (Jin *et al.* 1997, Kang *et al.* 1998a, Kang *et al.* 1998b, Seo *et al.* 2000, Okado-Matsumoto *et al.* 2000). Trxs and glutathione (GSH) provide the most important reducing power in the cells both having a broad substrate capacity. Reduction of Prxs is one task of Trxs: this is essential to restore the catalytical activity of Prxs.

In addition to their antioxidative role, Prxs and Trxs regulate signalling cascades affecting cell proliferation, differentiation, apoptosis and transcriptional regulation (Zhang *et al.* 1997, Kim *et al.* 2000, Sasagawa *et al.* 2001). Furthermore they can have an influence on the efficacy of drugs or other therapies based on ROS production (Park *et al.* 2000b, Chung *et al.* 2001). According to mouse mutant models, Prxs I, II or VI do not seem to be essential for normal development, but lack of Prx I or II causes haemolytic anaemia and Prx I seems to act as a tumour suppressor (Wang *et al.* 2003, Lee *et al.* 2003, Neumann *et al.* 2003). Lack of Prx VI renders especially the lung of mice susceptible to oxidant stress (Wang *et al.* 2003, Lee *et al.* 2003).

Prxs have been studied in human tissues only briefly, especially their expression and role in lung is unclear. There is however evidence for a connection between Prx levels and certain diseases; elevated Prx levels have been observed in some tumours but studies have mostly focused only to Prx I (Yanagawa *et al.* 1999, Yanagawa *et al.* 2000, Chang *et al.* 2001, Noh *et al.* 2001, Choi *et al.* 2002, Karihtala *et al.* 2003). Altered Prx levels have been also detected in neurological diseases characterised by oxidative stress, like Creutzfeld-Jacob disease, Alzheimer's disease, Pick's and Down syndromes (Kim *et al.* 2001b, Nicolls *et al.* 2003). There have been also proposals about their possible involvement to other diseases like atherosclerosis, but these issues need further clarification (Phelan *et al.* 2002). The role of Prxs in inflammatory diseases may be crucial, but at the beginning of this project there were no human studies on their involvement.

The lung provides an entry into the body for inhaled oxygen as well as bacteria, virus and many other harmful agents such as example pollutants or smoke. Oxygen and many chemicals give rise to ROS; furthermore the immune system of lung is dependent on active production of ROS by inflammatory cells. Therefore lung is continuously exposed to higher levels of ROS than most other tissues, which makes it especially important in the field of antioxidant research.

This study aimed at elucidating the expression, regulation and role of Prxs in normal human lung and during various lung disorders including pulmonary sarcoidosis, COPD, pleural mesothelioma and lung carcinomas.

2 Review of the literature

2.1 Reactive oxygen and nitrogen species

Reactive oxygen species can be defined as partially reduced oxygen intermediates. They contain free radicals like superoxide anion ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$) and hydroperoxyl ($HOO^{\bullet}$) radicals and non-radicals like hydrogen peroxide (H_2O_2) and ozone (O_3). In addition there are nitrogen-centred reactive species, which include peroxynitrite ($OONO^-$), nitric oxide ($NO^{\bullet}$) and nitric dioxide ($NO_2^{\bullet}$) radicals, whose formation is closely linked with ROS.

2.1.1 Hydrogen peroxide

Hydrogen peroxide is a small molecule that easily passes through all membranes of the cell. In contrast to most other ROS, it is stable and can therefore be transported far from site of synthesis. H_2O_2 is generated spontaneously or enzymatically by superoxide dismutation ($O_2^{\bullet-} + 2\ H^+ \rightarrow H_2O_2 + O_2$) and it can be converted to other ROS by Haber-Weiss reaction ($O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH- + OH^{\bullet}$) or Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH- + OH^{\bullet}$).

Even though H_2O_2 is considered to be toxic, its role as second messenger became evident decades ago. Its concentration inside the cell fluctuates in phase with proliferation and it is thought to have a crucial role in maintaining normal cell growth and metabolism (Burdon *et al.* 1995). H_2O_2 is produced in adipocytes after insulin exposure, but it can itself cause the same stimulatory effects even without the presence of insulin (May & de Haen 1979a, May & de Haen 1979b). Growth factors like transforming growth factor β (TGF-β1), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) are known to elevate the H_2O_2 concentration inside the cell and also some other signalling routes are known to associate with its concentration (Ohba *et al.* 1994, Li *et al.* 1995, Sundaresan *et al.* 1995, Bae *et al.* 1997, Bae *et al.* 2004). The mechanisms of this H_2O_2 production are still unclear, but probably it is mediated by Rac-sensitive system as discussed later (Sundaresan *et al.* 1996, Bae *et al.* 2004).

There are two possible consequences of H_2O_2 induction associated with signal cascades: binding of it or modification catalysed by it (Rhee 1999). Since the chemical structure of H_2O_2 is so simple, it seems unlikely that it could be recognised and bound specifically by some protein during a specific signalling cascade. Probably the effect of H_2O_2 is mediated by its ablility to oxidize other molecules, especially the sulfhydryl moiety of cysteine (Cys) residues is thought to be an important target (Cooper *et al.* 2002, Poole *et al*. 2004). Cys exists in nature in several different oxidation states like thiols, thiolates, thiyl radicals, disulphides and sulphenic/sulphinic/sulphonic acids. Each state has its own properties, which affect the Cys containing protein's stability, redox-sensitivity, binding and catalytical properties.

H_2O_2 is not however capable of oxidizing all Cys residues, because pKa values of most protein Cys-SH groups are above 8 due to the surrounding amino acids. Oxidation by H_2O_2 requires the pKa to be below 7. Nonetheless there are a number of candidate proteins possessing Cys-SH residues with low pKa values: Trx; protein disulfide isomerase; phosphatases; proteases and Prxs (Holmgren 1989, Freedman *et al.* 1994, Kortemme & Creighton 1995, Lohse *et al.* 1997, Rhee 1999). A common feature of these proteins is that they have two cysteines separated by two other residues (CxxC motif), In certain proteins, the carboxy-terminal Cys may be replaced by serine or threonine. Another possible target of H_2O_2 modification is a methionine (Met) residue that can be oxidised to sulfoxide, but there is no evidence for signal transduction associated with Met.

2.1.2 Sources of reactive oxygen species

Molecular oxygen is the most important oxidant in nature and the high oxygen pressure present in the lung favours the formation of ROS. Like other tissues exposed directly to the atmosphere, the lung is constantly exposed to many extracellular components, i.e. not only ROS but other chemicals promoting chain reactions leading to ROS production. Additionally harmful components of cigarette smoke, pollutants, asbestos, irradiation and several chemoterapeutics evoke ROS exposure to lung (Kinnula & Crapo 2003). A brief overview of ROS production in the human lung is shown in figure 1.

The major intracellular source of ROS is aerobic energy metabolism. This is based on oxidative phosphorylation in which adenosine triphosphate (ATP) is formed as electrons originating from food sources are transferred to molecular oxygen (O_2) by a multicomponent enzymatic system, in which cytochrome oxidase is the terminal electron donor. In eukaryotes, the oxidative phosphorylation takes place in the inner membrane of mitochondria by respiratory assemblies. However, during this transfer process, oxygen may be reduced only partially due to the sequential nature of reaction as shown in figure 2. The tendency towards partial reduction is a result of the molecular structure of O_2, which actually contains two unpaired electrons located in different orbitals and possessing different spins. This is not a common structure in other molecules or atoms and therefore it is difficult to find a matching molecule with electrons with opposite spins, and thus O_2 is generally reduced by one electron at a time. Cytochrome oxidase itself maintains partially reduced oxygen in a tightly bound fashion, but some other

components of the electron transfer system (NADH ubiquinone reductase, succinate ubiquinone reductase or cytochrome c reductase) may leak electrons to oxygen (McLennan & Esposti 2000).

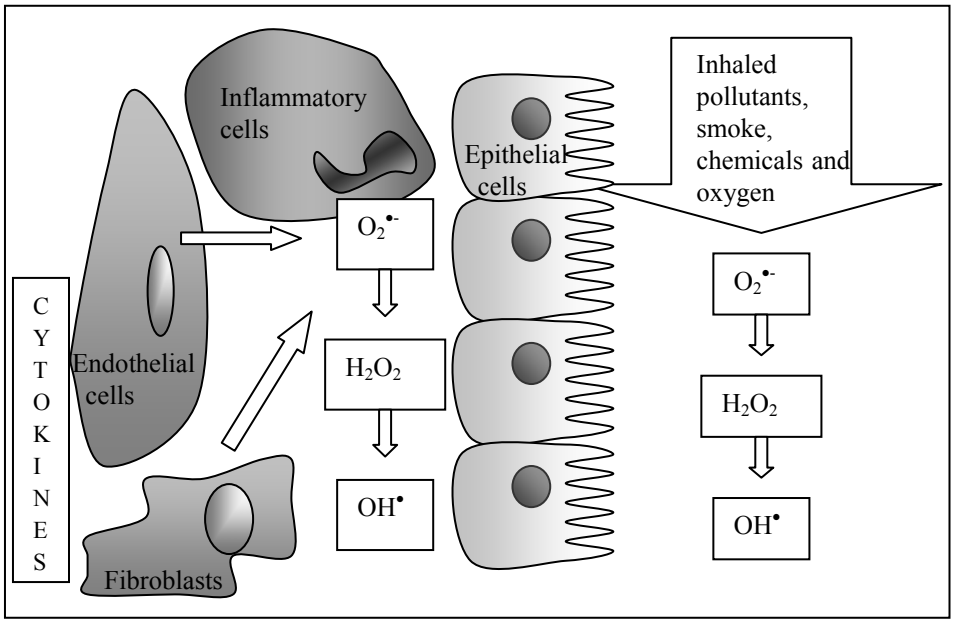

Fig. 1. Major sources of ROS in human lung (modified from Kinnula & Crapo 2003 and Kinnula & Crapo 2004).

One-electron reduction of O_2 yields $O_2^{\bullet-}$, which can be converted to other ROS like H_2O_2 or $OH^{\bullet}$. The amount of electrons leaking from the respiratory chain has been under debate, estimates varying from 0.1 % even to 2 % of total electron flow and even the whole concept of ROS generation in this way has been questioned (Boveris & Chance 1973, Imlay & Fridovich 1991, Forman & Azzi 1997). In addition to their location in the inner mitochondial membrane, cytochrome oxidases are also located in endoplasmic reticulum and participate in oxidizing several compounds like steroids, xenobiotics and fatty acids (Zangar *et al.* 2004)

In addition to the mitochondrial respiratory chain, peroxisomal β-oxidation may also generate H_2O_2 as a by-product (Kasai *et al.* 1989, Arnaiz *et al.* 1995). The first step it is the conversion of the fatty acyl-coenzyme A (-CoA) to *trans*-2-enoyl-CoA by acyl-CoA oxidase, which transfers electrons from the substrate to molecular oxygen, thus leading to H_2O_2 generation (Schulz 1991). The leakage of H_2O_2 out from peroxisomes may even be related to cell proliferation i.e. more H_2O_2 is claimed to be released in highly proliferating cells (Oikawa & Novikoff 1995). On the other hand, the destruction of H_2O_2 is very effective in peroxisomes, and thus there may be only minimal leakage of ROS to cytosol in general.

