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ALTERED GENE EXPRESSION IN THE HUMAN AIRWAYS DURING OXIDATIVE STRESS

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1 ABSTRACT

The atmospheric accumulation of pollutant gases, such as NO_2 and ozone, has been associated with an increased airways hyper-reactivity within the healthy population. Moreover, those with conditions such as asthma and chronic obstructive pulmonary diseases generally experience an exacerbation of their symptoms upon exposure to these noxious gases. Cells lining the respiratory tract can be considered as the primary target for the potential toxic effects of airborne chemicals, but the molecular mechanisms behind the injury occurring in these cells upon gas inhalation remain to be fully elucidated. It has been hypothesised that the toxicity of these gases relies on their reactivity towards macromolecules in the lining fluid of the lung and the formation of reactive secondary products, which cause a situation of oxidative stress. Cells posses a well-developed defence against reactive species, but when this defence is overwhelmed damage can occur to important cellular components, including DNA and proteins. The major aim of this thesis was to study the effects of oxidative stress on the redox-sensitive machinery of gene expression in human lung cells. Cells are able to sense oxidative damage, as well as changes in the cellular redox status and adapt their gene expression profiles in a manner either promoting protection against the insult, or taking the decision to induce cell death to prevent propagation of damaged genetic templates.

The pulmonary defence against inhaled antigens is based on the activation of immunological events, most of them dependent on cytokine signalling. Analysis of human alveolar macrophages exposed to NO_2 *in vitro* revealed inhibition of transcription and release of some inflammation-modulating cytokines. The inhibition was more pronounced in macrophages from habitual smokers. The effects of ozone on mRNA profiles were studied in human alveolar macrophages exposed to low-concentration of ozone *in vivo*. Large-scale screening of these events resulted in the detection of a discrete number of ozone-responsive genes belonging to disparate biological pathways, including nucleic acids synthesis and repair, inter- and intra-cellular signal transduction, cytoskeletal organisation, inflammation and protein modification. These findings also report on considerable biochemical effects of ozone in the lung at levels close to the safety limits recommended by international air quality guidelines and standards. Exposure of cells to the thiol-oxidising agent diamide *in vitro*, lead to increased mRNA levels and expression of stress responsive-genes and to the oxidation of GSH with the concomitant S-glutathionylation of cellular proteins. In contrast, *in vitro* exposure of cells to hydrogen peroxide failed to induce many of the diamide-responsive genes, significant oxidation of GSH or formation of protein-GSH mixed disulphides. Thus, redox-sensitive alteration of gene expression could be correlated to the oxidation of GSH and the concomitant formation of mixed protein-GSH disulphides. In addition, induced expression of protein and DNA chaperones, including heat shock proteins, by diamide exposure, resulted in cytoprotection against both heath shock and the DNA-damaging pro-oxidant potassium bromate. Exposure of the human A549 lung epithelial cell line to sub-toxic levels of hydrogen peroxide caused sustained arrest in cell cycle progression and activation of apoptotic events, including activation of caspase-3 and the augmentation of TRAIL-dependent caspase-3 activation. These effects may be in consequence of the DNA damage caused by the oxidant. Hydrogen peroxide treatment lead also to the alteration in mRNA profiles for a variety of genes, many of them known to be p53-dependent and other not previously associated with changes in cellular redox states. These alterations could be functionally related to cell cycle arrest, apoptosis and DNA damage.

In summary, airborne pollutants and their secondary oxidant products can damage important components of lung cells and alter their gene expression profiles. The results of these studies contribute to our understanding of the mechanisms of oxidative stress in human lung cells, as well as provide evidence for co-ordination in the regulation of the redox-sensitive machinery of gene expression.

2 LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to by their Roman numerals

I. Dandrea T, Tu B, Blomberg A, Sandström T, Sköld M, Eklund A, Cotgreave I. Differential inhibition of inflammatory cytokine release from cultured alveolar macrophages from smokers and non-smokers by NO_2. *Human and Experimental Toxicology.* **16**: 577-588 (1997)

II. Dandrea T, Björklund C, Blomberg A, Sandström T, Wärngård L, Cotgreave I. The identification of a novel ozone-responsive gene cluster in human alveolar macrophages following low-level *in vivo* exposure on individuals. *Manuscript.*

III. Dandrea T, Bajak E, Wärngård L, Cotgreave I. Protein S-glutathionylation correlates to selective stress gene expression and cytoprotection. *Archives of Biochemistry and Biophysics.* **406**: 241-252 (2002)

IV. Dandrea T, Hellmold H, Jonsson C, Zhivotovsky B, Hofer T, Wärngård L, Cotgreave I. The transcriptosomal response of human A549 lung cells to hydrogen peroxide: Relationship to DNA damage, cell cycle arrest and apoptosis. *Manuscript*

3 CONTENTS

4 LIST OF ABBREVIATIONS

8-oxodG	8-oxo-7,8-dihydro-2'-deoxyguanosine
AC	Ascorbic acid
AM	Alveolar macrophages
APC	Antigen presenting cell
ARE	Antioxidant response element
BAL	Bronchoalveolar lavage
COPD	Chronic obstructive pulmonary diseases
ELF	Epithelial lining fluid
GPX	Glutathione peroxidase
GRO-α	Growth-related oncogene protein-α
GSH	Glutathione (reduced form)
GSSG	Glutathione (oxidised disulphide form)
GST	Glutathione S-transferase
HSE	Heath shock element
HSF-1	Heath shock factor-1
HSP	Heath shock protein
ICAM-1	Intercellular adhesion molecule-1
IL-	Interleukin-
IP3	Inositol 1,4,5-triphosphate
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic chemokin-1
MIP-2	Macrophage inflammatory protein-2
NF-κB	Nuclear factor-κB
NO	Nitric oxide
NO_2	Nitrogen dioxide
NO_x	Nitric oxides
O_3	Ozone
PKC	Protein kinase C
PLCγ	Phospholipase Cγ
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TF	Transcription factor
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
TRAIL	TNF-related apoptosis-inducing ligand
TRX	Thioredoxin
UA	Uric acid
UFA	Unsaturated fatty acid
VCAM-1	Vascular cell adhesion molecule-1
VOC_x	Volatile organic compounds

5 INTRODUCTION

5.1 AIRBORNE ENVIRONMENTAL POLLUTANTS

Inhalation is a common route by which humans are exposed to xenobiotica and therefore, the respiratory tract can be considered as a target organ for the potential toxic effects of airborne chemicals. Many agents which can cause pulmonary injuries have been extensively studied. These include oxygen, smoke, various airborne particles and noxious gases including sulphur oxides, nitrogen oxides, phosgene, and ozone.

Many toxic organic and inorganic gases can cause acute pulmonary injury after inhalation of high concentrations, for example during incidents in industrial settings, whereas others are common pollutants causing low-level environmental exposure to large segments of the human population. Usually airborne oxidant gases are found in urban or industrial areas as components of the inhaled air and insufficient information is available on the potential health risks associated with exposure of humans to these chemical mixtures.

In urban areas where the formation of photochemical smog occurs, exposure to ozone, nitrogen dioxide, organic intermediates, and other reactive compounds is unavoidable. Inhalation of these gases can lead to a variety of pulmonary disorders such as inflammatory diseases, bronchitis, oedema, airway hypersensitivity, pulmonary fibrosis, and, potentially, lung cancers [1,2]

This thesis will be limited to the adverse effects of oxidant gases especially nitrogen dioxide (NO_2), ozone (O_3) and oxidant metabolites formed from them in the lining fluid of the human airways.

Several guidelines and standards exist for ozone and NO_2 in ambient air. Table I lists the most recent air quality guidelines and standards recommended by WHO, the US Environmental Protection Agency (EPA), and the European Union (EU). The EU standards are targets to be reached 2010. The most remarkable difference lies in the annual value for NO_2 since the WHO and EU value is only 40% of the US value.

Table I. WHO, US Environmental Protection Agency (EPA, and European Union (EU) air quality guidelines and standards for ozone and nitrogen dioxide. Modified from [3]

	Maximum concentration allowed when averaged over time		
	1 h	**8 h**	**1 year**
Ozone (μg/m^3)			
WHO		120	
EPA	235	157	
EU		120*	
NO_2 (μg/m^3)			
WHO	200		40
EPA			100
EU	200*		40*

*Proposed 2010

5.1.1 Nitrogen dioxide

Nitrogen dioxide (NO_2) is a dense, relatively insoluble brown gas and the most common of the oxides of nitrogen. NO_2 is a common air pollutant, frequently found in ambient air and produced mainly in industrial settings by arc welding, combustion of nitrogenous material, in the manufacture of nitric acid and in the production of silage [4].

In cigarette smoke, NO_2 is formed during the combustion of organic nitrogen-containing compounds, producing mostly nitric oxide (NO) but also some NO_2. Levels of 50-300 parts per million (ppm) of NO_2 have been reported in undiluted cigarette smoke [5,6].

For Sweden the exposure limits for NO_2 in workrooms are 2 ppm for an eight-hour work shift, with a peak exposure limit of 5 ppm for 15 minutes. The exposure limits for ambient air are 0.06 ppm and 0.04 ppm for 1 and 24 hours, respectively (Swedish Environmental Protection Agency, 1994). Atmospheric concentrations of NO_2 are usually expressed in ppm, but also as milligram per cubic meter (mg/m^3) and the two measurements can be inter-converted by the following formulae: **1 ppm = 1.8 mg/m^3**

NO_2 is a poorly water-soluble gas and dosimetric studies have estimated a relatively evenly distribution in the conducting airways after inhalation with a major deposition in

the terminal bronchioli. Only under conditions of energetic activity high concentrations of NO_2 have been demonstrated to reach the alveoli.

Duration of exposure and gaseous concentration of NO_2 determine the extent of injury to the respiratory tract. The chemical mechanisms of injury are related to its reactivity with proteins and nucleic acids, which results in nitration and functional depletion of cellular sulfhydryl groups [7].

5.1.2 Ozone

Ozone (O_3) is a pale blue gas mainly produced in the troposphere by a series of sunlight-driven reactions and serves as an important protective shield against solar radiation in the atmosphere. Close to the earth's surface, ozone is an unwanted oxidant, and is often regarded as the most toxic air pollutant. Ozone is formed in urban areas as a result of sunlight-driven photochemical reactions involving oxygen, nitric oxides (NO_x) and volatile organic compounds (VOC_x) arising largely from mobile and stationary combustion sources. During the summer-time, ground-level concentrations of ozone may exceed 0.2 ppm in Central Europe and other industrialised countries [8].

Atmospheric concentrations of ozone are usually expressed in part per million (ppm) but also as milligram per cubic meter (mg/m^3) and the two measurements can be inter-converted by the following formulae: **1 ppm = 2 mg/m^3**

Although humans generally are not exposed to acute poisoning by ozone, there is a large potential for chronic low-level exposure through ambient pollution in the environment. There is also increasing evidence of detrimental effects in humans due to exposure to this oxidant gas, particularly with respect to the pathogenesis of asthma. Ozone impairs the normal mechanical function of the human lung, producing symptoms such as chest tightness, cough, wheezing, and lung function decrements [9].

The biological effect of ozone is attributed to its ability to cause oxidation or peroxidation of biomolecules either directly or via free radical mechanisms [1,10].

5.2 THE HUMAN RESPIRATORY TRACT

The lung is an organ uniquely situated within the body, being interposed between the host and its environment. The respiratory tracts, or airways, may be considered as comprising three major regions, the nasopharyngeal, the tracheobronchial and the pulmonary regions. Well over 40 cells types are required to perform the diverse functions of the respiratory tract. The cells of greatest interest are those that are unique

to the respiratory tract, such as ciliated bronchial epithelium, non-ciliated bronchiolar epithelium or Clara cells, type I pneumocytes, type II pneumocytes and alveolar macrophages. In addition, some other cell types are of interest, endothelial cells and interstitial cells (fibroblasts and fibrocytes), which constitute the greatest percentage of lung population.

The lung is vulnerable to acute toxic injury to both its epithelial side, by inhalation, and to its endothelial side, via the circulation. Because these two compartments are only separated by a thin, delicate surface, inhalation toxicity can also encompass endothelial injury. The adverse effects of airborne toxicants and the site of injury in the respiratory tract depend on physical properties of the inhalant, such as chemical reactivity and aqueous solubility. Less soluble gases, such as NO_2 and ozone, are dispersed into the lower airways and injure the terminal bronchioles and adjacent alveoli. In addition to solubility differences among the noxious gases, they possess varying reactivities, which determine the concentrations necessary to cause injury [11].

5.2.1 The epithelium

Airway epithelial cells exist at the interface with the external environment, and are therefore among the first to be exposed to airborne pollutants. Epithelial cells, which differ in type and function at the various levels, line the conducting airways from the trachea to the respiratory bronchioles forming a physical barrier against inhaled pathogens. The bronchiolar epithelium consists primarily of two cell populations, ciliated and non-ciliated cells, supported by a basement membrane. Of the bronchiolar cells, ciliated cells are most vulnerable to injury. After severe injury, these cells lose cilia, die, and slough into the airway lumen. Non-ciliated airway cells have a secretory function, are more resistant to injury, and are the apparent precursor for the ciliated cells. Additionally, cells in the bronchiolar epithelium are capable of synthesising and releasing a large number of inflammatory mediators, including cytokines, eicosanoids, chemokines, and growth factors, all of which are thought to contribute to proliferation, differentiation, activation and chemoattraction of various inflammatory cells in the airway mucosa [12].

The alveolar epithelium consists of type I and type II pneumocytes, which rest on a common basement membrane with a huge surface area to allow for adequate gas exchange. Type I pneumocytes are large and flat and cover more than 95% of the alveolar surface. Type II pneumocytes are smaller cuboidal cells interspersed between type I cells and have a secretory function. Type II pneumocytes cover only 5% of the

alveolar surface but the ratio of type I to type II cells in the alveolus is 2:3 and the estimated cell numbers in the normal human lung are $19x10^9$ and $37x10^9$ respectively [13]. In general, toxic injuries to the epithelium cause type I pneumocytes to swell and slough off the basement membrane. As repair occurs, type II cells proliferate to cover the basement membrane, differentiating into type I cells over several days [14].

5.2.2 The endothelium

As mentioned above, damage to lung cells is not limited to the epithelial surface. Capillary endothelial cells compose approximately 40% of the lung and 30% of the cells in the alveolar region [13]. Micro-vascular damage in the lung produces swelling of the endothelial cells, oedema in the underlying tissue and may result in necrosis. Repair of the endothelium occurs primarily by cell proliferation, which often is accompanied by increases in fibroblasts and extra-cellular matrix in the interstitium. This results in thickened alveolar septa, which can impair pulmonary gas exchange [7].

