GLUTATHIONE, THE REDOX SENSITIVE TRANSCRIPTION FACTORS AP-1 AND NF-κB, AND EARLY ONE ADENOVIRAL PROTEIN IN HUMAN LUNG IN SMOKING RELATED LUNG DISEASE

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I declare that this thesis was written by me and that the work contained herein was performed by myself except where otherwise stated. The work has not been submitted for any other degree or professional qualification.

ABSTRACT

The major factor in the development of chronic obstructive airways disease (COPD) is cigarette smoking, although not all smokers develop chronic obstructive pulmonary disease. Inflammation and destruction has been shown in the lungs of smokers with chronic obstructive pulmonary disease. Oxidative stress, both from cigarette smoke and oxidants generated endogenously by cellular processes, contribute to the inflammation that occurs in the lungs in chronic obstructive pulmonary disease. It remains unclear why certain individuals appear susceptible to the effects of cigarette smoke and go on to develop inflammation and airflow limitation. The glutathione redox system is an important antioxidant protective system within the lungs, and this system may play a critical role in the development of inflammation. An alteration in the transcription of pro-inflammatory cytokines and mediators is also likely to contribute to the inflammation within the lung. Nuclear factor kappa-B (NF-κB) and activator protein-1 (AP-1) are both redox sensitive transcription factors, and are involved in the regulation of the gene transcription of many pro-inflammatory mediators. Activator protein-1 and nuclear factor kappa-B have a close relationship with γ-glutamylcysteine synthetase (γ-GCS) (glutamate cysteine ligase, GCL), the rate limiting enzyme in the synthesis of glutathione, with the γ-glutamylcysteine synthetase gene containing various elements including an Activator protein-1 binding site. Susceptibility to the effects of cigarette smoke is likely to explain why certain individuals develop chronic obstructive pulmonary disease, and this susceptibility may arise from an earlier viral infection such as adenoviral infection that lies dormant, but which in the face of an oxidant stimulus such as cigarette smoke augments the inflammatory process.

The in vivo studies herein have examined glutathione and γ-glutamylcysteine synthetase, gene transcription, oxidant/antioxidant imbalance, the redox sensitive transcription factors nuclear factor kappa-B and activator protein-1, and have

assessed for the presence of the early one adenoviral protein in human lung in smokers and patients with COPD.

The results show similar levels of total glutathione in the lungs of patients with and without airflow obstruction, and decreased γ-glutamylcysteine synthetase activity in patients with severe airflow obstruction who have undergone lung volume reduction surgery for emphysema compared to those with no airflow obstruction. Local lung oxidative stress as measured by malondialdehyde, and trolox equivalant antioxidant capacity a marker of systemic oxidative stress did not correlate with lung function. DNA binding of Nuclear factor kappa-B correlated with lung function as measured by percent predicted forced expiratory volume in one second (FEV_1), however no such relationship was found with Activator protein-1 DNA binding. Examination for early one adenoviral gene and protein in human lung tissue failed to reveal conclusive results.

In conclusion levels of glutathione in human lung tissue, oxidative stress including both lung and systemic oxidative stress, and the DNA binding of activator protein-1 in lung are not related to the degree of airflow obstruction present.

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ABBREVIATIONS

ARE	acute response element
4-HNE	4-hydroxy-2,3-nonenal
ANOVA	analysis of variance
AP-1	Activator Protein-1
BAL	broncho alveolar lavage
BCA	bicinchoninic acid
BSA	bovine serum albumin
CBP	CREB binding protein
cDNA	complementary DNA
COPD	chronic obstructive pulmonary disease
CREB	cyclic AMP response element binding proteins
CT	computerised tomography
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleatide triphosphate
DTT	dithiothreitol
E1A	early 1 adenoviral
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELF	epithelial lining fluid
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
EpRE	electrophile response element
FEV_1	forced expiratory volume in 1 second

FVC	forced vital capacity
fra1	fos-related antigen-1
G293	Graham 293
GAPDH	glyceraldehyde-3-phosphate
GCL	glutamate-cysteine ligase
GMCSF	granulocyte monocyte colony stimulating factor
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidised glutathione
GST	glutathione S-transferase
H_2O_2	hydrogen peroxide
HAT	histone acetyltranasferase
HDAC	histone deacetylase
ICAM-1	intracellular adhesion molecule –1
IgE	immunoglobulin E
IKK	I kappa B kinase
IL-1β	interleukin-1beta
IL-6	interleukin-6
IL-8	interleukin-8
IFN-γ	interferon-γ
IκB	inhibitory kappa B
LPS	lipopolysaccharide
MDA	malonyldialdehyde
mg	milligram
MIP-1α	monocyte inhibitory protein-1

MCP-1	monocyte chemoattractant protein-1
mRNA	messenger ribonucleic acid
μg	microgram
Na_2VO_3	sodium orthovanadate
NAC	N-acetyl-L-cysteine
NF-κB	nuclear factor-kappa B
NP-40	nonidet P-40
$^{\bullet}OH$	hydroxyl radical
O_2^{-}	superoxide anion
PAF	platelet activating factor
PBEC	primary bronchial epithelial cell
PBS	phosphate buffered saline
PCAF	p300/CPB associated factor
PCR	polymerase chain reaction
PI-3K	phosphatidylinositol 3-kinase
RANTES	regulated on activation, normal T-cell expressed and secreted
RNS	reactive nitrogen species
ROS	reactive oxygen species
rpm	revolutions per minute
SOD	superoxide dismutase
SDS	sodium dodecyl sulphate
TBE	Tris borate EDTA buffer
TE	Tris-Cl EDTA buffer
TEAC	trolox equivalant antioxidant capacity
TEMED	(M,M,M',M'- tetramethylethylemediamine)

TGF-β	transforming growth factor-beta
TNF-α	tumour necrosis factor-alpha
γ-GCS	gamma-glutamyl cysteine synthetase
γGT	gamma-glutamyl transferase

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CHAPTER 1

INTRODUCTION

1.1 Chronic Obstructive Pulmonary Disease (COPD)

Chronic obstructive pulmonary disease (COPD) is a disease, associated with progressive airflow limitation, which can ultimately lead to respiratory failure and death. The clinical symptoms of COPD include cough with sputum production and increasing breathlessness. Chronic bronchitis is defined in clinical terms as cough with sputum production for most days for 3 months for at least 2 years (chronic mucous hypersecretion). The symptoms of chronic bronchitis generally predate the development of airflow obstruction and breathlessness.

The characteristic physiological feature of COPD is airway obstruction, which is largely fixed (Pauwels *et al.,* 2001). COPD includes emphysema, which is defined anatomically by permanent, destructive enlargement of airspaces distal to the terminal bronchioli without evidence of obvious fibrosis, bronchitis and bronchiolitis (Snider., 1985; Snider., 1989). Airflow obstruction is determined by measuring the forced expiratory volume in one second ($FEV_{1)}$ and the forced vital capacity (FVC). The presence of airflow obstruction is defined by an $FEV_1 < 80\%$ predicted and an FEV_1 / FVC ratio of < 70% predicted (BTS guidelines 1997). The natural history of COPD has been described by Fletcher who showed that FEV_1 declines continuously with age and that sudden large irreversible falls in FEV_1 are rare (Fletcher and Peto, 1977). Non-smokers lose FEV_1 at a slower rate than smokers. Their study identified a group of smokers who lost FEV_1 at a faster rate, and in so doing identified a group of smokers who appeared to be more susceptible to the effects of cigarette smoke. However, it is not known why certain individuals develop COPD.

The most important factor in the development of COPD is cigarette smoking (Doll *et al.,* 1994). Not all smokers will go onto develop COPD. The proportion of smokers that go onto develop clinically significant COPD is approximately 15-20% (Tashkin *et al.,* 1984), however a more recent report suggests this is higher at around fifty percent (Lundback *et al.,* 2003). Smoking cessation is the only intervention that can modify the natural history of COPD (Anthonisen *et al.,* 1994). Traditionally cough

and sputum have been considered to be less important, and that mortality and morbidity in those suffering COPD is related to airflow obstruction rather than mucus hypersecretion (Traver *et al.,* 1979; Wiles *et al.,* 1991; Vestbo *et al.,* 1989). However, this idea has been challenged in more recent work from the Copenhagen City Heart Study which demonstrated an association between hypermucus secretion and mortality and the development of exacerbations of COPD (Pistelli *et al.,* 2003). FEV_1 is a powerful predictor of mortality from COPD (Thomason *et al.,* 2000; Lange *et al.,* 1990), and longitudinal population based studies have shown that impaired pulmonary function is a strong predictor of increased mortality (Tockman and Comstock 1989; Hole *et al.,* 1996). Also, the effect that smoking cessation has on reducing mortality is not just restricted to those with impaired lung function (Pelkonen *et al.,* 2000).

Clinical airflow obstruction develops from a lengthy and initially asymptomatic process. In susceptible individuals that go on to develop COPD, a complex interaction between genetic and environmental factors occurs. There are a number of questions which remain unanswered with regard to the development of COPD. Inflammatory responses may be different in susceptible smokers that go onto develop COPD, or alternatively the protective mechanisms may be impaired.

1.2 The Anatomy of the Human lung

The function of the respiratory system is to extract oxygen from the inhaled air into the pulmonary circulation, and to eliminate the waste gas carbon dioxide. The respiratory tract is divided into an upper respiratory tract consisting of nose, pharynx and larynx, and lower respiratory tract consisting of the airways and the lungs. The larger, upper airways of the lungs consist of the trachea, main and lower lobe bronchi and these airways progressively divide into smaller airways, and as they do so the anatomical structure changes. The larger upper airways the trachea and bronchi contain cartilage, but the more distal smaller airways or bronchioles are without

cartilage. In the respiratory bronchiole there are increasing numbers of alveoli, and the final lung unit is the acinus, in which gaseous exchange takes place.

Within the acinus, the alveolar wall is composed of type I (very thin) and type II (more cuboidal in shape) alveolar cells, basement membranes, interstitial tissue and capillary endothelial cells. Type I cells are fewer in number than type II, but because of their flattened shape have a larger surface area. They are derived from type II cells and do not undergo mitosis. The function of type I alveolar epithelial cells is for the diffusion of gas from alveolus to capillary. Type II cells are more numerous than type I and have microvilli on their surface. Their cytoplasm is rich in mitochondria and endoplasmic reticulum, and lamellar bodies are present. They are the precursor of type I cells and they tend to proliferate in acute alveolar injury. Endothelial cells line the alveolar capillaries.

The bronchial wall consists of mucosa which is pseudostratified, the submucosa and a fibrocartilaginous layer. Most of the mucosa is covered by ciliated cells (which convey secretions towards the pharynx), interspersed with non-ciliated cells. Goblet cells secrete mucin and their numbers reduce in the more distal as the airways. Clara cells are found predominantly in the bronchioles and distal bronchi and their role is secretory. The serous cell is a secretory cell and has microvilli present on its surface.

Alveolar macrophages are the main phagocytic cell of the acinus. They are derived from bone marrow promonocyte stem cells, enter the circulation as monocytes and reach the lung through the capillary endothelium and are able to penetrate the alveolar epithelium into the alveolar space. They have phagocytic roles, secretory and immunological functions.

The inner layer of the alveolar epithelium is separated from the alveolar gas by a thin layer of surfactant. Surfactant consists of dipalmitoyl lecithin and dipalmotoyl phosphatidyl ethanolamine and proteins.

The amount of mucous secreting goblet cells present in the airways decreases as the alveolus is approached. There are no goblet cells and hence no mucous in the

alveolus, and the fluid within the alveolus is known as alveolar epithelial lining fluid. The epithelial lining fluid (ELF) is an important area as it is in direct contact with the type I and type II cells, and macrophages and may offer protection to these cells from various stresses.

1.3 Pathological changes in the lungs in COPD

Effects of cigarette smoke on the lungs

In this section the pathology of the airway structure is described with particular reference to cellular and morphological changes that occur in cigarette smoking and clinical airflow obstruction.