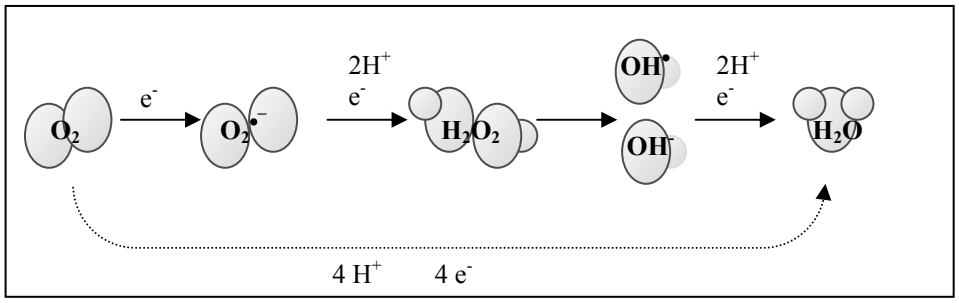

Fig. 2. Schematic representation of the sequential reduction of molecular oxygen to water.

There are also several other enzymes like NADPH oxidase (Nox), and xanthine oxidase (XO) that may produce ROS. Nox was originally defined as a phagocytic leukocyte specific enzyme, but subsequently similar oxidase complexes have been characterised in non-phagocytic cells such as vascular endothelial, smooth muscle cells and fibroblasts (Meier *et al.* 1991, Zulueta *et al.* 1995). In phagocytes, Nox is activated by chemokines or phagocytic particles, which induce assembly of cytosolic subunits (Rac2, $p47^{phox}$ and $p67^{phox}$) with membrane bound compartments ($p22^{phox}$ and gp91) to generate $O_2^{\bullet -}$ from O_2 and NADPH. This so called respiratory burst leads to the release of ROS out of the cell, which is an important feature of immune defence against bacterial and fungal infections (Holmes *et al.* 1967). Released $O_2^{\bullet -}$ may be converted to H_2O_2, which in turn is a substrate for myeloperoxidase that produces hypochlorous acid and other strong oxidants (Winterbourn *et al.* 2000).

In non-phagocytotic cells, endogenous ROS production by Nox has not been as extensively characterized. Several different homologues of gp91 are found in mammalians with tissue specific expression and Rac2 has a homologue Rac1, while other subunits seem to be expressed widely in different cells. Several factors can initiate ROS production by Nox e.g. interleukin 1β (IL1β), tumour necrosis factor α (TNFα), menadione, insulin, TGFβ or other growth factors (Nisimoto *et al.* 1988, Chiu *et al.* 2001, Li *et al.* 2002b, Baea *et al*. 2004, Chena *et al*. 2004, Talior *et al*. 2005).

XO, a key enzyme in purine catabolism, possesses the capability of generating $O_2^{\bullet -}$. It is located in cytoplasm, but is has been also detected on the outer surface of endothelial cells in an asymmetric manner (Rouquette *et al.* 1998). There are possibly several other systems also capable of generating ROS under certain circumstances. In addition, non-enzymatic reactions like radiolysis of water can result in the production of $H^{\bullet}$ and $OH^{\bullet}$ radicals.

2.1.3 Effects of reactive oxygen species

Reactive oxygen species can cause damage to living organisms via different targets as briefly reviewed in table 1. In multicellular organisms, ROS damages to DNA can be very harmful since they can cause mutations, which are then passed on to future cell

generations. However, more commonly ROS damage evokes apoptosis or necrosis, which can be very severe and extensive leading to major tissue damage.

In addition to H_2O_2, other ROS have also been linked to several signalling pathways and may have important regulatory functions. They have been associated with mitogen-activated kinase (MAPK), stress-activated protein kinase (SAPK) and protein kinase C (PKC) activities (Janssen-Heininger *et al.* 1999, Liu *et al.* 2000, Majumder *et al.* 2001). These provide tools for wide regulatory possibilities in cell growth, proliferation, apoptosis, differentiation and cellular metabolism.

Table 1. Targets of ROS damage in the cells.

Target	Damage	Primary consequence	Secondary consequence
DNA	Base damage Strand break	DNA repair Mutations	Cell cycle arrest Apoptosis Cancer or other diseases
RNA	Base damage Strand break	Translational errors Inhibition of protein synthesis	Alterations of cell growth and proliferation
Proteins	Oxidation of Cysteine or Methionine	Modified enzyme activity Modified protein stability Modified ion transport	Alterations of cell growth and proliferation
Lipids	Loss of unsaturation Formation of reactive metabolites	Membrane damage Altered membrane permeability Modified affinity of membrane bound proteins Chain reactions	Alterations of cell growth and proliferation
Carbohydrates	Formation of reactive metabolites	Disruption of other molecules Chain reactions	Alterations of cell growth and proliferation

Several transcription factors have been reported to be redox sensitive e.g. nuclear factor κB (NFκB), activator protein 1 (Ap1) and specificity protein 1 (Sp1) (Toledano & Leonard 1991, Li *et al.* 1994, Ammendola *et al.* 1994). However, the participation of endogenously produced ROS in the activation of NFκB was recently questioned, as in TNFα stimulated cells, the activation occurs independently of Rac/NADPH oxidase and is terminated before the ROS concentration has become elevated (Hayakawa *et al.* 2003). This suggestion is supported by earlier observations indicating that H_2O_2 does not activate NFκB in all cell types (Anderson *et al.* 1994, Bowie *et al.* 1997). Clearly further studies are needed to clarify the role and mechanism of the elevated ROS concentration during cell signalling processes.

Oxidative damage to lipids, proteins and especially to DNA is capable of activating the repairing machinery inside the cell. Oxidative DNA damage is predominantly repaired by base excision repair, nucleotide excision repair or direct ligation of single strand break. The first step in base excision repair is to remove oxidised nucleotides from

DNA by glycosylases, which cleave the bond between the base and the sugar. The resulting site without a base is removed by apurinic/apyrimidinic endonuclease and the subsequent gap is filled by DNA polymerase and ligated by DNA ligase (Kubota *et al.* 1996). Nucleotide excision repair removes a stretch of DNA, which is replaced after the synthesis of a new patch of DNA. This system requires the presencec of several components including replication factors, DNA polymerases, ligases and nucleases.

2.2 Antioxidants

Reactive oxygen species are detoxified by antioxidants, which include both enzymatic and non-enzymatic systems e.g. molecules like vitamin C, vitamin E and carotenoids. The most important enzymatic systems are superoxide dismutases (SODs), catalase and the glutathione peroxidases. There are also other important systems participating in the defence against oxidants.

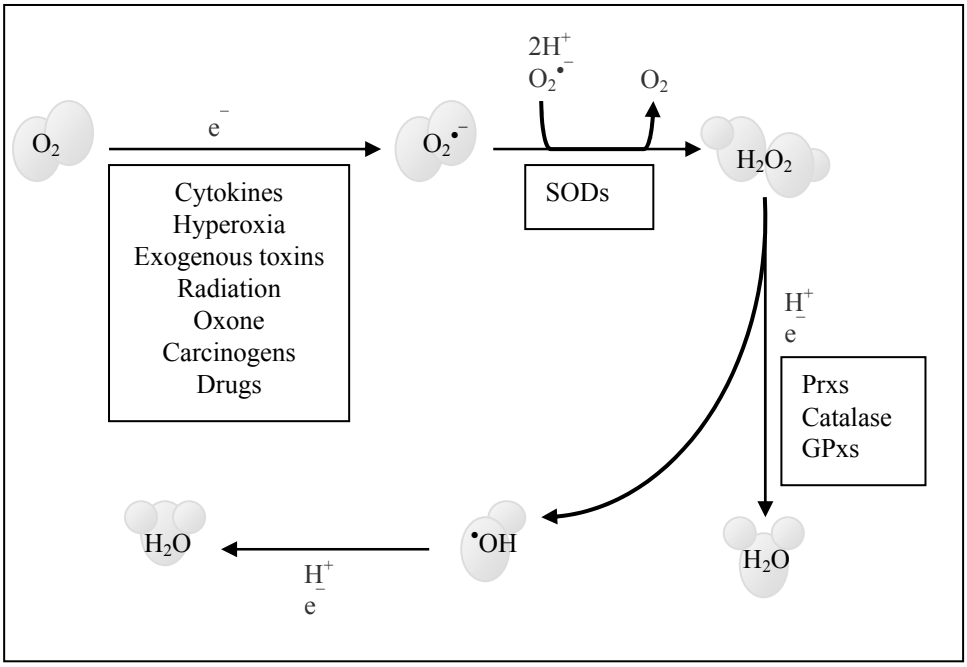

Fig. 3. Overview of ROS production and catabolism.

The first group comprises of enzymes associated with GSH including GSH S-transferases, glutamate-cysteine ligase (GCL, also known as γ-glutamyl-cysteine synthase) and glutaredoxins. The second group is formed by Trxs and Prxs, which are enzymes belonging to the thioredoxin system (Fig 3).

Oxidative stress may result not only from increased oxidant generation, but also from decreased antioxidant production or defective oxidative damage repair. Therefore the regulation of AOE expression is important and has been studied widely during recent years as have the effects of dietary antioxidants. Expression of several AOEs is regulated by transcription factors Ap1, NFκB, hypoxia inducible factor 1 (Hif1), Sp1 and/or nuclear factor erythroid 2 related factor 2 (Nfr2). Regulatory areas of these genes contain the so-called antioxidant response element (ARE), which binds Nfr2 (Jaiswal 1994, Ren & Smith 1995, Jones *et al.* 1995, Mulcahy & Gipp 1995).

2.2.1 Superoxide dismutases

Superoxide dismutases decompose $O_2^{\bullet-}$ to the less reactive H_2O_2. There are three different SODs in humans: cytosolic copper-zinc SOD (CuZnSOD); mitochondrial manganese SOD (MnSOD) and extracellular SOD (ECSOD). Mitochondrial metabolism is the major source of $O_2^{\bullet-}$ and therefore it is not surprising that MnSOD located in mitochondrial matrix seems to be the most essential of the SODs. According to mouse models, MnSOD seems to be crucial since knockout mice die within three weeks after birth, while CuZnSOD and ECSOD knockout mice are nearly normal (Carlsson *et al.* 1995, Li *et al.* 1995).

MnSOD is dramatically induced by oxidative stress like exposure to cigarette smoke, while other SODs are less strictly regulated (Gilks *et al.* 1998). According to transfection studies in cultured cells, the overexpression of MnSOD induces resistance against hyperoxia, cigarette smoke, cytokines, irradiation or oxidants (St Clair *et al.* 1991, Hirose *et al.* 1993, Lindau-Shepard *et al.* 1994, St Clair *et al.* 1994).