Endothelial cells, by virtue of their capacity to express adhesion molecules and cytokines, are intricately involved in inflammatory processes [15]. Interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), produced by infiltrating inflammatory cells and/or by epithelial cells, can induce endothelial cells to express several cytokines as well as adhesion molecules. For instance, the intercellular adhesion molecule-1 (ICAM-1) is well known to be upregulated upon inflammation on endothelium and have been shown to be important for transendothelial migration of lymphocytes [16]. Similar to ICAM-1, P-selectin and the vascular cell adhesion molecule (VCAM-1) are also well documented to be induced in endothelial cells during inflammation [17].

5.2.3 Inflammatory cells

In addition to epithelial and endothelial cells, inflammatory cells reside in the lumen and in the interstitium of the respiratory tract in order to protect the lung from inhaled antigens. The major initiators and regulators of the immune functions in the lung are denditric cells, T-cells and macrophages.

The alveolar macrophages (AM) are the representative of the mononuclear phagocytic system, consisting of monocytes in the blood and macrophages in the tissue. Considering the massive colonisation of the lung by macrophages and the abundance of antigens in the pulmonary milieu one would expect a permanent activation of the pulmonary immune system. Alveolar macrophages constitute the major macrophage population present in the bronchoalveolar lavage fluid (BAL). However, the general

observation is that alveolar macrophages are poor antigen-presenting cells (APCs) for priming T-cells. In contrast AMs may be immunosuppressive. It has been reported that alveolar macrophages failed as professional APCs because they do not express CD80 and CD86, which are essential in activating T-cells to proliferate [18]. However, it has been shown that the number of alveolar macrophages was significantly higher in BAL fluid of smokers compared with non-smokers [19]. Macrophages are actively phagocytic cells capable of ingesting and digesting exogenous antigens such as microorganisms, insoluble particles, injured and dead host cells and cellular debris. Macrophages are normally in a resting state, but in the course of an immune response, a variety of stimuli can activate macrophages. An excessive inflammatory response may lead to the release of high levels of hydrogen peroxide, through respiratory burst activity, which causes cell swelling and cell lysis of the neighbouring cells with further release of cytosolic compounds, leading to widespread necrotic cell death. Activated macrophages not only produce a number of antimicrobial mediators, including reactive oxygen intermediates, reactive nitrogen intermediates and hydrolytic enzymes, but also secrete various inflammatory factors, such as cytokines and complement proteins which can activate or affect other cells [20].

In recent years, significant progress has been made in our understanding of dendritic cell function. In the lung, denditric cells form an inter-digitating, intra-epithelial network in the epithelium, reminiscent of the typical distribution of Langerhans' cells in the skin. They are more concentrated at sites of inflammation and are also present in the peribronchiolar connective tissue. Their representation fluctuates in the BAL fluid of normal subjects. However, in condition of local bacterial infection, their frequency in the BAL fluid increases significantly [21]. Recently, it has become evident that dendritic cells are the most potent APCs, that they are highly specialised and that they have the ability to stimulate naïve lymphocytes. They are capable of initiating cytotoxic T-cell responses and cytokines production in T-cells, as well as T-dependent B-cell responses. Critical to their function is the surface expression of co-stimulatory molecules for T-cells, such as CD40, CD80, and CD86. It is likely that in the lung these cells are the critical initiators and regulators of T cell-mediated immunity [22]. In spite of recent progress in understanding the immunostimulatory function of denditric cells and the potency of these cells as APCs, little is known about their role in pulmonary immunity. It is not yet clear whether antigen presentation by lung denditric cells is likely to preferentially generate either Th_1- or Th_2-type responses.

5.2.4 The epithelial lining fluid

The liquid layer lining the alveolar epithelium is composed of surfactant, which contains a number of surface-active compounds, primarily phospholipids. Surfactant, which is secreted into the alveolar space by the type II alveolar epithelial cells, lowers the work of breathing by lowering surface tension, thereby stabilising alveoli and preventing them from collapsing. The epithelial lining fluid (ELF) serves also as a protection for the underlying epithelium, indeed the ELF has been shown to contain high concentrations of reactive antioxidants, including glutathione (GSH), ascorbic acid (AA), uric acid (UA) and α-tocopherol. It has been suggested that the antioxidants found in the ELF function as the first line of defence and may protect the airways from oxidant injury induced by exposure to oxidant air pollutants, such as NO_2 and ozone [23]. The antioxidants act as sacrificial substrates scavenging oxidants penetrating the airways and, thereby, preventing oxidation of macromolecules such as lipids, proteins and carbohydrates. Increased concentrations of GSH in the human BAL fluid have been reported in a number of conditions where oxidative stress is thought to be involved, including cigarette smoking, adult respiratory distress syndrome and asthma.

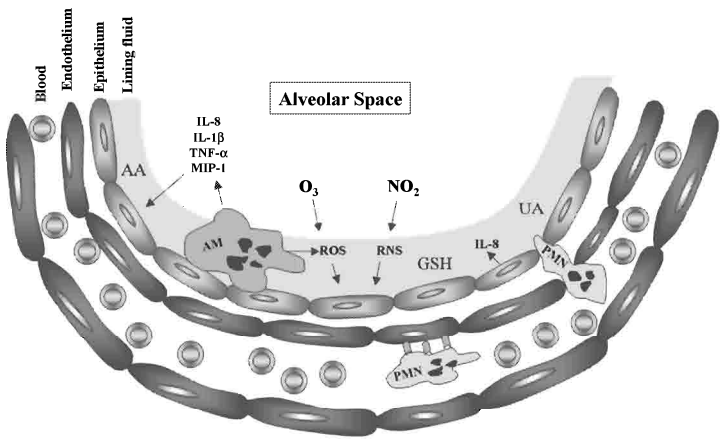

Figure 1. The Pulmonary Alveolar Space

Studies of BAL fluid from humans exposed to NO_2 or ozone have shown that uric acid and ascorbate are consumed in concentration- and time-dependent manners, suggesting that these compounds are important components of the protective antioxidant capacity

of the ELF. In contrast to the water-soluble antioxidant ascorbic acid and uric acid, the lipophilic α-tocopherol is believed to act as a chain-breaking antioxidant by scavenging peroxyl radicals and thus preventing the propagation of lipid peroxidation processes [24].

5.2.5 Pulmonary Inflammation/Hypersensitivity

The prevalence of allergic diseases has increased dramatically during the past few decades, both in industrialised as well as industrialising countries. This increase has occurred despite a decrease in the severity of grass pollen season. Similarly, the incidence of asthma has also gradually increased over the past four decades and these effects are suggested to be caused by environmental changes. Epidemiological studies have clearly shown a consistent and significant association between ambient levels of various air pollutants and an increasing incidence of allergic and/or inflammatory airway diseases, such as asthma and allergic rhinitis [12]

Although inflammation in the respiratory tract can be evoked by a diversity of agents and conditions, there are general mechanisms that contribute to the recruitment of inflammatory cells into the lung. In this respect, inflammation is regulated by lipid and protein mediators, which can be expressed proximal and distal to the site of the eliciting agent. Usually, inflammation is a protective response, serving to eliminate or isolate injurious agents and facilitate the repair and regeneration of damaged tissue. However, if the inflammatory response is excessive in its magnitude or persistence, it can per se become the cause of tissue damage. Cytokines are one class of molecules that regulate inflammation and, as such, are important factors influencing the response of the lung to noxious agents. Leukocyte recruitment into the lumen of the airways requires communication among infiltrating inflammatory cells and the endothelium. The secretion of early response or initiating cytokines, which are able to stimulate expression of cell-surface adhesion molecules, in the endothelium, and the production of chemotactic cytokines, mediates these events. The two most important cytokines acting as initiator of the inflammatory cascade are tumor necrosis factor α (TNF-α) and interleukin 1 (IL-1). In the context of the lung, there are several studies indicating that it is TNF-α that plays the predominant role in initiating inflammatory events upon inhalation of pneumotoxic materials. Although many cell types can express TNF-α, it appears that mononuclear phagocytes, and in particular alveolar macrophages, are the main sources of this cytokine in the lung [25,26].

Recruitment cytokines, including the chemokine TGF-β and IL-1, posses chemotactic activity for leukocytes and play a role in inflammatory cell recruitment and activation. Several studies have characterised the expression of chemokines in lung tissue and cells after exposure to pneumotoxic agents, demonstrating increased expression of these proteins in BAL fluid under exposure conditions leading to pulmonary inflammation. For example, exposure of humans to zinc oxide fumes or ozone results in increased BAL fluid levels of the chemokine IL-8, in association with neutrophil infiltration [27,28]. Evidence that the association between chemokines and lung inflammation represent a "cause-and-effect" relationship has been demonstrated in studies characterising the time-courses of chemokine expression and inflammatory cell recruitment. Zhao et al. [29] exposed mice acutely to ozone and analysed both the expression of the monocyte chemotactic chemokine (MCP-1) and the macrophage inflammatory protein-2 (MIP-2), concurrent with the changes in numbers and types of inflammatory cells in the BAL fluid. The temporal pattern of ozone-induced increases in MIP-2 and MCP-1 and in the respective infiltration of neutrophils and macrophages into the lung, clearly support a role for these chemokines in mediating inflammatory cell recruitment. The degree and persistence of inflammation in the lung and in other tissues is influenced by the balance between pro-inflammatory factors and other processes that serve to down-regulate inflammation. Several cytokines, such as TGF-α, IL-1RA, IL-4, IL-10, and IL-6 exhibit anti-inflammatory properties. For example, treatment of animals with TGF-α, IL-6 or IL-1RA attenuates the pulmonary inflammatory response caused by endotoxin exposure, an effect at least in part mediated by the attenuation of TNF-α or IL-1 expression [30,31].

An important concept in cytokine-mediated inflammatory events is that they result from a network of many different cytokines and cell-cell interactions influencing each other at different stages of the inflammation cascade. There is also increasing evidence that expression of several pro-inflammatory cytokines genes is regulated by mechanisms involving the generation of reactive oxygen species [32]. There is also a common consensus that auto-immune diseases may arise from a combination of genetic and environmental triggers and that free radical generation appears to play an important role in perpetuating the chronicity of inflammation and in producing potential auto-antigens, through biomolecule denaturation.

However, a full description of the broad orchestration of inflammatory events, from the initiation of the inflammatory response, recruitment of inflammatory cells, and down-

regulation of inflammation with the participation of a great numbers of mediators is out of the scope of this thesis. The mechanisms of inflammatory events in the lung of humans will be discussed only in relation to the effects of exposure to the airborne oxidant NO_2 and ozone, and in correlation to the development of oxidative stress within lung cells.

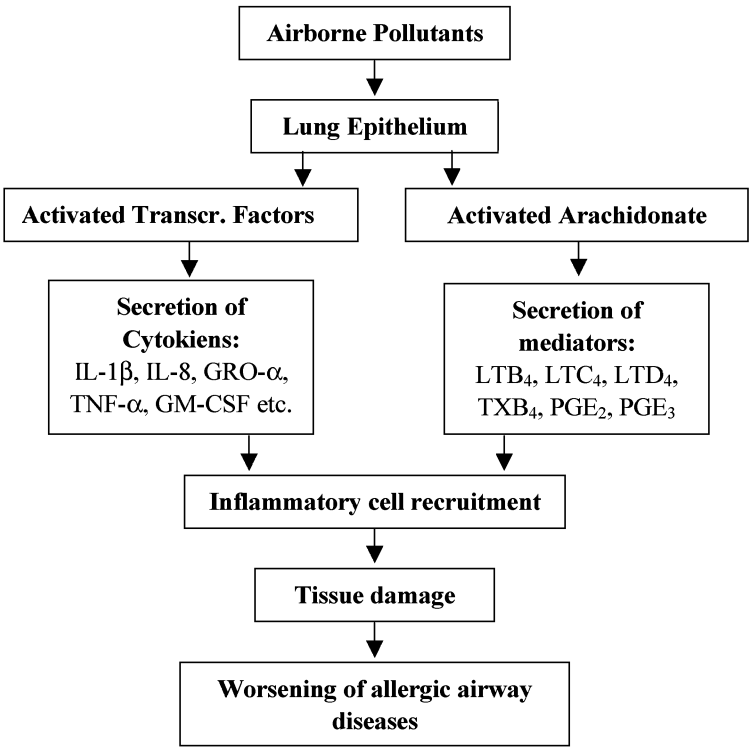

Figure 2. Role of airway epithelium in modulating allergic diseases (Modified From [12])

5.2.6 Effects of Nitrogen dioxide on lung cells

A discrete number of studies have been performed to investigate the mechanisms of the NO_2-induced inflammation in humans. A dose-dependent increase in mast cells and lymphocytes in BAL fluid after exposure to 2.25-5.5 ppm of NO_2 has been reported [33]. However, this effect was not observed at concentrations lower than 2 ppm [34]. After exposure to 2 ppm of NO_2 for 4 hours an increased concentration of the inflammatory cytokine IL-8 was detected in bronchial wash early after exposure with a subsequent recruitment of neutrophils 6 hour after the exposure. It was suggested that the neutrophil-response may occur due to increase in IL-8 secretion, but the mechanisms

behind the increase in IL-8 and its cellular origin are still unclear [35]. The kinetics of the NO_2-dependent antioxidant reactions in the lung have been examined in subjects exposed to 2 ppm for 4 hours. In both bronchial wash and BAL, decreased concentrations of ascorbate and uric acid were detected, but the levels returned to normal 6 hours after exposure. In contrast, GSH levels increased in the bronchial wash and returned to control levels after 24 hours. This data suggest that antioxidants present in the ELF react with radicals formed upon NO_2 exposure and it is plausible that oxidative stress may be involved [36]. In the environment, humans are usually exposed to this gas at low concentrations and on several occasions. Studies on the effects of repeated exposure to NO_2 reveal significant decrements in forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) after the first exposure to 2 ppm of NO_2, but the effect was attenuated with repeated exposure. Moreover, the exposure resulted in a decreased number of neutrophils in the bronchial epithelium and signs of neutrophilic airway inflammation were found in the bronchial wash. This fact indicates that migration and activation of neutrophils occurs in the airways due to NO_2 exposure in humans [37].

5.2.7 Effects of ozone on lung cells

Ozone is another air pollutant that has been linked to negative effects on the human respiratory system. Epidemiological studies on humans, investigating the role of ozone as an inducer of lung dysfunctions, have demonstrated decreased lung function and increased airway hyper-reactivity in 20% of the healthy population. Moreover, those with conditions such as asthma and chronic obstructive pulmonary diseases (COPD) generally experience an exacerbation of their symptoms upon ozone exposure. Together, these observations suggest that there is a large interindividual variation in susceptibility to this oxidant gas among the exposed population [38].

