REGULATION OF ANTIOXIDANT DEFENSE IN CELLS DERIVED FROM THE HUMAN LUNG

by

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ACADEMIC DISSERTATION

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SUMMARY

Oxidative stress has been increasingly implicated in the pathogenesis of lung diseases. Antioxidant mechanisms and their regulation are poorly characterized in the human lung, but they may be important in the development and progression of pulmonary diseases. The present study was designed to investigate the regulation of the most important antioxidant enzymes (AOEs), superoxide dismutases (MnSOD, CuZnSOD), catalase, and glutathione peroxidase (GPx), in human lung cells by exposing them to agents and states (cytokines, asbestos fibers, oxidants, and hyperoxia), which may play an important role in the pathogenesis of lung diseases.

Enzyme regulation was investigated in cultured human bronchial epithelial cells (BEAS-2B), pleural mesothelial cells (MET-5A), alveolar macrophages, monocytes, and neutrophils. Enzyme expression was assessed at the level of mRNA, protein, and/or activity. Cell injury was measured by lactate dehydrogenase (LDH) release and/or depletion of high-energy nucleotides. Copper-zinc superoxide dismutase was constitutively expressed in all cell types investigated. The specific activity of MnSOD was quite low, but similar in all cell types. It was induced by TNF-α but not by high oxygen tension or asbestos fibers in BEAS-2B and MET-5A cells, respectively. Induction of MnSOD by TNF-α offered no protection against subsequent oxidant exposure. Catalase, glutathione peroxidase and reductase were constitutively expressed in BEAS-2B cells, with no change during exposure to any agent. The activity of catalase was highest in neutrophils, whereas glutathione levels and enzymes of the glutathione cycle were highest in monocytes. Catalase was the main scavenger of exogenous H_2O_2 in alveolar macrophages and also appeared to explain the high resistance of neutrophils against exogenous H_2O_2. During the respiratory burst of alveolar macrophages, the glutathione redox cycle was mainly responsible for the maintenance of cellular integrity. Low levels of glutathione and related enzymes may account for the short survival of human neutrophils.

In conclusion, MnSOD is induced by TNF-α, but cytokine-induced MnSOD does not protect lung cells against oxidant-induced damage. The enzymes associated with the scavenging of H_2O_2 are not upregulated by cytokines or oxidants, but they play an important role in scavenging of both exogenous H_2O_2 and endogenous oxidants in a cell-specific manner. Because transformed cell lines or cell culture conditions for primary cells were used, the results should be extrapolated with caution with respect to the situation in vivo.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals:

I Kinnula, V. L., P. Pietarinen, K. Aalto, I. Virtanen, and K.O. Raivio. Mitochondrial superoxide dismutase induction does not protect epithelial cells during oxidant exposure in vitro. Am. J. Physiol. 268:L71-L77, 1995.

II Pietarinen-Runtti P., K. O. Raivio, M. Saksela, T. M. Asikainen, and V. L. Kinnula. Antioxidant enzyme regulation and resistance to oxidants of human bronchial epithelial cells cultured under hyperoxic conditions. Am. J. Respir. Cell Mol. Biol. 19:286-292, 1998.

III Pietarinen-Runtti, P., K. O. Raivio, K. Linnainmaa, A. Ekman, M. Saksela, and V. L. Kinnula. Differential effects of tumor necrosis factor and asbestos fibers on manganese superoxide dismutase induction and oxidant-induced cytotoxicity in human mesothelial cells. Cell Biol. Toxicol. 12:167-175, 1996.

IV Pietarinen, P., K. O. Raivio, R. B. Devlin, J. D. Crapo, L. Y. Chang, and V. L. Kinnula. Catalase and glutathione reductase protection of human alveolar macrophages during oxidant exposure in vitro. Am. J. Respir. Cell Mol. Biol.13:434-441, 1995.

V Pietarinen-Runtti P., E. Lakari, K. O. Raivio, and V. L. Kinnula. Expression of antioxidative enzymes in human inflammatory cells. Am. J. Physiol. Cell Physiol. In Press.

LIST OF NONSTANDARD ABBREVIATIONS

AMOS	amosite
AOE	antioxidant enzymes
ARDS	adult respiratory distress syndrome
ATZ	aminotriazole
BAL	bronchoalveolar lavage
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BPD	bronchopulmonary dysplasia
BSO	buthionine sulfoximine
CDNB	1-chloro-2,4-dinitrobenzene
COPD	chronic obstructive pulmonary disease
CuZnSOD	copper-zinc superoxide dismutase
ECSOD	extracellular superoxide dismutase
EDTA	ethylenediamine tetra-acetic acid
FMLP	formyl-methionyl-leucyl-phenylalanine
G6PDH	glucose-6-phosphate dehydrogenase
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione, reduced
GSSG	glutathione, oxidized
H_2O_2	hydrogen peroxide
IFN-γ	interferon-γ
IL-1	interleukin-1
LDH	lactate dehydrogenase
MnSOD	manganese superoxide dismutase
NAC	N-acetylcysteine
$O_2^{-\cdot}$	superoxide anion
$^1O_2$	singlet oxygen
$OH^{\cdot}$	hydroxyl radical
$ONOO^-$	peroxynitrite
PEG	polyethylene glycol
$RO^{\cdot}$	alkoxyl radical
$ROO^{\cdot}$	peroxyl radical
ROOH	lipid hydroperoxide
ROS	reactive oxygen species
SOD	superoxide dismutase
TNF-α	tumor necrosis factor α
XO	xanthine oxidase

INTRODUCTION

Oxygen-derived free radicals and other reactive oxygen species (ROS) are produced during normal cellular metabolism. ROS are potentially harmful because they have the ability to react with and alter all principal components of the cell, including lipids, proteins, carbohydrates, and nucleic acids. To prevent oxidant damage, cells have evolved antioxidant enzymes (AOEs), such as superoxide dismutase, catalase, and glutathione peroxidase, as well as nonenzymatic scavengers. An imbalance between oxidants and antioxidants leads to oxidative stress, which has been proposed to play an important role in the pathogenesis of many diseases. Although damaging, ROS also have beneficial functions as part of the host defense against micro-organisms and as cellular signaling agents.

Reactive oxygen species appear to play an important role in the lung both in health and in disease. Lung cells are the first to encounter inspired oxygen, and the oxygen concentration in the lung is relatively high compared to levels in other organs. The generation of ROS is further increased by inhaled toxic particles and gases. Considerable evidence suggests that ROS, produced both intracellularly by lung parenchymal cells and extracellularly by lung macrophages and infiltrating neutrophils, play a central role in the pathogenesis of various lung diseases, such as adult respiratory distress syndrome (ARDS), asthma, chronic obstructive pulmonary disease (COPD), bronchopulmonary dysplasia (BPD), granulomatous lung diseases, and asbestos-induced diseases (Heffner and Repine 1989, Barnes 1990, Sibille and Reynolds 1990, Kamp et al. 1992, Kinnula et al. 1995).

The lungs have efficient antioxidative defense mechanisms. The primary defense mechanisms against oxidant stress are AOEs. Based on animal models, various lung cell types appear to differ in their resistance against oxidants, which may in part be explained by differences in the antioxidant defense mechanisms of the cells. Although various studies conducted on animal-cell models in vivo and in vitro have shown that increased activities of pulmonary AOEs confer resistance on oxidants, and that the regulation of AOEs is complex, little is known about the role and regulation of AOEs in the human lung. Furthermore, the protective effect of the induction of various AOEs has remained unclear.

This series of studies aimed to compare the expression and regulation of various AOEs as well as their relative importance in human bronchial epithelial cells, pleural mesothelial cells, and alveolar macrophages. In these experiments the cells were exposed to factors relevant in the pathogenesis of oxidant-related lung diseases, such as high oxygen tension, cytokines, and asbestos fibers. Furthermore, the significance of AOE induction in cell protection was investigated.

REVIEW OF THE LITERATURE

1. Reactive oxygen species (ROS)

1.1. Chemistry of ROS

A free radical is defined as any molecule or atom which contains one or more unpaired electrons in its outer orbit (Halliwell and Gutteridge 1989). The presence of an unpaired electron makes the species highly reactive. The lifetime of various radicals is only a fraction of a second, but there is considerable difference between various radicals (Pryor 1986). These compounds can be formed by the loss of a single electron from a non-radical, by gain of a single electron by a non-radical, or by homolytic fission, in which a covalent bond is broken and each fragment retains its own electron. A free radical is shown in chemical formulas by a dot, $R^{\cdot}$. The most important radicals in biological systems are the superoxide anion ($O_2^{-\cdot}$), hydroxyl radical ($OH^{\cdot}$), nitric oxide ($NO^{\cdot}$), and the lipid-derived peroxyl radical ($ROO^{\cdot}$) and alkoxyl radical ($RO^{\cdot}$).

The term ”reactive oxygen species” (ROS) is often used to refer to free radicals and other oxygen-related reactive compounds, such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroperoxides (ROOH) (Halliwell and Gutteridge 1989). All these compounds have the potential to cause free-radical reactions (Pryor 1986). When a free radical reacts with a non-radical molecule, the target molecule is converted to a radical, which may further react with another molecule.

The primary formation of most of the ROS is the reduction of molecular oxygen with the formation $O_2^{-\cdot}$ (Fridovich 1983, Halliwell and Gutteridge 1989). Although the reactivity of $O_2^{-\cdot}$ is quite low, it is capable of initiating free-radical chain reactions. Superoxide undergoes a dismutation to form H_2O_2 spontaneously or enzymatically (I). The spontaneous dismutation rate is slow, whereas the reaction catalyzed by superoxide dismutases (SOD) is 10^4 times as fast (Halliwell and Gutteridge 1989). Superoxide can also react with nitric oxide ($NO^{\cdot}$) to form peroxynitrite ($ONOO^-$) (Beckman et al. 1994).

(I) $$O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide is more stable than $O_2^{-\cdot}$. It can diffuse through the plasma membrane, and if not scavenged locally by catalase or glutathione peroxidase, can promote radical reactions far from its origin (Halliwell and Gutteridge 1989).

The most reactive and potentially harmful radical has been considered to be $OH^{\cdot}$ (Halliwell and Gutteridge 1989). Because the lifetime of $OH^{\cdot}$ is extremely short, it can thus be expected to react at or close to its site of

formation. The hydroxyl radical is generated from H_2O_2 through the Fenton reaction catalyzed by the transition metals iron or copper (II):

(II) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^-$

or from $O_2^{-\cdot}$ and H_2O_2 through the Haber-Weiss reaction catalyzed by iron or copper (III) (Halliwell and Gutteridge 1989):

(III) $O_2^{-\cdot} + H_2O_2 \xrightarrow[\text{catalyst}]{\text{metal}} O_2 + OH^{\cdot} + OH^-$

1.2. Sources of ROS in the lung

Reactive oxygen species are generated in the normal metabolism of all aerobic cells. Lung cells are the first to encounter inspired oxygen, and oxygen concentration in the lung is higher than levels in other organs. The basic findings on ROS generation in lung cells have come mostly from rabbit and rat lungs, and the generation of ROS has also been investigated in human alveolar macrophages (Kinnula et al. 1995). Production of ROS is increased as a result of exposure to high oxygen concentrations and to exogenous oxidants like ozone, asbestos fibers, cigarette smoke, radiation, and certain drugs (Freeman and Crapo 1982, Sibille and Reynolds 1990, Crystal 1991, Kamp et al. 1992). Superoxide generation in the mitochondrial respiratory chain is associated with NADH dehydrogenase and the ubiquinone cytochrome b complex (Freeman and Crapo 1982). Findings of increased mitochondrial ROS generation after exposure to hyperoxia suggest that mitochondria are an important source of ROS production in the lung (Freeman and Crapo 1981, Turrens et al. 1982). Superoxide generation is highest when the respiratory chain carriers located in the inner mitochondrial membrane are greatly reduced (Freeman and Crapo 1982). Mitochondrial H_2O_2 is mainly derived from the dismutation of $O_2^{-\cdot}$. Approximately 1 to 2% of the oxygen consumed by the mitochondria is estimated to be converted to $O_2^{-\cdot}$ and H_2O_2.

Cell cytoplasm is also an important site of ROS production. One non-enzymatic source of ROS is the generation of $OH^{\cdot}$ by iron-catalyzed reactions (Fenton and Haber-Weiss reactions), and in addition, ROS are formed by intracellular enzymes. Xanthine oxidase (XO) is a cytoplasmic enzyme which under normal conditions is in its dehydrogenase form with no detectable ROS generation. In ischemic tissues, it is converted to oxidase, leading to the production of $O_2^{-\cdot}$ and H_2O_2 during reoxygenation (McCord 1985). Because the level of XO in lung tissue is low, its role in ROS production is probably not significant (Linder et al. 1999). Neutrophils contain myeloperoxidase, which in the presence of halide ions converts H_2O_2 to hypochlorous acid (HOCl), a relative

long-lived oxidant (Halliwell and Gutteridge 1989). Other sources of ROS possibly relevant in the lung are microsomal cytochrome P-450 enzymes and peroxisomal oxidases.