2.2.2 Catalase and glutathione peroxidases

There are three classes of enzymes that detoxify H_2O_2, namely catalases, GPxs and Prxs. Catalase is located mostly in peroxisomes, but detected to some extent also in cytoplasm and mitochondria. It decomposes high concentrations of H_2O_2 ($H_2O_2 \rightarrow H_2O + ½O_2$) with catalytical efficiency of about 10^6 $M^{-1}s^{-1}$ but it may also oxidize molecules like ethanol at low H_2O_2 concentrations (Hillar *et al.* 2000). Catalase tightly binds heme and NADH/NADPH, though the latter compounds do not affect the enzymatic reaction but rather protect it from inactivation (Kirkman & Gaetani 1984). It has been recently suggested that catalase may have a dual role in oxidant systems as it has been shown to generate ROS in response to UVB radiation in keratinocytes (Heck *et al.* 2003). This observation gives another interesting perspective on the vulnerable balance of oxidants and antioxidants i.e. even the same enzyme may be both a protecting and damaging component depending on the prevalent circumstances.

GPxs are enzymes that receive their reducing power from glutathione, which in turn is reduced by glutathione reductase ($H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$). The catalytical efficiency of GPxs is high, about 10^8 $M^{-1}s^{-1}$. There are several GPxs, which have different locations (Brigelius-Flohe 1999). GPx1 and GPx2 are intracellular but GPx2 is found

only in the epithelium of gastrointestinal tract while GPx1 has a more widespread distribution in human tissues. GPx1 knockout mice develop normally and show no sensitivity to hyperoxia (Ho *et al.* 1997). However these mice suffer some abnormalities in their brain during cold-induced injury or stroke, pointing to a role for GPx1 in the regulation of cell death and inflammatory responses (Flentjar *et al.* 2002, Crack *et al.* 2003). GPx3 is extracellular and located mainly in plasma (Schwaab *et al.* 1998). GPx4 is widely expressed but its catalytical activity may be targeted to hydroperoxides integrated in membranes (Kelner & Montoya 1998). GPx5 is epididymis-specific secreted protein (Rigaudiere *et al.* 1992).

2.2.3 Antioxidant related enzymes

The most important reductants in cells are GSH and Trx. Trxs are small proteins of 10-12 kDa. Their major role is to reduce oxidised ribonucleotide reductase, Prxs and certain transcription factors like NFκB (Hayashi *et al.* 1993, Chae *et al.* 1994a). Trxs contain two Cys residues separated by two amino acids at their catalytical site. Sulphydryl groups of these Cys undergo reversible oxidation providing reducing power for the substrate. The formed disulphide of Trx is reduced by thioredoxin reductase (TrxR) with electrons originating from NADPH.

In human cells, Trx and TrxR can exist in two isoforms, one mitochondrial and the other cytosolic. The processed form of Trx may also be secreted out from the cell, where it binds to the outer plasma membrane (Balcewicz-Sablinska *et al.* 1991). A nuclear localisation has also been detected in certain cancer cells, even though there is no nuclear localisation signal sequence, but the small size of these proteins allows them to pass through nuclear pores by passive diffusion (Hirota *et al.* 1997, Grogan *et al.* 2000). The number of different Trxs or TrxR may however be even higher, as recently new Trx like homologues have been found but their catalytical relevance is still unclear. It is feasible that there might be several Trxs with different properties, e.g. different substrate affinities.

Due to Trx's important role as a reductant, they have several biological tasks. Trx itself is able to break down H_2O_2, thus it can be considered as an antioxidant (Spector *et al.* 1988). It has growth factor like properties, being able to stimulate growth of fibroblasts and certain tumour cells (Wakasugi *et al.* 1990, Oblong *et al.* 1994). Trxs also regulate kinase activity and thus can modify cell signalling in several different pathways and e.g. the apoptosis pathways (Saitoh *et al.* 1998). Trx is highly expressed in several carcinomas and this may be related to their developing resistance against anticancer treatments (Gasdaska *et al.* 1994, Kahlos *et al.* 2001a).

GSH belongs to a family of low molecular weight antioxidants since it is a tripeptide. The Cys residue of GSH is responsible for redox status and it nonenzymatically forms conjugates with reactive electrophilic compounds like aldehydes or peroxides. The rate limiting enzyme in GSH formation is GCL, which is formed from two subunits: the heavy subunit with catalytical activity and the light subunit with a regulatory role. The GSH and Trx systems were originally considered as two distinct systems, but there is a

clear interaction between them as GSH can inactivate Trx (Casagrande *et al.* 2002). Oxidation of GSH can be reversed by GSH reductase.

2.3 Peroxiredoxins

2.3.1 Peroxiredoxins in different organisms

Peroxiredoxins (Prxs) are a family of small proteins that catalyse the reduction of peroxides using their conserved Cys residues as catalytical centres. They do not require any co-factors, unlike the heme-dependent catalase and the selenium-dependent GPxs. The best characterised enzymes of the Prx family are the alkyl hydroperoxide reductases (Ahp) from *Escherichia coli* and *Salmonella typhimurium* (Greenberg & Demple 1988, Jacobson *et al.* 1989). They were characterised as protectors of DNA against oxidative injury and they use NADH or NADPH as their reducing agent, but act independently of GSH.

There are five different Prxs in the yeast *Saccharomyces cerevisiae*, namely thiol specific antioxidant enzyme 1 (Tsa1, known also as cytosolic thioredoxin peroxidase I cTPx I), cTPx II, cTPx III, nTPx and mTPx (Chae *et al.* 1993, Verdoucq *et al.* 1999, Jeong *et al.* 1999, Lee *et al.* 1999, Pedrajas *et al.* 2000, Park *et al.* 2000a). Tsa1 is the most abundant Prx in the cytoplasm and mTPx is located in mitochondria. Genetic studies carried out in *S. cerevisiae* showed that Tsa1 is not essential for normal growth under anaerobic conditions (Chae *et al.* 1993). However, under aerobic conditions, the growth rate of a mutant yeast lacking *Tsa1* becomes depressed especially under oxidative pressure. The importance of Tsa1 as antioxidant is further confirmed by the fact that its expression is upregulated in normal yeast if they are shifted from anaerobic to hyperaerobic conditions (Kim *et al.* 1989).

In addition to microorganisms, Prx family members have been found in mammalians and plants (Chae *et al.* 1994b, Baier & Dietz 1996). There may be several different Prxs present in the same organism, up to eight in some plants with some being targeted to mitochondria and some to chloroplasts, where they are important in protecting proteins against oxidative injury and in maintaining normal photosynthesis (Baier & Dietz 1999). Some Prxs have a protective role also against reactive nitrogen species (Wong *et al.* 2002, Sakamoto *et al.* 2003).

In human cells, there are six different Prxs, which are named Prx I-VI. Prxs were originally characterized from mammalian cells mostly recognized for properties other than their antioxidant effects. They have been identified for example as proliferation associated gene (PAG), natural killer enhancing factor (NKEF), macrophage 23 kDa stress protein (MSP23), peroxisomal membrane protein (PMP), torin and 23 kDa heme binding protein (HBP23) (Ishii *et al.* 1993, Shau *et al.* 1994, Iwahara *et al.* 1995, Yamashita *et al.* 1999, Harris *et al.* 2001).

There is now convincing evidence for a role for H_2O_2 in cellular signalling, but the regulation of its concentration is poorly understood. As the concentrations needed for signalling are thought to be low, it seems unlikely that catalase could be the enzyme responsible for H_2O_2 removal. The catalytical efficiency of Prxs is weaker than that of

catalase or GPxs, but the optimal H_2O_2 concentration for Prxs is relatively low, and for this reason, these enzymes have been considered as potential regulators of endogenously produced H_2O_2. According to overexpression studies, cytosolic Prxs are known to break down H_2O_2 generated as second messengers of TNFα or growth factors and Prx III participates in apoptosis signalling in mitochondria (Kang *et al.* 1998b, Kang *et al.* 2004, Chang *et al.* 2004a).

Human Prx genes have been poorly characterized, but the murine genes have been analysed in some detail. Some properties of human Prxs are summarised in table 2. In addition to the indicated chromosomal locations, there are several pseudogenes found in the human genome e.g. in positions 9p22, 13q12 and 4q35.2.

2.3.2 Catalytical mechanism of peroxiredoxins

Peroxiredoxins can be divided into three categories according to their structure and catalytical mechanism, namely 2-Cys Prxs, atypical 2-Cys Prxs and one cysteine (1-Cys) Prxs. Human Prxs I-IV belong to the first category, Prx V is an atypical 2-Cys Prx and Prx VI is 1-Cys Prx (Kang *et al.* 1998a, Kang *et al.* 1998b).

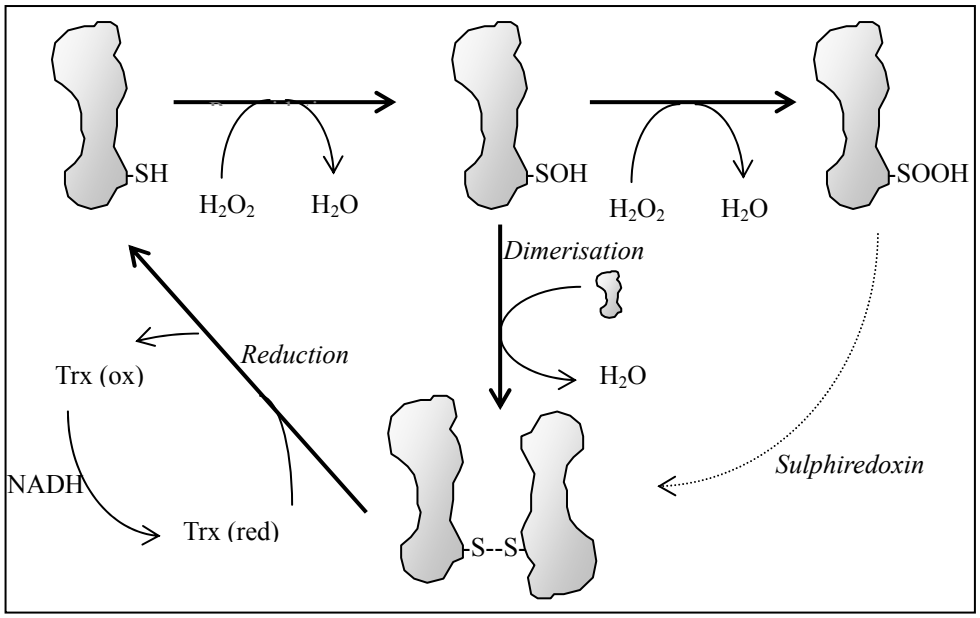

Fig. 4. Model of typical 2-Cys Prx catalytical mechanism partially modified from Wood and co-workers (2002).

2-Cys Prxs seem to be mostly present as homodimers, but they may also form heterodimers and several dimers may further group together to form even decamers

(Schroder *et al.* 2000, Harris *et al.* 2001, Wood *et al.* 2002). Prxs oligomerisation is dependent on ionic strength, pH, magnesium/calcium concentrations and most of all, the redox state of the active Cys. It has been suggested that the peroxidase reaction of all Prxs is initiated when the thiol group of cysteine (–SH) attacks peroxide (figure 4).