Cigarette smoke is the major aetiological factor in the development of COPD. Pathological changes can be found throughout the entire respiratory tract in response to cigarette smoke (Jeffery, 1998; Cosio *et al.,* 1980; Niewoehner *et al.,* 1974), even before the development of symptoms of chronic bronchitis or breathlessness. Niewoehner examined the lungs of healthy people who had died in road traffic accidents and found an inflammatory reaction in the peripheral airways of smokers (Niewoehner *et al.,* 1974). Cigarette smoke leads to increased numbers of macrophages and neutrophils within the airway lumen (MacNee and Selby, 1993; Kuschner *et al.,* 1996). Chronic smoking leads to an increase in size of the submucosal glands, an increase in the number of goblet cells in the surface epithelium, and also the presence of goblet cells in the smaller more distal airways (mucous metaplasia) (Ebert and Terracio, 1975). In smokers Clara cells are replaced by mucus secreting cells with the potential for increased amounts of mucus in the smaller peripheral airways (Ebert and Terracio, 1975). The mucociliary clearance mechanism is affected by cigarette smoking (Wanner, 1985) with reduction in numbers of ciliated cells and cilia length. In normal subjects the mucus is in discrete flakes, but in smokers with bronchitis it is found in a more continuous layer over the cells. These features lead to pooling of secretions and ultimately to an environment that favours bacterial growth.

The precise mechanism for increased mucus production remains unclear. IL-4 is known to be important in the pathogenesis of asthma and also in airway wall eosinophilia (Gross *et al.,* 1993, Cho *et al.,* 2005). IL-4 can also induce mucin production and secretion (Dabbagh *et al.,* 1999), and in smokers with chronic bronchitis the IL-4 gene is strongly expressed in the inflammatory cell infiltrate in the bronchial sub-epithelium and the submucous secreting glands (Zhu *et al.,* 2001). Oxidative stress has also been shown to promote mucus hypersecretion (Takeyama *et al.,* 2000). It is possible that in the early stages of lung inflammation there is upregulation of IL-4 and this may be responsible for the clinical symptom of increased mucus secretion in chronic bronchitis. No difference in m RNA expression for either IL-4 or IL-5 is found in lung biopsy tissue from healthy non smokers, patients with COPD and patients having an exacerbation of COPD (Zhu *et al.,* 2001).

Th2 cytokines may have a role in the activation of proteolytic pathways and contribute to the development of COPD. In an inducible transgenic animal model with over expression of IL-13, the phenotype resembles that of human COPD (Zheng *et al.,* 2000).

The protease antiprotease hypothesis of the development of airway inflammation and subsequent COPD and emphysema postulates that there is a defect in the protective antiprotease shield. It has been suggested that emphysema is the product of an abnormal increase in locally produced proteases and/or reduction in pulmonary antiproteases and in the absence of normal repair, proteolysis leads to increased tissue destruction and airspace enlargement. Human neutrophil elastase (HNE) is one such protease, consisting of 267 amino acids and is capable of breaking peptide bonds with catalytic activity against matrix substrates such as elastin which provide the structural stability of the lungs. Certainly animal models have shown that neutrophil elastase contributes to cigarette smoke-induced emphysema in mice (Shapiro *et al.,* 2003). In the human lung studies of the role of HNE have been inconsistent with reports of HNE localized in areas of diseased lung (Damiano *et al.,*

1986) and others reporting an inverse relationship of HNE to airspace destruction (Eidelman *et al.,* 1990). HNE can induce mucin production (Voynow *et al.,* 1999), and this may occur by virtue of the proteolytic activation of an epidermal growth factor receptor (EGFR) signaling cascade involving TGF-β (Kohri *et al.,* 2002).

IL-13 may also have a role in the mucus hypersecretion, and increased expression of IL-13 has been shown in bronchial wall cells of chronic smokers with chronic bronchitis compared to asymptomatic smokers (Miotto *et al.,* 2003).

The immune system plays a vital role in protecting the respiratory mucosa from infection, but these systems need to be under tight control so that undesirable effects do not ensue. The cells involved in this immune response are T cells, and there is a network of T cells and dendritic cells that present antigen. CD8+ T cells are MHC class I restricted whereas CD4+ cells respond to antigenic peptides presented to the MHC class II cleft. MHC I pathway collects antigen present in the cytosol of the cell such as viral or broken down self proteins. Soluble protein antigens enter the MHC class II pathway. These two pathways are not completely exclusive when processing antigen. CD4+ cells can be classified according to the cytokines produced, with those that secrete interferon γ (IFN-γ), IL-2, and tumour necrosis-β (TNF-β), but not IL-4, IL-5, IL-6, and IL-10 or p600 (IL-13) (Th 1) and those that make IL-4, IL-5, IL-6, and IL-10 and IL13 (Th 2) (Mosmann *et al.,* 1986). CD4+ T cells have a better survival, less susceptibility to apoptosis and appear to be resistant to cytokine-induced anergy compared to CD8+ T cells. Th1 responses are associated with infection with pathogenic organisms, but can go on to cause inflammation and chronic tissue damage.

T Cell Sub-populations in COPD

The inflammatory reaction in COPD may be in part the result of uncontrolled Th1 responses leading to chronic tissue damage. Immunohistochemical examination of human lung tissue has been helpful in attempting to define pathological changes, and

T cell types, and inflammatory cell profiles in COPD; although some reports have shown conflicting results (see table 1.1 for summary of findings).

Table 1.1
Summary of T cell findings, neutrophils and macrophages in smokers and COPD subjects

Biopsy Specimen	subject groups	Cell findings	Reference
Bronchial	**chronic bronchitics** vs normal	↑ total lymphocytes neutrophils, eosinophils, macrophage ↔	Saetta *et al.*,1993
Bronchial	**smokers, chronic bronchitis and airflow obstruction** vs no obstruction	↑ T lymphocytes, ↑ macrophages submucosa neutrophil ↔ lymphocyte subpopulations ↔	di Stefano *et al.*, 1996
Bronchial	**Severe airflow obs** vs mild/mod airflow obs vs no airflow obs	CD3, CD4, CD8 ↔ } subepithelium ↑ neutrophils ↑ macrophages ↑MIP-1α	di Stefano *et al.*, 1998
Bronchial	**severe obstruction** vs mild vs no obs	↓ CD8, ↓ CD3 (↓CD3 CCR5)	di Stefano *et al.*, 2001
Bronchial	**airflow obs** vs no obs	↑ CD3, ↑CD8, neutrophils ↔	O'Shaughnessy *et al.* '97
Bronchial	**exsmokers with airflow obs** vs without obs	CD8=CD4 } submucosa	Rutgers *et al.*, 2000
Bronchial	**symptomatic smokers** vs never smokers	↑CD68+ } submucosa	Amin *et al.*, 2003

Table 1.1 continued
Summary of T cell findings, neutrophils and macrophages in smokers and COPD subjects cont'd

Biopsy Specimen	subject groups	Cell findings	Reference
Resected lung (bronchial ca)	**airflow obstruction** vs no airflow obstruction	↑CD8, ↔CD4, ↔ macrophages ↔ neutrophils	Saetta *et al.*, 1998
Resected lung	**smokers with chronic bronchitis** vs Smokers with no chronic bronchitis	(in epithelium) ↑ neutrophils CD4+↔ CD8 ↔ eosinophils ↔ macrophages ↔	Saetta *et al.*, 1997
Resected lung	**smokers with COPD**/smokers no COPD/ never smokers	↑CD8 pulm parenchyma & pulm arteries	Saetta *et al.*, 1999
Resected lung	**smokers with airflow obstruction/** Smokers with no airflow obstruction/ Non smokers with normal lung function	↑Goblet cells in smokers COPD versus non smokers ↑ CD45 smokers ± COPD versus non smokers ↑ macrophages smokers ± COPD vs non smokers ↑ CD8+ smokers + COPD vs non smokers neutrophils ↔ CD4+ ↔	Saetta *et al.*, 2000

Bronchial biopsies from subjects with both mucus hypersecretion and airflow obstruction have demonstrated significantly higher numbers of T lymphocytes and macrophages in the bronchial submucosa, but no difference in lymphocyte subpopulations or in neutrophil counts, compared to those with out airflow obstruction (di Stefano *et al.,* 1996). In severe airflow obstruction bronchial biopsies have shown increased neutrophil, macrophage and NK lymphocyte numbers and an increase in MIP-1α positive epithelial cells, but within the subepithelium there was no significant difference in the numbers of CD3, CD4 and CD8 T lymphocytes compared to those with no obstruction (di Stefano *et al.,* 1998). A later study by Di Stefano *et al* in which bronchial biopsies from patients with severe airflow obstruction were examined by immunostaining appears to show conflicting results with reduced numbers of CD-8 and CD-3 lymphocytes compared to the groups with mild or no airflow obstruction, and reduced CD-3+ cells expressing the chemokine receptor CCR5 (Di Stefano *et al.,* 2001).

O'Shaughnessy (O'Shaughnessy *et al.,* 1997) examined the sub epithelial zone in bronchial biopsies and showed an increased numbers of CD3+ T lymphocytes and CD8+ cells in biopsies from subjects with airflow obstruction compared to normals. No difference in number of neutrophils, mast cells, eosinphils or B lymphocytes was found in those with and without airflow obstruction. Examination of bronchial biopsies in exsmokers with and without airflow obstruction in another study failed to show a predominance of CD8+ over CD4+ in the submucosa (Rutgers *et al.,* 2000). It is possible that cigarette smoke has a direct affect on airway CD8+ cells. Peripheral blood lymphocytes are affected by cigarette smoking (Hughes *et al.,* 1985) and mild to moderate smokers have been shown to have increased CD4+ and CD8+, and heavy smokers reduced CD4+.

The above studies have examined bronchial biopsies and so largely represent the more proximal airways. The subject numbers that made up each of the groups have also tended to be small with approximately 10 in each. A larger study by Amin and

co-workers examined bronchial biopsies in 16 never smokers and 29 asymptomatic smokers and found that asymptomatic smokers had increased CD68 (macrophage marker) positive, IL-8 positive, human neutrophil lipocalin (neutrophil marker) positive and eosinophil peroxidase (eosinophil marker) positive cells in the mucosa compared to never smokers (Amin *et al.,* 2003). Saetta (Saetta *et al.,* 1998) examined the more peripheral airways by using lung tissue from patients undergoing resection for peripheral lung tumours. The numbers were small in each group, and immuno staining showed no difference in numbers of CD 4+ T cells, macrophages or neutrophils, but an increase in CD 8+ lymphocytes was found in those with airflow obstruction. This finding conflicts with that of Bosken (Bosken *et al.,* 1992) who found that patients with airway obstruction had more B-lymphocytes in the airway than control subjects and that the number of submucosal polymorphonuclear leukocytes related to the amount smoked. Hogg and co-workers have examined resected human lung tissue and have shown an increased absolute volume of B cells and CD8 cells with increasing airflow obstruction (Hogg *et al.,* 2004).

Neutrophil and lung inflammation

It is likely that the neutrophil plays an important role in airway inflammation in COPD. In response to cigarette smoke, peripheral blood neutrophils are less deformable and so are more likely to be retained within the pulmonary circulation (Drost *et al.,* 1992). Increased neutrophils have been shown to be present in the lung by immunostaining in those with severe airflow obstruction (di Stefano *et al.,* 1998), however this has not been the case when airflow obstruction is mild or moderate (Saetta *et al.,* 1999; Saetta *et al.,* 1998; O'Shaughnessy *et al.,* 1997; di Stefano *et al.,* 1996). Cigarette smoke leads to increased numbers of neutrophils and macrophages within the airway lumen (MacNee *et al.,* 1993). Neutrophil function is altered in COPD with increased chemotactic activity (Burnett *et al.,* 1987) and enhanced expression of adhesion molecules (Noguera *et al.,* 1998). There is also enhanced

peripheral neutrophil response to pro-inflammatory agents and enhanced respiratory burst in patients with COPD, compared to non smokers (Noguera *et al.*, 2001)

1.4 Free Radicals, Reactive Oxygen species

Oxidants are formed as a normal product of aerobic metabolism and biological systems are continuously exposed to oxidants. Reactive oxygen species (ROS) are metabolites of oxygen, and the lung is particularly susceptible to oxidant-mediated damage because it is the site of gaseous exchange and hence is an environment of high oxygen levels.