Rather high concentrations of ozone (i.e. 0.4 and 0.6 ppm for two hours) induce a pronounced inflammatory response in the human airways, characterised by increases in neutrophils, proteins, albumin, and prostaglandin-E_2 in the BAL fluid [39]. Other studies evaluating exposure to ozone at levels similar to ambient air concentrations encountered in many European countries (i.e. 0.08 and 0.1 ppm) demonstrate that these low concentrations are sufficient to initiate an inflammatory response [40]. Data from several studies show that exposure to ozone, at ambient air concentrations, induces a neutrophil-dominated airways inflammation, detectable in both endobronchial biopsies and in airway lavage fluid. The neutrophil influx was preceded by an upregulation of

the expression of the adhesion molecules P-selectin and ICAM-1 in the vascular endothelium, which is necessary for the recruitment of inflammatory cells into the lung. Furthermore, increases in cytokines IL-8 and growth-related oncogene protein-α (GRO-α) have also been reported. In summary, all these effects could be of critical importance in the development of airway inflammation after ozone exposure [24].

Ozone, although not a free radical itself, is a highly reactive molecule that can react primarily with unsaturated fatty acids and amino acids. Reaction with naturally-occurring reducing agents in the ELF, such as GSH, ascorbic acid, vitamin E, and uric acid, can also directly scavenge the ozone molecules and prevent damage to other cellular components. Indeed, increased levels of GSH and oxidised GSH (GSSG), and decreased levels of ascorbate has been detected in the ELF of subjects exposed to 0.2 ppm of ozone [41].

Ozonation of unsaturated fatty acid, which are present in the ELF and in cell membranes, produces aldehydes, hydrogen peroxide, and other ozonides. It has been hypothesised that the ozone molecules are unlikely to penetrate the liquid lining of the respiratory tract, suggesting that the reaction products and their metabolites or the release of ROS from activated inflammatory cells may exert many of the negative effects in the lung [38].

5.3 OXIDATIVE STRESS

The toxicity of a variety of xenobiotica appears to be related to their ability to induce intracellular "oxidative stress", characterised by increased free radical production and/or stimulation of cellular oxidant responses. The molecular mechanisms of acute and/or chronic lung toxicity resulting from many toxic agents are poorly understood, but a common general mechanism usually involves oxidative stress. Toxic gases, such as nitrogen dioxide and ozone, are capable of forming reactive oxygen species directly and/or cause an inflammatory response, which may amplify the oxidant injury.

The term "oxidative stress", in general, has been defined as a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to a potential damage of biological molecules [6]. In biological systems, oxidative stress can arise from the generation of free radicals which are formed either by normal biological processes, such as oxygen-linked mitochondrial electron transport, the action of lipooxygenase and cyclooxygenase in eicosanoid metabolism, the response of inflammatory cells against invading micro-organisms, and the production of certain regulatory chemicals

(e.g. nitric oxide). However, these processes do not usually produce free radicals in such amounts to create a situation of severe oxidative stress. In contrast, exposure to ionising radiation (IR) [42], exposure/metabolism of exogenous compounds, or pathological metabolic disorders [43] can alter the redox balance in cells so that the cellular defence mechanisms are overwhelmed and oxidative damage of essential cellular components can occur. If the insult is either sustained or of sufficient intensity to acutely overcome cellular defences, damage to macromolecules can accumulate, leading to loss of cell function, membrane damage and, ultimately, to cell death.

Oxidative stress can result in severe cellular dysfunction due to peroxidation of membrane lipids, protein modification, depletion of nicotinamide nucleotides, rises in intracellular free Ca^{2+} ions, cytoskeletal disruption and DNA damage, in form of single-stranded breaks, double-stranded breaks, chromosomal aberrations or base modifications [44]

All the above mentioned effects can result in alterations of the expression of specific redox-responsive genes, due to the ability of biological systems to sense and adapt to changes in the surrounding redox-state. Hence gene expression is regulated by oxidant and/or antioxidant responses, but the mechanisms by which the transcription of specific eukaryotic genes is modulated by intracellular redox-state are very complex and still poorly understood [32]. The phenomenon of altered gene expression due to changes in the redox-status of the cell is one of the most important aspects of this thesis and will be discussed in more detail later.

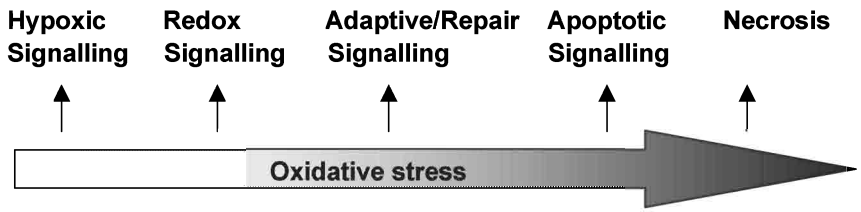

Figure 3. Oxidative stress and biological responses

5.3.1 Oxidants, radicals and reactive oxygen species

As explained above, oxidative stress is a situation of imbalance between oxidants and antioxidants. By definition, an oxidant is the electron acceptor in a redox reaction. A

radical is an atom or group of atoms with one or more unpaired electrons and may have positive, negative or zero charge [45].

Table II. Reactive nitrogen- and oxygen species including radical and non-radical compounds

Name	Abbr.	Comments
Superoxide radical anion	$O_2^{\bullet-}$	One-electron reduction state, formed in many autoxidation reactions, relatively stable, dismutates to H_2O_2 by SOD-catalysed reaction, reductant of transition metal ions.
Hydrogen peroxide	H_2O_2	Two-electron reduction state, formed either from $O_2^{\bullet-}$ by dismutation or directly from O_2, source of $HO^{\bullet}$ in the presence of transition metals.
Hydroxyl radical	$HO^{\bullet}$	Three-electron reduction state, formed either by metal ion-catalysed Fenton reaction or from $NO^{\bullet}$, highly reactive, extremely short half-life but capable of causing great damage within a small radius of its site of production.
Hydroperoxyl	$HO^{\bullet}_2$	Protonated and more lipid-soluble form of $O_2^{\bullet-}$, more powerful oxidant, but present in low concentrations at physiological pH.
Peroxy radical	$ROO^{\bullet}$	Formed from organic (e.g. lipid) hydroperoxide, ROOH, by hydrogen extraction.
Alkoxyl Radical	$RO^{\bullet}$	Highly reactive, readily propagate lipid peroxidation by abstraction of hydrogen from unsaturated lipids.
Ozone	O_3	Naturally occurring oxidant gas, formed as a result of sunlight-driven photochemical reactions.
Singlet oxygen	$^1O_2$	High-energy state of O_2, generated *in vivo* by light in the presence of a suitable sensitiser (chlorophyll, porphyrins) or during lipid peroxidation, very reactive.
Hypochlorous acid	HOCl	Formed by myeloperoxidase in the presence of H_2O_2 and Cl^- or I^-. Highly reactive, being able to oxidise many biological molecules.
Nitric oxide	$NO^{\bullet}$	Produced by vascular endothelium and other cells in the body from the amino acid L-arginine. Endogenous $NO^{\bullet}$ is also called endothelium derived-relaxing factor (EDRF), reacts with $O_2^{\bullet-}$ to give $ONOO^-$.
Nitrogen dioxide	NO_2	Brown, poorly water-soluble gas, formed during combustion processes. Nitrogen-centred powerful oxidising reactive radical.
Peroxynitrite	$ONOO^-$	Powerful oxidant, which damages many biological molecules, can decompose to release $HO^{\bullet}$ independent of metal catalysis.

5.3.2 Reactive nitrogen species (RNS)

As already mentioned, NO_2 is one of the most common air pollutant in industrialised countries and its toxicity is related to the reaction with proteins and nucleic acids, which results in nitration and functional depletion of sulfhydryl groups.

Nitric oxide (NO) is formed endogenously from the oxidation of L-arginine to L-citrulline by a family of NADPH-dependent enzymes, the NO synthases. NO exists in different chemical forms (NO^-, $NO^\bullet$ and NO^+) and, thus, has a wide-ranging degree of chemical reactivity and functions in a variety of different biological roles [46].

$NO^\bullet$ is a free radical and produced by the vascular endothelium. Endogenous $NO^\bullet$ is also called endothelium derived-relaxing factor (EDRF), which is an important mediator of vascular responses as well as a regulator of cell function and tissue viability [47]. The toxicity of $NO^\bullet$ involves its reaction with $O_2^{\bullet -}$ to yield $ONOO^-$, (peroxynitrite) inducing DNA damage [48].

5.3.3 Reactive oxygen species (ROS)

The ROS that cause oxidative damage may be divided into two categories: Radicals and non-radicals ROS. [49]. It is important to note that when radicals reacts with non-radicals in a redox reaction, the result is a new radical, which may result in a chain reaction of radical formation. Common oxygen radicals include $HO^\bullet$ (hydroxyl radical) and $O_2^{\bullet -}$ (superoxide radical anion). Non-radical ROS include molecules such as H_2O_2 (hydrogen peroxide) and O_3 (ozone). Each of this ROS are capable of damaging cellular molecules, including DNA, either by direct action or through reactions with other cellular constituents to produce new ROS.

5.3.4 Hydrogen peroxide/ hydroxyl radical

Hydrogen peroxide is produced by a variety of intracellular reactions, particularly oxidative electron transport in the mitochondria, and is normally present in cells at a concentration of roughly 1.0×10^{-8} M [50]. The peroxide appears to play a role in normal metabolism and is required for a number of cellular events such as thyroid hormone biosynthesis and the anti-microbial activity of macrophages [32]. Hydrogen peroxide can be formed *in vivo* when $O_2^{\bullet -}$ dismutates and, also, by the action of many oxidase enzymes, including monoamine and amino acid oxidases [51]. Xantine oxidase converts hypoxantine to xantine and xantine to urate by simultaneously reducing O_2 to both $O_2^{\bullet -}$

and H_2O_2 [52]. By itself, H_2O_2 is relatively nonreactive toward nucleic acids. Most of the H_2O_2-mediated DNA damage is due to the production of $HO^{\bullet}$ via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\bullet}$) [53].

The hydroxyl radical $HO^{\bullet}$ is a highly reactive oxygen-centred radical with an estimated life in cells of only 10^{-9} seconds. $HO^{\bullet}$ attacks all proteins, DNA, polyunsaturated fatty acids in membranes and many other biological molecules. When $HO^{\bullet}$ reacts with a molecule the result is the formation of another radical species with a lower reactivity than the $HO^{\bullet}$ itself.

The formation of aldehydes and H_2O_2 has been shown to occur by the reaction of ozone with unsaturated fatty acids (UFA) *in vitro*. Since the lining fluids of the respiratory tract contains rather high concentrations of UFA, in an aqueous solutions ozonation would be expected to produce aldehydes and H_2O_2 even in this environment. These radicals are suggested to be responsible for initiating lipid peroxidation of polyunsaturated fatty acid, both in the ELF and in cell membranes. Thus, aldehydes, H_2O_2, and directly produced organic radicals are suggested to be mediators of ozone-induced injury in the lung [54].

Low levels of H_2O_2 have been shown to increase rates of DNA replication and cell division. An important point to consider here is that these effects are only observed at very low concentrations of peroxides, and the dose-response curve is typically biphasic with inhibition of cell growth and increased cell death occurring at high concentrations [55,56].

Hydrogen peroxide is also able to regulate gene expression by displacing an inhibitory subunit from the cytoplasmic gene transcription factor NF-κB at least in some cell types. The activated factor then migrates into the nucleus and causes expression of multiple gene families [57]. Expression of the subunits of AP-1, c-fos and c-jun is increased in rat epithelial cells due to H_2O_2 exposure [58]. Futhermore, bcl-2 and mdm-2 have been shown to be induced in the human bronchial epithelial cell line HBE1 by H_2O_2 treatment [59].

5.3.5 Superoxide radical anion

The superoxide radical anion ($O_2^{\bullet-}$) is an oxygen-centred radical with selective reactivity. This species is produced by a number of enzyme systems, by autoxidation reactions and by non-enzymatic electron transfer that univalently reduce molecular oxygen. In aqueous solutions $O_2^{\bullet-}$ can oxidise ascorbic acid [60].

Immune-reactive cells, especially phagocytes, such as macrophages, neutrophils, eosinophils, and monocytes can all generate large amounts of $O_2^{\bullet -}$ as part of their killing mechanisms directed against microorganisms. The enzyme responsible for the formation of $O_2^{\bullet -}$ is the membrane-bound NADPH oxidase, which becomes activated when immune-reactive cells encounter foreign particles. However, sustained activation of phagocytes leads to excessive production of $O_2^{\bullet -}$ and can cause oxidative damage to the surrounding tissue.

5.4 BIOLOGICAL REACTIVITY OF RADICALS

5.4.1 Initiation and propagation of oxidative damage

Reactions of superoxide, nitric oxide, and metals induce oxidative injury by forming secondary oxidants that are believed to be responsible for causing most of the biological oxidative damage. These reactions and some of their intermediates are key control points in the initiation and propagation of oxidative damage. Superoxide itself is a relatively weak oxidant, and most of its "oxidant" properties are attributed to more reactive oxidants formed during its decomposition. Enzyme-catalysed and non-enzymatic dismutation yield hydrogen peroxide, a non-radical that may freely diffuse across membrane and, via Fenton reaction, forms hydroxyl radicals, which are the most reactive radicals known in biological systems. Superoxide may also react with nitric oxide to form the highly reactive non-radical peroxynitrite [61]. Transition metal ions may also enhance oxidative damage by catalysing the cleavage of hydrogen peroxide and alkyl hydroperoxides to hydroxyl and alkoxyl radicals, respectively. Moreover, metal-catalysed cleavage of lipid hydroperoxides is probably the major cause for most lipid peroxidation studied *in vitro* [62].