The thiol group becomes itself sulphenic acid (-S-OH), which is highly unstable and quickly forms either a disulphide bridge (-S-S-) with another thiol or is converted to sulphinic acid (-SOOH) or even sulphonic acid (-SOOOH). Disulphide bridges are generally reduced by thioredoxin or possibly by glutathione, but these reductants are not able to reduce sulphinic acid forms of cysteines. Previously these so called over-oxidised forms were thought to be irreversible, but recent data suggests that they are reduced by a protein called sulfiredoxin (Biteau *et al.* 2003, Woo *et al.* 2003). The catalytical mechanism of Prxs exhibits similarities to other thiol proteins (Poole *et al*. 2004).

Atypical 2-Cys Prxs probably form an intramolecular disulphide bridge instead of dimerization as shown in figure 5 (Seo *et al.* 2000). 1-Cys Prxs bind some other molecule than Prx.

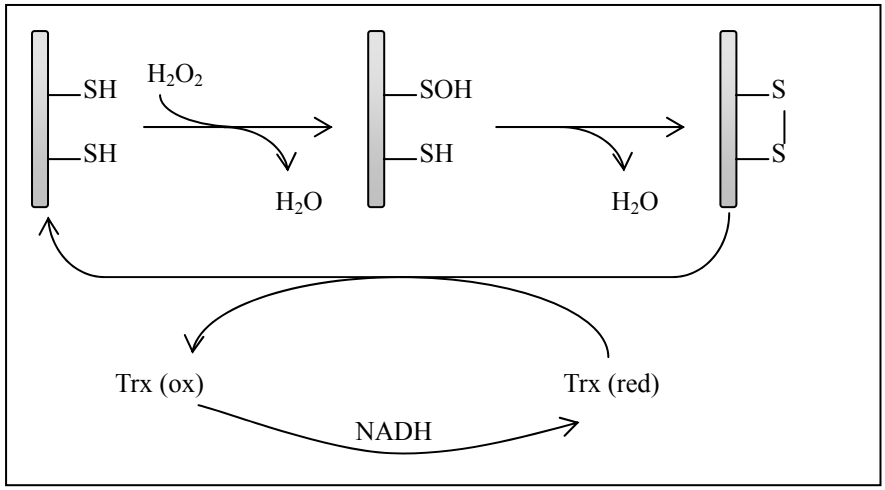

Fig. 5. Model of atypical 2-Cys Prx catalytical mechanism as proposed by Seo and co-authors (2000).

Prxs can undergo a direct interaction with cyclophilin A. Cyclophilins are abundant proteins first characterised for their ability to act as a cyclosporine A receptor. They have *cis-trans* isomerase activity fitting with their proposed role as chaperones (Ivery 2000). Additionally cyclophilins seem to act as mediators in signalling and have growth factor like effects after oxidant exposure (Jin *et al.* 2000).

2.3.3 Sulfiredoxin

Sulfiredoxin was recently characterized from budding yeast as an enzyme with an ability to reduce Cys-SOOH (Biteau *et al.* 2003). Sulfiredoxin, like Trxs and Prxs, is a thiol protein whose activity is based on its Cys residue. It has conserved homologues in higher eucaryotes, but they are so far poorly characterized. No homologue has been found in procaryotes, except in certain cyanobacteria. Bacterial Prxs are indeed much less sensitive to overoxidation, as they generally have a slightly different structure at their C-terminal end. Budding yeast has both sensitive and insensitive Prxs for overoxidation and sulfiredoxin was shown especially to affect the activity of sensitive forms. Yeast sulfiredoxin was proposed to act both as a phosphotransferase and a thioltransferase, as ATP hydrolysis, Mg-ion and dithiotreitol was essential for the function. Mammalian sulfiredoxins seem to have different mechanisms of action as they prefer using GTP hydrolysis as power and Trx or GSH as co-factor (Chang *et al.* 2004b). The substrate specificity of sulfiredoxins is still unclear but they cannot rescue the overoxidised forms of atypical 2-Cys Prxs or 1-Cys Prxs (Woo *et al.* 2004) but on the other hand, their activity may not be targeted only to Prxs, but they may be involved in repairing several other damaged proteins as well.

2.3.4 Peroxiredoxin I

Human Prx I is a 22 kDa protein expressed ubiqitously throughout the body. It has been known by the names PAG-A, NKEF-A and thioredoxin peroxidase A (TpX-A or TDPX-A) (Prosperi *et al.* 1993, Prosperi *et al.* 1994, Shau *et al.* 1994, Pahl *et al.* 1995). Its mouse homologue is known also as MSP23 or osteoblast specific factor (OSF-3) (Ishii *et al.* 1993, Kawai *et al.* 1994). It has been considered to be a cytoplamic enzyme, but a recent report has suggested additionally nuclear, mitochondrial and peroxisomal locations (Immenschuh *et al.* 2003). Its expression is induced by serum, oxidative stress and in some cases by H_2O_2 but not in every cell type (Ishii *et al.* 1993, Prosperi *et al.* 1993, Prosperi *et al.* 1994, Kim *et al.* 2000). In cultured murine cells, Prx I expression is cell cycle dependent being highest at the S phase (Prosperi *et al.* 1998). This has not been observed in human cells, but instead Prx I phosphorylation is affected by cell cycle state so that it occurs during mitosis (Chang *et al.* 2002). This Cdc2 mediated phosphorylation on threonine 90 inhibits Prx I activity, which may have an important role in regulating the H_2O_2 concentration during mitosis. However, only a fraction of Prx I is phosphorylated, which may in theory even result in a H_2O_2 gradient inside the cell.

Prx I expression is upregulated in the rat lung during late gestation being highest at the time of birth (Kim *et al.* 2001a). This upregulation seems to occur at the translational level as the amount of mRNA is even lowered. In newborn rats, the expression is lowered within a few days to adult levels but can be again induced by hyperoxia. Prx I expression has also been studied in the fetal baboon lung, but only at the mRNA level, where it remained constant during the last trimester (Das *et al.* 2001). The amount of mRNA was however increased after birth in an oxygen dependent manner via PKC regulation. Sodium arsenate, an agent inducing oxidative stress, increases the level of Prx I

expression as well acting via a PKCδ mediated mechanism in murine osteoblasts (Li *et al.* 2002a). Very little is known about the details of Prx I induction, but transcription factor Nrf2 is essential in this induction response, at least in mouse macrophages (Ishii *et al.* 2000).

In cultured cells, the overexpression of Prx I protects cells against apoptosis induced by H_2O_2 but not by other agents such as doxorubicin (Berggren *et al.* 2001). Murine Prx I has been shown to interact with the SH-3 and kinase domains of c-abl and to reverse c-abl´s cytostatic effects (Wen & Van Etten 1997). It also interacts with the Myc Box II (MBII) region of the c-Myc transactivation domain (TAD), which is highly conserved among all Myc oncoprotein members (Mu *et al.* 2002). Prx I and c-Myc also confer resistance to oxidative stress. In contrast, Prx I inhibits tumorigenesis by c-Myc overexpressing fibroblasts and cause a selective loss of c-Myc target gene regulation, which suggests a role for Prx I as a tumor suppressor. This is also supported by results from a transgenic mouse model: *Prx I* knockout mice develop lymphomas, sarcomas and carcinomas (Neumann *et al.* 2003). Mice lacking Prx I suffer also from haemolytic anaemia, which shows the importance of Prx I for erythrocyte antioxidative defence (Immenschuh *et al.* 2003).

2.3.5 Peroxiredoxin II

Prx II (22 kDa) is also known as TDPX-1 or -B, TpX-B, PAG-B, NKEF-B, protector protein (PRP), TSA, torin and calpromotin (Harris & Naeem 1981, Kim *et al.* 1988, Moore *et al.* 1990, Prosperi *et al.* 1994, Shau *et al.* 1994). It is very similar to Prx I in the structure, subcellular location and catalytical properties (Lim *et al.* 1994). Prx II has some anti-apoptotic characteristics as its overexpression leads to resistance against cisplatin, irradiation, serum starvation, ceramide and etoposide (Zhang *et al.* 1997, Park *et al.* 2000b, Chung *et al.* 2001). Manipulations of Prx II levels have also shown that Prx II inhibits NFκB activation after TNFα or H_2O_2 treatment and they have also shown the importance of the H_2O_2 concentration in several signalling cascades downstream to TNFα (Kang *et al.* 1998b, Kang *et al.* 2004). Artificial Prx II down-regulation in cultured cells makes the cells more susceptible to apoptosis by cisplatin treatment, and in that way it can be considered as a chemosensitiser, which makes it extremely important in the cancer treatment strategies (Yoo *et al.* 2002).

Prx II expression in the rat lung during perinatal period is constant at the protein level but transcriptional induction is observed at the time of the birth (Kim *et al.* 2001a). The regulation of Prx II expression is poorly characterised, but it does not seem to be altered by H_2O_2 (Seo *et al.* 1999, Kim *et al.* 2000). Prx II expression is induced by ultraviolet B radiation in rat skin and by a high glucose content in glomerular mesangial cells (Lee *et al.* 2000a, Morrison *et al.* 2004).

Prx II is an abundant protein in erythrocytes, *Prx II* knockout mice develop hemolytic anemia (Lee *et al.* 2003). It binds membrane proteins or membranes by its C-terminus (Moore & Shriver 1997, Cha *et al.* 2000).

2.3.6 Peroxiredoxin III

Prx III is a mitochondrial enzyme and it may have a significant role in the protection against ROS produced by aerobic metabolism. In mouse, it is known by the names antioxidant protein 1 (AOP1) and Mer5 (Tsuji *et al.* 1995). Prx III has a mitochondrial localisation signal sequence that is cleaved. Antisense experiments performed on murine cells have revealed that it is essential for normal mitochondrial function as its depletion decreases mitochondrial mass, membrane potential and affects mitochondrial morphology (Wonsey *et al.* 2002). Prx III activity is negatively regulated by protein synthesis inhibitor Abrin A-chain (ABRA), which is an inducer of apoptosis (Shih *et al.* 2001). This interaction can influence the ROS levels in the mitochodria thus resulting in the release of cytochrome c that activates the caspase cascade. Cyclophilin 18 binds and stimulates its activity, but the significance of this interaction is not clear as they have different subcellular localizations (Jaschke *et al.* 1998). *Prx III* is regulated by c-Myc and on the other hand it is required for c-Myc mediated proliferation, transformation and apoptosis (Wonsey *et al.* 2002).

2.3.7 Peroxiredoxin IV

Prx IV, which is also called antioxidant enzyme 372 (AOE372) and TPx related activator of NF-κB (TRANK), is partly extracellular and the intracellular precursor is proposed to be inactive (Okado-Matsumoto *et al.* 2000). The location and the role of Prx IV have been under debate due to conflicting reports, but probably the intracellular or membrane bound form of 31 kDa is cleaved to the secreted form of 27 kDa in certain but not in all cell types. In cultured rodent cells, it has been detected only in the intracellular space, where its overexpression prevents ROS production by p53 or EGF (Wong *et al.* 2000). Prx IV seems to have cytokine-like properties, as Prx IV exposure of the cells induces iNOS by receptor-mediated mechanism (Haridas *et al.* 1998). It also activates c-Jun N-terminal kinase and induces proliferation in fibroblasts.