Oxidants can be produced at elevated rates under patho-physiological circumstances and include partially reduced oxygen metabolites, free radicals which have an unpaired electron in their outer orbit, and non-radical reactive oxygen species, which are capable of subtracting an electron from another compound so changing the oxidative state of both compounds. A free radical is a species capable of independent existence with one or more unpaired electrons, is usually unstable and can react with a wide range of compounds by extracting an electron, or more rarely donating an electron (Halliwell., 1991). ROS are metabolites of oxygen, and many are also free radicals. During respiration, bimolecular oxygen is reduced by 4 electrons, and partial reduction generates the superoxide anion ($O_2^{\bullet-}$).

$$O_2 + e^- \longrightarrow O_2^{\bullet-}$$

Free radicals in cigarette smoke are derived from both the gas and tar phase. Short acting oxidants such as $O_2^{\bullet-}$ and nitric oxide (NO) are predominantly found in the gas phase. NO and $O_2^{\bullet-}$ immediately react to form the highly reactive peroxynitrite ($ONOO^-$). The tar phase is also a metal chelator and can bind ions and generate H_2O_2. Also the tar and lung epithelial lining fluid contain metal ions which take part in the Fenton reaction to produce hydroxyl radicals ($^{\bullet}OH$). Hydrogen peroxide and

hydroxyl radical ($^{\bullet}OH$) are formed from the superoxide anion by the action of superoxide dismutases (SOD) on the superoxide anion. Hydrogen peroxide can also be generated by the Haber Weiss reaction in which ferrous ion and superoxide anion react together.

$$Fe^{3+} + 2H^{+} + O_2^{\bullet-} \longrightarrow H_2O_2 + Fe^{2+}$$

The Fenton reaction which involves the addition of an electron from a trace metal such as iron (Fe^{2+}) to hydrogen peroxide produces the hydroxyl radical (OH).

$$H_2O_2 + Fe^{2+} \longrightarrow O_2^{\bullet-} + HO + Fe^{3+}$$

This burden of oxidants in smokers lungs is enhanced further by the release of oxygen radicals from the influx and activation of inflammatory leukocytes, both neutrophils and macrophages. It has also been shown that leukocytes from smokers release increased amounts of oxidants such as superoxide anion and hydrogen peroxide compared to non smokers (Morrison *et al.,* 1999).

Within the environment of the lung, activated neutrophils, macrophages and eosinophils will generate ROS, with the formation of superoxide anion ($O_2^{\bullet-}$) which is rapidly converted to hydrogen peroxide (H_2O_2). Circulating neutrophils from cigarette smokers and patients undergoing an exacerbation of COPD release more $O_2^{\bullet-}$ (Rahman *et al.,* 1996). In animal models of lung inflammation, sequestered neutophils in the pulmonary microcirculation release more ROS than circulating neutrophils (Brown *et al.,* 1995). Not only do infiltrating inflammatory cells produce ROS, but the pulmonary epithelium can produce ROS (Rochelle *et al.,* 1998; Kinnula *et al.,* 1992). Piotrowski has also shown that differentiation from type II to type I alveolar epithelial cells is accompanied by an increase in hydrogen peroxide production (Piotrowski *et al.,* 2000). ROS can be generated intracellularly from the NADPH oxidase system and xanthine/xanthine oxidase. The primary ROS-generating enzyme system is NADPH oxidase which is present in phagocytes and

epithelial cells. There is also evidence that airway smooth muscle cells, and vascular smooth muscle and endothelial cells generate ROS from low activity NADPH oxidases homologous to phagocytic NADPH oxidases which act as intermediates in signaling in pathways (Brar *et al.*, 2002; Griendling *et al.*, 2000).

Detoxification of ROS occurs by a number of antioxidant defense systems which detoxify reactive products or convert them to products that are easily quenched by other antioxidants. Superoxide dismutate (SOD) is a cellular enzyme which forms part of the antioxidant defense which catalyses the conversion of superoxide anions (O_2^-) to hydrogen peroxide (H_2O_2) (Fridovich and Freeman, 1986).

$$2\,O_2^{\bullet-} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

Hydrogen peroxide is eliminated by the action of catalase (Chance *et al.*, 1979). Glutathione peroxidase also reduces hydrogen peroxide to water by oxidizing glutathione to glutathione disulfide (Cohen and Hochstein, 1963). Free radical scavengers are found in the tissues and serum and aid detoxification of oxidants. Low molecular weight free radical scavengers found in the tissue include vitamin E, vitamin C, reduced glutathione, α-tocopherol, taurine, uric acid and α-carotine. Higher molecular weight scavengers are found in the serum and include α-antitrypsin inhibitor, transferrin, albumin, caeruloplasmin, and amino acids methionine and cysteine.

Oxidative Stress

Oxidative stress results from an imbalance in the oxidant-antioxidant equilibrium in favour of oxidants. If the oxidant burden is so great these highly reactive oxygen species are not be detoxified by the oxidant defense mechanisms and they will damage cell components such as lipid membranes, proteins, carbohydrates and DNA.

Cell membranes are damaged by oxidative stress by virtue of their electron rich environment due to the presence of multiple carbon-carbon double bonds, with resultant lipid peroxidation. Reactive oxygen species including superoxide anion radical ($O_2^{\bullet-}$), hydroxyal radical (OH), hydrogen peroxide (H_2O_2), and nitrogen species, such as nitric oxide and peroxynitrite ($ONOO^-$), can all affect cellular function. Hydrogen peroxide is an important oxidant as it passes through lipid membranes relatively easily, and in so doing can generate further oxidants both intra and extracellularly. The local pH during the process of macrophage phagocytosis can help the release of transition metals from their stores with enhanced hydroxyl radical formation. Free iron in the ferrous form catalyses the Fenton reaction and also the superoxide driven Haber-Weiss reaction, which generates the $^{\bullet}OH$, a very aggressive oxidant capable of cellular damage with a half life of 10^{-9} seconds (Sies, 1991).

Oxidative stress can lead to an enhanced inflammatory process with generation of ROS, which can increase activation of transcription factors (Schreck and Baeuerle, 1994; Adcock *et al.,* 1994), alter chromatin remodeling and increase gene expression of pro-inflammatory mediators (Iwata *et al.,* 2002, Rahman *et al.,* 2002).

1. 5 Inflammatory mediators in Chronic Obstructive Pulmonary Disease

Cytokines

Cytokines are pluripotent mediators of inflammation that are produced and secreted by a wide range of cells including neutrophils, monocytes, macrophages, endothelial cells and epithelial cells. Cytokines and the chemoattractant cytokines (chemokines) act by binding to one or more cellular transmembrane receptors, and initiate intracellular signaling pathways that are involved in recruitment of leucocytes to sites of inflammation and injury (Strieter *et al.,* 1996). Chemokines are divided into two groups depending on the position of the first conserved cysteine residues at the amino-terminus; those which are adjacent are termed C-C and those that are separated by one amino acid are termed C-X-C. The cytokines associated with

COPD include TNF-α, interferon (IFN)-γ and (IL)-1β and IL-6 (Chung, 2001; Majori *et al.,* 1999).

Cellular and cytokine relationships in the lung

The alveolar and bronchial epithelium forms the first line of defence in the lungs to inhaled environmental stimuli, allergens and microbes. Neutrophils and macrophages are vital to the clearance of microbes, and it is well documented that there are increased neutrophil numbers in the airway lumen in smokers with COPD (Hunninghake and Crystal, 1983; Lacoste *et al.,* 1993; Pesci *et al.,* 1998). Cigarette smoke leads to recruitment of alveolar macrophages and neutrophils into the lungs (Kuschner *et al.,* 1996), and these infiltrating inflammatory cells produce endogenous factors such as TNF-α and interferon-γ. These primary mediators can provoke the production of secondary mediators in the local epithelium and also in infiltrating cells, and include lipid mediators such as prostaglandins, leukotrienes and platelet activating factor (PAF). These secondary mediators are chemotactic for neutrophils and eosinophils and macrophages, and can also activate macrophages and eosinophils and alter vascular and epithelial permeability (Rola-Pleaszczynski *et al.,* 1993; Cohn *et al.,* 1997).

BAL from smokers show increased inflammatory mediators compared to non smokers including IL-8, IL-1β, IL-6 and MCP-1 (Kuschner *et al.,* 1996). However blood derived monocytes/macrophages from smokers show reduced levels of these cyokines (Brown *et al.,* 1989; Soliman *et al.,* 1992; Yamaguchi *et al.,* 1989; Ohta *et al.,* 1998). Such conflicting studies demonstrate the complexity of the micropulmonary area and the likely contribution from surrounding cells and inflammatory mediators.

TNF-α can stimulate the epithelium to produce secondary mediators such as lipid mediators, reactive oxygen species (ROS), reactive nitrogen species (RNS), and cytokines (Martin *et al.,* 1997). These secondary mediators can affect epithelial cells and other local cells and lead to further inflammatory responses. Neutrophils and

macrophages can cause detrimental effects by causing airway wall damage and remodelling via the actions of secreted proteases. It is still unclear whether the macrophage or the neutrophil is the predominant cell implicated in the development of smoking related lung disease and emphysema. Churg et al (Churg *et al.,* 2003) have demonstrated in a recent mouse model that macrophage metalloelastase mediates acute cigarette-induced inflammation via TNF-α release.

Tumour Necrosis Factor alpha (TNF-α)

TNF-α interacts with two transmembrane receptors TNFR1 (TNFR p55) and TNFR2 (TNFR2 p75). In the majority of cells these receptors are co expressed, but there is evidence to suggest that TNF-R55 is primarily found on the surface of cells of epithelial origin and TNF-R75 on the cell surface of cells of myeloid origin (Brockhaus *et al.,* 1990). Proteolytic shedding of the extracellular cytokine binding domains of the TNF-α receptors occurs in response to various inflammatory stimuli, including endogenous TNF-α (Lantz *et al.,* 1990), and have been used as a marker of inflammation. Sputum obtained from COPD patients have increased neutrophils (Vernooy *et al.,* 2002; Peleman *et al.,* 1999) and increased IL-8 levels compared to healthy smokers (Keatings *et al.,* 1996; Yamamoto *et al.,* 1997). Keatings and co-workers (Keatings *et al.,* 1996) have also shown increased TNF-α in induced sputum in COPD, but Vernooy failed to confirm these findings in a later study; however Vernooy did show increased soluble TNF-α receptor TNF-R55 in induced sputum (Vernooy *et al.,* 2002). The source of the s TNF-R55 is not clear, but it may originate from neutrophils as activated neutrophils in vitro shed sTNF-R55 (Porteu and Nathan, 1990) and the neutrophil is the predominant cell type within the airway lumen (Keatings *et al.,* 1996).

Cultured bronchial epithelial cells secrete TNF-α in response to cigarette smoke (Mio *et al.,* 1997). Polymorphisms of the TNF-α gene result in constitutively higher levels of TNF-α (Wilson *et al.,* 1997). TNF-α can induce airway mucous cell metaplasia in vitro and vivo (Takeyama *et al.,* 2001), a reduction in interepithelial binding and cell death (Kampf *et al.,* 1999), deposition of collagen in alveolar walls in murine models of emphysema (Sulkowska *et al.,* 1997), and can also induce IL-1, TNF-α, IL-8, and MCP-4 expression (Cromwell *et al.,* 1992) and IFN-γ receptors on epithelial walls (Wu *et al.,* 1996). IFN-γ inhibits the proliferation of epithelial cells and this may contribute to the loss of epithelial integrity that is present in emphysema (Kampf *et al.,* 1999). TNF-α has multiple pro inflammatory actions such as neutrophil degranulation and associated release of proteolytic enzymes eg lysozyme, and stimulation of the respiratory burst (Richter *et al.,* 1989).