Reactive oxygen species play an important role in the host defense against microbicidal agents. During phagocytosis, activated inflammatory cells generate ROS through membrane-bound NADPH oxidase - the phenomenon called respiratory burst (Babior 1984). Respiratory burst is induced by various endogenous and extracellular factors such as opsonized bacteria, endotoxin, cytokines, fibrous material, phorbol esters, and formyl-methionyl-leucyl-phenylalanine (FMLP) (Rossi 1986). Recent studies have detected NADPH oxidase not only in inflammatory cells but also in endothelial cells (Jones et al. 1996).

1.3. Comparison of ROS production between various cells types in the lung

The major sources and the amount of ROS produced vary between species and organs. Although comparison of ROS production between cells is difficult, some studies which have quantified the generation of ROS in various cell types of the lung. The amount of H_2O_2 released by stimulated neutrophils is an order of magnitude higher than that of monocytes (Ruch et al. 1983). Unstimulated rat alveolar macrophages release H_2O_2 at a rate of 3.1 nmol/min/mg protein; rat type II pneumocytes, 0.7 nmol/min/mg protein; and cultured endothelial cells, 0.06 nmol/min/mg protein (Kinnula et al. 1991). Rat pleural mesothelial cells generate minor amounts of H_2O_2 extracellularly (less than 0.1 nmol/min/mg) (Kinnula et al. 1992c).

Exposure to hyperoxia enhances the formation of ROS in rat lung homogenates, in isolated rat lung mitochondria, and in rat alveolar macrophages (Freeman and Crapo 1981, Freeman et al. 1982, Jamieson et al. 1986, Kinnula et al. 1992d). Generation of H_2O_2 by bovine vascular endothelial cells is dependent on oxygen tension, but it is already saturated at low concentrations (10% O_2) (Kinnula et al. 1993). Adult rat lung microsomes and mitochondria produce more ROS than do organelles isolated from neonatal lungs, indicating that ROS-generation is age-dependent (Frank 1991).

1.4. Effects of ROS

Reactive oxygen species are potentially harmful, because they interact with each other and modify cellular biomolecules (Figure 1). Lipid peroxidation is a radical-mediated chain reaction initiated by abstraction of a hydrogen atom from a polyunsaturated lipid and terminated by chain-breaking antioxidants such as α-tocopherol (Halliwell and Gutteridge 1989). Hydroxyl radical, $ROO^{\cdot}$, $RO^{\cdot}$, and some iron complexes, but not $O_2^{-\cdot}$ or H_2O_2, are sufficiently reactive to initiate lipid peroxidation. Excessive peroxidation of membrane lipids disrupts the bilayer arrangement, decreases membrane fluidity, increases membrane permeability, and modifies membrane-bound proteins.

Reactive oxygen species can damage DNA, proteins, and carbohydrates. Radicals induce DNA strand breaks as well as oxidation of purine and pyrimidine bases, and increase the occurrence of mutations (Freeman and Crapo 1982). The appearance of DNA strand breaks activates poly-ADP-ribose polymerase, a DNA repair enzyme, which leads to depletion of nicotinamide adenine dinucleotide (NAD), depletion of adenosine triphosphate (ATP), and alteration in energy balance (Schraufstatter et al. 1986, Hyslop et al. 1988). In addition, ROS can activate or inactivate proteins by oxidizing sulfhydryl groups and modifying amino acids (Davies 1987, Davies and Delsignore 1987).

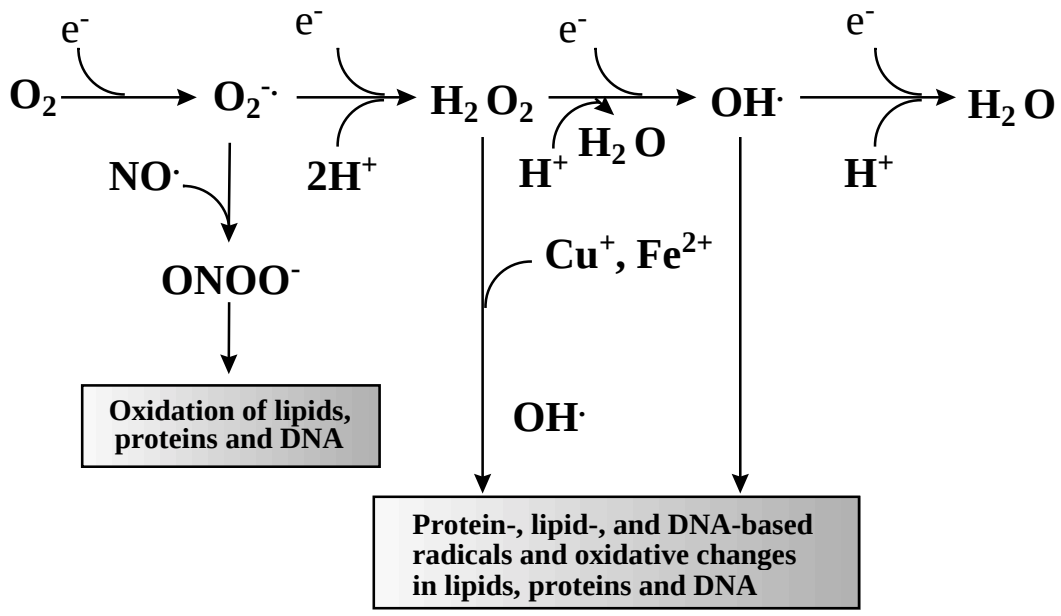

Figure 1: *Effects of reactive oxygen species.*

Severe oxidative stress with lipid peroxidation, protein oxidation, DNA damage, and ATP depletion leads to cell death by necrosis, which is characterized by disruption of the cell membrane and cellular organelles. Another mechanism of cell death is apoptosis, a complex cell-suicide mechanism which is beyond the scope of the present study. It has to be emphasized, however, that ROS and changes in the cellular redox state, especially at the level of the mitochondria, may play an important role in regulating and initiating reactions closely associated with apoptosis (Kroemer et al. 1998, Mignotte and Vayssiere 1998).

Although most interest has focused on the damaging effects of ROS, they also have beneficial effects on cells. The importance of ROS as a defense mechanism against microbes has already been mentioned. $NO^{\cdot}$ acts as a relaxing factor in the vascular endothelium (Moncada and Higgs 1993). Moreover, ROS have been shown to play a critical role in redox-regulation of gene expression (Sen and Packer 1996), and in cell growth and proliferation (Burdon 1996).

1.5. Lung injury caused by ROS

In the normal lung, the upper airways are lined by pseudostratified ciliated columnar cells, goblet and basal cells. In the distal airways, the epithelium becomes progressively thinner and simplifies, with ciliated and Clara cells predominating. The alveolar epithelium is composed of type I pneumocytes that cover over 90% of the surface area, and cuboidal type II pneumocytes about equal in number. Type II pneumocytes are producers of surfactant and capable of cell division is why they act as progenitor cells of the alveolar epithelium. The respiratory epithelium is covered by epithelial lining fluid containing inflammatory cells, alveolar macrophages, T-lymphocytes, and neutrophils, which play a central role in the defense of the lung. Alveolar macrophages are the predominant cells in the alveolar space, >90% of the cells being macrophages. The pleural mesothelium is covered by a single layer of epithelial cells, called mesothelial cells (Crystal 1991).

The lung is the organ most severely damaged by exposure to hyperoxia. Morphological changes in response to high oxygen tension are very similar among different animals (Crapo 1986, Klein 1990) (Figure 2). Exposure of rats to a lethal dose of hyperoxia (e.g. 100%) is associated with an initiation phase (first 24 to 72 h) with no morphological changes but with increased production of ROS (Freeman et al. 1982). The earliest morphological changes in the hyperoxic rat lung are changes in endothelial cell structure, leading to increased capillary permeability (Crapo et al. 1980). This is followed by accumulation of inflammatory blood cells (platelets and neutrophils) into intravascular and interstitial spaces (Barry and Crapo 1985) and release of inflammatory mediators. The final stage of lethal oxygen toxicity is a destructive

phase with endothelial cell damage after 80 h of hyperoxic exposure. No changes in the number of type I or II pneumocytes are observed in rats, but after 4 days of hyperoxia exposure in monkeys, there is almost complete destruction of type I pneumocytes and hyperplasia of type II pneumocytes (Kapanci et al. 1969). The progression of lung damage is basically similar in sublethal hyperoxia (60-85% O_2), but the onset of the various phases is delayed for up to a week. Proliferation of type II pneumocytes and fibroblasts leads to interstitial fibrosis and development of pulmonary hypertension due to thickening of the walls of pulmonary arteries (Crapo et al. 1980). The most common early manifestations of pulmonary oxygen toxicity in humans are symptoms of tracheobronchitis, occurring as early as 3 h but usually within 12 to16 h of exposure to >95% O_2 (Deneke and Fanburg 1980, Davis et al. 1983, Crapo 1986, Erzurum et al. 1993).

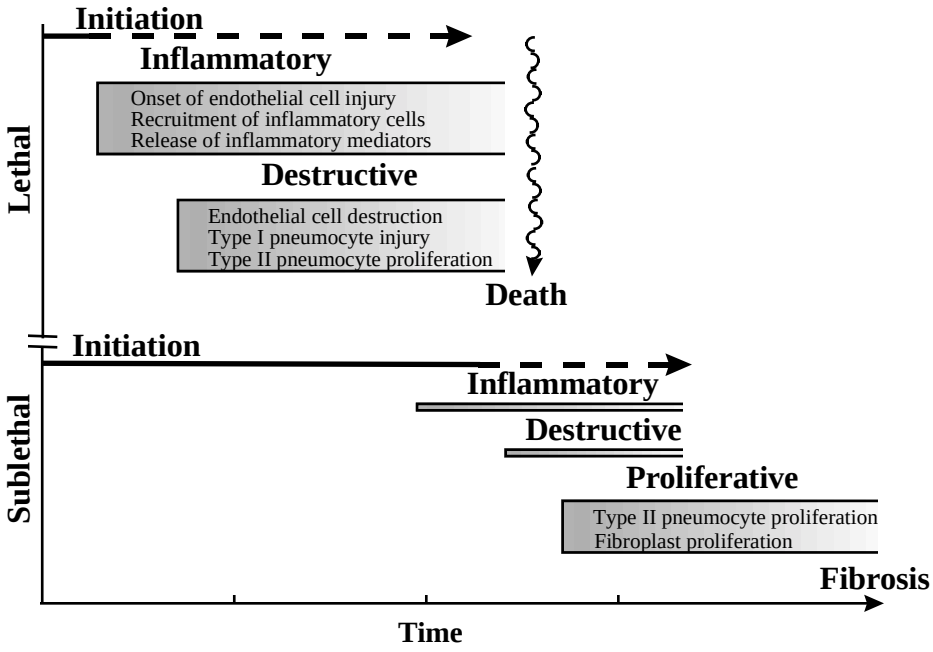

Figure 2: *Relative time course of each phase of lung injury caused by lethal and sublethal exposure to hyperoxia. Metabolic events associated with initiation of lung injury cause no morphologic changes (see text). These metabolic events continue throughout most or all of the duration of exposure to hyperoxia. Modified from Crapo 1986.*

2. Antioxidant defense mechanisms

Cells contain several antioxidant defense mechanisms to prevent injury. Lung cells differ profoundly in their resistance to oxidant stress, which may be due to differences in their antioxidant capacity or in the balance between oxidants and antioxidants in these cells. These enzymatic and non-enzymatic antioxidant defenses are discussed below. However, prevention of ROS formation and repair of oxidative damage are also essential for the survival of aerobic organisms. Sequestration of transition metals, e.g., by binding to storage and transport proteins, minimizes the ”free” iron within the cells and prevents the formation of highly reactive radicals (Fridovich and Freeman 1986, Heffner and Repine 1989).

2.1. Superoxide dismutases

Superoxide dismutases are metalloenzymes which catalyze the dismutation of $O_2^{-\cdot}$ to H_2O_2 and O_2 at a rate 10^4 times as fast as than spontaneous dismutation at neutral pH (Halliwell and Gutteridge 1989). Three different kinds of SODs have been characterized in eukaryotes; a copper- and zinc-containing form (CuZnSOD) localized in the cytosol, a manganese-containing form (MnSOD) in the mitochondria (Fridovich 1986, Tsan 1997), and a copper- and zinc-containing form in the extracellular matrix (ECSOD) (Marklund 1982). SOD has been shown to play a central role in protecting cells and tissues against oxidant stress (Fridovich and Freeman 1986, Tsan 1997).

Copper-zinc superoxide dismutase, a homodimer with a molecular weight of ca. 32 000, was found by McCord and Fridovich (1969). CuZnSOD is mainly localized in the cytosol, but is also found in the nucleus and peroxisomes, at least in human cells (Crapo et al. 1992). CuZnSOD contains both Cu(II) and Zn(II) at its active sites. CuZnSOD is encoded by a single gene located on chromosome 21. In human cells are found two mRNA transcripts (0.7 and 0.9 kb) (Sherman et al. 1984).