Prx IV can bind to heparan sulphate, which is located on the cell surface. It has been proposed that Prx IV is anchored on the extracellular surface, where its function is regulated by extracellular signals like redox status (Okado-Matsumoto *et al.* 2000). Prx IV may have an effect on rat spermiogenesis during membrane rearrangement as cleaved and membrane bound forms seem to be present in different phases (Sasagawa *et al.* 2001).

2.3.8 Peroxiredoxin V

Prx V is the smallest (22 kDa and cleaved form 17 kDa) but most widely subcellularly distributed of the Prxs; it is cytoplasmic, nuclear and organellar (Knoops *et al.* 1999, Seo *et al.* 2000, Zhou *et al.* 2000). It was first identified as a gene that is downregulated in adenovirus-infected cells and it has been known by names like antioxidant enzyme 166

(AOE166), PMP20 or ARC1 (Wattiez *et al.* 1999, Kropotov *et al.* 1999). It has the capability to bind DNA and to repress RNA polymerase III catalysed transcription *in vitro*, but the *in vivo* significance of these abilities remains unclear (Kropotov *et al.* 1999). In mouse, there are different transcripts produced tissue-specifically but some of them are possibly translationally inactive (Lee *et al.* 2000b, Sparling & Phelan 2003). Prx V expression is induced by calcium in keratinocytes but in other cell types the regulation is unclear (Seo *et al.* 2002). Also Prx V overexpression prevents p53 induced ROS generation and apoptosis, furthermore when targetted to the nucleus it can protect DNA (Zhou *et al.* 2000, Banmeyer *et al.* 2004). Downregulation of Prx V increases the amount of etoposide-induced DNA double-strand breaks (Kropotov *et al.* 2004). In the rat lung inflammation increases Prx V transcription pointing to protective role for Prx V in lung during inflammatory processes (Knoops *et al.* 1999). Prx V is the only one of human Prxs known to act also as a peroxynitrite reductase (Dubuisson *et al.* 2004).

2.3.9 Peroxiredoxin VI

Prx VI has been known by names AOP2, ORF6, phospholipase A_2 (PLA2), KIAA0106 and non-selenium glutathione peroxidase (Nagase *et al.* 1995, Jin *et al.* 1997, Kim *et al.* 1997, Kim *et al.* 1998, Phelan *et al.* 1998). Prx VI is cytosolic, but it differs remarkably from Prxs I and II as it belongs to the group of 1-Cys Prxs and it is not reduced by Trx but rather GSH (Fisher *et al.* 1999, Fratelli *et al.* 2002, Manevich *et al.* 2004). It has not only peroxidase but also possesses phospholipase A_2 activity and has been implicated in repairing oxidised lipids (Manevich *et al.* 2002). Animal models have indicated that it is possibly the most important Prx in the lung and also highly expressed there (Kim *et al.* 1998, Kim *et al.* 2002, Wang *et al.* 2003, Wang *et al.* 2004).

Prx VI knock-out mice have a shorter life span, severe tissue damage and increased protein oxidation compared to normal mice (Wang *et al.* 2003). Also knock-in mice have been developed and they have a decreased H_2O_2 accumulation (Phelan *et al.* 2003), but the relevance of this finding to the normal life span or diseases is not clear, even though oxidative stress in the rat lung is known to induce Prx VI expression (Kim *et al.* 2003). Adenovirus mediated transfer of *Prx VI* into murine lung protects its lung against hyperoxic injury thus supporting results obtained from transgenic animals (Wang *et al.* 2004).

2.3.10 Human peroxiredoxins

Increased oxidative stress in tissues and cells has to be combalted by increased antioxidant capacity. Indeed, Prx expression is known to be increased in several different carcinomas. One preliminary study performed in two different cell lines originating from human lung and in four homogenate samples of lung carcinoma indicated that Prx I expression might be altered in lung carcinoma (Chang *et al.* 2001). Prx I expression is high also in thyroid, oral and breast carcinomas (Yanagawa *et al.* 1999, Yanagawa *et al.* 2000, Noh *et al.* 2001, Karihtala *et al.* 2003). Mouse models support the idea for an

important role for Prxs in several diseases. Prx level alterations have been also noted in Creutzfeld-Jacob disease, Alzheimer's disease, Pick's and Down syndromes (Kim *et al.* 2001b, Nicolls *et al.* 2003, Sanchez-Font *et al.* 2003). The properties of human Prxs are summarised in table 2.

Table 2. Properties of human Prxs.

Prx	Length (amino acids)	Calculated size (kDa)	Cleaved form (kDa)	Genebank accession number	Interactions with	Chromosome
I	199	22	-	GI 440306	C-myc Heme c-Abl Macrophage migration inhibitory factor Cyclophilin	1p34.1
II	198	22	-	GI 440308	Membrane Cyclophilin	19p13.2
III	256	28	21	GI 682748	ABRA Cyclophilin	10q25-26
IV	271	31	27	GI 799381	Heparin Cyclophilin	Xp22.11
V	214	22	17	GI 6103724	DNA Cyclophilin	11q13
VI	224	25	-	GI 285949	Cyclophilin	1q25.1

2.4 Human lung diseases associated with reactive oxygen species and antioxidant enzymes in human lung

ROS have been linked to several both non-malignant and malignant lung diseases e.g. obstructive lung diseases (asthma and chronic obstructive pulmonary disease COPD), interstitial lung diseases (granulomatous diseases and idiopathic pneumonias), carcinomas, dysplasias and respiratory distress syndrome (Saetta *et al*. 2001, Kinnula & Crapo 2003, Kinnula & Crapo 2004, Kinnula *et al*. 2004). Cigarette smoke contains free radicals and other ROS, but it also activates inflammatory cells to produce the ROS and thus a high proportion of patients suffering lung disease will have a smoking history.

Normal human lung is well protected against ROS. The epithelial lining fluid contains GSH, vitamin E, vitamin C and several important proteins like metal binding transferrin (Cantin *et al.* 1987, Cantin *et al.* 1990, Kinnula *et al.* 2004). Furthermore, several AOEs including CuZnSOD, MnSOD, ECSOD and catalase are expressed in the epithelium of the human airways (Marklund 1984, Oury *et al.* 1996, Lakari *et al.* 1998, Lakari *et al.*

2000). All of these enzymes are detected in both bronchial and alveolar epithelium. MnSOD and ECSOD are also located in macrophages. All of these enzymes, except MnSOD, can be found in vascular endothelial cells and MnSOD and CuZnSOD are also found in fibroblasts.

2.4.1 Chronic obstructive pulmonary disease

One common disease generally caused by smoking is COPD. It is a severe and progressive disease characterized by inflammation in airways, obstruction of peripheral airways and emphysema. It consists of morphological changes in both central and peripheral airways. Histopathological changes include increased amounts of macrophages, neutrophils, Goblet cells and enlarged mucous secreting glands, but fewer ciliated cells. Tissue proteolysis results in loss of lung elasticity and reduces the amount of alveolar epithelial cells. Oxidant stress plays an important role in injury and the inflammatory responses of COPD and thus the regulation of antioxidant enzymes may have a crucial effect on the initiation and progression of this disease (Ichinose *et al.* 2000, Paredi *et al.* 2000, Saetta *et al.* 2001, MacNee 2001, Langen *et al* 2003).

2.4.2 Pulmonary sarcoidosis

Sarcoidosis is a granulomatous disorder of unknown origin. In addition to granulomas, it is characterised by inflammation. It is capable spreading over several organs and its grade can vary from spontaneous recovery to fatal. ROS production especially from inflammatory cells increases the oxidative stress during sarcoidosis and presumably can affect the pathogenesis of this disease (Calhoun *et al* 1988). MnSOD is highly expressed in granulomas and is upregulated also in bronchoalveolar lavage (BAL) of patients with sarcoidosis (Lakari *et al.* 1998, Lakari *et al.* 2000)

2.4.3 Lung carcinomas

Lung carcinomas can be divided to small cell and non-small cell lung carcinomas. Small cell carcinomas are usually aggressive but sensitive to anti-cancer drugs. Non-small cell carcinomas include adenocarcinomas, large cell carcinomas and squamous cell carcinomas, which are often resistant to drugs. In addition to smoking, asbestos exposure is considered as a risk factor for their promotion (Rahman *et al.* 1977, Mossman & Gee 1989). ROS are important in initiation, progression and even treatment of lung carcinomas as ROS affect mitogenic signalling, cell motility, tumour invasiveness and apoptosis (Behrend *et al.* 2003, Knaapen *et al.* 2004). In particular, p53 mediated apoptosis and hypoxia-inducible factor stabilisation with resulting angiogenesis are important ROS sensitive mechanisms associated with tumour initiation and progression (Green & Reed 1998, Chandel & Schumacker 2000). The levels of antioxidant enzymes

in lung caner are variable, but there is evidence that at least the Trx-system is highly elevated in this tumor and this is claimed to have an association to patient prognosis (Kahlos *et al.* 2001a, Raffel *et al.* 2003).

2.4.4 Malignant pleural mesothelioma

Malignant pleural mesothelioma is often attributable to asbestos exposure. Asbestos causes DNA strand breaks and damage to cells (Jackson *et al.* 1987, Kamp *et al.* 1992). Even though it is rather rare tumour, its resistance to therapeutics makes it an important target of cancer research. Mesothelioma contains elevated levels of several AOEs including MnSOD, catalase and GCL and Trx (Kahlos *et al.* 1998, Kahlos *et al.* 2001b, Järvinen *et al.* 2002, Kinnula & Crapo 2004).

3 Aims of the study

The purpose of the study was to understand the mechanisms of Prx expression, action and regulation in human lung. The more specific aims of the study were:

1. To determine the cell specific expression and subcellular localization of Prxs at the protein level in normal human lung and in various cultured human lung cell lines including malignant A549 cells and five different mesothelioma cell lines , and non–malignant bronchial Beas2B cells and mesothelial cells Met5A.
2. To assess the cell specific expression of Prxs during inflammation (sarcoidosis).
3. To examine Prx during different malignancies of the lung (especially squamous cell carcinoma and adenocarcinoma) and pleura (malignant pleural mesothelioma).
4. To study the responses of Prx, Trx and TrxR expression to smoking and in patients with COPD.
5. To assess the regulation of Prxs at the transcriptional and translational levels, and the post-translational oxidation state in lung cells exposed to oxidants or cytokines that are relevant in human lung diseases.