The importance of TNF-α in cigarette smoke induced emphysema is shown by the observation that mice lacking TNF-α receptors do not develop smoke induced inflammation (Churg *et al.,* 2002).

Interleukin-8 and COPD

Interleukin-8 (IL-8) is a 16 kDa protein that is a member of the C-X-C family of cytokines. The potential role of IL-8 in the development of COPD comes from studies that have examined IL-8 levels in BAL, induced sputum and lung tissue which have demonstrated increased levels of IL-8 (Pesci *et al.,* 1998; Keatings *et al.,* 1996; Fuke *et al.,* 2004) in the lungs of smokers with COPD. Tanino (Tanino *et al.,* 2002) has also shown that BAL IL-8 levels can be used to identify a population of smokers with subclinical emphysema on CT scanning. Immunostaining and *in situ* hybridization techniques for IL-8 have also shown that bronchiolar and alveolar epithelium stains more intensely in smokers with COPD than those without COPD (de Boer *et al.,* 2000). There is *in vitro* evidence to suggest that large airway

epithelium behaves differently to smaller peripheral airway epithelium in terms of oxidative stress and chemokine release (Witherden *et al.,* 2004; Mio *et al.,* 1997; Masubuchi *et al.,* 1998). There is increased IL-8 release from bronchial epithelial cells in response to cigarette smoke (Mio *et al.,* 1997). However, type II bronchial epithelial cells isolated from human lung tissue show inhibition of IL-8 release in response to cigarette smoke extract (Witherden *et al.,* 2004). Further, BAL obtained from proximal airways contain higher concentrations of IL-8 compared to BAL from more distal airways, and BAL IL-8 concentrations correlate with neutrophil counts in BAL, suggesting that neutrophils may be the source of increased IL-8 release (Mio *et al.,* 1997). The presence of increased levels of IL-8 in large airways in COPD exacerbations is suggested by elevated sputum levels of IL-8 (Aaron *et al.,* 2001), however BAL IL-8 levels in exacerbations of COPD are not altered compared to baseline levels (Drost *et al.,* 2005).

IL-8 is synthesised and released by bronchial epithelial cells (Nakamura *et al.,* 1991), macrophages, monocytes and neutrophils (Stieter *et al.,* 1990; Chung, 2001). IL-8 is a potent chemoattractant that is needed to recruit and activate neutrophils (Baggiolini, 1998) and is fairly resistant to changes in pH and proteolytic degradation compared with other chemoattractants (Steiter *et al.,* 1996). It is regulated primarily at the gene transcription level (Sica *et al.,* 1990), although post transcriptional control does occur (Yu *et al.,* 2003). The cytokines TNF-α and IL-1β usually cause the most expression of IL-8 within human cells (Brasier *et al.,* 1998), but reactive oxygen species (ROS) (Lakshminarayanan *et al.,* 1997) and viral and bacterial infection can also induce IL-8 production (Khair *et al.,* 1994). Stimulation of cells leads to rapid induction of IL-8, and there is evidence that production of IL-8 mRNA occurs within 60 minutes (Lakshminarayanan *et al.,* 1997). Rapid induction of IL-8 gene expression is mediated by transcription factors that bind to the IL-8 promoter region. The IL-8 promoter area contains binding sites for inducible transcription factors which are pro-inflammatory responsive elements important for IL-8 gene expression

(Mukaida *et al.,* 1994). In some circumstances there is both stimulus specific and cell type specific expression of the IL-8 gene, and this is probably related to the preferential activation and binding of transcription factors to the IL-8 promoter region (Lakshminarayanan *et al.,* 1998). There are two distinct promoter regions that are important for IL-8; a distal promoter element comprised of an AP-1 binding site and a proximal promoter area which has binding sites for both NF-IL-6 and NF-κB (Brassier *et al.,* 1998).

Relationship of IL-8 and NF-κB

The NF-κB binding site is the predominant cis acting element in the IL-8 gene (Roebuck, 1999). For optimal IL-8 gene expression, cooperation of NF-κB with either AP-1 or NF-IL-6 is needed (Mukaida *et al.,* 1994). NF-IL-6 alone binds only weakly to the IL-8 promoter, but in the presence of NF-κB the binding increases (Kunsch *et al.,* 1994). There is competition for the IL-8 NF-κB site and the classic NF-κB site. These two sequences are different and IL-8 NF-κB binding site binds the different NF-κB subunits Rel-A, NF-κB1 and c-Rel. The classic NF-κB hetero (p50/p65) is unable to transactivate the IL-8 site (Kunsch *et al.,* 1993). IL-8 can be down regulated through the NF-κB binding site, and IFN-β, nitric oxide and glucocorticoids inhibit cytokine induced IL-8 expression in cell lines (de Caterina *et al.,* 1995; Oliveira *et al.,* 1994; Wertheim *et al.,* 1993).

Oxidative stress can induce IL-8 expression, and this can occur through the activation of an oxidant-responsive element in the IL-8 promoter. Genes that code for protective antioxidant enzymes such as glutathione-S-transferase contain an ARE within the promoter of the gene. The IL-8 gene promoter has a similar sequence to the core ARE.

Oxidants increase the transcription of c-fos and c-jun genes, and the associated proteins have a redox sensitive cysteine residue that must be in the reduced state to permit DNA binding. This relationship has been demonstrated in an epithelial cell

line where H_2O_2 induced IL-8 correlated with activation and binding of AP-1 to the IL-8 promoter (Lakshminarayanan *et al.*, 1997).

The role of RANTES, MIP-1α, MIP-1β and MCP-1 and IL-8 lung inflammation

The neutrophil and the macrophage are central to the inflammatory process in COPD. Important chemotactic and activating cytokines for neutrophil and macrophage inflammatory cells include TNF-α, IL-8, Monocyte chemotractant protein (MCP-1), and Macrophage inflammatory protein (MIP-1α). MCP-1 and MIP-1α are both C-C chemokines.

MCP-1 is a chemoattractant for mononuclear phagocytes, T lymphocytes, B cells and NK cells. MCP-1 has a role in monocyte migration across epithelial and endothelial cell layers. Preferential release of MCP-1 and regulated on activation, normal T-cell expressed and secreted (RANTES) from the apical compartment of the epithelial cell with the creation of a chemotactic gradient across the epithelial cell and an increase in monocyte traffic in the basal to apical direction has been shown *in vitro* in alveolar type I and II cell lines (Rosseau *et al.*, 2000). Human lung macrophages release IL-8, MCP-1, and MIP-1α (Goodman *et al.*, 1998, Fuke *et al.*, 2004). *In vitro* studies examining leucocyte migration across an endothelial monolayer using fresh alveolar type II epithelial cells also show constitutive basal MCP-1 levels which are upregulated by TNF-α (Eghtesad *et al.*, 2001). Type II alveolar cells isolated from human lung produce detectable basal levels of MCP-1, IL-8 and GRO-α, but low or undetectable basal levels of MIP-1α and RANTES (Witherden *et al.*, 2004). TNF-α stimulated A549 cells release monocyte chemotactic factors with MCP-1 being the predominant factor compared to GMCSF, RANTES or TGF-β (Koyama *et al.*, 1999).

The importance of these cytokines in smoking related lung disease is suggested by an increase in cytokine expression within the lung environment. The expression of TNF-α, IL-8, MCP-1 and MIP-1α cytokines is increased in sputum (Keatings *et al.*,

1996), BAL fluid (Capelli *et al.,* 1999), plasma (Nguyen *et al.,* 1999) and lung tissue in patients with COPD (de Boer *et al.,* 2000). Also cigarette smoke, even in healthy individuals can lead to increased MCP-1 levels (Kuschner *et al.,* 1996).

RANTES and MIP-1α are both β chemokines that are involved in the migration of inflammatory cells within the lungs. RANTES is a T-cell product and attracts memory T-cells (Conlon *et al.,* 1995) and is also a chemoattractant for moncytes, basosphils and eosinophils (Schall *et al.,* 1990, Bischoff *et al.,* 1993). In stimulated blood-derived lymphocytes, CD8+ lymphocytes are the predominant source of MIP-1α and RANTES (Conlon *et al.,* 1995). There are however many other cellular sources of RANTES including monocytes, platelets, epithelial cells, fibroblasts and dendritic cells (Kameyoshi *et al.*, 1994; Berkman *et al.,* 1996; Rathanaswami *et al.,* 1993; Nagorsen *et al.,* 2004). RANTES is released in the lung in response to noxious stimuli such as bacteria and viruses (Fillion *et al.,* 2001). In particular, respiratory syncytial virus upregulates RANTES in-vitro in epithelial cell lines (Becker *et al.,* 1997), and this viral induction of RANTES within the lung may be important in exacerbations of COPD.

Increased expression of mRNA RANTES is found in lung biopsies in subjects undergoing an exacerbation of COPD, with strong mRNA RANTES expression in the surface epithelium (Zhu *et al.,* 2001). The expression of RANTES is under the control of various transcription factors including the redox sensitive transcription factor NF-κB (Song *et al.,* 2000).

1.6 Cell Membrane Lipid peroxidation

Within the cell membrane lipid peroxidation occurs in response to ROS, which are generated in smoking related lung disease, and produce a chain reaction in which unstable lipid hydroperoxides and secondary carbonyl compounds such as malondialdehyde (MDA) are formed. As well as in lung disease lipid peroxidation has been implicated in a number of other disease processes including atherosclerosis (Palinski *et al.,* 1989), diabetes (Shah *et al.,* 1994) and genetic haemochromatosis (Young *et al.,* 1994).

Malondialdehyde and cigarette smoking

High levels of lipid peroxidation products are found in the blood and epithelial lining fluid of patients with COPD compared to controls (Rahman *et al.,* 1996; Morrison *et al.,* 1999). During exacerbations of COPD levels of peroxidation products are increased (Rahman *et al.,* 1997). It has also been shown that serum MDA levels return to normal after the exacerbation of COPD has abated (Sahin *et al.,* 2001).

Significantly higher levels of MDA are found in resected lung tissue from smokers with a heavy smoking history compared to non smokers (Fahn *et al.,* 1998). Lee (Lee *et al.,* 1999) measured MDA in lung tissue and found that MDA levels increased with increasing age of the patient, and that MDA increased with cumulative smoke exposure. Petruzzelli (Petruzzelli *et al.,* 1990) found that the cumulative smoke exposure (adjusted for age) did not correlate with the level of MDA in lung tissue, but did find higher levels of MDA in patients who had smoked within 30 days of their operation for lung resection.

4-HNE and Cigarette smoking related lung disease

Aldehydic end-products such as malonyldialdehyde (MDA), 4-hydroxy-2,3-nonenal and 4-hydroxy-2,3-alkenals (HAKs) are generated from lipid peroxidation. 4-HNE, a stable end product of lipid peroxidation, is highly reactive and capable of diffusion

away from the original site of production (Esterbauer *et al.,* 1991), and affects both intracellular and extracellular biological targets. 4-HNE and other such aldehydic compounds can affect cellular functions including signal transduction, gene expression, apoptosis, cell proliferation and cell responses (Parola *et al.,* 1999). 4-HNE and MDA can modify proteins through covalent alkylation of lysine, histidine and cysteine amino acid residues, and it is thought that these intermolecular aldehyde-protein adducts are central to the cytotoxic events attributable to uncontrolled lipid peroxidation. Removal of 4-HNE can be achieved by direct interaction with glutathione (Canuto *et al.,* 1994), transformation into the metabolite 4-hydroxy-nonenoic acid by alcohol dehydrogenase isoforms into 1,4-dihydroxynonene, and the formation of conjugates between GSH and 4-HNE catalysed by glutathione S-transferase (GST) (Ketterer *et al.,*1998) or with end products of 4-HNE metabolism (Siems *et al.,* 1998).