Manganese superoxide dismutase, which constitutes approximately 10 to15% of total cellular SOD activity, is a homotetramer with a molecular weight of ca. 88 000 (Fridovich and Freeman 1986). MnSOD, having manganese (III) at its active site, is localized in the matrix of the mitochondria, this location being optimal for removal of $O_2^{-\cdot}$ produced by the respiratory chain. The human MnSOD gene, on chromosome 6, is transcribed into two major mRNA species of 4 and 1 kb having identical coding regions but different lengths of their 3’ untranslated regions (Wispe et al. 1989, Church et al. 1992). MnSOD activity can be distinguished from that of CuZnSOD by its resistance to 1 mM potassium cyanide or by the difference between their pH optima. The activity of CuZnSOD

is approximately 10 times as great at pH 10 as at pH 7.8, whereas MnSOD is only twice as active at the higher pH.

Extracellular superoxide dismutase is a secretory tetrameric Cu/Zn-containing glycoprotein, with a molecular weight of around 135 000 (Marklund 1982). ECSOD is the least abundant of the SODs in tissues, but it is the major SOD in extracellular fluids such as plasma and extracellular matrix (Marklund 1984, Sandstrom et al. 1993, Oury et al. 1994). Although most animals have relatively high ECSOD activity in their lungs compared with other tissues, ECSOD accounts for only 5 to 10% of total lung SOD activity (Marklund 1984).

2.2. Catalase

Catalase, a tetrameric hemoprotein with a molecular weight of 240 000, decomposes H_2O_2 to water and oxygen. Catalase is mainly localized in the peroxisomes (Davies et al. 1979), but is also found in the cytosol of human neutrophils (Ballinger et al. 1994) and in rat-heart mitochondria (Radi et al. 1991). The Michaelis-Menten constant (K_m) of catalase is higher than the K_m of glutathione peroxidase (GPx), which suggests that catalase scavenges H_2O_2 efficiently at high H_2O_2 concentrations (Jones et al. 1981).

2.3. Glutathione redox cycle

The glutathione redox cycle is a mechanism which scavenges lipid peroxides and H_2O_2 (Figure 3). The enzymes in the cycle include glutathione peroxidase (GPx) and glutathione reductase (GR). Glutathione peroxidase is a tetrameric selenoprotein which uses reduced glutathione as cosubstrate. Its molecular weight is 85 000. Glutathione peroxidase is localized in the cytosol and mitochondria, at least in rat liver and in human fibroblasts (Mbemba et al. 1985, Behne et al. 1990), with a distinct form found in human plasma (Takahashi et al. 1987). Two other known forms of GPx are selenium-dependent GPx in the intestine (Chu et al. 1993) and phospholipid hydroperoxide GPx (Schuckelt et al. 1991). The regeneration of glutathione through GR requires NADPH, which in turn is regenerated through glucose-6-phosphate dehydrogenase (G6PDH) (Figure 3) (Halliwell and Gutteridge 1989). The glutathione cycle is complementary to catalase in scavenging H_2O_2. The K_m value of GPx for H_2O_2 is lower than that for catalase, and in contrast to catalase, GPx also reduces lipid peroxides.

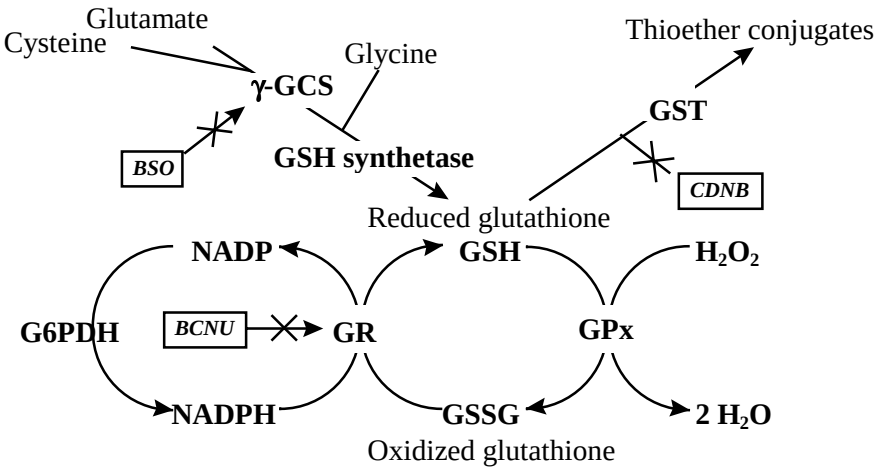

Figure 3: *Glutathione redox cycle modified from Harlan et al. 1984. γ-GCS: γ-glutamylcysteine synthetase, GST: glutathione S-transferase, G6PDH: glucose-6-phosphate dehydrogenase, GR: glutathione reductase, GPx: glutathione peroxidase, BSO: buthionine sulfoximine, CDNB: 1-chloro-2,4-dinitrobenzene, BCNU: 1,3-bis(2-chloroethyl)-1-nitrosourea.*

2.4. Antioxidant enzymes in the lung

The localization of different AOEs in airways and in lung parenchyma has been investigated by immunohistochemistry and by in situ hybridization, both performed on tissue biopsies and freshly isolated cells derived in most cases from rat lungs (Coursin et al. 1992, Kinnula et al. 1992a, Kinnula et al. 1992c, Clyde et al. 1993, Oury et al. 1994, Chang et al. 1995). CuZnSOD has been shown to be expressed throughout the lung, the highest levels in normal rats being in the bronchial epithelium (Coursin et al. 1992, Chang et al. 1995, Gilks et al. 1998). The expression of MnSOD in healthy lung appears to be low. In situ hybridization of normal rat lung has revealed a consistent pattern of MnSOD mRNA distribution in the lung, the most prominent labeling occurring in the septal tips of alveolar ducts, around arterioles near airways, in alveolar type II pneumocytes, and mesothelial cells (Clyde et al. 1993). According to immunohistochemical studies, MnSOD protein is present in the mitochondria of all rat lung cells, with the highest staining in type II epithelial cells and bronchial epithelium (Coursin et al. 1992, Chang et al. 1995). Comparison of the enzyme activities of rat alveolar type II pneumocytes and alveolar macrophages has shown that the activity of MnSOD is higher in alveolar macrophages (Housset et al. 1991). Both of these SODs are expressed at low levels in human bronchial epithelium, alveolar type II pneumocytes, and alveolar macrophages (Kinnula et al. 1994, Coursin et al. 1996, Lakari et al. 1998).

Extracellular SOD mRNA is found in airway epithelial cells, type II pneumocytes, and fibroblasts of human lung (Su et al. 1997). After its synthesis, ECSOD is secreted, and the ECSOD protein is primarily localized around larger vessels and airways, and to a lesser degree in the intercellular junctions of epithelial cells, and around smooth muscle cells (Oury et al. 1994). Furthermore, rat macrophages and neutrophils invading the lung during inflammation have shown intensive staining for ECSOD protein (Loenders et al. 1998).

Rat alveolar type II pneumocytes, Clara cells, and alveolar macrophages contain large amounts of catalase, whereas endothelial cells and pleural mesothelial cells have very low catalase protein labeling and activity (Kinnula et al. 1991, Kinnula et al. 1992c). In healthy human lung, catalase is mainly expressed in alveolar type II pneumocytes, and to a lesser degree in the bronchial epithelium and alveolar macrophages (Erzurum et al. 1993, Yoo et al. 1994, Coursin et al. 1996, Lakari et al. 1999). Very little is known about the expression of the glutathione cycle enzymes in the lung. Glutathione peroxidase mRNA and protein are expressed in the alveolar and bronchial epithelia of rat lung. However, the most prominent labeling of GPx protein is seen in the extracellular connective tissue of the lung (Coursin et al. 1992, Gilks et al. 1998). Only one study is available on human lung, where the distribution of GPx protein has been investigated. In that study the GPx was localized in the respiratory epithelium (Coursin et al. 1996). Because the activity of catalase, as well as other AOEs, decreases during in vitro culture (Panus et al. 1989, Kinnula et al. 1992a), the results obtained in culture models cannot, therefore, be used in assessment of the levels of various AOEs in human lung.

2.5. Regulation of AOEs in the lung

The regulation of pulmonary AOEs in response to hyperoxia is complex and has been intensively studied in experimental animals (Crapo and Tierney 1974, Freeman et al. 1986, Baker et al. 1989). Exposure of adult rats to 85 to 95% oxygen results in increased mRNA expression of MnSOD (Clerch and Massaro 1993, Ho et al. 1996) and GPx (Ho et al. 1996), but catalase and CuZnSOD mRNAs remain unchanged (Clerch and Massaro 1993, Ho et al. 1996). Simultaneously, the specific activities of these AOEs have been found to increase in some, but not all studies (Kimball et al. 1976, Freeman et al. 1986, Clerch and Massaro 1993, Ho et al. 1996). MnSOD activity in alveolar type II pneumocytes is significantly increased in rats exposed to sublethal hyperoxia (85%) (Freeman et al. 1986). In situ hybridization has shown that a major difference in the distribution of MnSOD mRNA between air- and hyperoxia-exposed rat lung occurs at the pleura (Clyde et al. 1993). However, hyperoxia in vitro does not increase AOE levels or activities in rat or rabbit alveolar epithelial cells (Panus et al. 1989, Housset et al. 1991). Furthermore, recent studies with human bronchial

epithelial cells have shown that total SOD and catalase activities do not increase, or the increase is not sufficiently high enough for cell protection after acute hyperoxia exposure in vivo or in vitro (Erzurum et al. 1993, Yoo et al. 1994).

The regulation of AOEs under oxidant conditions is more complex in vivo than in vitro. The mRNA expression, protein concentrations, and enzyme activities do not correlate with each other (Jornot and Junod 1992, Clerch and Massaro 1993). The induction of MnSOD in rat lungs in vivo is maximal at days 3 to 5 of hyperoxia exposure (Ho et al. 1996), which is also the time required for the recruitment of activated inflammatory cells into the lungs (Barry and Crapo 1985). It can therefore be hypothesized that AOE induction in the lung is related to cytokine release and inflammation rather than to the high oxygen tension itself. It has to be emphasized that the effects of inflammatory cytokines and inflammation are associated with an increased oxidant burden at the cellular level. Manganese superoxide dismutase mRNA or protein or both are induced by several cytokines and oxidants. These include tumor necrosis factor α (TNF-α) (Wong and Goeddel 1988, Tsan et al. 1990b, Visner et al. 1990, Warner et al. 1991, Visner et al. 1991), interferon-γ (IFN-γ) (Harris et al. 1991), interleukin-1 (IL-1) (Masuda et al. 1988, Visner et al. 1990, Tsan et al. 1991), endotoxin (Visner et al. 1990, Visner et al. 1991, Clerch and Massaro 1993, Tang et al. 1994), cigarette smoke (Gilks et al. 1998), and various oxidants, such as H_2O_2, thioredoxin, and peroxynitrite (Das et al. 1995, Warner et al. 1996, Jackson et al. 1998). Furthermore, exposure to asbestos fibers or to various fibrogenic materials has been shown to induce MnSOD in tracheal epithelial cells (Mossman et al. 1986) and in alveolar type II pneumocytes in vivo (Holley et al. 1992), and cause MnSOD mRNA upregulation in mesothelial and bronchial epithelial cells in vitro (Janssen et al. 1994a, Marks-Konczalik et al. 1998). It should be pointed out that in contrast to in vitro studies, in which only MnSOD is induced after cytokine exposure, in rats treated with cytokines and hyperoxia the activities of lung CuZnSOD, GPx, and catalase are also induced (White et al. 1989, Tsan et al. 1990a). In addition, ECSOD is upregulated by lipopolysaccharide but not by hyperoxia (Loenders et al. 1998). Most of these studies have been conducted on animal cell models or on various malignant cell lines.

2.6. Importance of AOEs in oxidant defense

Considerable evidence supports the importance of AOEs in pulmonary defense against oxygen toxicity. Animals pre-exposed to sublethal hyperoxia have shown resistance to consequent exposure to 100% O_2, and this resistance correlates with increased total SOD activity (Crapo and Tierney 1974). Tracheal insufflation of liposome-encapsulated or polyethylene glycol (PEG) -conjugated CuZnSOD or catalase or both protects rats against oxygen toxicity (Freeman et al. 1985, Padmanabhan et al. 1985, Tang et al. 1993). Genetic manipulation that causes an

elevation in or an absence of expression of SODs results, respectively, in increased or decreased tolerance to O_2 (Hassan 1988). Antisense oligonucleotides to MnSOD increase the sensitivity of leukemic cells to the growth-inhibitory effects of TNF-α (Kizaki et al. 1993). Transfection of cultured rat lung L2 cells with human CuZnSOD cDNA protects against xanthine/XO-induced cytotoxicity (Komada et al. 1996). Furthermore, tracheal insufflation of endotoxin, TNFα, or IL-1 results in prolonged survival of adult rats during hyperoxia exposure, with a simultaneous increase in AOEs, most prominently MnSOD (Tsan et al. 1990a, Tsan et al. 1991, Clerch and Massaro 1993). On the other hand, resistance to hyperoxia in vivo is not necessarily related to changes in lung AOEs (Baker et al. 1989).

The possible mechanisms by which endotoxin, cytokines, and hyperoxia enhance MnSOD mRNA are by increased rate of transcription (Visner et al. 1991) or increase in the stability of MnSOD mRNA (Clerch and Massaro 1993). The transient fall in MnSOD activity during exposure to O_2 seen in some studies seems to be due to impaired translational efficiency and to a post-translational effect, i.e., low MnSOD-specific activity (Clerch et al. 1998).