The radical species formed during the reactions described above react with lipids, proteins and DNA or other biomolecules either by addition, hydrogen abstraction, or electron transfer to form carbon-centred radicals. In aerobic environments, these carbon-centred radicals reversibly add oxygen to form peroxyl radicals, which then react with adjacent biomolecules. This process is termed propagation and is normally considered in terms of lipid peroxidation, which can contribute to spread the oxidative damage to proteins and DNA. A free-radical chain reaction initiated by a single hydroxyl radical in a lipid membrane thus may lead to over 20 propagation cycles before the chain is terminated by particular scavenger molecules [63]

5.4.2 Lipid peroxidation

Lipid peroxidation, a marker of cellular oxidative stress, has long been recognised as a potential contributor to the oxidative damage caused by xenobiotic compounds, as well as inflammatory processes, ischemia/reperfusion injury and chronic diseases, such as atherosclerosis, and cancer [64]. The free radical species, either formed in cells by exogenous agents, or produced endogenously, can readily attack unsaturated lipids. As described in previous reviews, lipid peroxidation is evoked through one-electron reductions, where an initiating radical species combines with an unsaturated lipid and in turn with oxygen to form a lipid peroxyl radical [65].

$$LOOH \rightarrow LO^{\bullet}$$

$$LO^{\bullet} + LH \rightarrow LOH + L^{\bullet}$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$$

Reactions with lipids are unique among the cell constituents since the formation of lipid peroxidation products leads to the propagation of free radical reactions. The lipid peroxidation products contribute to cell injury by reacting with cell components similarly to the primary and initiating oxidants and free radical species. Cell damage and death is manifested through disruption of membrane structures, which are rich in lipids, and lead to the alteration or inactivation of membrane-associated enzymes and proteins [66,67]

Although a number of compounds initiate lipid peroxidation through direct production of radicals or other ROS, further peroxidation may take place by indirect processes involving enzyme activation, where lipid peroxidation products serve as substrates. Among the best studied examples are lipoxygenases, which are activated in response to disrupted membrane structure or composition and utilise polyunsaturated fatty acids from oxidised phospholipids as co-substrates for the metabolic activation of xenobiotica. [68,69].

5.4.3 Protein modification

Another important target for the action of radicals are proteins, although, until relatively recently, such reactions received little attention in comparison with radical-mediated lipid and DNA oxidation. The ROS commonly implicated in protein

modification include a number of free radical species, such as hydroxyl radical ($HO^{\bullet}$), superoxide radical ($O_2^{\bullet -}$), thiyl radical ($RS^{\bullet}$) and nitric oxide radical ($NO^{\bullet}$), as well as several non-radical species, such as hydrogen peroxide, alkyl peroxides, ozone, singlet oxygen ($^1O_2$), peroxynitrite ($ONOO^-$) and hypochlorous acid (HOCl) (**Table II**). A wide range of oxidation products may be formed as a result of radical attack on proteins. These products include a range of modified and degraded amino acids, which can contain new functional entities such as hydroxyl- and carbonyl groups. These alterations may result in secondary effects including protein fragmentation, cross-linking and unfolding, which is associated to alteration or loss of protein activity and function [70]. Protein modification by oxidants and the resulting changes in protein functionality is a complex matter and only briefly touched in this thesis, giving some specific examples of protein modification in general.

All amino acid residues are potentially targets for attack by $HO^{\bullet}$, which is the most reactive and, hence, the least selective of the radicals [71]. However, protein modification by different forms of ROS exhibits a high degree of specificity.

Reaction of cysteine with radicals such as $HO^{\bullet}$ occurs extremely rapidly and preferentially at the sulphur centre, by hydrogen abstraction, to yield a thiyl radical ($RS^{\bullet}$) [72]. Cysteine residues of proteins are particularly sensitive to intra- or inter-protein disulphide cross-linked derivatives, but also to the formation of mixed disulphide adducts with glutathione. In some cases oxidation to higher oxidation states, such as to sulfinic, sulfenic, and sulfonic acid derivatives, may occur [73]. Radicals also very readily damage the thioether side-chain of methionine and current evidence suggests that this reaction proceeds mainly via an initial reaction at the sulphur centre. Most forms of ROS are able to convert methionine residues of proteins to methionine sulfoxide (MeSOX) residues [74]. Significantly, the oxidation of cysteine residues to disulphides and the oxidation of methionine residues to MeSOX residues are the only ROS-mediated modifications of proteins that can be reversed. However, some evidence also suggests that protein cysteinyl sulfinic acids may also be reduced under physiological conditions. The regeneration of methionine and cysteine is mediated by the action of NADPH-dependent dehydrogenases (MeSOX + NADPH + H^+ → Methionine + H_2O + NAD^+). In the case of protein disulphides (Pr-SS-Pr), reversal is also catalysed by glutaredoxin(s) in a glutathione-dependent reaction (Pr-SS-Pr + 2 GSH → 2Pr-SH + GSSG).

The aromatic amino acids and histidine are preferred targets of most ROS. Tryptophan residues are particularly sensitive to oxidation. When proteins are exposed to ionising radiation or high concentration of hydrogen peroxide and copper, the tyrosine residues are converted to 3,4-dihydroxyphenylalanine and tyrosine-tyrosine cross-linkage may be formed [75,76].

It is also well known that protein backbones can be selectively cleaved as a result of radical attack by $HO^{\bullet}$ and a variety of other radicals such as peroxyl species. A number of different mechanisms have been proposed for the occurrence of polypeptides breaks that may lead to protein denaturation and have been reviewed by Garrison et al 1987 [73].

Various cross-linking reactions can be initiated upon oxidative modification of amino acid side chains, which alter configuration of proteins and protein complexes. Hydroxyl radicals readily oxidise cysteine residues to the corresponding thiyl radicals. In the absence of oxygen, two of these radicals can dimerise (comproportionation) to form S-S bridges. Tyrosine is another amino acid prone to undergo cross-linking, which occurs when a pair of tyrosine phenoxyl radicals dimerises. Theoretically, covalent cross-links can involve interactions of carbon-centred radicals of any two identical or non-identical amino acid residues in the same or in two different protein molecules, but such cross-links are very difficult to characterise and are not yet fully defined [71,73].

5.4.4 DNA damage

The causal relationship between oxidative DNA modifications and diseases, cancer and aging is postulated, but awaits full, direct corroboration. However, there is convincing indirect evidence that oxidative damage to DNA causes or contributes to inflammation, neurodegenerative diseases, apoptosis, cancer and aging. For example, oxidative mutagenesis contributes to cancer initiation, promotion and progression [77], and overexpression of antioxidative enzyme results in decreased DNA damage and increased longevity [78]. The threat that oxidants impose on DNA is also supported by the fact that all nucleated cells posses a sophisticated repair machinery for oxidative DNA modifications in the nucleus, and that defects in repair enzymes are a major risk factor for the survival of the cells [79].

ROS and RNS can cause base modifications (oxidation and deamination), base losses, (apurinic, apyrimidinic sites), single- and double-strand breaks, and cross-links in DNA. Around 100 oxidative DNA modifications have been identified and the mechanisms of reaction between ROS and DNA have been described in several

reviews [80,81], whereas reactions between RNS and DNA have not been elucidated to the same extent.

As could be expected from the different reactivities of ROS species, the pattern of DNA modifications depends on the nature of the ROS under consideration. Thus, $O_2^{\bullet -}$ and H_2O_2 do not readily react with DNA but are important reactants in the formation of $HO^{\bullet}$, which can react with all the four bases and generate a large number of products, among them 8-oxodG (8-oxo-7,8-dihydro-2'-deoxyguanosine). This modified base is also formed by the selective reaction of $^1O_2$ with DNA. Hydroxyl radicals react mainly with DNA bases but also, to a lesser extent, with the sugar-phosphate backbone causing cleavage of the DNA strand. The most frequently base modification, caused by several different oxidants, is probably 8-oxodG, which can lead to G:C →T:A transversion during replication. 8-oxodG can be removed from DNA by cellular repair enzymes and may be detected in urine where they serve as a convenient in vivo bio-markers of oxidative DNA damage. Other modified bases produced by $HO^{\bullet}$ attack on DNA are 8-hydroxyadenine, 2-hydroxyadenine, and formamidopyrimidines (Fapy lesions). Additional examples of oxidative base modifications are thymine glycols, 5-hydroxymethyluracil, and 5,6-dihydroxycytosines.

Apurinic/apyrimidinic sites (AP sites) can be formed by normal, spontaneous hydrolysis and by oxidation due to the attack at carbons 1, 2, or 4 of the sugar residues in the sugar-phosphate backbone of the DNA. AP sites are non-instructive lesions, block DNA replication, and frequently result in deletion, presumably because they are easily transformed to strand breaks [82].

Strand breaks result from either the cleavage of the sugar moiety in DNA, leaving a portion of the sugar molecule on the 3' end of the break and a 5' phosphate on the other, or the loss of a nucleoside moiety, leaving a phosphate group on both the free 5' and 3' termini. Proximate breaks on both strands result in complete disruption of the molecule and are believed to be the main cause of cell death from ionising radiation. Single-strand breaks are not very dangerous because they can be repaired, whereas double-strand breaks are very critical and may be responsible for many chromosomal aberrations [79]

Nitric oxide and its congeners mainly cause DNA-deaminations, but in the form of $ONOO^-$, also causes damage similar to that induced by $HO^{\bullet}$ [46,83].

The magnitude of oxygen-induced toxicity and tissue damage is extensive. Floyd and co-workers [84] have estimated that a human cell metabolises approximately 10^{12}

molecules of molecular oxygen per day, generating 3 x 10^9 molecules of hydrogen peroxide per hour. Based on the amount of oxygen-damaged altered nucleotides detected in human urine it has been estimated that approximately 2 x 10^4 oxidative DNA lesion occur per human genome each day [85]. Because of the mutagenic potential of ROS, it seems indeed reasonable to postulate that they make a significant contribution to somatic mutations and, thereby, play a role in carcinogenesis, aging and a range of other human diseases.

5.5 ANTIOXIDANT DEFENCE AND REPAIR MECHANISMS

Adaptation and defence against oxidative insult is one of the fundamental mechanisms in cell biology, which enables cells and organisms to survive in a predominantly oxidising environment. The evolution of bioactivation processes that form biological reactive intermediates, and the continual formation of ROS as unavoidable consequence of oxygen metabolism, probably necessitated the concomitant evolution of cellular defence and repair systems. The prevention of oxidation is an essential process in all the aerobic organisms, as decreased antioxidant protection may lead to cytotoxicity, mutagenesis and/or carcinogenesis. Since defence systems are present and functioning under normal conditions, endogenous free radicals do not necessarily constitute a risk.

All tissue and cells contain defence systems for detoxification of biological reactive molecules and to prevent or minimise cellular damage though the action of antioxidants. Antioxidants are substances that delay or prevent the oxidation of cellular substrates and can be divided in two categories: 1) Enzyme-mediated antioxidant systems, including superoxide dismutase(s), catalase, glutathione peroxidase(s), metallothionein, thioredoxin(s)/thioredoxin reductase, glutaredoxin(s), peroxiredoxin(s), nitric oxide synthase oxygenase, eosinophil peroxidase, etc.; 2) Small molecules-mediated antioxidant systems, including glutathione, ascorbic acid, α-tocopherol, vitamin A, β-carotene, NADPH, coenzyme Q-10, urate, flavonoids etc. [86]

A general observation is that the enzyme-mediated antioxidant defences are directed against the primordial initiator superoxide and the less reactive secondary mediator hydrogen peroxide and organic hydroperoxides. Small-molecule, chain-breaking antioxidants are directed primarily against peroxyl radicals involved in radical propagation instead [66].

5.5.1 Major detoxifying enzymes

5.5.1.1 Superoxide dismutase

Superoxide dismutase (SOD) destroys the radical superoxide by converting it to molecular oxygen and hydrogen peroxide that can, in turn, be destroyed by catalase or glutathione peroxidase reactions.

$$2\ O_2^{\bullet -} + 2\ H^+ \rightarrow H_2O_2 + O_2$$

In humans, there are three forms of SOD, cytosolic Cu, Zn-SOD, mitochondrial Mn-SOD and extra cellular-SOD (EC-SOD) [87].

Cu, Zn-SOD belongs to a class of enzyme which is usually composed of two identical subunits, each containing a metal cluster at the active site, constituted by a copper and a zinc atom bridged by a common ligand: His 61 [88].

Mn-SOD is a homotetramer containing one manganese atom per subunit that cycles from Mn(III) to Mn(II) and back to Mn(III) during the two-step dismutation of superoxide. The respiratory chain in mitochondria is a major endogenous source of oxygen radicals and Mn-SOD is the enzyme that functions to remove superoxide radicals formed in this cell compartment. The mitochondrial Mn-SOD is highly inducible by cytokines, such as TNF-α and by mediators of acute oxidative stress, such as superoxide or hydrogen peroxide [89-91]

EC-SOD is a secreted, tetrameric, copper- and zinc-containing glycoprotein found in the interstitial spaces of tissue and also in extracellular fluids, accounting for the majority of the SOD activity of plasma, lymph, and synovial fluid. EC-SOD is the only known extracellular enzyme designed to scavenge the superoxide anion and is not inducible by its substrate or other oxidants. Its regulation in mammalian tissue primarily occurs in a manner co-ordinated by cytokines, rather than as a response to oxidants [92,93].

5.5.1.2 Catalase

Catalase is a tetrameric heme-enzyme consisting of four identical, tetrahedrally-arranged subunits. Therefore, it contains four ferriprotoporphyrin groups per molecule. Catalase is one of the most efficient enzymes known and cannot be saturated by hydrogen peroxide at any concentrations. [94]. Catalase reacts with H_2O_2 to form water

and molecular oxygen, and with H^+ donors (such as methanol, ethanol, formic acid, phenol etc.) using 1 mole of peroxide in a type of peroxidase activity.

$$2\,H_2O_2 \rightarrow 2\,H_2O + O_2$$

$$ROOH + AH_2 \rightarrow H_2O + ROH + A$$

Catalase protects from H_2O_2 generated in the cells and even though catalase is not essential for some cells type under normal conditions, it plays a role in the acquisition of tolerance to oxidative stress and the adaptive response of cells [95]

5.5.1.3 Glutathione peroxidases

The GSH peroxidases (GPXs), the most important being selenium-dependent glutathione peroxidase (GPX-1), catalyse the reduction of a variety of hydroperoxides (ROOH and H_2O_2) using GSH, thereby protecting mammalian cells against oxidative damage.

$$ROOH + 2\,GSH \rightarrow ROH + GSSG + H_2O$$

There are at least five GPX isoenzymes found in mammals (GPX-1-5). Although their expression is ubiquitous, the level of each isoforms varies depending on the type of tissue. GPXs share the substrate H_2O_2 with catalase, but GPXs alone can react effectively with lipid and other organic hydroperoxides. The glutathione redox cycle is a major source of protection against low levels of oxidant stress, whereas catalase becomes more significant in protecting against severe oxidant stress [96].

5.5.1.4 Thioredoxins

Thioredoxins (TRXs) are ubiquitous, thiol-reactive proteins. Mammalian thioredoxins include thioredoxin-1 (TRX-1), mitochondrial (TRX-2) and a thioredoxin-like protein $p32^{TrxL}$. They act to reduce oxidised cysteine residues in proteins. Cysteine residues, present in a conserved redox catalytic site, within all thioredoxins, become oxidised during this process. Thioredoxin reductases, acting in concert with NADPH, recycle the thioredoxins by returning these cysteine residues to their reduced form. TRX-1 is predominantly located in the cytosol, but agents such as phorbol esters, UV radiation

and hydrogen peroxide promote its translocation into the nucleus [97]. In addition to their role in reducing and refolding oxidised proteins, thioredoxins have the capacity to act at several levels in respect of redox-regulated gene expression. TRX-1 appears to regulate the activity of transcription factors within the nucleus by altering their thiol redox state and consequent DNA-binding affinities [98]. In addition, thioredoxins can also interact with and modulate the function of protein kinases, which are central to many cell-signalling pathways [99].