In human lung tissue 4-HNE is found predominantly in the cytoplasm of airway epithelial cells, endothelial cells, neutrophils and macrophages. Higher levels of 4-HNE adducts are found in airway epithelial cells of smokers with COPD compared to those without COPD (Rahman *et al.,* 2002). There is a positive correlation between levels of 4-HNE and γ-GCS mRNA in alveolar epithelium (Rahman *et al.,* 2002). In murine models, acute cigarette exposure leads to increased levels of 4-HNE, particularly in type II alveolar epithelial cells (Aoshiba *et al.,* 2003). 4-HNE induces cell signalling via the mitogen-activated protein kinase pathway leading to the induction of AP-1 mediated genes (Uchida *et al.,* 1999; Leonarduzzi *et al.,* 2000). 4-HNE can also induce γ-GCS mRNA in alveolar epithelial cells (Liu *et al.,* 2001), suggesting that 4-HNE is intimately related to redox sensitive genes controlled by AP-1. *In vitro* work has shown that 4-HNE inhibits NF-κB activation in monocytes by selectively blocking signalling events that are required for IκB phosphorylation (Page *et al.,* 1999). In emphysema in which there is destruction of alveolar walls and permanent enlargement of distal airspaces, increased numbers of alveolar

macrophages are retained within the alveolar interstitial space (Jeffery, 1998). Lipid peroxidation products may have a role in retention of macrophages by virtue of modification of the extracellular matrix. Kirkham (Kirkham *et al.,* 2003) has shown increased macrophage adhesion to both cigarette smoke modified collagen IV and similarly collagen that has been modified with acrolein and 4-HNE. In vitro, Kirkham and coworkers (Kirkham *et al.,* 2004) have also shown that human macrophages interacting with carbonyl or cigarette smoke modified extracellular matrix proteins dramatically down regulates their ability to phagocytose apoptotic neutrophils.

1.7 Glutathione

The glutathione redox system is the major antioxidant defence system within the lungs. Glutathione is a low molecular weight tripeptide (L-γ-glutamyl-L-cysteinylglycine) which is synthesised from three amino acids (cysteine, glutamate and glycine), and is found both intra and extracellularly (figure 1.1, molecular structure GSH). GSH homeostasis is crucial to the maintenance of normal cellular physiological processes. GSH reacts both enzymatically and non enzymatically with cigarette smoke, highly reactive nitrogen oxides and electrophilic compounds such as quinines, aldehydes, and peroxides, so rendering them less toxic to cells. By virtue of strong nucleophilic properties, GSH can inactivate electrophilic reactive compounds by the formation of GSH conjugates. GSH is also an important vehicle for stabilizing, detoxifying and transferring cysteine.

The main source of GSH is the liver, which tends to export the majority of newly synthesised GSH into the circulation. The kidney and the lungs also have a high turnover of GSH, but the precise mechanism within the lung is not fully understood. This is further complicated by the probability of individual cell types within the lung differing in their GSH transport systems across the cell and may explain why particular cells within the lung are more susceptible to certain noxious stimuli.

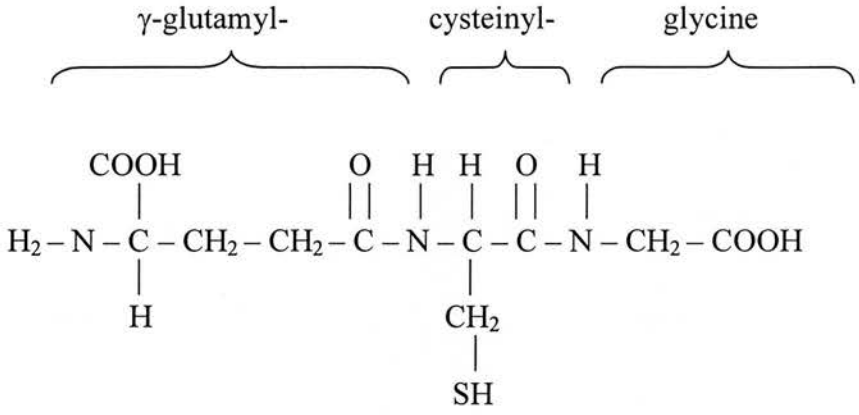
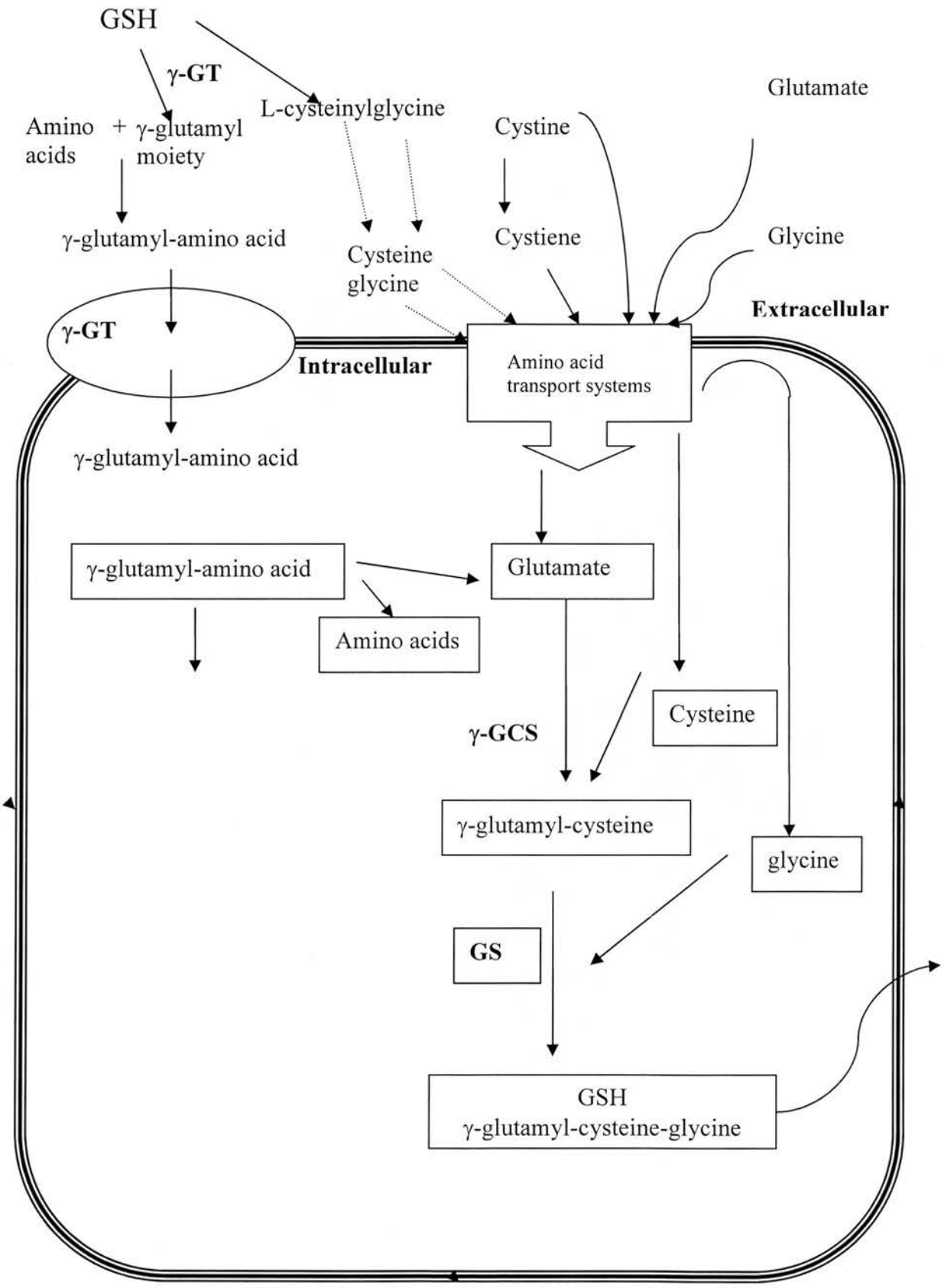


Figure 1.1 Molecular structure of glutathione

Glutathione Synthesis

Most GSH within the cell results from the transfer of its constituent amino acids from outside the cell. The γ-glutamyl cycle (figure 1.2) is a series of reactions in which GSH is both synthesized, and degraded, and together with various key amino acids is able to move into and out of the cell.

Figure 1.2 The γ-glutamyl cycle



Extra-cellular GSH under the influence of the transmembrane enzyme γ-glutamyl transferase (γ-GT), is broken down into a γ-glutamyl moiety and L-cysteinylglycine. Again under the influence of γ-GT, γ-glutamyl moiety with amino acids forms γ-glutamyl-amino acid. The γ-glutamyl-amino acid is taken up into the cell, where it is cleaved to free amino acids and glutamate. The L-cysteinylglycine at the cell surface, under the influence of peptidases will release the amino acids cysteine and glycine. These released amino acids will be free to move through the cell membrane utilizing the amino acid transport system in a similar fashion to cysteine, glycine and glutamate that are already present in the extracellular environment from the plasma. There are also means for cellular uptake of intact GSH, intracellular GSH synthesis and degradation and efflux of intact GSH and oxidized GSH (GSSG).

Cysteine, synthesized in the liver and found in low concentrations in plasma, is the rate limiting substrate for GSH synthesis, particularly under conditions of oxidative stress (Bannai, 1984; Deneke *et al.,* 1992). Glutamate and glycine are rarely rate limiting substrates in GSH formation (Bannai *et al.,* 1984; Bannai *et al.,* 1986). The redox status of the extracellular area influences the cellular uptake of cysteine (Bannai, 1984), and as extracellular GSH affects the redox status of the cell, then extracellular GSH levels will also affect intracellular GSH levels. Cysteine uptake into the cell is by a sodium dependent mechanism (ASC system) which is non-inducible, and is not influenced by amino acid starvation or oxidative stress (Bannai, 1984). In a reduced extracellular environment, cystine is converted to cysteine which is then taken up by the cell by the ASC amino acid system. Cystine is taken up into the cell via the x_c^- system of amino acid transport which has both a sodium dependent and non dependent system (Bannai *et al.,* 1982). The x_c^- system is inhibited by arginine, lysine and ornithine and competively inhibited by glutamate (Bannai and Ishii 1982; Bannai and Kitamura, 1980). There may be species or cell type differences in the x_c^- cystine transport system as bovine pulmonary artery endothelial cells have an inducible (by diethyl maleate DEM) sodium-independent system

(Deneke *et al.,* 1989). In type II epithelial cells only the non-inducible sodium dependent cystine transport system is identified (Bukowski *et al.,* 1995).

The liver is not able to uptake intact GSH directly, and GSH first undergoes degradation by γ-glutamyl tranpeptidase, and the constituent amino acids are taken up and used for intracellular GSH synthesis. In the lung however, there is evidence for direct cellular uptake of GSH, as addition of GSH to the perfusion media of rat lungs maintains lung GSH levels. Also inclusion of GSH in the medium of type II epithelial cell isolates gave protection against paraquat, and this protection persisted even in the presence of a γ-glutamyl tranpsptidase inhibitor (Hagen *et al.,* 1986). An active transport system for GSH uptake in rat type II epithelial cells has been proposed by Bai (Bai *et al.,* 1994). Others (Cheek *et al.,* 1994; Joshi *et al.,* 1986) however have failed to show evidence of direct GSH uptake in lung cells.

Gamma glutamyltransferase (γ-GT)

Gamma glutamyltransferase (γ-GT) is an enzyme that has two subunits; a 55 kDa membrane bound unit and a smaller (XXkDa) catalytic unit (Meister *et al.,* 1981). The active site of this enzyme is directed toward the outside of the cell (Griffith and Meister, 1979), and it is involved in both synthesis and degradation of GSH. It can break the γ-glutamyl bond of GSH and so release cysteinyl-glycine and a glutamyl moiety. The glutamyl moiety can be transferred to either an amino acid, a dipeptide or GSH to produce a glutamyl derivative.

$$\text{GSH} + \text{amino acid} \xrightarrow{\gamma\text{-GT}} \gamma\text{-glutamyl-amino acid} + \text{cysteinyl-glycine}$$

There is evidence that γ-GT has a protective role against oxidative stress, as specific γ-GT inhibitors can prevent the protective effect afforded by extracellular glutathione against added oxidants (van Klaveren *et al.,* 1997).