It has been suggested that cytokine-induced MnSOD protects cells and tissues during oxygen exposure in vivo and in vitro (Wong et al. 1989, Warner et al. 1991, Lewis-Molock et al. 1994, Tsan 1997), but contradictory results with vascular endothelial cells have also been obtained (Marcho et al. 1991). The combination of different AOEs may be more important than any individual enzyme (Amstad et al. 1991, Amstad at al. 1994). For example, tolerance to hyperoxia of transgenic mice overexpressing CuZnSOD is associated with increased activity of GPx (White et al. 1991). It should also be noted that H_2O_2-scavenging enzymes are increased simultaneously with MnSOD induction (Freeman et al. 1986, Jornot and Junod 1992).

Recent studies with genetically manipulated mice have provided further information about the importance of AOEs in resistance to oxygen toxicity. Transgenic mice that overexpress MnSOD in alveolar type II pneumocytes and in non-ciliated bronchial epithelial cells are tolerant to hyperoxia (Wispe et al. 1992). Homozygous MnSOD knock-out mice die within the first 10 days of life with a dilated cardiomyopathy, accumulation of lipid in the liver and in skeletal muscle, and metabolic acidosis (Li et al. 1995). Heterozygous knock-outs are viable, and are not more susceptible to 100% oxygen than are their normal littermates (Tsan et al. 1998). Transgenic mice, which constitutively express elevated levels of human CuZnSOD, have, upon exposure to hyperoxia, shown increased survival and decreased morphological evidence of lung damage (White et al. 1991), but for unknown reasons, this protective effect has been noted only in a subgroup of young, female transgenic mice. Contradictory results have also been obtained: no protection against hyperoxia in transgenic mice overexpressing CuZnSOD or MnSOD (Ho et al. 1998). Knockout mice lacking ECSOD develop normally, but are more

susceptible to the toxic effects of oxygen (Carlsson et al. 1995), whereas transgenic mice overexpressing ECSOD, when exposed to hyperoxia, show decreased morphological evidence of lung damage and a reduced number of recruited inflammatory cells than do wild-type mice (Folz et al. 1999). Due to its ideal localization in the areas where $O_2^{-\cdot}$ is released by phagocytic leukocytes, ECSOD may be important in protecting cell surfaces and extracellular matrix proteins from superoxide-mediated damage.

Catalase and the glutathione-redox cycle are two H_2O_2-scavenging pathways. Glutathione plays an important role in maintaining intracellular redox balance and in cellular defense against oxidative stress (Rahman et al. 1995). Glutathione is present in high concentrations in human lung epithelial lining fluid (Cantin et al. 1987). Extracellular glutathione has also been shown to protect rat alveolar macrophages against hyperoxia, and that this protection is abolished by an inhibitor of γ-glutamyl transpeptidase suggest the importance of the glutathione cycle in alveolar macrophage protection (Forman and Skelton 1990). Furthermore, pulmonary depletion of glutathione in lungs of mice or rats exacerbates oxygen toxicity (Deneke et al. 1985, Smith et al. 1990), and intraperitoneal supplementation of glutathione protects preterm rabbits against oxidant stress (Brown et al. 1996). Inhibition of glutathione synthesis by buthionine sulfoximine (BSO) increases hyperoxic cell injury in rat type II pneumocytes in vitro (Aerts et al. 1992). The significance of H_2O_2-scavenging pathways varies considerably in different cell types. Human alveolar macrophages and freshly isolated rabbit or rat type II pneumocytes protect themselves against exogenous H_2O_2 mainly by a catalase-dependent pathway (Engstrom et al. 1990, McDonald et al. 1991, Kinnula et al. 1992a). On the other hand, vascular endothelial cells or pleural mesothelial cells, both of which contain low AOE levels (Kinnula et al. 1991, Kinnula et al. 1992c), are protected against exogenous H_2O_2 mainly by the glutathione redox pathway (Harlan et al. 1984, Suttorp et al. 1986, Kinnula et al. 1992c). In addition, we have previously shown that the glutathione cycle is important in maintaining intracellular energy metabolism and protecting the cell membrane against oxidant damage in human bronchial epithelial cells (Aalto et al. 1996).

2.7. Nonenzymatic antioxidants

A number of compounds act as non-enzymatic antioxidants. α-tocopherol (vitamin E) is lipophilic, and its major role is to block the chain reaction of lipid peroxidation (Sies et al. 1992). Pre-treatment with vitamin E has been shown in rabbits to prevent lung injury caused by hyperoxia (Jacobson et al. 1990). α-tocopherol and ascorbic acid (vitamin C) cooperate in cellular defense against ROS. Ascorbic acid reduces the oxidized α-tocopheryl radical back to α-tocopherol (Freeman and Crapo 1982). In addition, vitamin C, a hydrophilic

scavenger of ROS, is considered the most important antioxidant in extracellular fluids (Sies et al. 1992). On the other hand, ascorbic acid can promote ROS-injury in the presence of free transition metals (Heffner and Repine 1989). Dietary carotenoids and ubiquinol are also thought to act as antioxidants, but their precise role in vivo is unclear (Halliwell and Gutteridge 1990). In addition to being essential in the glutathione redox cycle, glutathione can scavenge ROS non-enzymatically (Freeman and Crapo 1982), as well as detoxify xenobiotics via the glutathione S-transferase reaction. Glutathione protects SH-groups of proteins from oxidation and maintains vitamin-C levels (Meister 1992). The precursor of glutathione, N-acetyl-cysteine, scavenges ROS and has been used in various clinical trials (Aruoma et al. 1989, Bernard et al. 1997).

3. Clinical implications of antioxidant defense in the lung

3.1. Adult respiratory distress syndrome (ARDS)

Adult respiratory distress syndrome (ARDS) is characterized by neutrophil accumulation, diffuse pulmonary infiltrates, and pulmonary edema, and in mechanically ventilated patients its severity is increased by the use of high oxygen tensions. It has been suggested that increased production of ROS by activated neutrophils combined with decreased antioxidant capacity plays a central role in the pathogenesis of ARDS (Chabot et al. 1998). Patients with ARDS have elevated concentrations of H_2O_2 in their exhaled air (Sznajder et al. 1989), and the serum of ARDS patients scavenges H_2O_2 more efficiently than that of patients without ARDS, a finding associated with the increased AOE activities in the serum of ARDS patients (Leff et al. 1992, Leff et al. 1993). The glutathione content of alveolar lining fluid is decreased in ARDS (Pacht et al. 1991), and ARDS patients show significantly lower plasma levels of non-enzymatic antioxidants such as α-tocopherol (Metnitz et al. 1999).

3.2. Inflammatory airway diseases

In the pathogenesis of chronic obstructive pulmonary disease (COPD), generation of ROS by activated inflammatory cells plays a central role. Cigarette smoke itself contains ROS. One puff of cigarette smoke has been estimated to contain 10^{15} radicals (Church and Pryor 1985). Cigarette smoke also contributes to the recruitment of inflammatory cells to the airways. Furthermore, this inflammation is stimulated by other inhaled pollutants and by cytokines. A protease-antiprotease imbalance plays an important role in the progression of COPD, but an imbalance between oxidants and antioxidants has also been proposed as playing a role in the pathogenesis of this disease (Rahman and MacNee 1996). The data on AOE activities in the lung of cigarette smokers are contradictory.

Alveolar macrophages of smokers and smoke-exposed hamsters have increased activities of SOD and catalase, with no change in GPx (McCusker and Hoidal 1990). Catalase and GPx activities in the bronchoalveolar lavage (BAL) cells from smokers are increased, without any difference in SOD activity (Hilbert and Mohsenin 1996). Another study reports that SOD activity of BAL fluid is lower in smokers than in controls (DiSilvestro et al. 1998), and that increased production of $O_2^{-\cdot}$ by alveolar macrophages of elderly smokers is associated with a post-translationally modified reduction in AOE activities when compared to those of nonsmokers (Kondo et al. 1994). The mRNA expression of MnSOD is transiently increased in bronchial epithelial cells of smoke-exposed rats. After prolonged exposure, the mRNA level of GPx is also elevated (Gilks et al. 1998).

Bronchial asthma is defined by reversible airflow obstruction, by bronchial hyper-responsiveness, and by chronic inflammation characterized by influx and activation of inflammatory cells and epithelial cell damage (Barnes 1990). Cells obtained from the peripheral blood and lungs of patients with asthma generate increased amounts of ROS (Vachier et al. 1992, Jarjour and Calhoun 1994, Kinnula et al. 1995, Majori et al. 1998). In contrast to what might be expected, peripheral blood cells from asthmatic patients exhibit reduced or unchanged antioxidant defense including reduced GPx and SOD activities (Vachier et al. 1992, Joseph et al. 1993, Misso et al. 1998). In addition, it has recently been shown that in the airway epithelium of asthmatic patients, CuZnSOD, but not catalase or GPx, is decreased (De Raeve et al. 1997, Smith et al. 1997). Decreased antioxidant defense in the airways of asthmatic patients may be associated not only with the increased oxidant burden but also with the epithelial injury of these patients.

3.3. Interstitial lung diseases

Reactive oxygen species may play a central role in the pathogenesis of a variety of interstitial lung diseases such as idiopathic pulmonary fibrosis (usual interstitial pneumonia, UIP, and desquamative interstitial pneumonia, DIP), asbestosis, and granulomatous lung diseases (sarcoidosis and allergic alveolitis). Alveolar macrophages from patients with pulmonary fibrosis produce significantly more $O_2^{-\cdot}$ (Strausz et al. 1990). Furthermore, it has been found that patients with idiopathic pulmonary fibrosis have higher serum CuZnSOD protein levels than do controls, and that these levels correlate with disease severity (Borzi et al. 1993). Recent studies conducted on human lung tissue have shown high expression of MnSOD in the granulomas of pulmonary sarcoidosis and allergic alveolitis (Lakari et al. 1998), and induction of MnSOD and consistent expression of catalase in the well-preserved alveolar regions of various interstitial lung diseases (Lakari et al. 1999). The role of ROS in the pathogenesis of asbestos-related diseases is supported by the fact that asbestos fibers enhance ROS

generation from inflammatory cells (Kamp et al. 1992), and that the AOEs, especially MnSOD, are induced by asbestos fibers (Mossman et al. 1986, Holley et al. 1992). In addition, treatment of rats with PEG-conjugated catalase reduces lung injury caused by the fibers (Mossman et al. 1990).

3.4. Bronchopulmonary dysplasia (BPD)

At birth the infant is exposed to increased oxidative stress. Preterm infants are at high risk for developing chronic lung disease: bronchopulmonary dysplasia (BPD) (Saugstad 1998). Among factors playing an important role in its development are prematurity, barotrauma, volutrauma, and oxygen toxicity. AOE defense against high inspired oxygen levels after birth in preterm infants may be poor, since many investigators have demonstrated that in various animals the expression of MnSOD, CuZnSOD, catalase, and GPx increase toward term in the fetal lung (Tanswell and Freeman 1984, Frank 1991, Clerch and Massaro 1992). Very few studies have reported a similar profile, at least for catalase in human ontogenesis (McElroy et al. 1992, Asikainen et al. 1998). When exposed to hyperoxia, preterm rabbits and baboons (Frank and Sosenko 1991, Morton et al. 1999), but not rats or guinea pigs (Sosenko and Frank 1987, Chen et al. 1994), in contrast to term rats, are unable to induce AOE activities in the lung. Not only AOEs but also surfactant deficiency and ventilator treatment are factors contributing to the development of BPD (Saugstad 1998). In experimental animal models, the morphological changes in BPD, such as endothelial and epithelial damage, alveolar disruption, bronchial smooth muscle hyperplasia, and hyaline accumulation in alveolar spaces, as well as interstitial fibrosis, are also seen in oxygen toxicity (Crapo 1986, Margraf et al. 1991). Moreover, inflammatory cells which are recruited into the lungs (Margraf et al. 1991) can further enhance ROS production, as well as induce the release of inflammatory cytokines. Therefore, it is not surprising that the following results are in line with the cytokine- and hyperoxia-induced MnSOD expression discussed previously. Pulmonary MnSOD is significantly elevated in the lungs of baboons representing an animal model of BPD, and the protein expression is regulated, at least partly, at the post-transcriptional level (Clerch et al. 1996). In human BPD lungs, proliferating type II pneumocytes and alveolar macrophages show intense staining for MnSOD protein (Dobashi et al. 1993, Ohbayashi et al. 1997). Surfactant replacement, antenatal steroid therapy, and more gentle ventilatory techniques have improved the outcome for preterm infants, and new therapeutic approaches with antioxidants and AOE supplementation have also given promising results, although their use in practice needs further investigation.