5.5.1.5 Peroxiredoxin

This recently discovered class of antioxidant enzymes functions in similarity with the glutathione peroxidases, reducing hydroperoxides utilising thioredoxin as co-substrate. The class contains 4 isoenzymes (peroxiredoxins-1 to-4), whose expression varies from tissue to tissue [100,101].

5.5.1.6 Other enzymes

Detoxifying enzymes also include GSSG reductase, conjugation enzymes such as glutathione S-transferases (GSTs), UDP-glucoronyl transferase and sulfotransferase, as well as NADP-quinone oxidoreductase and DNA repair enzymes. Genes encoding various detoxifying enzymes are usually ubiquitously expressed and co-ordinately induced in response to antioxidant and xenobiotica [102].

5.5.2 Non-enzymatic antioxidants

Two main types of small-molecule antioxidants exist in biological systems, water-soluble- and lipid-soluble species.

The most important small-molecule antioxidants include GSH, ascorbic acid, uric acid, some plasma proteins, vitamin E, carotenoids, ubiquinol and flavonoids.

5.5.2.1 Glutathione

The tripeptide glutathione GSH (γ-L-glutamyl-L-cysteinyl-glycine), is the most abundant low molecular weight thiol and is present in most cells, including micro-organisms, plants and animals, at concentrations varying from 5 to 10 mM [103]. Under normal conditions, approximately 1 % of the total cellular glutathione is in the oxidised form (GSSG), with the rest being in the reduced form (GSH) [104]. The GSH system is probably the most important cellular defence mechanism that exists in the cell. GSH not only acts as an ROS scavenger but also functions in the regulation of the

intracellular redox state. The system consists of GSH, glutathione peroxidase and glutathione reductase. As already mentioned above, glutathione peroxidase catalyses the reduction of peroxides and converts GSH to its oxidised, disulphide form (GSSG), which is then reduced back to GSH by glutathione reductase. The ability of the cell to regenerate GSH is an important factor in the efficiency of managing oxidative insults. Under normal conditions, more than 95% of the GSH in the cell is reduced and so the intracellular environment is highly reducing. A depletion of intracellular GSH has been reported to occur with the onset of apoptosis and is often accompanied by a concomitant increase in ROS [105]. In addition, depletion of GSH has been shown to be sufficient to induce apoptosis in a number of cell types and can also render cells more susceptible to apoptosis induced by subsequent stimuli [106,107].

The lung ELF contains high concentrations of reduced GSH [108] and oxidative pollutants such as ozone are known to react with GSH. A loss of lung GSH has been shown in rats exposed to 0.4 ppm of ozone. However, no increase in GSSG could be shown suggesting the formation of mixed disulphides of protein and GSH instead [109]. In contrast, increased GSH concentrations in the BAL fluid have been reported in a number of conditions thought to be mediated by oxidative stress, including cigarette smoking, adult respiratory distress syndrome (ARDS) and asthma [24].

5.5.2.2 Ascorbic acid (vitamin C)

Ascorbic acid (AC) is an important water-soluble antioxidant capable of scavenging a variety of free radicals in vitro. In humans, the ELF contains varying amounts of ascorbate and the different dietary intake of vitamin C may possibly explain the wide range of BAL fluid ascorbate concentrations. [110]. Ascorbate has also been shown to represent a major reactant towards ozone. Studies of BAL fluid, from humans exposed to ozone, have shown that ascorbate is consumed in a concentration- and time-dependent manner, suggesting that this antioxidant is an important component of the protective antioxidant screen present in the ELF [111]. In guinea pigs, it has been shown that ascorbic acid deficiency decreases the defence mechanisms against the toxic effects of NO_2 [112].

5.5.2.3 Uric acid

Uric acid (UA) is an oxidised purine base, which acts as an antioxidant by directly scavenging reactive species but also by chelating iron and copper. UA is present in plasma at concentrations of 250-450 μM and *in vitro* studies have shown that UA is

utilised preferentially to other oxidants in plasma exposed to high concentrations of ozone [113]. UA is also present in the human ELF at high concentration and is consumed in time- and concentration-dependent manners during exposure of the ELF to NO_2 or ozone [111,114].

5.5.2.4 α-Tocopherol (vitamin E)

In contrast to the water-soluble antioxidants, α-tocopherol is lipid-soluble and does not provide protection by acting as a sacrificial oxidant. Instead, protection by α-tocopherol is believed to be due to its ability to act as a chain-breaking antioxidant, scavenging peroxyl radicals and, thus, preventing the propagation of lipid peroxidation processes [115]. It is important to highlight the fact that α-tocopherol is only one form out of the eight naturally-occurring forms of vitamin E, but it has been suggested that α-tocopherol is the most efficient antioxidant among them. However, several studies have shown that various forms of vitamin E have different effects on cell signalling systems [116].

5.5.2.5 Carotenoids

The most abundant member of the family of carotenoids is β-carotene, which is a strong antioxidant and quenches $^1O_2$ rapidly due to its conjugated double-bound-containing chain. Other properties of carotenoids, not related to the antioxidant activities, have been reported in recent studies, including stimulatory effects on gap junctional communication, expression of connexins, and induction of xenobiotic metabolising enzymes. Anyhow, the mechanisms underlying these effects are not known at present. It has been reported that carotenoids, especially lycopene, which has an exceptionally high activity, inhibit the growth of mammary, endometrial and lung cancer cells, probably by inducing the expression of the transcription factor AP-1 [116,117].

5.6 MOLECULAR AND CELLULAR EFFECTS OF OXIDATIVE STRESS

5.6.1 Redox signalling

Under conditions of oxidative stress, cellular response to ROS or RNS is critical in helping to maintain cellular functions, and in making the decision between cell survival and death. However, increasing evidence suggests that the balance of oxidants, antioxidants, and the redox status within the cells, is important in the regulation of gene expression, even in the absence of severe stress. Indeed, changes in cellular redox may

be associated with cell differentiation, cell cycling and ageing processes [118]. In some instances, radicals may act as second messengers and hence, bioavailability of antioxidants may modulate their influence on patterns of gene expression. Extracellular ROS can initiate cellular signalling by activation of growth factor and cytokine receptors in a manner that does not require the presence of the receptor ligands or via generation of lipid peroxides within cell membranes [119,120]. Well over 100 mammalian genes have been identified that can be regulated by cellular redox state, among them important transcription factors such as NF-κB, AP-1 and p53 [103,118,121]. Total cell GSH levels may act as a signal transducer or modulator and the background redox state of the cell will modify the specific response. Much of the redox-sensitive regulation of gene expression is transcriptional although alteration in mRNA or protein stability can also be influenced [122,123].

5.6.2 Protein glutathionylation

Thiol groups are central to most, if not all, redox-sensitive cell signalling mechanisms. Formation of mixed disulphides between glutathione and cysteines in proteins (glutathionylation) has long been known to occur during oxidative stress [124]. Oxidation of thiol groups is a reversible process that represents a sensitive redox-regulated functional switch. Specifically, oxidation/reduction of cysteine thiol groups, within key proteins may control much of the dynamics of redox-sensitive gene expression [125].

Glutathionylation takes place by thiol-disulphide exchange between protein sulphydryls and oxidised glutathione (GSSG), a reversible process that can be catalysed by glutaredoxin. ROS can also provoke the formation of intra- or intermolecular disulphide bridges between cysteines. This process may cause conformational changes or generate dimers or multimers that have the potential to alter protein function [118]. The ratio between GSSG and reduced GSH is an indicator of the redox status of the cell, and the extent of protein glutathionylation will vary accordingly to this ratio. A higher ratio will promote glutathionylation/binding, whereas a lower ratio will result in deglutathionylation/release of glutathione from the proteins of interest [126]. This finding also suggests a likely compartmentalisation of protein glutathionylation, as GSSG/GSH ratio is very high in the endoplasmic reticulum, where an oxidising environment favours sulfhydryl oxidation [127]. A number of proteins have been reported to undergo glutathionylation including important enzymes, transcription factors, and oncogenes [128].

5.6.3 Redox-sensitive signal transduction pathways

ROS can act through several different pathways of signal transduction, making use of signalling molecules such as calcium, protein tyrosine kinases (PTKs), protein tyrosine phosphatases (PTPs), serine/threonine kinases and phospholipases.

5.6.3.1 Protein kinases

Phosphorylation cascades are involved in many mechanisms for the transmission of cellular signals from the plasma membrane to the cell nucleus. Numerous studies have demonstrated that oxidants are capable of inducing protein phosphorylation and protein kinase activities [129-132].

The mitogen-activated protein kinases (MAPKs) are one of the most extensively studied families of protein kinases. Several MAP-kinase-related pathways have been identified (ERK, JNK/SAPK, p38 kinase, and BMK/ERK5 etc.). These kinase families exist in all eukaryotes, and control essential processes such as proliferation, differentiation and apoptosis [133]. Components of each of the MAP kinases contain redox-sensitive sites that provide the potential for modulation of the signalling via cellular redox status. ROS can activate MAP kinases via Ras-dependent mechanisms. There is also evidence that NO can react with cysteine 118, on the surface of Ras and that this reaction may represent a redox-sensitive switch. Once activated, Ras stimulates the small G protein Rac that, in turn, activates membrane-bound NADPH oxidase to produce ROS, which are required to mediate mitogenic stimulation [134,135].

Another important kinase signalling pathway involves members of the protein kinase C (PKC) family. PKCs also contains structural motifs that are susceptible to redox-modification and can be activated by various ROS (Newton 1997). Hydrogen peroxide, for example, can induce tyrosine phosphorylation of PKC, enhancing its activity [136]. In contrast, oxidative modification within the carboxy terminal domain inactivates PKC and this may explain why PKC appears to be subject to dual redox regulation with lower levels of ROS stimulating its activity and higher levels inhibiting it [137].

Taken together, redox regulation of cellular functions by protein phosphorylation occurs by a number of distinct processes, which may be activated by oxidative stress at multiple levels and "cross-talk" between the pathways may provide the potential for modulation of the signalling via cellular redox status.

5.6.3.2 Protein phosphatases

Protein phosphorylation is balanced by the action of protein kinases and phosphatases. Both serine/threonine phosphatases and protein tyrosine phosphatases (PTPs) are known to be redox-sensitive [138]. The mechanism is best understood for the PTPs, where the catalytic domain contains a conserved motif with a single cysteine residue. Oxidation or mutation of this cysteine renders the enzyme inactive. Furthermore, hydrogen peroxide has been shown to be a potent inhibitor of PTPs [139]. Inhibition of PTPs may be responsible for the activation of some PTKs following oxidant treatment [140]. In contrast, oxidative stress can induce the expression of protein phosphatase CL100, which is capable of dephosphorylating ERK. The inactivation of ERK could represent a negative feedback loop for ROS-induced activation of ERK.

5.6.3.3 Lipids and phospholipases

Several classes of phospholipases can be activated by oxidative stress. Activation of phospholipase Cγ (PLCγ) leads to the release of diacyl glycerol and inositol 1,4,5-triphosphate (IP3). Diacyl glycerol activates PKC while IP3 provokes release of calcium into the cytosol from intracellular pools. Oxidants, such as hydrogen peroxide and vanadate, have been shown to lead to PLCγ phosphorylation and concomitant increase in IP3 [141]. Oxidants as well as lipid peroxidation, PKC, PLCγ, calcium and/or Rac, may be involved in the activation of phospholipases A_2 and D. Arachidonic acid, the predominant reaction product of PLA_2, and its metabolites are, in turn, potent mediators of inflammatory processes [142-145].

5.6.3.4 Antioxidant response element

Redox-dependent protein modifications occurring in the cell during oxidative stress may serve to trigger the expression of specific genes needed to adapt to the changed conditions. Interestingly, oxidative stress induces the co-ordinated expression of a number of genes including several phase II detoxifying enzymes. The promoter region of such genes contains a *cis*-acting sequence, termed the antioxidant (or electrophile) response element (ARE/EpRE), which is important for the induction of expression. Examples of genes regulated by ARE are NADPH-quinone oxidoreductase, glutathione-S-transferase, UDP-glucuronosyl transferase, thioredoxin, heme oxygenase-1 and ferritin, reviewed in [116]. Transcription factors, such as Nrf1 and Nrf2, heterodimerise with small Maf proteins and binds to the ARE element. In particular,

Nrf2 seems to be important for regulation of ARE/EpRE-mediated gene transcription [146]. The importance of Nrf2 for defence against oxidative stress is clearly demonstrated in studies of Nrf2-deficient mice. These mice are rapidly depleted of hepatic glutathione when challenged with inducers of oxidative stress [147].

5.6.3.5 Intracellular calcium signalling

Intra-cellular calcium concentrations modulate many cellular processes including gene transcription. Oxidants stimulate an increase in intracellular free calcium concentration in most models.

Ca(II) Is a co-factor for PKC, activates intracellular proteases, such as calmodulin and calpain and also activates endonucleases responsible for DNA degradation during apoptosis. Intracellular calcium can be increased in two ways: By the release of intracellular calcium stored within the endoplasmic reticulum and/or mitochondria and by the increased uptake of extracellular calcium. Increased uptake of calcium can be caused by the activation of cytoplasmic membrane-associated Ca (II) transport channels, probably by a phosphatidyl inositol-depend mechanism [148]. Release of intracellular stores of calcium from within the endoplasmic reticulum can be facilitated by the redox activation of the membrane transport protein MTP.

5.6.4 Redox sensitive transcription factors (TF)

As already mentioned in other parts of this thesis, numerous TFs exhibit redox sensitivity and this is believed to be one of the most fundamental mechanisms of regulation of gene expression. The three most characterised are NF-κB, AP-1, and p53, but the number of studies reporting implication of other TFs in situations of oxidative stress is constantly increasing.

5.6.4.1 NF-κB

Nuclear factor kappa-B (NF-κB) has, for some time, been held as the paradigm model of a redox-sensitive transcription factor [149]. A substantial body of experimental data links NF-κB activity to cellular oxidative status. NF-κB regulates the transcription of a host of genes including acute phase genes, cytokines and cell surface receptors [150]. Various stimuli, such phorbol esters, pro-inflammatory cytokines, endotoxin and some ROS can activate NF-κB. Exposure to hydrogen peroxide activates NF-κB in many cell

types [151], but in other systems, oxidants have been demonstrated to reduce or block NF-κB activation [152].