In intracellular glutathione synthesis, the rate limiting enzyme γ-glutamylcysteine synthetase (also called glutamate-cysteine ligase-GCL) (Meister *et al.,* 1985; Grifffith *et al.,* 1979), catalyses the bond between glutamate and cysteine, and is inhibited by L-buthionine-[S,R]-sulphoximide (BSO). γ-GCS (GCL) activity is regulated by negative feedback inhibition from GSH, but not GSSG. High cellular levels of cystine can stop the negative feedback of GSH production on γ-GCS (Kang *et al.,* 1994). γ-GCS (GCL) has 2 subunits, a modifier (GCL-M) and a catalytic (GCL-C).

GSH can be exported out of the cell and this pathway is important in determining final cellular GSH concentrations. This transport is affected by the thiol status of the extracellular environment. In a more oxidised extracellular environment then the cell retains GSH, and releases GSH in a more reduced environment (Lu *et al.,* 1993; Lu *et al.,* 1994; Garcia-Ruiz *et al.,* 1992). The origin of GSH in the ELF is not known. It seems unlikely to be the result of simple diffusion from within the cell. Other cells such as lymphocytes, macrophages, fibroblasts and endothelial cells can take GSH from the ELF and effectively transport it to other areas. GSH exists in an oxidised and reduced state. Usually there is only a tiny percentage of oxidised GSH (GSSG), less than 0.01%, unless there is an oxidant stimulus. Under these conditions there is export of GSSG out of the cell, but the precise details of this transport system are not clear. Some cells (type II cells, kidney cells and small intestine cells) have been found to uptake intact GSH (Hagen *et al.,* 1987; Hagen *et al.,* 1988; Brown *et al.,* 1992; Bai *et al.,* 1994; Jenkinson *et al.,* 1994). In type II cells the uptake of intact GSH is by an active mechanism that is sodium dependent. Type I cells do not have the ability to take up intact GSH (Deneke and Fanburg *et al.,* 1989). The total glutathione level (GSH and GSSG) in plasma is low with levels of 3 ± 0.6 μM, and

there is a 140 fold rise in the level found in the ELF with values of 429 ± 34 μM, with the majority of the GSH being in the reduced form (Cross *et al.,* 1994). Smokers have 80% more GSH in the ELF compared to non-smokers and the majority of this (98%) is in the reduced from (Cantin *et al.,* 1987). The higher ELF GSH concentrations may be a protective mechanism for epithelial cells in the face of an oxidant load. It is known that the erythrocytes from smokers have higher GSH concentrations and give more protection to endothelial cells from hydrogen peroxide than erythrocytes from non smokers (Toth *et al.,* 1986). In pulmonary fibrosis the ELF GSH is decreased four fold compared to normal individuals (Cantin *et al.,* 1989).

The glutathione depletion is not restricted to the lung. CT proven emphysema patients have reduced muscle antioxidant capacity as demonstrated by reduced resting muscle glutathione (Engelen *et al.,* 2000). Reduced GSSG, lipid peroxidation, γ-GCS (GCL modifying or catalytic) heavy subunit mRNA have been measured in muscle from COPD patients and healthy controls, both at rest and following an exercise programme. Interestingly healthy subjects were able to adjust their redox status, but this was not so in the COPD patients (Rabinovich *et al.,* 2001)

1.8 Glutamate-cysteine ligase (GCL) (γ-Glutamylcysteine Synthetase (γ-GCS))

GSH is synthesised by two enzymes, γ-GCS (the rate limiting enzyme) and glutathione synthetase (Seelig and Meister 1984). The γ-GCS enzyme is a haloenzyme and exists as a dimer, which is composed of a heavy (γ-GCS-HS; 73 kDa) and a light (γ-GCS-LS; 28 kDa) subunit (Seelig *et al.,* 1984). The heavy subunit possesses all of the catalytic activity (Huang *et al.,* 1993). γ-GCS nomenclature has changed and it is now referred to as glutamate-cysteine ligase (GCL) with a catalytic subunit (GCLC) and a modifier subunit (GCLM). The GCLC (γ-GCS-HS) gene is induced in alveolar epithelial cells after stimulation with oxidants, phenolic antioxidants and tumour necrosis factor (TNF-α) (Rahman *et al.,*

1996; Rahman *et al.*, 1996; Rahman *et al.*, 1998; Rahman *et al.*, 1999). In human alveolar cells an oxidant stress in the form of cigarette smoke condensate results in an initial depletion of intracellular GSH, followed by a rebound increase in glutathione synthesis (Rahman *et al.*, 1995). In chronic smokers there are elevated levels of GSH in the ELF, compared with non smokers (Cantin *et al.*, 1987).

Down regulation of GCL (γ-GCS) can occur in response to both TGF-β and oxidant stress in vitro (Arsalane *et al.*, 1997). GCL (γ-GCS) expression has been localized predominantly to bronchial epithelium (Soini *et al.*, 2001), and Rahman *et al* have demonstrated increased expression in the peripheral airways of COPD patients compared to healthy smokers using in situ hybridisation (Rahman *et al.*, 2000). Harju and co-workers (Harju *et al.*, 2002) performed immunohistochemical analysis of the GCL (γ-GCS) enzyme subunits in non smokers, smokers without COPD and smokers with COPD. This group found that in all groups, staining for GCLC and GCLM (γ-GCS HS and LS) was stronger in central airways compared to peripheral airways. In central and peripheral airways no difference in GCLC or GCLM (HS or LS) was found within the groups, except that GCLC (γ-GCS-LS) was increased in the central airways in the non smoking group. In contrast to Rahman, no staining was demonstrated in the alveolar epithelium. Harju and co-workers showed that alveolar macrophages had significantly more GCLM and GCLC (HS and LS) in the non smokers group. Rahman showed GCLC (γ-GCS-HS) mRNA was localized to bronchial, bronchiolar, and alveolar epithelial cells (Rahman *et al., 2000)*, and it is interesting that Harju et al (Harju *et al.*, 2002) found that alveolar epithelium failed to show any immuno-reactivity. Thus, although GCL (γ-GCS) message appears to be present in the alveolar epithelium, enzyme protein has not been demonstrated in the alveolar epithelium.

Human GCL (γ-GCS) is activated by the formation of reversible disulphide bonds, with at least two of these bonds required for optimal enzyme activity. One pair of disulphide bonds links the heavy and light subunits, and depletion of GSH promotes

formation of this disulfide bond and ultimately optimal enzyme activity. The second disulphide bond is intramolecular. GCL (γ-GCS) is transcriptionally, post transcriptionally and post translationally regulated (Soltaninassab *et al.,* 2000). In cell lines (human small cell line, lung, and Hep G2, hepatocytes) there is higher constitutive expression of the GCLC (γ-GCS light subunit) compared to that of the GCLM (heavy subunit) (Kurokawa *et al.,* 1995; Huang *et al.,* 1993, Soltaninassab *et al.,* 2000). Similarly these studies have also demonstrated tissue specific differences in the translational rates of these subunits. Stimuli that affect production of the heavy subunit will there by affect the synthesis of GSH. Certain stimuli produce signals that not only induce transcription but also result in message stabilization. There are a multitude of chemical, physical agents, and cytokines that are capable of inducing the subunits of GCL (γ-GCS), and these are shown in table 1.2.

Table 1.2 Compounds capable of inducing GCL (γ-GCS) subunit activity

Metals	arsenite, cadmium, copper, zinc, mercury
Reactive oxygen species production	menandione, hydrogen peroxide 2,3-dimethoxy-1,4-naphthoquinone, t-butyl-hydroquinone ionizing radiation oxidized low density lipoproteins pyrrolidine dithiocarbamate
Glutathione Depleting agents	diethyl maleate (DEM) ethacrynic acid 4-hydroxy-2-nonenal (4-HNE) nitric oxide prostoglandins BSO
Antioxidants	butylated hydroxytoluene
Cytokines	TNF-α, IL-1
Miscellaneous	heat shock azetidine cisplatin okadaic acid cigarette smoke

GCLC and GCLM genes (γ-GCS-HS and γ-GCS-LS genes)

The genes for GCLC (γ-GCS-HS) and GCLM (γ-GCS-LS) are single copy genes which have been mapped to 6p12 and 1p22.1 respectively (Griffith 1999; Wild and Mulcahy 2000). Loss of 1p21-22 is associated with malignant mesothelioma, and breast cancer and loss of this region results in loss of GCLM (γ-GCS-LS) which could limit GCL (γ-GCS) activity (Lee *et al.,* 1996); Vance *et al.,* 1997). Polymorphisms of the GCL (γ-GCS) subunits may be important in susceptible individuals who develop COPD.

As stated earlier, a multitude of agents can induce the subunits of GCL (γ-GCS). GCL (γ-GCS) gene transcription is an extremely complex process that is not fully unraveled. Groups interested in GCL (γ-GCS) gene transcription have used various cell lines including alveolar epithelial cells, Hep G2 cells, COS-1 cells, and mouse endothelial cells, with the inherent problem of not knowing whether various observations are cell or species specific. There is evidence of preferential DNA binding of particular transcription factors with the whole, or part of the EpRE sequence of the GCL (γ-GCS) promoter in response to different stimuli.

Inducers of phase II protective enzymes such as β-napthoflavone that initiate a protective response, the so called 'electrophile counterattack' have been useful in understanding GCL (γ-GCS) regulation (Prestera *et al.,* 1993; Prestera and Talalay, 1995). This counterattack leads to increased intracellular glutathione and upregulation of detoxification genes via antioxidant response elements (ARE)/electrophile response elements (EpRE) located in the promoter region of the respective genes. The EpRE core sequence is 5'(G/C)TGA(C/G)NNNGC(A/G)-3' and often contains an AP-1 binding site and is often flanked by an AP-1 or an AP-1 like binding site. The sequence of the EpRE is similar to that of other response elements such as the TRE-type Maf recognition elements (T-MARE) and the NF-E2-binding sites (Kataoka *et al.,* 1994). Thus transcription factors that work through

these sites will, or may also bind to and regulate expression through EpRE sequences. Such transcription factors are termed *trans*-acting factors.

Glutamate cysteine Ligase (GCL) and AP-1 binding sites

There is a close association between GCL (γ-GCS) and the redox sensitive transcription factors activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB). The 5'-flanking region of the γ-GCS-HS gene contains various elements (antioxidant response element (ARE), metal response element (MRE)), and putative transcription factors (AP-1, NF-κB, AP-2, SP-1). Functional AP-1 elements have been identified in the heavy and light subunits of γ-GCS, and these AP-1 elements are important for constitutive and inducible GCLC (γ-GCS-HS) and GCLM (γ-GCS-LS) activity (Moinova and Mulcahy 1998; Wild *et al.,* 1998). Cho has shown that oxidised low-density lipoprotein induced GCLC (γ-GCS-HS) expression, and that this was regulated by an AP-1 binding site located at -365 and -241 base pairs (Cho *et al.,* 1999). Rahman *et al* have also demonstrated in an epithelial cell line that an AP-1 element located between -269 and 263 base pairs is required for the oxidant mediated (menadione and hydrogen peroxide) regulation of GCLC (γ-GCS-HS) promoter activity (Rahman *et al.,* 1998).

Glutamate Cysteine Ligase (GCL) and electrophile response element (EpRE)

The promoter of GCLC (γ-GCS-LS) contains an electrophile response element (EpRE), which is located 0.3 kb upstream of the translational start site, and this has an AP-1 element within it, and in Hep G 2 cells it has been shown to direct constitutive and β-naphthoflavone induced γ-GCS-LS activity (Moinova *et al.,* 1998). There is also an apparent redundant AP-1 element located more distal to the EpRE on the GCLM (γ-GCS-LS) gene that is also able to direct β-naphthoflavone induced GCLM (γ-GCS-LS) activity. Similarly, the GCLC (γ-GCS-HS) gene has an

EpRE, designated EpRE4 (Mulcahy *et al.,* 1997) and this is located approximately 3.1 kb upstream of the translational start site.