3.5. Therapeutic implications for improving antioxidant defense in the lung

Considerable effort has been made in recent years to enhance AOE activities and oxygen tolerance by therapeutic utilization of antioxidants. Problems in the protective role of various AOEs are their poor penetrance into cells and extremely short plasma half-lives. Various manipulations of enzymes and means of delivery have thus been tested. Premature infants are known to be deficient in glutathione and selenium (Jain et al. 1995), but glutathione delivery by intravenous or intratracheal routes has failed to raise cellular levels of glutathione, and has not been shown to be protective (Meister 1991). N-acetylcysteine (NAC) administration in adults with ARDS offers some evidence of reduced lung injury (Bernard et al. 1997). A study on the treatment of preterm infants with NAC to improve survival and prevent development of chronic lung injury is ongoing in this hospital. In animals, intravenous or intratracheal administration of liposome-encapsulated or PEG-conjugated SOD and catalase are able to moderate hyperoxia- or asbestos fiber-induced lung damage (Freeman et al. 1985, Padmanabhan et al. 1985, Mossman et al. 1990).

For genetically modified mice, SODs are critically important in protecting the lung from the damaging effects of hyperoxia (Wispe et al. 1992, Carlsson et al. 1995), and lung injury caused by hyperoxia can be prevented by intratracheal administration of SOD (Padmanabhan et al. 1985, Davis et al. 1993, Tang et al. 1993). Recent evidence indicates that SOD may have beneficial effects in preventing lung injury also in humans. When preterm infants are treated with intratracheally delivered recombinant SOD, the concentration and activity of SOD increase in serum, intratracheal fluid, and urine, and the parameters of lung injury decline (Davis et al. 1997). However, preliminary results of a controlled clinical trial fail to suggest any beneficial effect (Davis et al. 1999). Molecular biology has opened new avenues to manipulate genes and express a variety of important proteins. Intratracheal injection of an adenovirus containing SOD and catalase cDNAs have been used successfully to increase enzyme expression in airway epithelium and alveolar macrophages, and thus prevent rats from hyperoxia- or irradiation-induced lung damage (Danel et al. 1998, Epperly et al. 1999).

AIMS OF THE STUDY

The formation of ROS and the consequent oxidative stress has been suggested to be important in the pathogenesis of various lung diseases. Because the antioxidant enzyme system is a primary defense mechanism of the lung, this present study was designed to investigate the role and regulation of the most important AOEs in human lung cells by exposing the cells to agents and states which may play an important role in the pathogenesis of lung diseases (cytokines, asbestos fibers, and hyperoxia). Furthermore, the study investigated the protective role of various AOEs in different cell types of the human lung. The specific aims were to investigate:

1) the effect of cytokines (TNF-α and IFN-γ) on MnSOD induction and oxidant-related cytotoxicity in human bronchial epithelial cells (I)

2) the effect of hyperoxia on various antioxidant pathways and on oxidant-related cytotoxicity in human bronchial epithelial cells (II)

3) the effect of asbestos fibers and cytokines on MnSOD induction and oxidant-related cytotoxicity in human pleural mesothelial cells (III)

4) the importance of different AOE pathways in oxidant-related cytotoxicity of alveolar macrophages (IV)

5) and to compare different AOE pathways in human inflammatory cells and assess their potential effects on cell survival and oxidant-related toxicity (V)

MATERIALS AND METHODS

1. Cells and culture

1.1. Cell lines (I-III)

Transformed human bronchial epithelial cells (I, II), BEAS-2B, and transformed human pleural mesothelial cells (III), MET-5A, were obtained from the National Cancer Institute, Laboratory of Human Carcinogenesis (Dr. C. Harris, Bethesda, MD, USA). BEAS-2B is an SV40-transformed epithelial cell line established from healthy bronchial epithelium which has been shown to maintain typical epithelial morphology and functional characteristics (Ke et al. 1988, Reddel et al. 1988). BEAS-2B cells contain lower AOE levels than do bronchial epithelial cells in situ, but they express all AOEs, and are therefore an appropriate stable in vitro model to investigate the role and the regulation of AOEs in epithelial cell defense and injury (Kinnula et al. 1994). BEAS-2B cells were cultured on uncoated 4-well tissue-culture plates or 35-mm petri dishes in serum-free hormone-supplemented medium according to the manufacturer's instructions (bronchial epithelial growth medium, BEGM, Clonetics Inc., San Diego, CA, USA). MET-5A is an SV40-transformed mesothelial cell line established from healthy mesothelium. MET-5A cells exhibit features of mesothelial cells, including sensitivity to the cytotoxic effects of asbestos fibers. Because they remain nontumorigenic, they are therefore a useful model for in vitro studies of fiber cytotoxicity (Ke et al. 1989). MET-5A cells were cultured on uncoated 24-well tissue-culture plates or 35-mm petri dishes in RPMI-1640 medium (Gibco Europe, Paisley, UK) containing 10% fetal bovine serum (FBS, Gibco), penicillin (100 U/ml), streptomycin (100 μg/ml) (Gibco), and L-glutamine (Gibco).

1.2. Primary cell isolation and culture (IV-V)

Peripheral monocytes (V) were isolated from buffy coats (Finnish Red Cross, Helsinki, Finland) by a modification of the method described by Böyum (1968). Briefly, buffy coats were obtained 1-2 h after venipuncture. After dilution 1:3 with phosphate-buffered saline (PBS, Orion, Espoo, Finland) and mixing with a glass rod, the cell suspension was layered over Ficoll-Paque (Pharmacia, Biotech AB, Uppsala, Sweden) and centrifuged at 900 x g for 30 min. Mononuclear cells were collected at the interface, washed twice with PBS, and suspended in the standard medium of RPMI-1640 supplemented with L-glutamine, penicillin-streptomycin, and 20% human AB-serum (Finnish Red Cross). Viability tested by trypan blue exclusion was over 90%. Mononuclear cells were incubated in collagen-coated 4-well tissue-culture plates or 35-mm petri dishes (Falcon,

Becton Dickinson U.K. LTD, Plymouth, UK) for 1 h at 37ºC. After incubation, non-adherent cells were removed by washing with PBS. Adherent cells were cultured under 5% CO_2 at 37ºC for up to 5 days in standard medium, which has been shown to induce maturation of monocytes into so-called monocyte-derived macrophages.

Neutrophils (V) were isolated from the heparinized venous blood of healthy volunteers as previously described (Böyum 1968). Briefly, after sedimentation with 3% dextran (Pharmacia), Ficoll-Paque centrifugation at 400 x g for 40 min, and hypotonic lysis of red blood cells, neutrophils were suspended in standard medium and pelleted immediately or cultured in 4-well tissue culture plates or petri dishes for up to 24 to 72 h. Viability after isolation was over 90%.

Alveolar macrophages (IV, V) were isolated from bronchoalveolar lavage fluid (BAL), obtained from patients with minor respiratory symptoms. BAL was centrifuged at 330 x g, and the cells were washed twice with RPMI-1640 medium. Cytocentrifuge preparations indicated that over 90% of the cells were macrophages. For further enrichment, cells were layered in petri dishes or 4-well tissue-culture plates in RPMI-1640 medium containing 10% FBS (Gibco), and incubated at 37ºC for 1 h or overnight. Non-adherent cells were removed by washing.

Study protocols utilizing patients' samples were approved by the Ethics Committees of the Department of Internal Medicine, University of Helsinki and the Finnish Red Cross.

2. Pretreatments and oxidant exposures (I-V)

2.1. Inactivation of AOEs (IV, V)

To assess the role of different antioxidant enzymes, the cells in culture media were pretreated with 10 μM 1-chloro-2,4-dinitrobenzene (CDNB, E.Merck, Darmstadt, Germany) for 40 min to conjugate glutathione (Buckley et al. 1991), with 100-500 μM buthionine sulfoximine (BSO) (Sigma) for 6 h or 16 h to inhibit γ-glutamylcysteine synthetase and deplete glutathione (Griffith and Meister 1979), with 100 μg/ml 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) for 15 min to inhibit GR (Babson and Reed 1978), or with 30 mM aminotriazole (ATZ) for 60 min to inactivate catalase (Margoliash et al. 1960). These compounds were removed by washing with PBS before subsequent oxidant exposures. All these inhibitors are widely used, and under these experimental conditions, nontoxic.

2.2. Exposures to cytokines (I-III), hyperoxia (I, II), and asbestos fibers (III)

Subconfluent cultures in standard media were exposed to 10 or 100 ng/ml recombinant human tumor necrosis factor-α (TNF-α) (I-III), to 10 or 100 ng/ml human recombinant interferon-γ (IFN-γ) (I), or to a combination of these cytokines (I) each for 16 to 72 h, or to 2 μg/cm^2 amosite asbestos fibers (AMOS) (III) or to a combination of TNF-α and AMOS (III) each for 48 h. The cytokines were provided by Boehringer Ingelheim, Europe (Vienna, Austria). In hyperoxia experiments (I, II) the cultures were exposed to 95% O_2 and 5% CO_2 for 16 to 72 h. Subconfluent cells in culture medium were placed in a Plexiglas chamber through which gas flow was adjusted so that the high oxygen concentration remained stable during the incubation.

2.3. Oxidant exposures (I-V)

Control and pretreated cells were washed and subsequently exposed to 0.1 to 5 mM H_2O_2 (Merck AG, Darmstadt, Germany) for 2 to 4 h (I, II, IV, V), to 5 to 500 μM menadione (Sigma) for 2 to 4 h (I-III), to hyperoxia (95% O_2) for 48 h, or to 10^{-7} M formyl-methionyl-leucyl-phenylalanine (FMLP, Sigma) for 2 h (IV, V). The exact H_2O_2 concentration was determined spectrophotometrically using the molar extinction coefficient of 44 M^{-1} cm^{-1} at 240 nm.

3. Measurement of enzyme activities (I-V) and glutathione concentrations (II, V)

The cells were detached with trypsin-EDTA (Gibco), pelleted, and immediately frozen at -80ºC until analysis. Total SOD activity (I, II, III, V) was assayed spectrophotometrically by measuring the inhibition of the reduction of cytochrome c in the xanthine oxidase reaction at 550 nm (McCord and Fridovich 1969). MnSOD activity was distinguished from CuZnSOD by its resistance to 1 mM potassium cyanide. Catalase activity (I, II, IV, V) was determined polarographically by measuring the oxygen production rate with a Clark oxygen electrode in a stirred chamber (Kinnula et al. 1992a). Glucose-6-phosphate dehydrogenase (G6PDH) activity (I) was quantitated spectrophotometrically by following the oxidation of NADP (Löhr and Waller 1974). GR activity (I, II, IV, V) was analyzed by measuring the oxidation of NADPH at 340 nm in the presence of oxidized glutathione (Beutler 1975), and GPx activity (I, II, IV, V) by measuring NADPH oxidation at 340 nm in the presence of *t*-butylhydroperoxide, glutathione, and GR (Beutler 1975). Enzyme activities are expressed as units per milligram protein. Protein was determined by the method of Lowry et al (1951) (I-IV) or by Bio-Rad DC assay (Bio-Rad Laboratories, Hercules, CA, USA) (V).

For the analysis of total glutathione levels (II, V), the cells were collected in 2 N perchloric acid containing 2 mM ethylenediamine tetra-acetic acid (EDTA). After neutralization with a solution containing 2 M KOH and 0.3 M N-morpholinopropanesulfonic acid (MOPS), total cellular glutathione content was determined spectrophotometrically by measuring the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (Sigma) by NADPH in the presence of glutathione reductase (Sigma) (Akerboom and Sies 1981). Cellular glutathione content is expressed as nmol/mg protein.

4. Northern blotting analysis (II, III, V)

Cells were collected in 4 M thiocyanate buffer, and the samples were frozen immediately at -80°C. Total RNA was extracted from the cells by the acid phenol-chloroform method (Chomczynski and Sacchi 1987). RNAs (10 or 20 μg/lane) were electrophoresed on a 1% agarose gel containing 0.36 M formaldehyde. Samples were capillary transferred onto Hybond-N nylon filters (Amersham International, Amersham, UK) and cross-linked to the filters by UV illumination (UV Stratalinker 1800; Stratagene, La Jolla, CA, USA). Prehybridization was done at 58.5°C for > 1 h. Filters were hybridized with ^{32}P-labeled cRNA probes representing nucleotides 596-987 of human MnSOD (Ho and Crapo 1988), 127-457 of human CuZnSOD (Hallewell et al. 1985), 537-2218 of human catalase (Quan et al. 1986), or 533-624 of rat GPx (Ho et al. 1988), each cloned into the pSP65 vector (Promega Co., Southampton, UK). The transcripts were purified by NucTrap columns (Stratagene) and added to the prehybridization solution at $2x10^6$ cpm/ml. Hybridization was then carried out at 58.5°C overnight with shaking. After washing, autoradiography was performed at -80°C by use of Kodak BioMax MR film (Eastman Kodak Co, Rochester, NY, USA). Following autoradiography, the filters were rehybridized with a β-actin control probe transcribed from pTRI-β-actin plasmid (Ambion, Austin, TX, USA). Antioxidant enzyme mRNA expressions were quantified relative to actin expression by a X-Rite 331 Transmission densitometer (II, V) (X-Rite, Grandville, MI, USA) or NIH Image 1.52 software (III). The human MnSOD, CuZnSOD, catalase, and rat GPx cDNAs were kindly provided by Dr. Y.-S. Ho, Wayne State University, Detroit, MI, USA.