4 Materials

4.1 Biopsies and bronchoalveolar lavage (I, II, III, IV)

Biopsies used were retrieved from the files of the Department of Pathology. Clinical information of all patients was received from patient records from Oulu University Hospital. Parenchymal biopsy specimens with sarcoidosis from ten patients and four control samples with normal histopathology from non–smokers were studied by immunohistochemistry (I). Samples with malignant mesothelioma from 36 patients and four apparently normal control samples of pleura (II), 92 carcinoma samples from patients with lung cancer and six histopathologically normal controls (III) were analysed by immunohistochemistry. Fifteen patients with COPD, 9 nonsmoking and 17 smoking controls (IV) were studied by immunohistochemistry so that a central and peripheral sample of each patient were analysed.

Tissue carcinoma samples from five patients with adenocarcinoma and six with squamous cell carcinoma were analysed by Western and/or RT-PCR (III). Seven peripheral tissue samples from patients with COPD and three healthy controls were studied by Western analysis (IV). The cells from the bronchoalveolar lavage were studied from four patients with sarcoidosis and two controls (I). The controls were suffering from minor respiratory symptoms, but their BAL cell profile was normal. Bronchoscopic examinations have been conducted as differential diagnostic investigations under local anesthesia.

Usage of patient samples in this study has been approved by Ethics committee of Northern Ostrobothnica District and TEO as required.

4.2 Cell cultures (I, II, IV and V)

Malignant lung adenocarcinoma cell line A549 derived from type II pneumocyte, non-malignant SV40 transformed bronchial epithelial Beas 2B cells and non-malignant SV40 transformed pleural mesothelial Met5A cells have been purchased from American Type Culture Collections or from National Cancer Institute (Laboratory of Human

Carcinogenesis, Dr Harris, Bethesda, USA). These cell lines were selected as they are well characterised and studied. Five different continuous mesothelioma cell lines (M14K, M24K, M25K, M28K and M38K) have been originally established from the tumour cells of five patients with untreated malignant mesothelioma (Pelin-Enlund *et al*. 1990).

4.3 Antibodies (I, II, III, IV and V)

Polyclonal anti-Prx antibodies were kindly provided by Professor Sue Goo Rhee (Laboratory of Cell Signaling, National Institute of Health, Bethesda, MD, USA) and Professor Sang Won Kang (Ewha Womans University, Seuol, Repulic of Korea). They are well characterised antibodies and have high specificity (Jin *et al.* 1997, Kang *et al* 1998a, Kang *et al*. 1998b, Seo *et al*. 2000). Monoclonal anti-β-actin antibody (Sigma-Aldrich) has been used to normalize the detection. Trx and TrxR antibodies (Sigma-Aldrich) were used in some cases. Polyclonal MnSOD and GCS heavy chain antibodies were generous gifts from Professor J.D. Crapo and T. Kavanagh and they were used as controls of cell culture treatments. Ki–67 antibody (Zymed) was used to estimate the proliferation state of cells (II) since Ki–67 is expressed only in proliferating cells but is missing from resting cells (Gerdes *et al.* 1984).

5 Methods

5.1 Cell culture treatments (I, II, IV and V)

The cells were cultured according to the suppliers' instructions. Cultured A549 and Beas2B cells were exposed to variable concentration of H_2O_2 to create direct oxidative stress and to menadione or cisplatin to cause endogenous ROS production (IV and V). TNFα was used because it is an important cytokine and is known to upregulate MnSOD expression. TGFβ was selected as it is a growth factor whose function is associated with ROS and it downregulates GCL expression. BSO was used to deplete GSH, which affects the redox state of the cell. Generally cells were passaged the day before the initiation of exposure so that growth phase had started. The agent was added to normal growth medium and cells were cultured there for 1-72 hours. For some overoxidation analysis, the cells received a 30-60 min pulse of H_2O_2, after which the medium was replaced with fresh growth media.

In the serum starvation and stimulation assay, the cells were passaged normally and on the next day the normal medium was replaced with medium containing no serum. Cells were cultivated for 72 h to synchronise the cells, after which the medium was replaced with normal media.

5.2 Protein analysis

5.2.1 Immunohistochemistry (I, II, III and IV)

The tissues were fixed in 10 % formalin and embedded in paraffin after dehydration. Four μm thick sections were cut from paraffin embedded biopsies and processed routinely through xylene and ethanol series. Immunostainings were done by Histostain™ –Plus Kit (Zymed Laboratories Inc., USA) according to the manufacturer's instructions. This staining is based on biotinylated secondary antibody in conjuction with streptavidin peroxidase. Counterstaining was performed with Mayer-hematoxylin. Stain intensities have been evaluated semiquantitatively in collaboration with two or three pathologists.

Negative controls containing PBS or isotype control serum from non–immunised host animal instead of primary antibody were prepared in each staining series and confirmed to be negative. Dilutions of Prx antibodies varied slightly in different series due the different lots used.

5.2.2 Western blotting (I, II, III, IV and V)

For Western analysis, the biopsies were stored in liquid nitrogen and cultured cells were trypsinated and the cell pellets were stored at -70°C after washing with PBS. Western blotting was used to detect Prxs after routine SDS-PAGE or after non-reducing electrophoresis. Non-reducing electrophoresis was performed as standard SDS-PAGE, except that the sample buffer contained no β-mecaptoethanol. This left the subunits of the dimeric forms associated together, since to break the disulphide bridge would require addition of a strong reductant. An enhanced chemilumenescence system was used for detection.

5.2.3 Immunoelectron microscopy (II)

Immunoelectron microscopy was performed to study the subcellular localisation of Prxs in lung cells. Cultured cells were fixed in 4 % paraformaldehyde, immersed with 2.3 M sucrose and frozen. Cryosections were treated with Prx antibodies and thereafter with Protein A-gold conjugate. Methyl cellulose embedded sections were analysed with a transmission electron microscope.

5.3 Transcript analysis

5.3.1 Extraction of RNA (III and V)

For RNA isolation, the cell pellets were stored at -70°C and tissue samples were incubated with RNAlater™ (Ambion) prior to storage in liquid nitrogen. The mRNAs of the cultured cells were extracted with the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia). Alternatively total RNA of cultured cells or human tissues was purified by Rneasy Mini Kit (Qiagen).

5.3.2 Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) (III and V)

The RT-PCR primers were designed to be cDNA specific and to contain at least one predicted intron sequence in order to avoid contamination of genomic DNA. Competitive

RT-PCR controls for Prxs were prepared by PCR using recombinant primers, which caused a 50-100 bp deletion into the product leaving the RT-PCR primer binding sites intact. These products were subcloned into pGem-3zf and amplified in *E. coli* XL1-blue strain. Plasmids were purified from the bacteria and RNA was produced by the Riboprobe *in vitro* transcription system (Promega) according to the manufacturer's instructions. Produced control RNA was used in the same RT-reaction as the mRNA under investigation. By preparing serial dilutions of the control RNA and comparing the amounts of PCR products from the control and studied mRNA it was possible to obtain an estimate of the amount of mRNA level in the sample.

5.4 Assessment of proliferation and apoptosis (II)

Proliferation of tumour cells was estimated immunohistochemically by staining with Ki–67 antibody. Apoptosis was detected by the ApopTag Kit (Oncor) that stains the 3'–ends of DNA fragments present in apoptotic cells.

5.5 Computational and statistical analysis

Alignment analyses were performed by BLAST programs available at www.ncbi.nlm.nih.gov against nucleotide, expressed sequence tags of human (human-EST) or genomic nucleotide databases for designing proper primers for RT–PCR (Altschul *et al.* 1990). Genetic structure was predicted also to help primer design by comparing genomic sequences with cDNAs (GeneFinder, URL www.bcm.edu). Promoter prediction and computational analysis was done by using the TFFACTOR, TFSITE and Eucaryotic Promoter Database (URL www.embl–heidelberg.de) to help in the Prx regulation studies. Statistical analysis was performed with SPSS 10.0 or 11.5 for Windows (Chicago, IL, USA). Data was analysed using ANOVA and *post hoc* comparison (continuous data) or using Fisher's exact test (categorical data). Kaplan-Meier curves were used for survival studies and log-rank, Breslow and Tarone-Ware tests were used to test the statistical significance. Generally, probability values below 0.05 were considered statistically significant. The immunohistochemical evaluations of two pathologists were compared using Cohen's κ statistics to ensure good agreement.

6 Results

6.1 Peroxiredoxins in healthy human lung (I, II, III and IV)

All six Prxs were clearly detectable in homogenates of apparently normal lung by Western analysis and by RT-PCR. Immunohistochemical staining revealed the expression to be cell-specific according to table 3. Prxs I and III had a similar cell specific expression, that was highest in bronchial epithelial cells and alveolar macrophages, but lower expression could be detected in alveolar epithelium and vascular endothelium. Prx II was seen in epithelial cells and macrophages. Prx IV was expressed in bronchial epithelium and alveolar macrophages. Prxs V and VI were located in epithelium and a lesser extent in macrophages.

Table 3. Cell-specific expression of Prxs in normal human lung.

Cell type	Prx I	Prx II	Prx III	Prx IV	Prx V	Prx VI
Bronchial epithelium	Moderate	Low	High	Low	High	High
Endothelium	Low	No	Low	No	No	Low
Alveolar epithelium	Low	Low	Moderate	No	High	High
Alveolar macrophages	Moderate	Low	High	Low	Moderate	Moderate

Immunohistochemistry and immunoelectron microscopy revealed that the Prxs had different subcellular locations in the manner shown in figure 6. Even though Prxs can be detected in several compartments of the cell, their nuclear location can be seen only occasionally and not for example in cultured A549 cells.

6.2 Peroxiredoxins in pulmonary sarcoidosis (I)

The expression pattern of Prxs in parenchymal tissue and BAL fluid cells of pulmonary sarcoidosis was very similar to that seen in normal lung, but there was high expression of

Prxs I and III in granulomas detected by immunohistochemistry (table 4). Also Prxs IV, V and VI were detected in granulomas but less intensively.

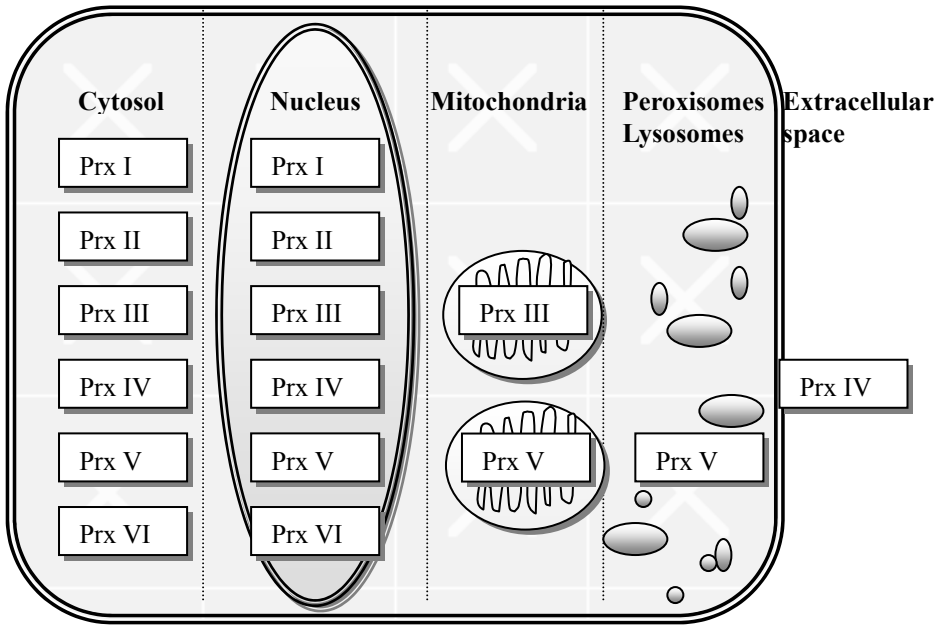

Fig. 6. Subcellular locations of Prxs in cultured human lung cells.