Glutamate Cysteine Ligase (GCL) and relationship with other nuclear proteins

It has been shown that in COS-1 cells, the bZIP transcription factors TCF11/Nrfl and Nrf2 cause increased expression of GCLC (γ-GCS-HS) by interacting with AREs within the GCLM (γ-GCS-HS) gene (Myhrstad *et al., 2001*).

Jeyapaul and co-workers showed that there is competition between antioxidant response elements of different genes for the DNA binding of the nuclear proteins Nrf and jun (Jeyapaul *et al.,* 2000). This group also demonstrated that over expression of Nrf1, Nrf2 and jun proteins caused a significant up regulation of GCL (γ-GCS) ARE-mediated basal and beta-naphthoflavone induction of the CAT gene transfected hep G2 cells. Also the c-Jun level within cells was shown to determine the level of ARE mediated GCL (γ-GCS) gene expression (Jeyapaul *et al.,* 2000).

Wild (Wild *et al.,* 1998) has postulated that the binding of the transcription factor Nrf2 to the EpRE site in the promoter is critical to transcriptional upregulation of GCLC (γ-GCS-HS) gene. This group has shown that xenobiotic exposure of HepG 2 cells does not lead to an increase in Nrf2 protein levels and that it is likely that this transcription factor merely translocates from the cytosol to the nucleus under the influence of oxidative stress. Wild *et al* has also suggested that small Maf proteins dimerise with jun or fos proteins and this dimerisation is essential for the DNA binding of the Nrf2 to the EpRE site, but that Jun proteins were not required for the inducible response. This group has also postulated that the enzyme protein product is likely to exert both a negative and positive effect. The negative effect is mediated by the inhibition of the dimerisation of the jun-maf proteins, and the positive effect on the actual gene transcription. Binding at the ARE-4 promoter area may stimulate dimerisation of small maf proteins that exert a negative effect on the DNA binding of Nrf2 at the ARE-4 site. It is possible that there are multiple putative transcription

sites in the promoter of GCL (γ-GCS) gene that are involved in response to different stimuli. The depletion of GSH in human alveolar epithelial cells in response to TG-Fβ has been shown to involve the AP-1/ARE-4 DNA binding site and that the AP-1 complex consists of c-Jun and Fra-1 dimers (Jardine *et al.,* 2002). Although Jardine and co-workers have failed to identify Nrf2 DNA binding by Supershift experiments in resting alveolar cells (and TGF-β stimulated), this does not preclude Nrf2 promoter binding at the ARE in human alveolar epithelial cells in response to oxidative stress.

It is thus possible that certain stimuli lead to preferential binding of a particular form of AP-1 complex, and some of these complexes, although binding may not cause gene transcription.

Constitutive and basal Glutamate cysteine Ligase (GCL) gene transcription

There is both constitutive and inducible GCL (γ-GCS) gene transcription, with possible differences in the associated pathways. There is evidence in HepG cells to suggest that basal expression of GCL (γ-GCS) subunit genes are under the influence of AP-1 binding sites in the promoter region (Moinova *et al.,* 1998; Wild *et al.,* 1998). Rahman and co-workers (Rahman *et al.,* 1998) have shown that in alveolar epithelial cells, the -511 to +82 of the 5'- flanking region of the GCLC (γ-GCS-HS) promoter is involved in the constitutive expression, and that the -303 to -201 base pair sequence of the promoter is involved in oxidant induced transcriptional upregulation of GCLC (γ-GCS-HS) gene. Rahman *et al* (1998) used menadione, which induces oxidative stress by the generation of oxygen radicals, and hydrogen peroxide in a series of CAT reporter deletion constructs. In contrast Mulcahy, who used β-napthoflavone and HepG2 cells, has reported an ARE present in the area -3147 to -3137 base pairs that is involved in GCLC (γ-GCS-HS) gene transcriptional upregulation (Mulcahy *et al.,* 1997).

1.9 Transcription Factors

Nuclear Factor-κB (NF-κB)

NF-κB is likely to play a pivotal role in the development of inflammation within the lungs. This section aims to discuss the complex relationship that exists between the transcription factor NF-κB and pro-inflammatory cytokine production, and antioxidants within the lung. There is evidence that the pathways for NF-κB activation may be different depending upon the stimulus, the presence of associated antioxidants in the local cellular environment, and also on the type of cell (Janssen-Heininger *et al., 2000;* Brennan *et al.,* 1995; Bonizzi *et al.,* 1999; Korn *et al., 2001).*

Nuclear factor kappa (NF-κB) is a member of the Rel family of transcription factors which all share a sequence homology over a 300 amino acid sequence (the NF-κB/Rel domain) (Baldwin, 1996), and can exist as hetero or homo dimmers. The classical activated form of NF-κB is a heterodimer consisting of a 65 kD heavy subunit and a 50 kD light unit. NF-κB can be formed from other subunits such as p105, p50, p100, p52, c-Rel, and Rel B. Although dimers formed from these subunits will have the same 300 amino acid homology, they are likely to activate different genes and have different stimuli for translocating to the nucleus from the cytoplasm. NF-κB that is found in the cytoplasm is in an inactive form (unable to bind to DNA) in association with an inhibitory protein, IκB.

Inhibitory kappa B (IκB)

There are several isoforms of IκB such as IκB-α, IκB-β, IκB-γ, IκB-δ, and IκB-ε, but the most abundant is IκB-α (Baldwin, 1996; Baeuerle, 1998). An appropriate cell stimulus such as a cytokine will allow the inhibitory protein IκB-α to be phosphorylated at specific serine residues by specific IκB Kinases (IKK). This leads to the transfer of an ubiquitin molecule to the IκB-α and NF-κB complexes. In the case of IκB-α, ubiquitination serves as a signal to proteolytic enzymes to degrade

IκB-α, and so releasing the NF-κB (p105/p65 complex) from the inhibitory affect of IκB-α (Karin and Ben-Neriah, 2000). In the case of NF-κB (p105/p65 complex) the ubiquitination of the p105 subunit leads to it increasing the rate at which p105 is processed to p50 and thus the formation of the p50/p65 complex, which can then translocate to the nucleus where it binds to specific areas on the promoter of target genes. The p50/p50 homodimer can bind to the NF-κB binding site, but will inhibit transcription rather than stimulate it. The IκB-α gene (MAD-3) has several κB sequences in its promoter region, so that when NF-κB translocates to the nucleus it also induces the synthesis of IκB-α. The newly formed IκB-α then moves into the nucleus and binds to the activated NF-κB and induces the export of NF-κB into the cytoplasm, thus rendering it inactive once again (Arenzana-Seisdedos *et al.,* 1995). Both IκBα and IκBβ in the post induction phase can enter the nucleus and repress NF-κB DNA binding, but only IκBα can cause nuclear export of NF-κB. Changing the N-terminal region of IκBβ to that of the IκBα sequence allows the nuclear export of NF-κB (Huang and Miyamoto, 2001) and this property of IκBα allows for rapid reactivation of NF-κB within cells.

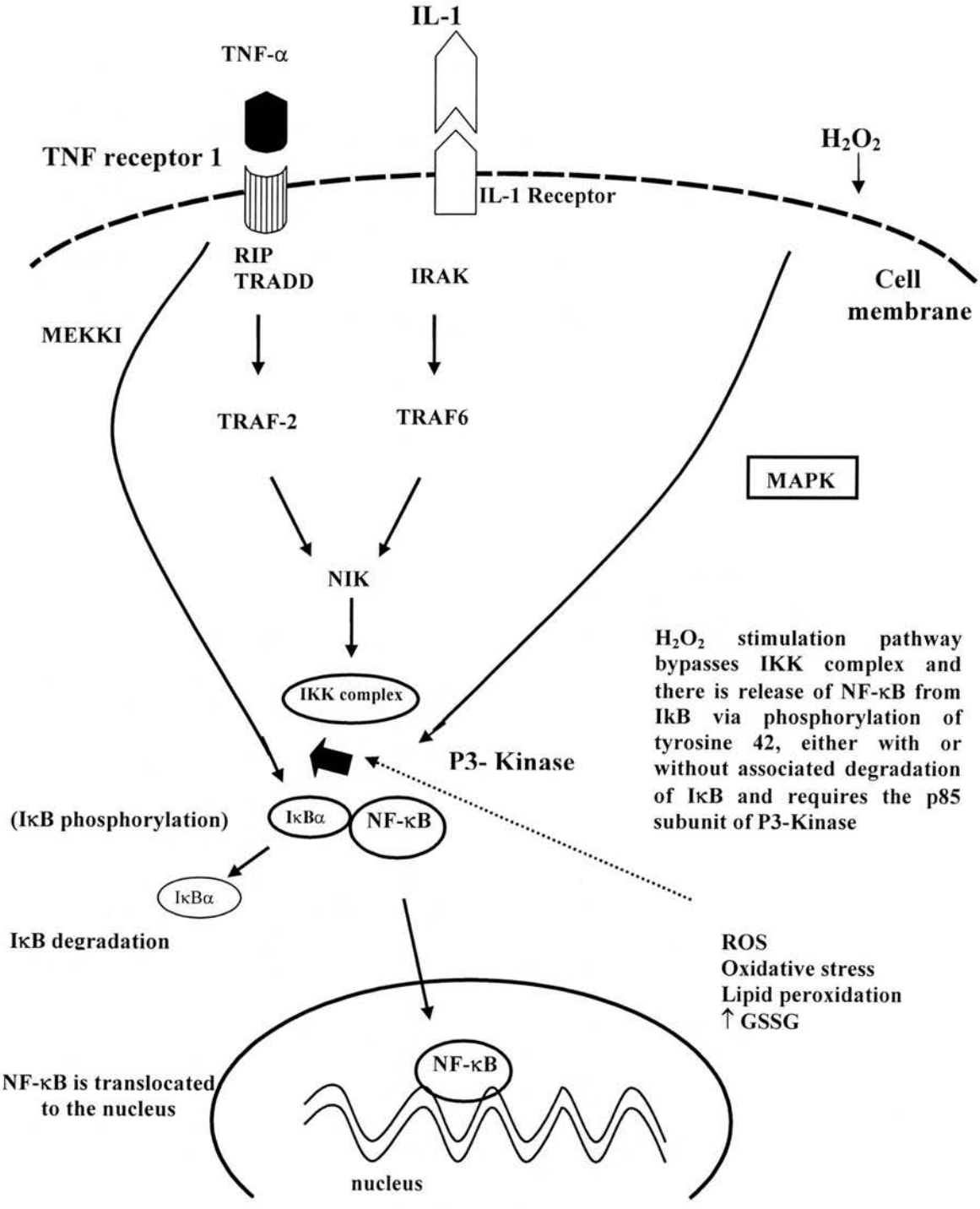
IKK is composed of two catalytic subunits IKKα and IKKβ and a regulatory subunit, IKKγ (Zandi *et al.,* 1999; Rothwarf *et al.,* 1998), see figure 1.3 NF-κB activation pathways. Negative and positive NF-KB regulator pathways converge at the level of IKK. There are a number of upstream Kinases including Mitogen-activated protein kinase/Extracellular signal-regulated kinase Kinase Kinase 1 (MEKK1), NF-κB Inducing Kinase (NIK) and protein Kinase B (Akt) that activate IKK by leading to the phosphorylation of a critical number of serine residues (phosphorylation of IκB on serines 32 and 36) in the IKKα and IKKβ subunits. The IκB Kinase (IKK) complex and its activation is a crucial component of the activation of NF-κB in response to various stimuli.