5. Western blotting analysis (I,V)

The cells were detached, centrifuged, and washed with PBS. The cell pellet was mixed with the electrophoresis sample buffer and boiled for 5 min at 95°C. 50 or 75 μg of cell protein was applied per lane to a 10-12% sodium dodecyl sulfate (SDS) -polyacrylamide gel (Laemmli 1970). The gel was electrophoresed for 1.5

h (90 V) (V) or 3.5 h (40 mA) at room temperature, the protein was transferred (45 min, 100 V or 3 h, 400 mA) onto Hybond ECL nitrocellulose membranes (Amersham, Arlington Hights, IL, USA) in a Mini-PROTEAN II Cell (Bio Rad Laboratories). The blotted membrane was incubated with rabbit antibody to recombinant human MnSOD (1:10 000) (gift from Professor J.D. Crapo, National Jewish Medical and Research Center, Denver, CO, USA), followed by donkey anti-rabbit secondary antibody (V) or goat anti-rabbit immunoglobulin G (1:30 000) conjugated to horseradish peroxidase (Amersham). MnSOD was detected by an enhanced chemiluminescence system (ECL, Amersham), and the luminol excitation was imaged on X-ray film (Eastman Kodak). Cell protein was determined by Bio-Rad DC assay (Bio-Rad Laboratories).

6. Immunohistochemistry (IV)

Immunolocalization of catalase was performed on sections of cell pellets from freshly isolated and cultured macrophages. Cell pellets from four healthy volunteers (two samples cultured, the other two freshly isolated) were fixed with 2% paraformaldehyde plus 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After 1 h, they were transferred to 2% paraformaldehyde without glutaraldehyde for an additional 24 h. Thereafter, the cells were washed three times with PBS and incubated in 10% gelatin at 37°C for 10 min. Blocks were cryoprecipitated with 2.3 M sucrose and frozen in liquid nitrogen. Cryoultrathin sections were prepared with a cryomicrotome, and immunolabeling was done by the method of Slot et al. (1986). Cells from each pellet were photographed, and the number of gold particles on peroxisomes, cytoplasm, and nucleus counted. The area occupied by these compartments was measured by point counting.

7. H_2O_2 consumption (II, IV)

H_2O_2 consumption by untreated and pretreated intact cells was analyzed fluorometrically by following H_2O_2-dependent oxidation of 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA, Sigma) in the presence of horseradish peroxidase (HRP, Sigma) by the modified method (Kinnula et al. 1992d) of Ruch et al. (1983). Incubations were conducted at 37°C in PBS. H_2O_2 (50 to 500μM) was added to the medium, and H_2O_2 consumption was analyzed on the basis of samples drawn during a 40-min incubation. The exact H_2O_2 concentration was determined spectrophotometrically using the molar extinction coefficient of 44 M^{-1} cm^{-1} at 240 nm.

8. Measurement of ROS production (I)

Reactive oxygen species were assessed by chemiluminescence, with an automated microcomputer-controlled luminometer. Untreated and cytokine-treated cells after the 48-h incubation were used. The reaction mixture at 37°C in 1 ml contained $0.5\text{-}1 \times 10^6$ trypsinized and washed cells in PBS, 700 µl 10^{-4} M luminol (5-amino-2,3-dihydro-1,4-phtalazinedione, LKB, Wallac), and in some experiments 5-500 µM menadione. Chemiluminescence was measured for 30 min in the presence and absence of cytokines from both untreated and cytokine-pretreated cells.

9. Measurement of cell injury

9.1. Trypan blue exclusion (V)

The cell suspension was incubated with trypan blue in PBS for 5 min at room temperature. The cells were counted in a Bürker cell-counting chamber, and trypan blue-negative cells were considered as viable cells.

9.2. Lactate dehydrogenase (LDH) release (I-III)

Lactate dehydrogenase release into the medium from cells cultured on 4- or 24-well tissue culture plates was measured spectophotometrically, with pyruvic acid (Sigma) as the substrate (Bergmeyer 1974). Total LDH was measured in cell lysates obtained by treatment with 1% Triton X-100. The results are expressed as percent of activity released into the medium.

9.3. Adenine nucleotide depletion (I-V)

For cytotoxicity experiments, in which cell injury was assessed by adenine nucleotide depletion, cells cultured in 4- or 24-well tissue-culture plates were preincubated with 0.1 mM ^{14}C-adenine (specific activity 51 to 55 mCi/mmol (I-IV) or 284-287 mCi/mmol (V); Amersham International) for 6 h (V), overnight (I, III, IV), or for 48 h (II). After incubation, the labeled medium was removed, and the cells were washed and exposed to H_2O_2, menadione, or FMLP in serum-free RPMI-1640 medium as described previously. Experiments were terminated by removing and freezing the medium at -20°C and extracting the cells with 0.42 N ice-cold perchloric acid. Purine nucleotides (ATP, ADP, and AMP) in neutralized cell extracts, as well as total nucleotides and nucleotide catabolic products (hypoxanthine, xanthine, and uric acid) in the medium were separated

by thin-layer chromatography (Aalto and Raivio 1990). The separated purine compounds were visualized on the chromatography sheets under ultraviolet light, cut out, and counted in Optiphase ”High Safe”3 (LKB Scintillation Products, FSA Laboratory Supplies, Loughborough, UK) by means of an LKB Wallac Rackbeta 1209 liquid scintillation counter. Results are expressed as percent distribution of radioactivity among nucleotides retained in the cell, nucleotides in the medium, and their catabolic products.

10. Statistical methods

All experiments were performed at least in duplicate. Data analyses were performed with the Stat View or SPSS for Windows software (SPSS Inc., Chicago, IL, USA). Two groups were compared by either the two-tailed Student´s t-test (I, III, IV) or nonparametric Mann-Whitney U-test (II, V), and multiple groups by analysis of variance (ANOVA) combined with Scheffé’s post hoc test (I, III, IV) P values of < 0.05 were considered significant.

RESULTS

1. Superoxide dismutases

1.1. Bronchial epithelial BEAS-2B cells

Bronchial epithelial cells (BEAS-2B) were used to study the regulation of MnSOD and CuZnSOD by cytokines and hyperoxia in vitro. TNF-α (16, 24 and 48 h), a well-known inducer of MnSOD in a number of animal cell types (Wong and Goeddel 1988, Harris et al. 1991), caused a significant increase in MnSOD mRNA also in BEAS-2B cells (II; Figure 1). The mRNA levels increased until 24 h and then decreased, levels after 48 h still being higher than in untreated cells. MnSOD protein was elevated by TNF-α and IFN-γ + TNF-α, but not by IFN-γ alone (I; Figure 1). The specific activity of total SOD did not change significantly after IFN-γ, TNF-α, or IFN-γ + TNF-α treatments (I; Table 1). The activity of MnSOD was increased significantly by TNF-α (Table 1, I; Table1). In contrast to the induction of MnSOD protein, MnSOD activity was further increased by the combination of these cytokines (Table 1, I; Table 1).

Because preliminary hyperoxia experiments with BEAS-2B cells indicated significant cell injury after 72 h exposure to hyperoxia (II), hyperoxia exposures of 16 to 48 h were selected to investigate AOE regulation in BEAS-2B bronchial epithelial cells. The mRNA levels and specific activities of MnSOD and CuZnSOD remained unchanged after the hyperoxia exposure (Table 1, II; Figures 1 and 2, Table 1). In conclusion, in human bronchial epithelial cells, MnSOD was induced by TNF-α but not by high oxygen tension.

1.2. Mesothelial MET-5A cells

Mesothelial MET-5A cells were used to study the regulation of MnSOD by TNF-α and asbestos fibers. TNF-α and amosite + TNF-α caused a significant upregulation of MnSOD mRNA (III; Figure 1) and of specific activity (Table 1, III; Figure 2) when compared to controls. The mRNA of MnSOD was not significantly increased after amosite exposure, and the specific activity of MnSOD showed no change. Total SOD activity remained unchanged in each case (III; Figure 2). Thus in these experimental conditions, only TNF-α but not asbestos fibers induced MnSOD activity.

		CuZnSOD	MnSOD	Catalase	GPx	GR	G6PDH
BEAS-2B	**unexposed**	100 ± 36	100 ± 24	100 ± 18	100 ± 22	100 ± 12	100 ± 12
	95% O_2 48h	81 ± 29	119 ± 38	121 ± 21	67 ± 26	109 ± 12	-
	IFN 10ng/ml	103 ± 7	124 ± 57	93 ± 11	149 ± 60	145 ± 11	135 ± 14
	TNF 10ng/ml	92 ± 22	273 ± 12	92 ± 5	102 ± 31	114 ± 4	132 ± 7
	IFN+TNF	86 ± 23	612 ± 297	75 ± 1	132 ± 22	113 ± 13	105 ± 31
MET-5A	**unexposed**	100 ± 46	100 ± 26	-	-	-	-
	TNF 10ng/ml	76 ± 16	379 ± 203	-	-	-	-
	AMOS 2mg/cm^2	87 ± 15	105 ± 32	-	-	-	-
	TNF+AMOS	92 ± 26	400 ± 103	-	-	-	-

Table 1: *Antioxidant enzyme activities of control BEAS-2B cells and cells exposed to hyperoxia for 48 h, IFN-γ (10ng/ml), TNF-α (10ng/ml), or IFN-γ + TNF-α, and of control MET-5A cells and cells exposed to TNF-α (10ng/ml), AMOS (2μg/cm^2), or TNF-α + AMOS. Results are means ± SD from 3-6 separate experiments and expressed as % of the activity of control cells.*

1.3. Inflammatory cells

When MnSOD and CuZnSOD mRNA and protein levels and/or activities were compared in freshly isolated neutrophils, monocytes, and alveolar macrophages, and these levels were compared to those of differentiated monocytes expressing features typical of macrophages, freshly isolated neutrophils had markedly higher levels of MnSOD mRNA than mononuclear cells; neither the specific activity nor protein, however, was higher (Table 2, V; Figure 1). The mRNA levels and specific activities of CuZnSOD were similar in all cells investigated (Table 2, V). SOD activities were not measured in alveolar macrophages, since the number of BALs performed was limited, but the amount of immunoreactive MnSOD protein was similar to that in freshly isolated monocytes (V; Figure 1). MnSOD mRNA, immunoreactive protein, and specific activity were upregulated in macrophages derived from monocytes upon in vitro culture (Table 2, V; Figure 1). Taken together, the levels of MnSOD varied in different inflammatory cells, whereas CuZnSOD appeared to be very similar in all cell types investigated.

1.4. Effect of cytokine-induced MnSOD on oxidant-related cytotoxicity

To investigate the protective role of MnSOD induction in cytokine-pretreated cells, the cells were pretreated with TNF-α (BEAS-2B and MET-5A), with IFN-γ, or with IFN-γ + TNF-α (BEAS-2B), with amosite asbestos fibers, or with a combination of TNF-α and amosite (MET-5A). These cytokines themselves were not toxic to the cells (I). Both untreated and cytokine-pretreated BEAS-2B cells were very resistant to hyperoxia (I). However, pretreatment of these cells with TNF-α or with TNF-α + INF-γ to cause MnSOD induction potentiated H_2O_2-induced, menadione-induced (menadione, a compound which generates $O_2^{-\cdot}$ intracellularly), and hyperoxia-induced cell injury, which was assessed by LDH release and adenine nucleotide depletion (I; Figures 2-5). This effect was most significant with the combination of IFN-γ and TNF-α. Amosite and amosite + TNF-α, but not TNF-α alone, caused significant injury in MET-5A cells, as assessed by LDH release (III; Figure 3); and TNF-α caused significant adenine nucleotide depletion (III; Figure 4). MET-5A cells, in which MnSOD was induced by TNF-α and the amosite + TNF-α combination, were more sensitive to menadione than were untreated cells (III; Figures 5 and 6).

2. H_2O_2 scavenging mechanisms

2.1. Bronchial epithelial BEAS-2B cells

When the most important H_2O_2-scavenging antioxidant enzymes (catalase and GPx) were investigated in cells treated with cytokines or exposed to high oxygen tension, the activities of GPx, GR, and G6PDH underwent no change during cytokine treatments (IFN-γ, TNF-α, or IFN-γ + TNF-α for 48 h); if anything, the activity of catalase decreased after treatment of the BEAS-2B cells with IFN-γ + TNF-α for 48 h (Table 1, I; Table 1). The mRNA levels and specific activities of catalase, GPx, and GR remained unchanged after hyperoxia exposure when followed for up to 48 h (Table 1, II; Figures 3 and 4, Table 1). However, although total cellular glutathione content was significantly increased by hyperoxia (II; Figure 8), this did not explain the increased oxidant resistance observed during subsequent oxidant exposures in these cells, since depletion of glutathione by buthionine sulfoximine failed to exacerbate the cell injury (II). Moreover, H_2O_2 consumption by normoxic and by hyperoxic cells was similar (II; Figure 7). Thus, cytokines or hyperoxia in vitro induced none of the H_2O_2-scavenging enzymes.

2.2. Inflammatory cells

In comparative studies of different inflammatory cells, neutrophils were found to contain higher levels of mRNA and of specific activity of catalase than did monocytes, monocyte-derived macrophages, or alveolar macrophages (Table 2, IV, V; Figure 2). Catalase was primarily localized in microperoxisomes, but was also found in the cytoplasmic and nuclear matrices of alveolar macrophages (IV; Figure 1, Table 1). The mRNA level and specific activity of GPx (Table 2, V; Figure 3), as well as total glutathione (V; Figure 4), were lower in neutrophils than in monocytes.