6.3 Peroxiredoxins in malignant mesothelioma and lung carcinomas (II and III)

All of the Prxs except Prx IV were highly expressed in malignant mesothelioma, and their expressions were conspicuously higher than in normal pleura (table 4). In adenocarcinoma and squamous cell carcinoma Prxs I, II and IV were highly expressed compared to normal lung as reviewed in table 4. Additionally Prx VI was elevated in adenocarcinoma but not in squamous cell carcinoma. In particular high Prx IV expression was seen in adenocarcinoma and Prx VI expression was associated with high grade squamous cell carcinoma.

6.4 Peroxiredoxins, thioredoxin and thioredoxin reductase in smoker's lung and in chronic obstructive pulmonary disorder (IV)

Prxs were studied from lung homogenates of non-smokers, smokers and smokers with COPD. Since Prx VI is highly expressed in human lung and its role there is significant, Prxs V and VI were selected for more detailed analysis by immunohistochemistry. Trx and TrxR were also analysed in these cases.

Trx was significantly increased in the smokers' lung bronchial epithelium but other effects of smoking or COPD were quite small. Some increase in the expression of Prxs V and VI was seen in alveolar macrophages. A summary of Prx expression in the studied lung diseases is shown in table 4.

Table 4. Expression of peroxiredoxins in various disorders of the lung or pleura compared to apparently normal tissue

Prx	Pulmonary sarcoidosis, granulomas	COPD	Adeno-carcinoma, lung	Squamous cell carcinoma, lung	Pleural meso-thelioma
I	Highly expressed	No alterations in homogenates	Increased	Increased	Increased
II	Not expressed	No alterations in homogenates	Increased	Increased	Increased
III	Highly expressed	No alterations in homogenates	No clear alterations	No clear alterations	Increased
IV	Highly expressed	No alterations in homogenates	Increased	Increased	No clear alterations
V	Highly expressed	Mild induction in macrophages	No clear alterations	No clear alterations	Increased
VI	Highly expressed	Mild induction in macrophages	Increased	No clear alterations	Increased

6.5 Peroxiredoxins in cultured cells (I, II, IV and V)

All Prxs were expressed very similarly in both non-malignant bronchial Beas 2B cells and in malignant A549 cells, except that Prx II was almost totally missing from A549

cells as could be seen both by RT–PCR and Western analysis. Prx expression was also very constant in both of these cell lines and could be induced only by the oxidative stress generated by H_2O_2 exposure but not by agents like TNFα, TGFβ, BSO, cisplatin or menadione. Western analysis was performed generally 2, 4 or 6, 24, 48 and 72 hours after exposure. Quantitative RT–PCR was performed 30 min, 1, 4 or 24 hours after exposure but no alterations were seen. Also the catalytical cycle of Prxs was affected only by H_2O_2 Serum depletion/addition was tested, but also in these experiments the alterations were rather minor. A representative of oxidation state analysis is shown in figure 7. Oxidation state analysis was performed also from tissue samples of apparently normal lung tissue, adenocarcinoma, squamous cell carcinoma or COPD but there were no differences detected.

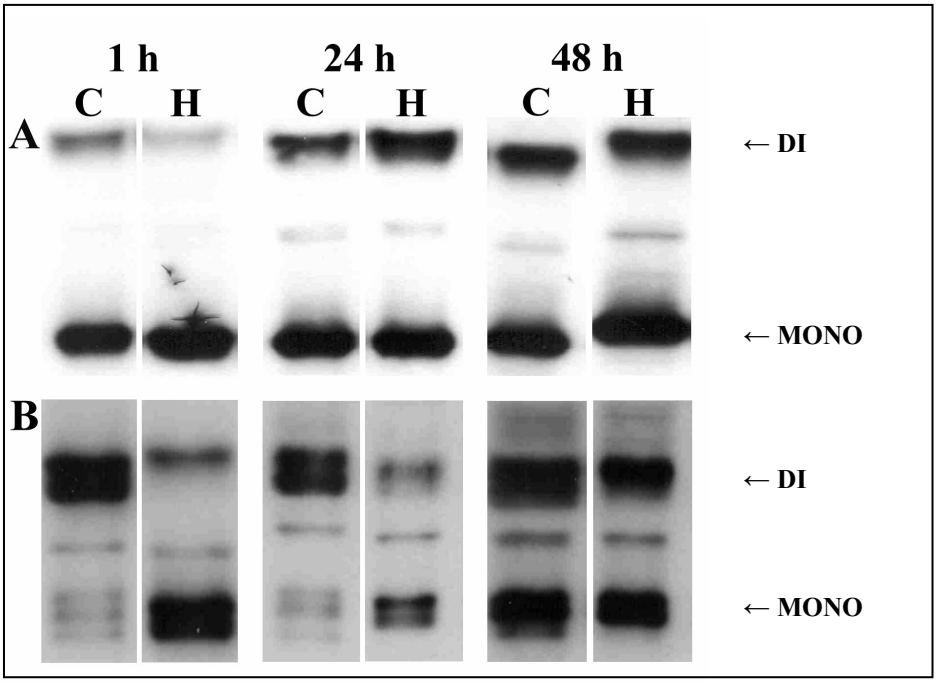

Fig. 7. Non–reducing SDS–PAGE analysis of Prx I (A) and III (B) 1, 24 or 48 hours after H_2O_2 exposure (H) or unexposed control cells (C). Sites of dimeric (DI) and monomeric (MONO) forms are indicated by arrows.

6.6 Predicted gene structures and properties of peroxiredoxins

Before planning the competitive constructs for RT-PCR, the exon/intron boundaries were predicted by computational analysis from published human genome project sequences. Furthermore the predicted promoter sequences were analysed for transcription factor

binding sites. The amount of predicted exons is shown in table 5, where also the existence of the ARE sequence and selected putative transcription factor binding site are indicated.

Table 5. Predicted properties of genomic sequences of human Prxs.

Prx	Number of exons	ARE	Putative binding sites					
			NFκB	Ap1	Ap2	c-Myc	Sp1	Hif1
I	6	+	+	+	+		+	+
II	6	+	+	+	+		+	+
III	7	+				+	+	+
IV	7	+	+			+	+	+
V	6	+	+					+
VI	6	+	+					+

7 Discussion

The main goals of this project were first to study the expression of Prxs in human lung under normal physiological conditions and during selected lung disorders and secondly to study the role and regulation of Prxs by using cultured cells as model systems. The results showed minor alterations in Prx expression during non-malignant diseases but a remarkable increase during malignancies. Regulation of Prxs in cultured human lung cells was found to be TNFα and TGFβ independent, but slightly upregulated by high concentrations of H_2O_2 Furthermore, the issue about the role of Prxs in human lung, i.e. they have a protective or regulatory nature, remained ambiguous.

7.1 Peroxiredoxins in normal human lung (I, II, III, IV and V)

This was the first study of Prxs in human lung revealing their expression in normal lung and in various lung disorders. Prxs are abundant proteins existing in six different isoforms, but the reasons for their multiplicity and their interplay are mostly unclear. One explanation for the existence of six different Prxs was noted already in our preliminary studies, when different Prxs were observed in different cell types of the lung (I). Furthermore their subcellular compartmentalisation was clear. Prxs III and V were known to be present in organelles, Prx IV in extracellular space and Prx III had been previously detected in the nuclear fraction of cultured cells. Our results confirmed these localisations in lung cells and in addition our studies indicated that also other Prxs may not only have a nuclear location but they also may be occasionally concentrated in the nucleus. This phenomenon was mostly detected in the bronchial epithelium, but more rarely also in other areas of the lung. This raises the question about their role in the nucleus. Prx V has been reported to have DNA binding properties and possibly to regulate retroposon transcription (Kropotov *et al.* 1999), but no other direct connections of Prxs to DNA have been reported. It is also possible that their only purpose in the nucleus is to protect DNA from being exposed to ROS. This is supported by the fact that a strong nuclear location was observed in the bronchial epithelium, which experiences a very strong oxidative stress compared to other areas. On the other hand, also cultured cells are cultivated in an oxidant rich environment as compared to human tissues, but, as stated, no nuclear

location has been detected except for Prx III. These results suggest that there may be some sort of targeting machinery for Prxs, as their capability to passively translocate to the nucleus does not explain their concentration there and no traditional nuclear localisation signals are present in their sequences. The possible targeting force remained unsolved, is it H_2O_2, the general redox state or for example the cell cycle state. Trx is known to regulate directly the activity of certain transcription factors and therefore one cannot exclude a similar regulatory role for Prxs.

7.2 Peroxiredoxins in non-malignant diseases (I and IV)

Analysis of lung samples from patients with pulmonary sarcoidosis or COPD suggested that Prxs may have a role during inflammation. First all of the Prxs except Prx II were highly expressed in sarcoidosis granulomas, even though their expression elsewhere in the lung was very similar to normal lung. BAL cells mostly contain inflammatory cells and they seemed to have a slightly different Prx profiles in healthy controls and in sarcoidosis patients. Secondly we observed that macrophages were very heterogenous in how they expressed Prxs V and VI expressions, which may indicate different expression at different activation states of inflammatory cells or in different macrophage populations. As this induction was clear only in a subpopulation of macrophages, it is probable that this induction is caused more by intracellular signals than by for example the increased oxidative stress of present in the lungs of smokers.

Prxs V and VI were surprisingly constantly expressed in the epithelium of the airways of non-smokers, smokers and smokers with COPD. Similar results would be expected for the other Prxs as microarray studies performed on bronchial epithelium revealed only a minor induction of Prx IV in smoker's lung (Hackett *et al.* 2003, Golpon *et al.* 2004). In contrast, a clear induction of Trx by smoking could be seen. Since Prxs are not induced by cigarette smoke, it is probable that they do not provide any extra defence against the elevated oxidative stress. This could indicate either that the role of Prxs is non-AOE-like or they could be one part of AOE machinery that does not respond to oxidative stress forcefully thus resulting in accumulation of H_2O_2. The lack of induction by cigarette smoke is in line with studies that show Prx upregulation immediately after birth in rat lung and also with the reports that Prx expression is high in normal lung at ambient oxygen tension.