However, many studies are contradictory and the precise role of ROS in the activation mechanism remains to be elucidated. Recently, one review has called into question the link between NF-κB and the redox status, and conclusive proof that redox signalling plays a central role in the regulation of NF-κB activity is still required [153].

5.6.4.2 AP-1

The transcription factor Activator Protein-1 (AP-1) regulates the expression of genes associated with growth, differentiation and stress. This factor is composed of either homo- or heterodimers of the Fos (c-Fos, FosB, Fra1 and Fra2) and Jun (c-Jun, c-Junb, and c-JunD) families [154]. Expression of the different members of these families varies throughout the cell cycle and since the different components possess distinct transcriptional properties, variation in AP-1 composition is likely to modulate its regulatory functions [155]. The classical form of AP-1 consists of c-Fos/c-Jun dimers. In many cell types, c-Jun and c-Fos expression is induced rapidly and transiently by a variety of stimuli including alteration in cellular redox state [156,157]. Phosphorylation of the AP-1 subunits influences its DNA binding and transcriptional regulatory properties [158]. Some studies have suggested that oxidative stress activates AP-1, but in other experiments, antioxidants and antioxidant enzymes have also been shown to stimulate AP-1 [152,157]. The reasons for these contradictory results remain to be elucidated. An alternative explanation could be that, like NF-κB, extracellular and pro-oxidant conditions tend to promote AP-1 activation, but reducing conditions within the nucleus are required for DNA binding.

5.6.4.3 p53

The p53 tumor suppressor gene encodes a transcription factor that has been termed the "guardian of the genome". Cellular stress can lead to p53 activation, which triggers transcription of p53-dependent genes. Many of these genes are involved in the regulation of cell cycle arrest and apoptosis. Expression of p53 and its DNA-binding properties can be stimulated under conditions of oxidative stress [159,160]. However, like the other transcription factors described above, studies of p53 effects on cells have shown contradictory results and it is far from clear that oxidative stress always promotes p53 activity.

Oxidation of p53 impairs its DNA-binding capacity via a cysteine-dependent mechanism. The redox regulator Ref-1 can reactivate DNA binding of oxidised p53 and, as for AP-1, may play an important role in p53 activation following cellular oxidative challenge. In addition, p53 activation may result from processes related to DNA damage. Detection of such damage promotes p53 protein phosphorylation and may even reduce the rate of p53 degradation [161-163].

5.6.4.4 HSF-1

Heath shock factor 1 (HSF-1) belongs to a family of heat shock factors whose activation and DNA-binding is mediated by stress stimuli that disrupts thiol homeostasis. Stress-induced transcription of heat shock (or stress) genes requires activation of HSF-1, which subsequently binds to the heat shock promoter element (HSE). Activation of HSF-1 is characterised by the conversion of this factor from a monomeric to trimeric state, a phenomenon induced by heath shock and a large variety of different chemicals or conditions that generates abnormally folded proteins. It has been hypothesised that HSF-1 is dually regulated by oxidants. On the one hand, hydrogen peroxide favours the nuclear translocation of HSF-1, whilst on the other, it alters HSF-1 DNA-binding activity, most likely by oxidising critical cysteine residues. Interestingly, hydrogen peroxide upregulates TRX before HSF-1 activation and it has been suggested that reducing conditions generated by TRX upregulation are required to trigger HSF-1 DNA-binding activity during oxidative stress [164].

Expression of the Heat Shock Proteins (HSPs) usually results in repair of damaged proteins and increased survival of the cells, mainly through their chaperone function.

The common signal generated by various stress stimuli is likely to be protein damage, but how the cellular stress sensing mechanism functions in the cell is not fully elucidated. The expression of small HSPs, especially HSP27, and the inducible HSP70 has been shown to enhance the survival of mammalian cells exposed to numerous types of stimuli that induce stress and apoptosis [165]

5.7 CELL PROLIFERATION AND CELL DEATH

The wide spectrum of cell signalling and transcriptional processes influenced by the redox state of the cell implies that oxidative stress is likely to be involved directly or indirectly in the pathophysiology of a number of diseases. Most interest has been focused on cancer, where the involvement of radicals as mutagens, and ROS as

regulators of cell proliferation and apoptosis are clearly important. However, redox-regulated alterations of cellular processes may play a role in other pathological conditions such as diabetes, autoimmune disorders and degenerative diseases, including Alzheimer's and Parkinson's disease, but firm data for many of these links are still lacking.

A common mechanism involved in these dysfunctions, is the response to oxidative stress and to endogenous free radical formation by increasing or decreasing cell proliferation, changing immune response and/or by induction of apoptosis. All appear to be regulated by closely integrated, ATP-dependent signalling pathways [166]. In contrast, extreme levels of oxidative stress may give rise to necrotic cell death, a pathological process, which is considered to be a passive process. In many cases, however, there is no clear-cut delineation between apoptosis and necrosis [167]. The effects of oxidative stress depend on the cell type, the level of oxidative stress experienced and the protective mechanisms existing in each cell types. Hence, the sensitivity of cells to oxidative challenge depends on their intrinsic antioxidant systems, in particular the levels of GSH within the cell. [168].

5.7.1 Cell proliferation

A range of pathways, which may be affected by radicals, control cell proliferation. Both Ras and PKC can be activated by exposure to ROS and in turn stimulate a range of transcription factors including c-Fos, c-Jun and c-Myc, either directly or via the MAP kinase pathway [169].

Oxidative stress at levels too low to cause apoptosis can, in certain cases, induce temporary growth arrest. Micromolar concentrations of hydrogen peroxide have been shown to induce slowing down of the cell cycle and partial depletion of GSH can induce arrest in G2/M in some cell types [170,171].

5.7.2 Cell death

It is now well established that the generation or addition of ROS or RNS can cause cell death, either by apoptosis or necrosis. Necrosis usually occurs in response to severe injury to the cell and is characterised by cytoplasmic and mitochondrial swelling, plasma membrane rupturing and release of the cellular contents into the extracellular space, with subsequent generation of an inflammatory response, which can cause further injury to neighbouring cells. Apoptosis, in contrast, is a tightly regulated form of cell death in which a cell effectively partakes in its own demise. The execution of the

death programme is characterised by morphological and biochemical changes. These include mitochondrial depolarisation and alterations in phospholipid asymmetry, chromatin condensation, nuclear fragmentation, membrane blebbing, cell shrinkage and the formation of membrane-bound vesicles termed apoptotic bodies. During inflammatory reactions, free radical-mediated signalling may modulate the expression of pro-apoptotic cell surface changes, such as the upregulation of fas (Apo1) and its ligand, and the appearance of phosphatidylserine on the outer cell membrane. These markers signal for the cell to be removed by neighbouring macrophages and other phagocytic cells.

Many of the morphological changes associated with apoptosis are orchestrated by activation of a cascade of proteases termed caspases [172,173]. The caspases are a family of cysteine proteases comprising at least 14 members, all of which contains an active site thiol group necessary for the activity. Caspases cleave various substrates, including caspase-dependent endonucleases, which enter the nucleus, where they cut DNA into oligonucleosomal fragments [174].

The redox status of the cell can have obscure and elaborate effects on apoptosis. While addition of exogenous ROS is sufficient to trigger the apoptotic cascade, the executioners of apoptosis (i.e. caspases) are extremely redox-sensitive and require a reducing environment in which to function. Apoptotic cell death can be switched to necrosis during oxidative stress by two possible mechanisms. Firstly, inactivation of caspase due to oxidation of their active site thiol group by oxidation or S-nitrosylation and secondly, by a drop in cellular levels of ATP due to the failure of mitochondrial energy production by oxidants. The triggering and modulation of apoptosis by oxidative stress is a delicate balance between a large number of cellular elements and conditions encountered in different stress situations and this issue has recently been reviewed by Chandra et al. [175].

The description of the multiplex pathways of induction of apoptosis has been reviewed elsewhere and only the two major pathways, which are related to oxidative stress, will be mentioned in this thesis. In both, the redox state of the cell plays an important effector or regulatory role. A well-recognised reason for the induction of apoptosis is seen as a response to DNA damage, including oxidative damage. Detection of DNA damage is mediated through p53, but the mechanism by which p53 detects damage and signals the induction of apoptosis is not fully elucidated. Activation of p53 can signal to the cell to slow down cell cycling, usually blocking it in G1 to allow DNA repair, or it may signal for the cell to enter an apoptotic demise. Whether the cell tries to repair

DNA or enter apoptotic cell death may depend on a number of factors including the amount of Bcl-2, or related proteins (Bcl-x, Bcl-w intrinsically expressed in the mitochondrial membrane), the level of activation of poly-ADP ribose polymerase (PARP) or c-myc expression [176]. This pathway causes the release of cytochrome C from the mitochondria into the cytosol, where it binds to Apaf-1 (Apoptotic protease activating factor 1) and causes the subsequent activation of caspase-9 and caspases-3 and -7.

The redox state of the cell may also alter the balance between death and repair, by affecting both the expression and the activation of relevant enzymes involved in DNA repair. As mentioned above, p53 regulates the transcription of a number of genes involved in the oxidative response.

The second major signalling pathway, namely the death-receptor pathway, involves the binding of ligands to the CD95 receptor (fas, Apo1, TNF), causing clustering of the receptor and formation of the death-inducing signalling complex (DISC) [177]. Binding of the pro-caspase to the complex leads to cleavage and release of caspase 8 into the cytoplasm. Caspase-8 then activates caspase-3 and may also act directly on other apoptotic substrates. Thus, redox potential appears to modulate apoptosis through both mitochondrially-driven and ligand-receptor-driven mechanisms.

6 THE PRESENT STUDY

6.1 THE AIM OF THE STUDY

The overall aim of the present study was to characterise the molecular effects of air-borne oxidant gases on human lung cells and to characterise alterations of gene expression caused by the oxidative stress which, is believed to occur in the lung upon inhalation of such oxidants.

6.1.1 Specific aims:

- To compare the capabilities of human alveolar macrophages from smokers and non-smokers for the expression and release of inflammatory cytokines after exposure to NO_2 *in vitro*.
- To characterise alterations in mRNA profiles in human alveolar macrophages upon exposure to ozone *in vivo*.
- To compare the effects on gene expression during two situations of oxidative stress caused by *in vitro* exposure to sub-toxic concentrations of diamide or hydrogen peroxide in two different cell lines.
- To correlate the alterations in gene expression profiles to the extent of protein S-glutathionylation in cells exposed to sub-toxic concentrations of diamide or hydrogen peroxide *in vitro*.
- To assess the thermotolerance and cyto-protective effects achieved by the induction of stress genes and proteins due to pre-exposure of cells to diamide.
- To analyse the effects of sub-toxic concentrations of hydrogen peroxide on the human lung epithelial cell line A549, with focus on DNA damage, cell cycle arrest and apoptosis.
- To perform a large-scale screening of alterations in mRNA profiles in the human lung epithelial cell line A549 after exposure to sub-toxic concentrations of hydrogen peroxide.

6.2 MATERIAL AND METHODS

6.2.1 BAL, ECV304 and A549 cells

Inhaled air-borne pollutants affect primarily lung cells, such as alveolar macrophages, lung epithelial cells and to some extent also endothelial cells. Therefore, the choice of cells, used for the different experiments included in this thesis, was extremely important and both primary human cells and cell lines have been used.

Bronchoalveolar lavage cells (BAL) consisting of ~90% of alveolar macrophages were used in **Papers I and II**. These cells are obtained by BAL, as described below, and can be collected by a relatively non-invasive procedure. Thus, this technique allows for the study of cells exposed under in vivo conditions, which is of great advantage in studying the effects of oxidant gases in the human lung.

In contrast, primary human lung epithelial cells were not available in the large amount needed for the experiments performed in **Papers III and IV** and, therefore, commercial, immortalised cell lines were used. The human A549 type II lung epithelial cell line was chosen in **Paper III and IV.** This cell line is considered to posses a pronounced resistance to oxidative stress. Moreover, in **Paper III** the human ECV 304 "endothelial cell line" was used on account of its more pronounced sensitivity to the pro-oxidative treatments compared with the A549. This cell line was originally defined as being derived from human umbilical vein endothelial cells, but later reports have cast doubt on the full endothelial character of this cell. However, for the purpose of these studies, the contrasting sensitivity to hydrogen peroxide-induced redox changes ands toxicity, as compared to A549 cells, was considered paramount.

6.2.2 Bronchoalveolar lavage (BAL)

Fiberoptic bronchoscopy with airway lavage gives rapid access to the lumenal contents of the lower airways, i.e. the brochi, bronchioli, terminal airways and the alveolar space. The BAL technique provides the opportunity to collect cells from the airways and alveolar space, which can be used to assess events taking place in the airway in vivo. This technique has been described elsewhere and was performed according to the appropriate international guidelines [178]. The fiberoptic bronchoscopy with bronchoalveolar lavage was performed at the Department of Respiratory Medicine and Allergy, Umeå University.

Briefly, the bronchoscope was carefully wedged into a middle lobe bronchus and three aliquots of 60 ml phosphate buffered saline, pH 7.3, were infused into the lung and

gently aspirated back into a siliconised container placed on ice. The chilled lavage fluid was filtered and the BAL cells obtained by this procedure were collected by centrifugation and counted in a hemocytometer. BAL cells used in **Paper I** were seeded into culture dishes and allowed to adhere to the plastic surface before exposure to NO_2. Cells used in **Paper II,** which were exposed to ozone *in vivo*, were stored at -70 °C immediately after centrifugation and used to prepare RNA samples used for the Affymetrix gene screening.

In healthy subjects, differential cell counts from the lavage fluids have shown that more than 90% of the inflammatory cells obtained by BAL consist of macrophages, with the rest represented by neutrophils, lymphocytes and mast cells [35]. Therefore, BAL cell samples are termed "alveolar macrophages (AM)" in this thesis, and it can be assumed that the gene expression analysis performed in **Paper I and II** largely reflects the gene expression events from this cell type.

6.2.3 *In vitro* exposure to NO_2

In **Paper I**, BAL cells were seeded into culture plates and allowed to adhere for two hours at 37°C in humidified air (5% CO_2 incubator) before their exposure to air or NO_2 (5 or 20 ppm) for 20 minutes. The exposure was performed in a specially designed, highly-humidified exposure chamber (100% humidity, 37°C), allowing direct exposure of the cells in the gas phase by inversion of the culture plates, after initial washing with medium supplemented with 0.3% BSA and 25 mM HEPES. After the exposure, cells were incubated in fresh medium for 4 hours at 37°C in humidified air (5% CO_2 incubator). After this time, the medium was collected and used to analyse the secretion of the inflammatory cytokines TNF-α, IL-1β, IL-8 and MIP-1α. Cells were collected and RNA from these cells was used to quantify the alteration of mRNA profiles for the same cytokines.