		CuZnSOD	MnSOD	Catalase	GPx	GR
Inflammatory cells	**Monocytes 0h**	10.7 ± 4.1	3.2 ± 2.6	88.0 ± 15.6	3.0 ± 0.9	45.4 ± 7.6
	Monocytes 24h	8.0 ± 3.5	2.8 ± 1.7	85.6 ± 10.7	3.5 ± 1.2	36.5 ± 8.1
	Monocytes 72h	7.5 ± 2.8	6.2 ± 2.6	68.6 ± 26.7	3.6 ± 0.9	38.8 ± 16.8
	Neutrophils	7.1 ± 4.5	2.6 ± 1.8	368.3 ± 111.6	0.9 ± 0.9	27.0 ± 2.4
	Alveolar macrophages[1]	-	-	76.8 ± 9.2	7.5 ± 1.8	31.0 ± 7.4

Table 2: *Antioxidant enzyme activities (U/mg protein, GPx, and GR mU/mg protein) of inflammatory cells. Results are means ± SD from 3-9 separate experiments.* [1] *Activities of alveolar macrophages are from a different study.*

2.3. Importance of different AOE pathways in oxidant-related cytotoxicity of inflammatory cells

Cell viability studies showed that few, if any, neutrophils survived for 72 h, whereas monocytes were able to maintain high-energy nucleotides for at least 120 h (V; Figure 5). On the other hand, monocytes and monocyte-derived macrophages were more sensitive to exogenous H_2O_2 than were neutrophils, whereas the respiratory burst induced by FMLP caused significant cell injury in neutrophils, but not in monocytes or monocyte-derived macrophages (V; Figures 6 and 7). Pretreatment of cells with aminotriazole to cause catalase inactivation, or with BCNU to inhibit GR, indicated that alveolar macrophages consumed exogenous H_2O_2 mainly by the catalase-dependent pathway, whereas both the GSH redox cycle and catalase were important in the maintenance of cellular high-energy nucleotides (IV; Figures 3 and 5). In contrast to results of H_2O_2 -exposure, the GSH cycle was more important than catalase in maintaining cellular integrity of alveolar macrophages during the FMLP-induced respiratory burst (IV; Figures 2 and 4).

DISCUSSION

1. Methodological aspects

Information about the molecular regulation of AOEs is contradictory and is mostly based on animal models, and the situation in vivo is even more complicated than in vitro, since inflammation can modulate the cellular responses of various AOEs. Furthermore, since the regulation of AOEs is species- and age-dependent, results from animal studies cannot be extrapolated to humans (Frank 1991). Healthy human tissue is difficult to obtain. In addition, ethical requirements for studies on human cells and tissues limit the feasibility of these kinds of studies. On the other hand, human bronchial epithelial cells change even in primary culture, for instance cilia and surfactant disappear in vitro, and AOE levels decrease within the first days in culture (Kinnula et al. 1992a).

Instead of primary cells, we used transformed human bronchial epithelial (BEAS-2B) and mesothelial (MET-5A) cell lines, which are easy to maintain and are therefore suitable for induction and cytotoxicity studies in vitro. Transformed cells can be used in studies on enzyme regulation, since they express all major AOEs (Kinnula et al. 1994), although not at levels comparable to those of their non-transformed counterparts. In any case, when cell culture methods are used, the extrapolation of results to in vivo conditions must be made with caution. It is also noteworthy that cells are routinely cultured at 21% O_2 concentration, which is normoxia for the airway epithelial cells and alveolar macrophages, but not for mesothelial cells, monocytes, or neutrophils.

The isolation and purification procedures of monocytes and macrophages are based on their ability to adhere to glass or plastic. The advantage of separating monocytes and macrophages by adherence is that the procedure is quick and easy and does not require complicated equipment. However, because adherence is an activation event which can induce both gene expression and protein secretion (Sporn et al. 1990), the induction of MnSOD during monocyte differentiation in vitro may result from this adherence. In vitro-matured macrophages were used in most experiments, since healthy human alveolar macrophages, for ethical reasons, are difficult to obtain. The levels of AOEs in freshly isolated cells are probably similar to their activities in vivo, since previous studies have shown that the activities of these enzymes remain stable during the first hours ex vivo (Kinnula et al. 1992a). Neutrophils may be activated during isolation. Another problem is large interindividual variation even when experimental conditions are exactly similar. However, the isolation and study of these cells are important in understanding their role in the pathogenesis of lung diseases.

The measurements of AOE activities have been well characterized. In our protocol, the activities of CuZnSOD and MnSOD were distinguished by

use of cyanide to inhibit CuZnSOD. This method has limited sensitivity, so in some experiments the activity of MnSOD was undetectable. Catalase activity was assayed by use of an oxygen electrode, which in the presence of excess H_2O_2 measures increased O_2 production by catalase. Compared to this polarographic method, a spectrophotometric method for assay of catalase was insensitive in our laboratory.

Measurement of the depletion of radioactive-labeled adenine nucleotides is a sensitive method for monitoring cell injury. Other indicators of cell damage were also used, including trypan blue exclusion and LDH release. Both of the latter methods measure lytic cell membrane injury. Adenine nucleotide depletion is reversible and occurs much earlier than any cell membrane injury. However, since nucleotide levels can be assessed only in viable cells which have incorporated ^{14}C-adenine, the values for adenine nucleotide depletion overestimate the energy state and the viability of the whole cell population. Adenine nucleotide depletion has been shown to correlate with cell AOE levels, whereas no such relationship exists with lytic cell injury (Aalto et al. 1996). For example, the exposure to asbestos fibers or the combination with TNF-α resulted in increased LDH release, which indicates irreversible cell membrane damage. On the other hand, adenine nucleotide depletion was observed only during simultaneous cytokine and amosite treatment, indicating that these two methods measure different phenomena that do not necessarily correlate with each other. The problem with the LDH-release assay is not only its relative insensitivity, but also the inactivation of this enzyme under severe oxidant stress, although this inactivation was not significant at the concentrations of LDH and H_2O_2 used in this study (data not shown).

2. Superoxide dismutases

2.1. CuZnSOD

The role of SOD in pulmonary defense against oxygen toxicity has, since the 1970's been extensively studied. Some of these studies have measured the activity of total SOD instead of CuZnSOD in particular. Animals exposed to sublethal hyperoxia show resistance to consequent exposure to 100% O_2, and this resistance correlates with increased SOD activity (Crapo and Tierney 1974). Likewise, rats pre-exposed to hypoxia (10% O_2) become tolerant to lethal hyperoxia (Frank 1982), which is associated with increased pulmonary levels of total SOD, catalase, and GPx activities. On the other hand, increased resistance to hyperoxia has also been reported without induction of any AOEs (Baker et al. 1989), which agrees with our results with BEAS-2B cells, in which neither increases in AOE-specific activities nor in glutathione concentration were

necessary for the development of increased oxidant resistance. That CuZnSOD is not usually induced by cytokines (Wong and Goeddel 1988, Visner et al. 1990, Tsan et al. 1992) was shown also in the present study. Because antioxidant enzymes other than CuZnSOD are induced by hyperoxia or cytokines, the increased levels of CuZnSOD may not be responsible for the increased tolerance to oxygen toxicity.

2.2. MnSOD

Some, but not all studies with adult rats have shown that the mRNA and/or the specific activity of MnSOD is upregulated by hyperoxia in lung homogenates and alveolar type II pneumocytes (Freeman et al. 1986, Clerch and Massaro 1993, Ho et al. 1996). However, that immunoreactive protein, enzyme activity, and mRNA level do not necessarily correlate with each other (Jornot and Junod 1992, Clerch and Massaro 1993, Ho et al. 1996) indicates the complexity of the regulatory mechanisms of this and other enzymes and may explain some of the discrepancies found. In experimental animal models, MnSOD mRNA and/or protein are shown to be induced by various cytokines (Tsan 1997), most importantly by TNF-α (Wong and Goeddel 1988). After exposure of rats to hyperoxia the induction of MnSOD in the lungs is maximal at days 3 to 5 (Ho et al. 1996), which is also the time required for the recruitment of activated inflammatory cells into the lungs (Barry and Crapo 1985). Because MnSOD, but not the other AOEs, is upregulated by many cytokines (TNF-α, IL-1, IL-6, IFN-γ) (Wong and Goeddel 1988, Visner et al. 1990, Kinnula et al. 1995), the in vivo data on hyperoxia can at least in part be explained by inflammation, nuclear factor-κB activation, and a subsequent increase in MnSOD transcription (Das et al. 1995). In the present study, with cultured cells, MnSOD mRNA was upregulated after 16 to 24 h of TNF-α treatment. In contrast to studies conducted on human fibroblasts and malignant cell lines (Harris et al. 1991), our results showed no elevation in activity of MnSOD by IFN-γ, whereas the level of activity was significantly elevated by TNF-α, and further elevated by the combination of IFN-γ + TNF-α. No induction was observed in cells cultured in a hyperoxic atmosphere. These results with BEAS-2B cells support the theory that in human airway epithelial cells in vivo, inflammatory cytokines, but not oxygen, are responsible for the regulation of MnSOD.

According to previous studies, asbestos fibers cause MnSOD mRNA upregulation, increased immunocytochemical labeling, and increased specific activity of this enzyme in isolated lung cells and in the intact lung upon inhalation (Mossman et al. 1986, Holley et al. 1992). One recent study also showed a dose-dependent increase in the steady-state mRNA levels of MnSOD in cells exposed to crocidolite asbestos fibers (Janssen et al. 1994b). The specific activity of this enzyme was not investigated in that study, but by Western blot analysis the

enzyme protein was not significantly elevated. Two alternative mechanisms of MnSOD upregulation by fibers have been considered: a direct fiber reaction or a cytokine-mediated effect due to the proinflammatory properties of the fibers (Kamp et al. 1992). The effect of asbestos fibers on MnSOD induction in vivo and in vitro is not necessarily similar, because inflammation may modulate cellular antioxidant response in vivo. In the present study, amosite had no effect on MnSOD-specific activity in MET-5A cells, but because the fibers showed a tendency for MnSOD mRNA upregulation, our results do not exclude the possibility of MnSOD induction during prolonged asbestos exposure. On the other hand, TNF-α and TNF-α + amosite caused a significant upregulation of MnSOD mRNA and specific activity in MET-5A cells when compared to that in unexposed cells. These data suggest that the MnSOD induction by inhaled fibers observed in experimental asbestos models may be due to the proinflammatory potential of the fibers rather than to a direct effect on the target cell.

Although inflammatory cytokines such as TNF-α are important in the upregulation of MnSOD, the significance of MnSOD induction on oxidant resistance is unclear. MnSOD induction by cytokines has been shown to confer resistance to exogenous oxidants in some (Warner et al. 1991, Tsan 1997), but not in all investigations (Marcho et al. 1991). Because hyperoxia results in ROS generation in the mitochondria and endoplasmic reticulum (Freeman et al. 1982), increased MnSOD activity can be expected to protect cells against hyperoxia. In fact, TNF-α and endotoxin have been shown to prolong the survival of experimental animals during hyperoxia exposure (Clerch and Massaro 1993, Tsan 1997), and transgenic and knock-out mice have shown that MnSOD expression contributes to oxygen tolerance (Wispe et al. 1992, Tsan et al. 1998). However, resistance against hyperoxia is not necessarily associated with AOE induction (Baker et al. 1989), a fact which agrees with our findings showing no MnSOD upregulation in hyperoxia-exposed bronchial epithelial cells, despite increased resistance to subsequent oxidant exposure. In addition, cytokine-induced MnSOD offered no protection against endogenously or exogenously generated oxidants. In contrast, increased sensitivity was observed both in BEAS-2B and in MET-5A cells pretreated with cytokines. This finding is in line with the study of Marcho and associates, which has shown enhanced sensitivity to oxidative stress in cells containing high MnSOD activity (Marcho et al. 1991), but it contradicts the finding that elevated MnSOD mRNA expression and activity increase resistance to paraquat toxicity in human pulmonary adenocarcinoma cells (Warner et al. 1991). These contradictory data may be due to differences between transformed and malignant human cell populations, to cell-specific effects of the cytokines, or to the cytokine concentrations used. The protective effect of SOD may also be related to SOD-level, since the ability of small doses of SOD to protect the ischemically injured, reperfused rabbit heart is reversed at higher doses (Nelson et al. 1994). A possible explanation may also be the overproduction of H_2O_2 and subsequent enhanced lipid peroxidation due to an imbalance in cellular

antioxidant mechanisms. In BEAS-2B and MET-5A cells, the effect of TNF-α may in fact contribute to the induction of other mechanisms, for instance those related to cell death (apoptosis). The balance of these reactions is complicated and largely beyond the scope of the present study.