7.3 Peroxiredoxins in malignant diseases (II and III)

Several Prxs were upregulated in both lung carcinomas and malignant mesothelioma as expected from previous reports of high expression of Prx I in some other human malignancies (Yanagawa *et al.* 1999, Yanagawa *et al.* 2000, Chang *et al.* 2001, Noh *et al.* 2001, Choi *et al.* 2002, Karihtala *et al.* 2003). Obviously the Prx expression pattern was different in different carcinoma subtypes such that Prxs III and V levels were high especially in pleural mesothelioma, Prx VI in pleural mesothelioma and adenocarcinoma but not in squamous cell carcinoma, Prx IV in adenocarcinoma and squamous cell

carcinoma but not in pleural mesothelioma. These results highlight the need for further studies to map their possible use in diagnostics, for example differentiating malignant pleural mesothelioma from metastatic carcinomas which have spread to pleura.

Prx I is a potent tumor suppressor, but it was highly expressed in lung carcinomas as well as in malignant pleural mesothelioma in a manner similar to that occuring in several other carcinomas. This apparent disagreement is explained by the different phases of tumor initiation and progression. At the very early stages, the lack of Prx I makes cells more susceptible to oxidative damage and thus increases the accumulation of mutations ultimately leading to malignant transformation. It is not known whether decrease of Prx I occurs in human tissues and is one of the factors leading to cancer, but it would be interesting to follow its behaviour as cells are transformed. Once the cell has been transformed, Prx I expression would however be favourable for cancer progression as it would protect cells from apoptosis, which explains why several tumors contain high levels of this enzyme. In some COPD patients there were dysplastic areas in their bronchi and in these regions, Prx V and VI expressions were lower than in the surrounding epithelium, but reliable results would require analysis of more samples.

Prxs II and VI, the other cytosolic Prxs, were generally induced in malignancies, except for Prx VI in squamous cell carcinoma which showed no difference from normal lung. These differences between various carcinoma types could be simply explained by the different origins of these cells. A more elaborate explanation would be the different proliferation rate of these cells or some other yet unidentified system affecting Prx expression. Ultimately the factors leading to high Prx expression in malignant cells remained unclear. It is possible that elevated metabolic activity, increased proliferation or improved ability of the cancer cell to protect itself against apoptosis would be directly or indirectly affecting the Prx expression.

7.4 Oxidation state of peroxiredoxins (IV and V)

In tissues, Prxs I-IV are present mostly as dimers, but in cultured cells a higher proportion seemed to exist as a monomer (IV and V). During the normal catalytical cycle, Prxs fluctuate between monomeric and dimeric forms, but when the cells were treated with more than 50 μM H_2O_2, the balance was disturbed so that the proportion of the monomeric form increased. This was presumably because of overoxidation, which transiently inactivates Prxs. The importance of this type of regulation in human tissues remains unclear. However, recently characterized sulfiredoxin, the rescucer of overoxidised forms of 2-Cys Prxs, poses several questions and explanations. Sulfiredoxin is not expressed in all cell types. Thus in some tissues, Prx overoxidation may be irreversible and thus allow H_2O_2 accumulation and its long-term usage as a second messenger. In other tissues, sulfiredoxin is expressed at high levels and thus allows efficient H_2O_2 removal constantly offering good protection against oxidative stress. Sulfiredoxin catalyses the reduction relatively slowly in cells, which could even give time for the initiation of apoptotic signaling after the H_2O_2 burst.

Even though overexpression studies on cultured cells have indicated that Prxs break down H_2O_2 generated after TNFα exposure (Kang *et al*. 1998b), we did not observe any

alteration in Prxs I-IV dimer/monomer ratio after TNFα, TGFβ or BSO treatments. It is however possible that they degrade H_2O_2, but do so in a way that their catalytic cycle is not affected. On the other hand, the differences of the redox state between cultured cells and tissue biopsies suggests that cultured cells grown under conventional conditions may not represent a good model for this kind of study, since under normal cell culture conditions, a high proportion of Prxs might be inactivated.

7.5 Regulation of peroxiredoxin expression (V)

One important aim of this study was to identify factors regulating Prx expression. However, the growth factors, cytokines or other treatments that regulate the traditional AOEs had no effect on Prx expression in cultured cells. The depletion of GSH by BSO or serum starvation of the cells also had surprisingly small effects on Prx expression, even though serum has been reported to induce Prx I expression in murine cells (Ishii *et al.* 1993, Prosperi *et al.* 1998).

Computational analysis of the predicted promoter areas of Prxs showed some putative binding sites for Ap1, NFκB, c-myc and Hif1. These predictions do not however actually determine, whether these sites are functional or not. Preliminary attempts were made for Ap1 and NFκB depletion by antisense techniques, but no effects on Prx expression were seen. Also the lack of induction by TNFα would suggest only minor NFκB mediated regulation. In mouse, c-myc regulates Prx III expression and as there are several putative binding sites of c-myc in the predicted Prx promoters, c-myc seems likely to be one of the transcription factors involved. Additionally Nrf2 regulated induction in inflammatory cells may be the most important mechanism also in non-malignant human lung (Ishii *et al.* 2000). Very impressive Prx expression was seen in malignant tissue samples, but the precise upregulator triggering this process remained unsolved. It is probably attributable to several different factors including a mixture of growth factors and other regulators leading to the high proliferation.

7.6 Methodological aspects (I, II, III, IV and V)

Human lung contains dozens of different cell types, which makes it a very challenging target for research. The best way to study the protein expression of the lung is obviously immunohistochemistry, which was the most important method also used in these studies. Samples of lung tissue homogenates or BAL fluid cells can be very heterogeneous, for example the amount of macrophages may vary greatly not only between individuals but also in various areas of the same lung. Therefore it is not surprising that Western analysis of homogenates did not invariably correlate with the more detailed immunohistochemical findings (III and IV). In addition, it must be kept in mind that Prx IV is extracellular and Prxs III and V are organellar, which make these enzymes difficult to measure by immunohistochemistry as a proportion of extracellular proteins may escape during sample preparation and granular staining of organelles is difficult to evaluate. It is most likely that especially Prx IV is underestimated by immunohistochemistry. Therefore

further studies were conducted using several methodologies with the same tissue specimens. Immunoelectron microscopy provides more specific information about localisation, but as also here, it can be performed only in a very limited area and thus reveales expression only in a few cells. One major disadvantage of immunohistochemistry is the method used for evaluation of staining, since the assessment of positivity is very subjective, and requires several independent assessments of the same sample. Thus it is both time-consuming and not particularly accurate. However, as the samples are very heterogenous with all different cell types, a great deal of information may be lost if samples are evaluated only by means of positive area.

In situ hybridization was viewed as a good method for detecting expression at the transcriptional level in the lung. Therefore constructs for probe production were subcloned for different *Prxs* for further studies. A quantitative RT-PCR system was set up for these studies and they provided information about transcript levels in cultured cells but also in few cases in lung homogenates. In particular lung homogenate studies by RT-PCR and Western anlysis suggested that some Prxs are strongly regulated at the translational level (III), therefore *in situ* hybridization might give interesting results to compare with immunohistochemistry. Especially Prx V expression was clearly different in tissue samples by RT-PCR and Western analysis (III), which pointed to strong translational regulation or that perhaps there are two distinct transcripts in human cells similarly to the situation in mice, which have short and long forms. This was not tested by Northern blotting, but attempts were made to amplify two forms from human lung cDNA library by RT-PCR, but only one size transcript was amplified. This does not exclude the existence of longer transcript, especially as PCR favors shorter products.

Prxs are regulated also at the posttranslational level by phosphorylation and oxidation. There are some antibodies available for specifically these modified forms, but at least the antibody against phosphorylated Prx I did not work properly in immunohistochemistry nor Western, probably due to low levels of this protein form. Activity measurements would provide information about Prx modifications as phosphorylated and overoxidised forms are inactive, but this is difficult from tissue samples, as the correct use of this method would need sample fractionation, which is limited by small size of biopsy samples and this procedure itself might alter the protein conformation/activity. Therefore Western analysis was performed after non-reducing electrophoresis, which shows monomeric and dimeric forms of Prxs I-IV in different positions. This method does not directly reveal Prx activity, but reflects more disturbances in the catalytical cycle and in its simplicity provides a useful alternative to 2–dimensional electrophoresis, which separates proteins both based on their size and isoelectric point. On the other hand, the monomeric form may contain functional thiol or sulphenic acid groups, but there can be also non-functional sulphonic or sulphinic acid. The monomeric form could also, in theory, represent the unfolded form of Prx, as in addition to transcriptional responses, the stress experienced by the cell not only reduces translation but also reduces protein folding leading to the accumulation of unfolded proteins in endoplastic reticulum. Also alterations in the redox state may occur during sample preparation and this factor must be carefully considered especially when using tissue samples.

There are several under- and overexpression studies with Prx but no systematic studies on their regulation in intact cells. Therefore, cultured cells were used as a model for Prx expression. They were highly expressed in cultured cells, at about the same magnitude as

in carcinoma tissue but this expression was hard to manipulate with any tested agent. Possibly Prxs are up-regulated already in conventional cell culturing conditions, i.e. cells under high oxygen pressure and with several growth factors or serum added to the medium. A high basal level of expression may make it impossible to detect significant induction even by factors that may have a crucial role *in vivo*. These results do not rule out the possibility of a regulatory role for TNFα, TGFβ or other factors *in vivo*. In future studies it should be possible to reduce Prx amount in the cells via antisense technology and this may be one way to obtain information about their expression and especially their importance in cultured lung cells.

In addition to the small sample size also number of available samples was a limiting factor. In common diseases like COPD, the samples were easier to obtain, but for example it proved difficult to aquire a suitable amount of samples from patients with usual interstitial pneumonia even though our preliminary results were very interesting. In particular, the number of healthy controls was very small in all of these studies and they mostly contained individuals with minor respiratory symptoms. There are also other problems in obtaining lung tissue or BAL samples, especially if one wishes to study the redox system. Anaesthetic agents and elevated oxygen pressure during operation are procedures that can affect the redox state and the expression of AOEs.

8 Conclusions

1. All the members of Prx family are expressed in human lung but they are expressed in a cell-specific manner. The expression is generally highest in the epithelium and in inflammatory cells.
2. Several Prxs are highly expressed in granulomas of sarcoidosis, while the expression in non-granulatomous inflammatory areas is not affected.
3. Several Prxs are highly expressed in malignant mesothelioma, adenocarcinoma and squamous cell carcinoma. The expression pattern differs between different carcinoma subtypes.
4. The Prx expression pattern in the lungs of smokers or COPD patients is rather similar to that found in healthy non-smokers. The only difference was seen in alveolar macrophages, which contained more Prx V and VI positivity in COPD patients' lung than in healthy non–smokers' lung. However, Trx expression was elevated in bronchial epithelium of smoker's lung compared to healthy bronchial tissue from non-smokers.
5. It was difficult to modify Prx levels in cultured cells. None of the tested growth factors or cytokines affected the transcription or translation, only very strong oxidative stress created by high concentrations of H_2O_2 increased the expression. Strong oxidative stress also affected the oxidation state of 2-Cys Prxs, presumably inactivating them.

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