6.2.4 *In vivo* exposure to ozone

As described above, bronchoalveolar lavage allows collection of BAL cells, which were exposed during inhalation in vivo. In **Paper II**, healthy, non-smoking volunteers with normal lung function were exposed to 0.2 ppm (400$\mu g/m^3$) ozone for 2 hours in an exposure chamber. During this two-hour period, light exercise on a bicycle ergometer was alternated with rest in a 15-minutes cycle. Each subject was exposed twice, once to filtered air and once to ozone. The two exposures occurred in random order and were

separated from one another by at least three weeks to reduce possible carry over effects, arising from the exposure or from the bronchoscopy.

The bronchoscopy with airway lavage was performed 4 hours after termination of each exposure and BAL cells were stored at -70°C until RNA extraction. The RNA from these cells was used to perform the Affymetrix gene expression analysis.

Both, exposure to ozone and fiberoptic bronchoscopy with bronchoalveolar lavage were performed at the Department of Respiratory Medicine and Allergy, Umeå University.

6.2.5 *In vitro* exposure to diamide or hydrogen peroxide: Two different situations of oxidative stress.

The hypothesis that the toxicity of inhaled oxidants relies on the formation of radicals and other oxidant species and, hence, on the induction of oxidative stress, together with the lack of knowledge regarding the effects of stress situations on gene expression, suggested a closer analysis of more controlled situations of oxidative conditions in cells. In **Paper III**, two types of immortalised cells were exposed *in vitro* to two different oxidative agents, diamide or hydrogen peroxide. Diamide is known as a thiol oxidising agent and, therefore, is able to alter the redox status of intracellular glutathione, which plays an important role in the maintenance of the redox state of the cell. On the other hand, exposure to hydrogen peroxide results in a situation of oxidative stress, which is associated with the formation of a variety of oxygen-centred radicals, which can directly react with and damage biomolecules in the cell.

As mentioned above, two types of cells were chosen for these analyses, the human type II lung epithelial cell line A549 and the human endothelial cell line ECV304. These two types of cells are known to differ in sensitivity against oxidative stress and, moreover, they represent cell types encountered in the lung.

The first step in the study-design was to establish standard exposure conditions at sub-toxic levels for both cell lines and both oxidants. The aim was to find a threshold-level of toxicity, at which cells experience a situation of oxidative stress but maintain their viability. The changes of levels of mRNAs to be analysed should reflect those in a situation in which cells are still able to adapt to the insult, either by enhancing the protection machinery or by inducing apoptosis. Another important aspect deserving attention is the fact that diamide may alter the redox state in the cell and, hence, influence gene transcription, by directly affecting redox-sensitive cellular components, including transcription factors.

For the large-scale Affymetrix-based transcript screening performed in **Paper IV,** only A549 cells exposed to hydrogen peroxide were used. In this study, the choice of hydrogen peroxide has been made not only based on its oxidative properties, but also based on the knowledge that this oxidant is formed in the lining fluid of the lung upon ozone inhalation. In previous experiments in our laboratory, *in vitro* exposure of these cells to ozone failed to give satisfactory results, probably due to the problematic of exposure procedures and difficulties in accurately assessing ozone concentrations at the surface of the cells. Thus, the addition of hydrogen peroxide as a primary aqueous-soluble metabolite could provide an acceptable substitute. Again, the concentrations of the oxidant used in this study were kept at levels which affected the cells, but that fail to cause severe stress conditions, leading to marked loss of viability or necrosis.

6.2.6 Semi-quantitative RT-PCR

Quantification of mRNA transcripts in cells has been conventionally analysed by Northern blot, a method generally requiring large amounts of RNA to be prepared from the cells to be analysed. In the case of non-smoker's BAL cells, in **Paper I**, the number of cells obtained from each individual was less than from smoker's and, hence, the yield of RNA prepared from these cells did not satisfy the request to perform the quantification of mRNA coding for several cytokines at several endpoints. Thus, an RT-PCR-based method, which was more sensitive for quantification of mRNA transcripts, was developed and used in **Paper I**.

PCR *per se* is not a quantitative method, by the virtue of its exponential amplification of cDNA molecules. Under ideal or theoretical conditions, the amount of product doubles during each cycle of PCR reaction. Experimentally, the efficiency of amplification is less than perfect and the amount of product can be calculated from the following equation:

$N = N_0 \times (1 + E)^n$, where N is the number of amplified molecules, N_0 is the number of starting molecules, E is the efficiency of amplification and n is the number of PCR cycles. If the efficiency of amplification is compromised (i.e. < 1), the yield of PCR products might differ from reaction to reaction, in spite of the same amount of starting material. In order to overcome this disadvantage several methods have been developed, including the use of an internal standard or MIMIC. The MIMIC molecule consists of a fragment of DNA containing the same gene-specific primer-binding sites as the target cDNA, but differs in size from the target cDNA fragment. A known number of MIMIC molecules is added to each of the samples containing target cDNA and amplified in the

same reaction and, hence, with the same efficiency. The PCR products can then be separated by conventional agarose gel electrophoresis and the amount of product quantified and compared. The ratio between the amount of MIMIC PCR-product and the amount of target-cDNA PCR-product allows calculation of differences between samples. In **Paper III**, a semi-quantitative RT-PCR method was used in order to verify the result from the ATLAS-gene screening. In this case, instead of a MIMIC an internal control, the gene coding for the ribosomal protein S9 was used to quantify the PCR products.

6.2.7 Gene screening methods: from Northern blot to Affymetrix

By definition, "gene-screening" methods are procedures used to simultaneously identify and quantify many mRNA transcripts present in the cell at one time. Even though gene expression consists of several steps, the first and probably most important is the initiation of gene transcription. The strict control of each of these steps determines the overall extent to which a gene is expressed, i.e. the total amount of functional gene product synthesised. However, alteration of mRNA transcription profiles often reflects the changes in gene expression occurring at a certain situation and provides important information about integrated molecular events taking place in the cell.

The evolution of a variety of gene screening methods developed during the last years probably reflects the demand from scientists for such tools. The first, and oldest, method developed for the quantification of mRNA transcripts is the Northern blot, which has been used in **Paper I**. The large amount of RNA needed, and the possibility to analyse only one gene at the time, are the major disadvantages linked to this procedure. The next "generation" of gene screening methods was based on the RT-PCR technique. These methods require less RNA, are less time-consuming and are suitable for the analysis of a limited number of genes at the time. A semi-quantitative RT-PCR procedure has also been utilised in **Paper I** for samples with limited number of cells and in **Paper III** for the verification of data obtained by ATLAS-array screening.

In the next "era" of gene expression analysis, various macro/micro gene array techniques have been developed which allow the parallel detection and quantification of a large numbers of messenger RNA transcripts. Micro-array expression analysis has become one of the most widely used functional genomics tools.

Gene arrays are solid supports (membrane or glass) upon which a collection of gene-specific nucleic acid fragments (target-DNA) have been bound at defined locations,

either by spotting or direct synthesis (lithography). In array analysis, an mRNA- or cDNA-containing sample (sample-DNA) is labelled and then allowed to hybridise with the gene-specific targets on the array. Based on the amount of sample-DNA hybridised to each target-DNA spot, information is gained about the specific mRNA composition of the sample. The major advantage of gene arrays is that they can provide information on thousands of gene expression in a single experiment, using relatively small amounts of mRNA.

The introduction of these new technologies, where thousand of genes can be investigated simultaneously, has created new possibilities to assess alterations in transcriptosomal profiles in order to investigate cellular processes and compare different samples. In these studies two different gene array techniques were employed: The ATLAS-cDNA expression array from Clontech (**Paper III**) and the Affymetrix GeneChip analysis (**Papers II and IV**).

The ATLAS array is a nylon membrane-based array on which a few hundred genes are represented. In this study the ATLAS cDNA Human Stress Gene Array, containing 234 target-DNAs, was used. The target-DNAs represented on this array correspond to genes known to be involved and/or be affected by different stress situations occurring in the cell, including oxidative stress.

The Affymetrix GeneChips are glass slide arrays manufactured using special photolithographic methods and combinatorial chemistry, which allow the oligonucleotide spots to be synthesised directly onto the array substrate. The analysis procedure specifies that the mRNA samples are converted into biotin-labelled cRNA and each sample is hybridises to a separate GeneChip. The hybridised cRNA is then stained with streptavidin-phycoerythrin conjugate and visualised with an array scanner. The data obtained from the scanning of control samples is used as a base-line to calculate the changes in amount of mRNAs in the treated samples.

Thousands of genes are represented by the gene probes (target-DNA) included in the Affymetrix GeneChip, and a large amount of data is generated for each experiment. The challenge consists in the analysis and interpretation of this data. Various software analysis programs are available to quantify the alteration in transcriptional profiles, and the fold-change for each of the analysed genes can be calculated. However, hitherto no software program exists which is capable of performing an interpretation or provide an explanation of the *en mass* changes detected by the screening. This part of the analysis is still not possible to perform by the use of electrons and is still limited to the ability of human neurons!

6.3 RESULTS AND DISCUSSION

6.3.1 Expression and release of inflammatory cytokines from alveolar macrophages from smokers and non-smokers (Paper I)

There are a number of indications, from epidemiological and *in vitro* studies, suggesting that air pollutants may increase the susceptibility to airway infections, especially in smokers. Additionally, previous studies have shown that smoking can impair pulmonary immune function and, hence, alter resistance to the development of lung diseases. The pulmonary defence against inhaled antigens is based on the activation of immunological events, most of them dependent on cytokine signalling. The expression and release of inflammatory cytokines in the lung is an intricate issue, since these potent biomolecules control both the onset and resolution of inflammation. A disturbance in the normal cytokine balance can lead to perpetuation of inflammatory events and the development of asthma and chronic obstructive pulmonary diseases (COPD).

Because of the strategic location, and their ability to express inflammatory mediators, the alveolar macrophages play a central role both in anti-bacterial and immunological defence systems in the lung. AMs From smokers may have an impaired capability to respond to inhaled antigens depending on the recurrent exposure to toxic compounds in tobacco smoke and their adaptation to the effects of these toxicants. Attenuation of an inflammatory response is probably occurring in the smoker's lung, since the toxicity of the inhaled compounds and the increased number of AMs residing in the lumen of their lungs would normally cause chronic inflammatory conditions.

With these considerations in mind, the aims of this study were to compare and contrast the effects of *in vitro* exposure of NO_2 on the expression and secretion of some inflammatory cytokines from AMs from healthy non-smokers and healthy habitual smokers.

Several important facts deservd consideration in this study design. Firstly, plating and adhesion into culture dishes may activate AMs obtained by BAL. Secondly, in spite of a certain degree of activation occurring in the AMs, due to the culturing condition in vitro, it should still be possible to induce a response, which was provided in a para-pathophysiological manner by LPS treatment. Thirdly, the amount of AMs obtained from non-smokers was less than obtained from smokers and the detection of mRNA levels required a more sensitive method than northern blot. For this purpose a RT-PCR-

based method was developed and a quantitative comparison of mRNA levels between smokers and non-smokers was, thus, not possible in this study.

Both the alteration of steady state mRNA levels and the release of the corresponding proteins into the culturing medium were quantified for the inflammatory cytokines TNF-α, IL-1β, IL-8 and MIP-1α in human AMs. Human AMs were obtained by BAL, cultured *in vitro*, as described above, and used to analyse the effects of the following treatments/conditions: 1) The adhesion and culture of human AM into cell culture dishes. 2) The exposure of the human AMs to air, 5 or 20 ppm of NO_2 *in vitro*. 3) The activation of AMs by LPS addition into the medium. 4) The effects of pre-exposure of human AMs to air or 20 ppm of NO_2 followed by LPS addition into the medium. 5) The aim was to study if the effects of the above-described treatments differ in AMs from smokers compared with AMs from non-smokers.

The results of this investigation are summarised in Table III, both for the effects on mRNA levels and protein secretion into the culture medium. The major findings can be defined as followed:

Quantification of mRNA levels: i) Adherence of BAL cells to plastic caused an increase in mRNA levels of all four cytokines tested in smoker's cells and that of IL-1β and TNF-α in non-smoker's cells. ii) The steady state levels of the respective mRNAs for each of the cytokines were not significantly affected in smoker's cells by exposure to NO_2, except for a negative, dose-dependent trend in the case of TNF-α. In non-smoker's cells NO_2 tended to diminish the levels, particularly that of IL-1β. iii) Induction of mRNA levels by bacterial LPS was not impaired by pre-exposure to 20 ppm of NO_2 in non-smoker's cells. Interestingly, IL-8 was not inducible by any of the treatments. In contrast, NO_2 pre-treatment of smoker's cells diminished the induction of mRNAs, for all cytokines tested, when cells were exposed to LPS.

Quantification of secreted protein: i) Exposure to 20 ppm of NO_2 caused an inhibition of IL-8, TNF-α and MIP-1α secretion from smoker's cells. In non-smoker's cells only the secretion of TNF-α was inhibited by NO_2 exposure. ii) Addition of LPS into the medium caused an increased secretion of all cytokines except for IL-8 in non-smoker's cells. This proved that cultured macrophages were still inducible. iii) However, when smoker's cells were exposed to 20 ppm of NO_2 before LPS treatment, the secretion of all four cytokines was diminished (compared with the air and LPS exposed cells). In non-smoker's cells the exposure to 20 ppm of NO_2 did not affect the induced secretion of cytokines by LPS.