MnSOD may contribute to the protection of macrophages during inflammation. Studies have investigated superoxide dismutases during in vitro differentiation of monocytes and leukemia HL60 cells, but have not systematically compared AOE activities in various inflammatory cell types or investigated their significance. Our results are in agreement with data showing induction of MnSOD during the differentiation of monocytes (Nakagawara et al. 1981, Asayama et al. 1985), but are in contrast with data on human leukemia HL-60 cells, in which MnSOD mRNA remains unchanged and its activity decreases during differentiation into granulocytes or macrophages (Speier and Newburger 1986, Kizaki et al. 1993). Our data on mRNA levels suggest, as the mechanism of increased activity, transcriptional activation of MnSOD. However, previous and present findings on the induction of MnSOD during monocyte differentiation in vitro cannot be extrapolated to the situation in vivo, since these changes may occur due to cell adherence (Sporn et al. 1990). Whether the induction related to differentiation has relevance to the protection of macrophages in vivo remains unclear.

In a number of inflammatory cell types, MnSOD is induced in inflammatory cells by cytokines (Niwa et al. 1996, Tian et al. 1998). This induction of MnSOD may thus play an important role in the resistance of inflammatory cells during inflammation and oxidant stress. Based on one recent study, MnSOD is induced in granulomatous diseases such as sarcoidosis, especially in alveolar macrophages and granulomas (Lakari et al. 1998), but the significance of this induction remains unclear. A previous study showed no induction of MnSOD by TNF-α in human neutrophils (Kettritz et al. 1997), whereas another study showed elevation of MnSOD immunoreactive protein in human neutrophils treated with conditioned medium of human bronchial epithelial cells (Cox et al. 1994). The higher levels of mRNA, but not the activity of MnSOD in neutrophils, may be associated with activation of these cells during cell isolation. Discrepancies between the mRNA levels and activities may be related to differences in mRNA stability or translational efficiency of various cells (Clerch and Massaro 1992, Dirami et al. 1999). Whether MnSOD is induced in pathological conditions and contributes to the survival of these cells during inflammation or infection remains to be investigated.

3. H_2O_2 scavenging mechanisms

3.1. Catalase

In addition to MnSOD, catalase may also play an important role in protecting the bronchial epithelium against oxidants. Although the regulation of SODs by hyperoxia varies, the regulation of H_2O_2-scavenging enzymes is even more contradictory. The changes in catalase have been marginal or insignificant (Rister and Baehner 1976, Ho et al. 1996). The only in vivo study available on humans showed no change in catalase levels after 16 h hyperoxia exposure (Erzurum et al. 1993), which is consistent with the present study, in which catalase was not induced by hyperoxia in BEAS-2B cells. In contrast to cell culture studies in which only MnSOD is induced after cytokine exposure (Wong and Goeddel 1988), the pulmonary activity of catalase in rats treated with cytokines, endotoxin, and/or with hyperoxia has also been found to be induced (Tsan et al. 1990a, Tsan et al. 1991, Clerch and Massaro 1993). In addition, the activity of catalase has been shown to be elevated in macrophages of human smokers and in hamster alveolar macrophages exposed to cigarette smoke (McCusker and Hoidal 1990). In the present study, treatment with the combination of TNF-α + IFN-γ caused a significant decrease in catalase activity. The observations that human bronchial epithelial cells have relatively low MnSOD and catalase levels and activities with no induction by hyperoxia (Erzurum et al. 1993, Kinnula et al. 1994, Yoo et al. 1994) suggest that these characteristics may contribute to the vulnerability of bronchial epithelial cells to toxic environmental effects.

Although cytokine treatment increases MnSOD activity, it may also change the sensitivity of the cells to exogenous oxidants. Because superoxide is rapidly converted to H_2O_2 nonenzymatically or by SODs in intact cells, increased MnSOD activity may therefore lead to overproduction of H_2O_2 and to H_2O_2-related injury. Our results are in agreement with findings on endothelial cells in which TNF-α increases cell susceptibility to H_2O_2 and decreases glutathione concentrations (Marcho et al. 1991). This can be explained by an imbalance between various AOEs. The importance of antioxidant balance is further supported by findings in which the hypersensitivity to oxidants of human CuZnSOD-transfected cells is compensated for by co-transfection with the H_2O_2-scavenging enzymes catalase or GPx (Amstad et al. 1991, Amstad et al. 1994). Furthermore, the tolerance to hyperoxia of transgenic mice overexpressing CuZnSOD is associated with a concomitant increase in GPx activity, and the endotoxin-induced tolerance to oxygen with increased activities of catalase and GPx (Clerch and Massaro 1993). In our experiments, both IFN-γ and TNF-α enhanced cytotoxicity of H_2O_2, which may be a result of decreased catalase activity. On the other hand, this explanation is questionable, because catalase was

reduced only in cells pretreated with the combination of TNF-α and INF-γ. A more likely possibility, therefore, is high MnSOD activity without a corresponding or with only a minimal change in H_2O_2-scavenging systems, leading to an imbalance in antioxidant capacity.

Catalase may play a prominent role in protection of inflammatory cells, especially against severe oxidant stress. Previous studies have shown that freshly isolated rat alveolar type II pneumocytes, which are highly resistant to exogenous oxidants, contain high catalase activity (Kinnula et al. 1992a), whereas more sensitive cells, bovine endothelial and rat mesothelial cells, have low catalase protein and activity (Kinnula et al. 1992b, Kinnula et al. 1992c). Human alveolar macrophages contain relatively high catalase activity (McDonald et al. 1991). The present study revealed that neutrophils contain the highest catalase activity of all phagocytes, and are more resistant to exogenous H_2O_2 than are mononuclear cells. This high activity may play a profound role in proper phagocytosis during the respiratory burst and the oxidant stress of activated neutrophils. The importance of catalase is further supported by the differences in the K_m of catalase and GPx for H_2O_2, since the K_m for catalase is markedly higher than the K_m for GPx (Jones et al. 1981), which suggests that catalase scavenges H_2O_2 efficiently at high concentrations. In fact, the present and previous studies have shown that alveolar macrophages consume exogenous H_2O_2 mainly by the catalase-dependent pathway (McDonald et al. 1991). Although it is also possible that mechanisms other than catalase may explain oxidant resistance of neutrophils, the role of catalase is probably the most important.

Our results show that catalase is responsible for scavenging high concentrations of H_2O_2 in alveolar macrophages. Although H_2O_2 is freely diffusible, the localization of catalase raises the question whether catalase can scavenge H_2O_2 of extracellular origin. Catalase is mainly localized in the peroxisomes of alveolar macrophages (Davies et al. 1979), but is also found in the cytosol of human neutrophils (Ballinger et al. 1994). We discovered that catalase is localized mainly in the microperoxisomes, with some labeling also in the cytoplasm and nuclei of alveolar macrophages. Whether the low concentration of cytoplasmic catalase plays a substantial role in H_2O_2 scavenging in human alveolar macrophages remains unclear. The catalase concentration in the peroxisomes was about 200 times as high as that of the nucleus and the cytoplasm. However, since the nucleus and the cytoplasm are much larger compartments, they may contain, in total, about as much catalase as do the peroxisomes. The fact that catalase-like activity scavenges exogenous H_2O_2 suggests also that there may be ATZ-inhibitable catalase-like activity in the cell membrane. In our study, no catalase protein could be detected in the cell membrane, but this negative finding does not mean that another form of ATZ-inhibitable catalase-like activity is not present on the cell membrane of these cells.

3.2. Glutathione redox cycle

Because the glutathione redox cycle is complementary to catalase in scavenging H_2O_2, it may therefore be responsible for the protection of bronchial epithelium against oxidant stress. In the present study, neither hyperoxia exposure nor cytokine treatment had any effect on levels of GPx, GR or G6PDH. Thus, indicating that other mechanisms seem to be involved in the protection of these cells after prolonged oxygen exposure. Another potent antioxidative factor in human bronchial epithelial cells has been suggested to be glutathione with the enzymes regulating cellular glutathione levels (Cantin et al. 1987, Forman and Skelton 1990). Rat alveolar macrophages are protected against hyperoxia by γ-glutamyl transpeptidase, an enzyme that participates in glutathione transport (Forman and Skelton 1990). That study showed that extracellular glutathione protects cells against hyperoxia, and that this protection can be blocked by an inhibitor of the transport enzyme, suggesting that the glutathione cycle is important during oxidant exposure. Furthermore, previous studies have shown increases in glutathione content, in cystine uptake, and in γ-glutamyl transpeptidase activity in other cell types exposed to hyperoxia (Deneke and Fanburg 1989, Knickelbein et al. 1996). In the present study, glutathione content increased after hyperoxia exposure, which is in line with these findings. However, the hypothesis that increased glutathione, and enzymes related to its synthesis, explains the increased oxidant resistance of hyperoxia-exposed cells was not supported by our findings in BEAS-2B cells, because glutathione depletion by BSO did not potentate the cell injury in hyperoxic cells. H_2O_2 consumption by normoxic and hyperoxic cells was also identical, which further suggests that mechanisms other than induction of AOEs or cellular glutathione explain the resistance observed under these experimental conditions. However, it has to be pointed out that glutathione may play a role in protection against menadione, because this quinone can conjugate with glutathione.

In conclusion, the present data show that neither increases in AOE-specific activities nor glutathione concentrations are necessary for the development of increased oxidant resistance in BEAS-2B cells. The resistance to oxidant stress and possibly also the resistance to hyperoxia may in part be determined by inducible defense mechanisms other than AOEs and glutathione, because the expression of several unidentified transcripts is known to be modulated after nonlethal oxidant exposure (Wiese et al. 1995).

Glutathione metabolism may play an important role both in cell viability and in protection against exogenous oxidants of inflammatory cells. This theory is supported by data showing that phagocytes derived from patients with a genetic deficiency in GSH synthetase or glutathione reductase are rapidly damaged during phagocytosis (Roos et al. 1979, Spielberg et al. 1979), or damaged when the reduction of oxidized glutathione is inhibited (Cohen et al. 1987). One previous study measuring the enzymes of the GSH cycle in

neutrophils and monocytes showed very similar activities of GPx and GR in these cells (Voetman and Roos 1980). In contrast, the present study showed that the mRNA and specific activity of GPx, as well as glutathione levels, were lower in neutrophils than in monocytes. The present study also showed that the deterioration of cellular high-energy nucleotides was most prominent in BSO-pretreated neutrophils, and that BCNU, which inhibits GR, was toxic only to neutrophils. On the other hand, glutathione depletion had no effect on the viability or energy state of oxidant-exposed inflammatory cells. Lower concentrations of GPx and GSH may contribute to the short life-span of neutrophils, since neutrophils lose their viability within 24 h ex vivo, and more rapidly if glutathione is depleted (Curi et al. 1998), as shown also in the present study.

The glutathione redox cycle may play a more important role in protecting cells against physiologically relevant oxidant stress than does catalase. GPx has a low K_m for H_2O_2 in comparison to catalase, which favors the predominant activity of GPx at low, physiologic H_2O_2 concentrations (Jones et al. 1981). Glutathione peroxidase is also distributed throughout the cytoplasm. Furthermore, the glutathione redox cycle is active not only in the disposal of H_2O_2 but also of the long-chain hydroperoxides and lipid peroxides which may play an important role in membrane-related cell cytotoxicity (Andreoli et al. 1992). Exogenous oxidants cause ATP depletion within minutes of exposure, whereas irreversible cell damage is apparent only after many hours (Spragg et al. 1985). Extracellular release of nucleotides reflects not only minimal cell injury but also damage to the cell membrane. In the present study, human alveolar macrophages were protected both by catalase and the glutathione redox cycle against exogenous H_2O_2. Relatively low (100 μM) H_2O_2 concentrations were used, but because H_2O_2 is freely diffusible, even this concentration may result in higher intracellular H_2O_2 levels than those achieved during the transient respiratory burst by FMLP. Nucleotide leakage into the extracellular space was always highest in BCNU-pretreated cells. These results support the fact of the primary importance of the glutathione cycle in the maintenance of the high-energy nucleotide pool and of cell membrane integrity in vivo in human alveolar macrophages.

CONCLUSIONS

The present study explored the regulation and role of different AOEs during oxidative stress in human lung cells. Based on the results presented in this thesis, the main conclusions are:

1) MnSOD is induced by TNF-α, but cytokine-induced MnSOD does not protect bronchial epithelial cells against endogenously or exogenously generated oxidants. In contrast, the cells with the highest MnSOD activity are those most sensitive to subsequent oxidant damage. The balance between various antioxidant enzymes, rather than the induction of one enzyme, is important for protecting cells during oxidant exposure.

2) AOEs are not directly upregulated by high oxygen tension. Increases in the specific activities of AOEs and glutathione concentration are not necessary for the development of increased oxidant resistance in human bronchial epithelial cells.

3) MnSOD is not induced by asbestos fibers alone in human mesothelial cells. Asbestos fibers and TNF treatments render these cells more vulnerable to oxidant-induced cell damage despite elevated MnSOD activity.

4) Human alveolar macrophages scavenge exogenous H_2O_2 mainly by a catalase-dependent pathway, whereas the glutathione redox cycle is responsible for the maintenance of cellular integrity during the respiratory burst.

5) Human inflammatory cells exhibit different antioxidant profiles. Monocytes have higher levels of glutathione and of enzymes of the GSH cycle, whereas in monocytes catalase activity is significantly lower than it is in neutrophils. The high catalase activity of neutrophils may explain their high resistance against exogenous H_2O_2, whereas low glutathione content and GSH-related enzymes may account for the poor survival of human neutrophils.

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