Table III. Effects on mRNA levels and secreted amount of protein for the inflammatory cytokines IL-1β, IL-8, TNF-α, and MIP-1α in human AMs from smokers (**A**) and non-smokers (**B**). The mRNA levels in adherent cells were compared with those of cells directly after their isolation by BAL. Cells exposed to air were compared with control cells (i.e. only cultured in medium). Cells exposed to 5 or 20 ppm of NO_2 were compared with air-exposed cells. Cells exposed to 20 ppm of NO_2 and then treated with LPS were compared with cells exposed to air and then treated to LPS. For more details see Paper I.
n.a. = not analysed, — = no changes, ↑ = increased, ↓ = decreased

A) Smokers

Treatment/Exposure	IL-1β		IL-8		TNF-α		MIP-1α	
	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein
Culture and adhesion	↑	n.a.	↑	n.a.	↑	n.a.	↑	n.a.
Air	—	—	—	—	—	—	—	—
5 ppm NO_2	—	—	—	—	—	—	—	—
20 ppm NO_2	—	—	—	↓	—	↓	—	↓
20 ppm NO_2 + LPS	↑	↑	↑	↑	↑	↑	↑	↑
Air + LPS	↑	↑	↑	↑	↑	↑	↑	↑

B) Non-smokers

Treatment/Exposure	IL-1β		IL-8		TNF-α		MIP-1α	
	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein
Culture and adhesion	↑	n.a.	—	n.a.	↑	n.a.	n.a.	n.a.
Air	—	—	—	—	—	—	n.a.	—
5 ppm NO_2	↓	—	—	—	↓	↓	n.a.	—
20 ppm NO_2	↓	—	—	—	—	↓	n.a.	—
20 ppm NO_2 + LPS	↑	↑	—	—	↑	↑	n.a.	↑
Air + LPS	↑	↑	—	—	↑	↑	n.a.	↑

Taken together, this study could show that the exposure to NO_2 did not increase the expression and release of important inflammatory cytokines from alveolar macrophages. In contrast, the release of some of the cytokine tested was inhibited by the exposure, but the AMs from non-smokers were less sensitive to the inhibitory effects of NO_2. It is of interest that smoker's cells, whose basal ability to release cytokines is already compromised, clearly exhibited higher sensitivity to the inhibitory effects of the gas. This could provide a plausible explanation of the increased frequency of chronic infections evident in smoker's lung.

6.3.2 Exposure of healthy individuals to a low-level concentration of ozone (Paper II)

As already described before, two healthy volunteers were exposed to 0.2 ppm ozone for 2 hours, during which time light exercise on a bicycle ergometer was alternated with rest in a 15-minute cycle. This exposure situation is comparable to a situation of summer-time, outdoor activity, experienced in many urban areas in Central Europe and USA (i.e. the concentration of ozone and the duration of the exposure). Bronchoscopy with BAL lavage has been performed 4 hours after termination of exposure. This single sampling time-point is perhaps not optimal, since changes in mRNA levels may occur both at earlier as well as later times points and important changes may be missed. However, it is known that alteration of mRNA levels, corresponding to early responsive genes, can be detected as early as one hour after treatment whereas other changes take place after many hours or days.

Each individual was exposed twice, once to air and once to ozone. The BAL cells obtained after air exposure were used to calculate the steady state levels of mRNA transcripts present in the BAL cells.

6.3.3 Analysis of altered mRNA profiles upon ozone exposure (Paper II)

The enhancement of allergic inflammations induced by exposure to ozone has been suggested to be mediated by the generation of reactive oxygen species, which can alter the redox state in affected cells. It is now clear that cells can sense changes in redox state and adapt their expression of genomic information in order to combat the insult. A number of important transcription factors have been shown to be redox-sensitive and their activation leads to changes in gene expression. However, information about alterations in mRNA transcription profiles in the lung, which may explain the molecular mechanisms behind ozone toxicity, are still lacking. Data from *in vivo* exposure of humans to low concentration of ozone has not been published yet and the results presented in this study may contribute to the understanding of the negative effects of ozone, in spite of the fact that only data from two individuals is available.

The large-scale screening, using Affymetrix GeneChip technology, revealed that up to 20% of the steady state mRNA levels for genes represented on the chip were altered in both individuals. Many of the affected genes could be classified to important pathways within the cell, such as, synthesis, modification and repair of nucleic acids, transcriptional initiation, inter- and intra-cellular signal transduction, cytoskeletal organisation, inflammation, and post-transcriptional protein modification. Interestingly,

many of these alterations were individual-specific, which is in accordance with earlier studies demonstrating that the sensitivity to the gas varies greatly between individuals. A sub-group of 10-20% of the population is thought to be "responders", whilst others are classified as "non-responders" when it comes to lung function decrements caused by ozone.

Common alterations were also detected and amongst these some interesting changes deserve a mention. Several genes whose products are involved in DNA and RNA processing, such as the damage-specific DNA binding protein 48 (DDB48), were noted, as well as genes coding for some important cell signalling kinases and receptors, including PRK2, CD43, OX40 and the acetylcholine receptor. Interestingly, the steady state levels of mRNA coding for the secreted protein Ficolin-1 were increased in both individuals after ozone exposure. This protein could provide a possible biomarker of ozone exposure in the lining fluid of human lungs.

In summary, these data present new insights in the molecular events occurring in human alveolar macrophages in response to ozone-induced toxicity and contribute to the evidence that these cells do participate in the development of the stress response. The data also provide evidence for complex biological responses in this pulmonary cell type, occurring close to the accepted safety exposure limits for this gas in the environment.

6.3.4 Protein S-glutathionylation due to exposure of cells to diamide or H_2O_2 (Paper III)

During situations of oxidative stress, changes in the redox state of the cell may lead to reversible interaction of glutathione with critical protein thiols, by the formation of GSH-protein mixed disulphides. The formation of mixed disulphides may, in turn, alter the functional properties of a number of target proteins vital to cellular regulation. As described above, ECV304 cells or A549 cells were exposed to two different agents of oxidative stress, diamide or H_2O_2. The intracellular concentration of reduced GSH and the formation of mixed disulphides were analysed during and after the exposures.

Exposure of A549 cells to diamide resulted in a considerable oxidation of cellular GSH during one hour of exposure. When the stimulus was removed cells recovered their intracellular GSH levels within 3 hours after exposure. The measurement of the formation of mixed disulphides, formed under the same conditions, revealed that circa

12% of the cellular GSH transiently associated with A549 cell protein, but the levels were restored to base line 3 hour after exposure.

The same treatment of ECV304 cells resulted in near-quantitative oxidation of cellular GSH. This was reflected in considerably more GSH-protein disulphides formation than in A549 cells. Even in ECV304 cells the levels of both GSH and GSH-protein disulphides returned to normal 3 hours after exposure showing that these effects were reversible. These results are in concordance to the different stress-sensitivity of these two cell types. It must also be noted that A549 cells were exposed to higher concentrations of diamide than ECV304 cells but these concentrations were comparable when effects on cell viability were balanced.

In contrast to the situation with diamide, H_2O_2 exposure had little influence on the cellular GSH content, which was reflected in limited formation of GSH-protein disulphides in either cell type.

6.3.5 Alteration of mRNA profiles after exposure to diamide or H_2O_2 (Paper III)

Following the same protocols of exposure employed for the analysis of GSH oxidation, cell samples were prepared and used to record changes in mRNA profiles for both cell lines and both exposures. Macro-array analysis, using ATLAS cDNA Human Stress Gene Array, revealed that diamide induced a wide variety of stress genes mRNA levels in both cell types. However, the highest number of discrete inductions and the highest magnitude in the changes occurred in the ECV304 cells. Interestingly, the gene screening analysis confirmed the constitutive expression of several stress genes in A549 cells, which may explain the ability of these cells to tolerate oxidative insult.

Among the alterations caused by diamide exposure, increased steady state mRNA levels for several heat shock proteins and various DNA repair enzymes were detected, including HSP27, HSP70 and GADD153. In contrast, exposure to H_2O_2 failed to alter the levels of many of the diamide-inducible mRNAs in either cell line.

6.3.6 Protection against heat shock and potassium bromate (Paper III)

The induction of an integrated molecular chaperone response (HSPs and DNA husbandry proteins) by diamide treatment resulted in increased thermotolerance and cytoprotective effects of oxidative stress induced by the DNA-damaging pro-oxidant potassium bromate.

In summary, the data in this work suggest a correlation between the degree of S-glutathionylation of cellular proteins during oxidative stress and the induction of genes involved in protein/DNA protection. Moreover, the results lend support to a molecular link between altered GSH and protein cysteinyl redox status and the co-ordinated regulation of complex patterns of altered gene expression during sub-lethal oxidative stress conditions. In turn, these molecular events provide functional cyto-protection against heat shock and oxidative stress.

6.3.7 Exposure of A549 cells to H_2O_2 causes cell cycle arrest, DNA damage and induction of caspase-3 (Paper IV)

The oxidative conditions caused by inhalation of oxidant gases may compromise different cell types in the human lung. The subsequent inflammatory conditions may be the result of intricate signalling events between these cells. However, the role of each individual cell type in these processes is still unknown. In previous studies, presented in this thesis, the effects of NO_2 and ozone exposure were analysed using AMs. In this series of experiments, the effects of H_2O_2 exposure were examined in the A549 human epithelial cell line. Hydrogen peroxide may be formed in the lining fluid of the lung either by the reaction of ozone with unsaturated fatty acids present in the ELF, or in cell membranes, but can also be released by activated inflammatory cells.

Exposure of A549 cells to sub-toxic concentrations of H_2O_2, resulted in DNA damage and a near-quantitative suppression of cell proliferation. These findings suggests that, in response to DNA damage, the cells delay cell cycle progression from G_1 to S and from G_2 to M by induction of anti-proliferative genes. When oxidative damage is severe, checkpoint signalling may cause cells to either undergo apoptosis, or to enter an irreversible G_0 state to prevent perpetuation of damaged genetic templates. Indeed, apoptotic cells were observed in the cell culture and increased caspase-3 activity was detected in H_2O_2 treated A549 cells.

6.3.8 Alterations of mRNA profiles in A549 cells after H_2O_2 exposure (Paper IV)

The major aim of this study was to perform a large-scale screen of alterations in steady state mRNA profiles in response to H_2O_2. For this purpose, cells were exposed to the oxidant for one hour and collected 1, 3, 6 and 12 hours after exposure. The experiment was performed twice maintaining the same culture and exposure conditions. RNA samples purified from control and exposed cells were used in a large gene screening

using Affymetrix micro-array technique. The results of the analysis of changes in mRNA levels revealed a cluster of strongly modulated gene transcriptions, whose concerted action can be functionally related to the findings mentioned above. Thus, induction or suppression of cell cycle-interacting genes, several regulated by the transcription factor p53, could be detected and confirmed in both experiments. As discussed in the general background-section, p53 activation may result from processes related to DNA damage. Notable inductions included mRNAs coding for p21 (WAF1) and the anti proliferative and DNA damage-induced BTG-2. Suppressions for the mRNAs for cell cycle-related genes, such as CDC25C, CDC20 and C20orf1, and cyclin2 were also recorded.

In addition, three of the induced mRNAs code for the potent mediators of apoptosis, BAX, GADD34 and TRAIL-receptor 2. BAX is known to be involved in the loss of membrane potential and the release of cytochrome C from the mitochondria. Expression and activation of the growth arrest and DNA damage-inducible protein GADD34 is a downstream event in apoptotic signalling pathways and may contribute to apoptotic processes. One of the most interesting pro-apoptotic proteins recently identified is the TRAIL-receptor 2 or DR5. Binding of the TRAIL (TNF-related apoptosis-inducing ligand) to the receptor triggers apoptosis by inducing the oligomerisation of the intra-cellular death domain followed by caspase activation. Thus, when the TRAIL-ligand was added into the medium of H_2O_2-pretreated cells, caspase-3 activation was more pronounced compared with cells treated with H_2O_2 only. This suggested that H_2O_2-pretreated cells might become more prone to undergo apoptosis via the TRAIL-receptor 2 pathway.

Furthermore, induced species also included Heme oxygenase 1, important signalling molecules in the inflammatory cascade, such as IL-8 and MIC-1 and other genes involved in signal transduction, transcription, protein synthesis and mitochondrial function. The group of genes whose mRNA levels were suppressed contained several members of structural proteins involved in the formation of the cytoskeleton, as well as species related to DNA synthesis and histone complex and genes belonging to signal transduction pathways.

The results in this study show a multi-faceted and highly coordinated response in A549 cells upon exposure to sub-toxic concentrations of H_2O_2. The p53-dependency of a great number of the affected genes suggests that the events occurring post-exposure are based on the ability of the cells to sense DNA damage, caused by the oxidant, and

induce a response which manifests and maintains arrest in cell cycle progression and primes the onset of apoptotic events.

6.4 CONCLUSIONS

The adverse effects caused by the inhalation of oxidant gases, such as NO_2, ozone and/or the formation of secondary oxidant products, were investigated at the level of various molecular end-points in human lung cells. The results presented and discussed in this thesis have been focused on the connection between the toxicity of the oxidative agents used in the different studies, and the changes in mRNA profiles in response to conditions of oxidative stress, that did not cause acute necrotic cytotoxicity.

The expression and secretion of some inflammatory mediators from human alveolar macrophages were inhibited by the exposure to NO_2 *in vitro*, in particular in cells derived from habitual smokers. This may provide a plausible explanation to the fact that NO_2 inhalation may impair resistance to airway disorders especially in smokers.

Exposure to low-concentration of ozone *in vivo* caused changes in mRNA profiles in human alveolar macrophages. The large-scale screening of these changes revealed a number of ozone-responsive genes belonging to a variety of important biological pathways, including: Synthesis, modification and repair of nucleic acids, inter- and intra-cellular signal transduction, cytoskeletal organisation, inflammation and post-translational protein modification. These findings also report on considerable biochemical effects of ozone in the lung at levels close to the safety limits recommended by international air quality guidelines and standards.

Expression of a number of heath shock proteins and other DNA-damage sensitive genes may depend on the changes in intracellular redox status. Exposure of cells to the thiol-oxidising agent diamide *in vitro* lead to increased mRNA levels and expression of stress responsive-genes and to the oxidation of GSH with the concomitant S-glutathionylation of cellular proteins. In contrast, *in vitro* exposure of cells to hydrogen peroxide failed to induce many of the diamide-responsive genes, which was accompanied with a modest oxidation of GSH or formation of protein-GSH mixed disulphides. Thus, redox-sensitive alteration of gene expression can be correlated to the oxidation of GSH and the concomitant formation of mixed protein-GSH disulphides. In addition, induced expression of protein and DNA chaperones by diamide exposure resulted in both cytoprotection against heat shock and the DNA-damaging pro-oxidant potassium bromate.

Hydrogen peroxide can be formed in the lung upon ozone exposure and/or be released into the surrounding tissue by inflammatory cells. Exposure of the human epithelial cell line A549, to sub-toxic levels of hydrogen peroxide, resulted in arrest in cell-cycle progression and activation of apoptotic events, including activation of caspase-3 *per se* and the augmentation of TRAIL-dependent caspase-3 activation. These effects may be a consequence of the DNA damage caused by the oxidant. Hydrogen peroxide treatment lead to the alteration in mRNA profiles for a variety of known p53-dependent genes, but also many novel changes in mRNA levels of many genes not previously associated with redox-dependent regulation, which can be functionally related to cell cycle arrest, apoptosis and DNA damage.

The overall response of the cell to macromolecular damage during oxidative stress contains a complex pattern of genetically derived stress response elements, comprising delicate "molecular language". Therefore, the results of these studies contribute to the understanding of the mechanisms of oxidative stress in human lung cells, as well as provide evidence for co-ordination in the regulation of the redox-sensitive machinery of gene expression.

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