Phosphatidylinositol 3- kinase (PI-3K) pathway for NF-κB activation

There is however another pathway for NF-κB activation via oxidants such as hydrogen peroxide, pervanadate, hypoxia and ultraviolet light which involves release of NF-κB complex by tyrosine phosphorylation of IκBα (Li *et al.,* 1998; Imbert *et al.,* 1996; Janssen-Heininger *et al.,* 1999). The phosphorylation involves tyrosine 42 instead of serines 32/36 as is the case for IKK dependent pathway of phosphorylation. In this the PI-3K/Akt pathway is able to activate NF-αB by dissociating tyrosine 42 phosphorylated IκBα from NF-κB without degrading IκBα. This alternative (PI-3K) pathway in which there is no activation of IKK has been associated with both degradation and lack of degradation of IκB (Imbert *et al.,* 1996; Beraud *et al.,* 1999).

Using a myeloid cell line Takada (Takada *et al.,* 2003) has shown that H_2O_2 induced tyrosine phosphorylation of IκBα occurs via syk protein kinase. Cells that do not express syk kinase do not activate NF-κB, and this may explain why there are conflicting reports when different cell types are used.

Figure 1.3 NF-κB activation pathways



Activators of NF-κB

There are many stimuli that can activate NF-κB (Rahman *et al.,* 1998), table 1.3. The cytokines interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), tumour necrosis factor α and β (TNF-α and TNF-β) can trigger activation of NF-κB (Siebenlist *et al.,* 1994; Barnes and Karin, 1997). Oxidant stress such as hydrogen peroxide, ozone and hyperoxia all activate NF-κB (Schreck *et al.,* 1991). The ability of ROS to activate transcription factors and therefore induce gene expression, has been described by many workers (Schreck and Baeuerle, 1994; Adcock *et al.,* 1994; Meyer *et al.,* 1993). ROS can activate NF-κB by oxidation of the cysteine-SH group or by ubiquitination and proteolytic breakdown of IκBα (Shang *et al.,* 1997). Oxidative stress, lipid peroxidation and increases in cytosolic oxidized GSH (GSSG) lead to increased ubiquitination of NF-κB. TNF-α, IL-1β, LPS and ultraviolet irradiation can also cause a cellular increase in ROS by mitochondria (Richter *et al.,* 1995). The reactive oxygen species signaling that regulates the transcription of IL-4 (Jeannin *et al.,* 1995), IL-6 and TNF-α (Gosett *et al.,* 1999) is mediated through a thiol dependent mechanism.

NF-κB may not be exclusively required in the redox mediated pathways governing pro-inflammatory cytokines in the alveolar epithelium. Haddad (Haddad., 2001) has examined LPS-induced TNF-α and IL-6 production in vitro in an alveolar epithelial cell line. Pretreatment of the cells with BSO (inhibitor of γ-GCS) prior to stimulation with LPS augmented the TNF-α and IL-6 levels, and this was associated with induction of intracellular accumulation of reactive oxygen species. BSO inhibited the phosphorylation/degradation of IκB-α, but BSO also upregulated cytokine biosynthesis, and this suggests again that NF-κB is probably not exclusively required in the redox mediated pathways governing pro-inflammatory cytokines in the alveolar epithelium. It has also been shown that BSO blocks the oxyexcitation (deltapO_2)-dependent nuclear localization of RelA (p65) the major transactivating

member of the Rel family (Haddad *et al.*, 2001). It is possible that the Iκβ-α/NF-κB pathway is independent of, or at least partially independent of the redox-dependent regulation of pro-inflammatory cytokines. Antioxidants (Matsumota *et al.*, 1998) and glutathione precursors have been shown to down regulate cytokine synthesis and down stream processes (Reimund *et al.*, 1998), but depletion of GSH is associated with augmentation of an inflammatory signal and exacerbation of inflammation (Haddad *et al.*, 2001).

Agents Capable of Activating NF-κB	**Inhibitors of NF-κB Activation**
Tumour necrosis family	N-acetyl-L-cysteine
Interleukin-1β	pyrrolidine dithiocarbamate
Intereleukin-2	Glutathione
Intereleukin-6	thioredoxin
Intereleukin-8	Intereleukin-4
Interleukin-17	Intereleukin-10
Interleukin-18	
Lipopolysaccharide	Corticosteroids
Hydrogen peroxide	Vitamin E
hypoxia	
ozone	
reactive oxygen species	
ceramide	
phorbol esters	
growth factors	
Ultraviolet radiation	
X-rays	
γ-radiation	
erythropoietin	
pervanadate	
silica	
Infectious agents	
virues (Rhino virus)	

Table 1.3 Agents that can activate NF-κB and agents that inhibit NF-κB activation

The cell type appears to influence the pathway for H_2O_2 induced NF-κB activation, and there is also some variability within a cell type (Bonizzi *et al.*, 1999; Bonizzi *et al.*, 2000). This is demonstrated in T cell lines where Wurzburg T cells, but not Jurkat T lymphocytes are responsive to H_2O_2 (Anderson *et al.*,1994). Similarly a different response to H_2O_2 is found in rat alveolar and mouse alveolar cell lines (Janssen-Heininger *et al.*, 1999, Korn *et al.*, 2001). The inter-relationship of NF-κB with antioxidants, antioxidant enzymes and cytokines is complex and not fully understood. Oxidants not only have an effect on IKK activity, but oxidants also influence NK-κB dependent gene transcription. Reactive oxygen species may act as second messengers leading to NF-κB activation (Schreck *et al.*, 1991; Flohé *et al.*, 1997). It is still unclear whether the redox nature of the environment is crucial to the activation of NF-κB or not, and this is further complicated, as work by Hayakawa *et al* (Hayakawa *et al.*, 2003) has shown that the antioxidants N-acetyl-L-cysteine and pyrrolidine dithiocarbamate inhibit NF-κB independently of their anti-oxidative action. Korn in contrast has shown that hydrogen peroxide inhibits the activity of activated IKK (Korn *et al.*, 2001). Undoubtedly epithelial cells of the lung will be exposed to both oxidants and inflammatory cytokines, and these *in vivo* situations are difficult to imitate *in vitro.*

Infectious agents such as bacteria and viruses, and in particular rhino virus are known to activate NF-κB (Zhu *et al.*, 1996). Inhibitors of NF-κB include corticosteroids (dexamethasone), antioxidants such as GSH, thioredoxin, N-acetylcysteine (NAC), vitamin E and the cytokines interleukin-4 and interleukin-10 (Scheinman *et al.*, 1995; Schenk *et al.*, 1994; Wang *et al.*, 1994). GSH and NAC act by inhibiting the inducible decay of IκB-α (Oka *et al.*, 2000). There have also been conflicting reports where thiols such as NAC and thioredoxin have been shown to activate NF-κB by the generation of ROS in particular circumstances, and this is thought to be due to the reduction of a cysteine residue on the NF-κB protein (Fernandez *et al.*, 1999).

NF-κB and Pro-inflammatory genes

NF-κB regulates the expression of a number of genes involved in the inflammatory process within the lungs, including pro-inflammatory cytokines and adhesion molecules (table 1.4).

Table 1.4 Genes regulated by NF-κB

Cytokines	TNF-α
	Interleukin 1β
	Interleukin 2
	Interleukin 3
	Interleukin 6
	Interleukin 12
Growth factors	
	Granulocyte macrophage colony-stimulating factor
	Granulocyte colony-stimulating factor
	Macrophage colony-stimulating factor
Chemokines	Interleukin 8
	Macrophage Inflammatory protein 1- alpha
	Macrophage chemotactic protein-1
	Gro-α, -β, -γ
	Eotaxin
	RANTES
Inflammatory mediators	
	Inducible nitric oxide (Inos)
	Inducible cyclo-oxygenase- 2
	5-lipoxygenase
	Cytosolic phospholipase A
	C reactive protein
	12-lipoxygenase
Adhesion molecules	
	Intracellular adhesion molecules
	Vascular adhesion molecule-1 (VICAM-1)
	E-selectin
Immunoreceptors	
	Interleukin-2-receptor (α-chain)
	T-cell receptor (β-chain)
	Platelet activating factor
	CD11b and CD48
Proto-oncognenes	
	P53, c-myc, ras

The genes controlling production of some inflammatory mediators are known to be upregulated in inflammatory lung diseases. Also, the products of some genes that are regulated by NF-κB, can also cause activation of NF-κB. This is well demonstrated by IL-1β and TNF-α which can both activate NF-κB, and be activated by NF-κB. Section 1.5 has already examined the role of TNF-α in lung inflammation.

In TNF and IL-1 induced NF-κB activation pathways, there are individual upstream processes that occur prior to the degradation of IκBα. Cell surface receptors and protein-protein interactions occur, in which the two pathways of NF-κB activation both utilise members of the TNF receptor associated factor (TRAP) family of adaptor proteins and both converge at the level of the protein kinase NIK (Malinin *et al.*, 1997). TNF dependent trimerisation of the TNF receptor leads to recruitment to the cell membrane of the following: adaptor protein which is a TNF-R associated death domain protein (TRADD), receptor interacting protein (RIP) which is a death domain containing serine/threonine kinase and subsequently TRAF2, and NIK (Rahman and MacNee, 1998). TRAF2 and RIP have been shown to be required for NF-κB and IKK activation (Devin *et al.*, 2000). With regard to IL-1, an IL-1 receptor interacts with TRAF6 and NIK with subsequent activation of IKK complex. Another signaling pathway is that of MEKKI or MAPKKK because MEKKI is recruited to the TNF-α activated IκB complex (Lee *et al.*, 1997).

Activator Protein-1 (AP-1)

AP-1 is a collection of related transcription factors belonging to the Fos (cFos, FosB, Fra1, Fra2) and Jun (c-Jun, JunB, JunD) families that dimerise in various combinations through their leucine zipper region. Dimerisation of fos-jun or jun-jun is a prerequisite for DNA binding via a 'leucine zipper' domain (Karin *et al.*, 1997). A leucine zipper domain is an area that regulates the expression of a number of genes. Fos/Jun heterodimers bind with the greatest affinity and are the predominant form of AP-1 in most cells. Jun/Jun homodimers bind with a low affinity. Activation

of the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) response element (TRE) is a prerequisite for AP-1 induced transcriptional regulation. Any agents that activate TRE such as serum responsive factor also activate AP-1 by a protein kinase C mediated pathway (Rahman *et al.,* 1998). Depletion of intracellular GSH in airway epithelial cells following exposure to cigarette smoke free radicals leads to increased AP-1 DNA binding and release of IL-8 (Rahman *et al.,* 1996; Mio *et al.,* 1997). Rahman has shown that the AP-1 binding site on γ-GCS gene that is present at -269 to -263 base pairs, is involved in transcriptional up-regulation of γ-GCS-HS gene under oxidative stress (Rahman *et al.,* 1998).

1.10 Chromatin remodeling and Gene Transcription

A key requirement in gene transcription is the accessibility of DNA to regulatory transcription factors. DNA is packaged into chromatin to facilitate storage of large quantities of this nucleic acid within the nucleus. Chromatin consists of the coiling of DNA around four core histone (H) proteins: H3, H4, H2A, and H2B. H3 and H4 are organized into a tetramer, whereas H2A/H2B is assembled as two dimmers (Beato, 1996). Chromatin remodeling occurs through the action of histone acetylases (HAT) and histone deacetylases (HDAC). HAT proteins transfer an acetyl group from coenzyme A onto lysine, resulting in the unwinding of DNA from the histones and increasing greater accessibility of transcription factors to genes, which in turn enhances gene transcription (Imhof and Wolffe, 1998).

Gene repression is associated with reversal of acetylation by a process of deacetylation which is controlled by histone deacetylases (HDACs). Histone deacetylases catalyse hydrolysis of the acetyl groups from the amino terminal lysine residues of the nucleosomal core histones, thereby causing chromatin condensation and displacement of transcription factors from gene promoters (Narlikar *et al.,* 2002; Fischle *et al.,* 2001). Histone acetylation is a dynamic process and so changes in acetylases or deacetylases can have dramatic effects on the overall histone

acetyltransferase activity (HAT). Thus increased gene activity may result from increased HAT activity or a reduction in HDAC activity.

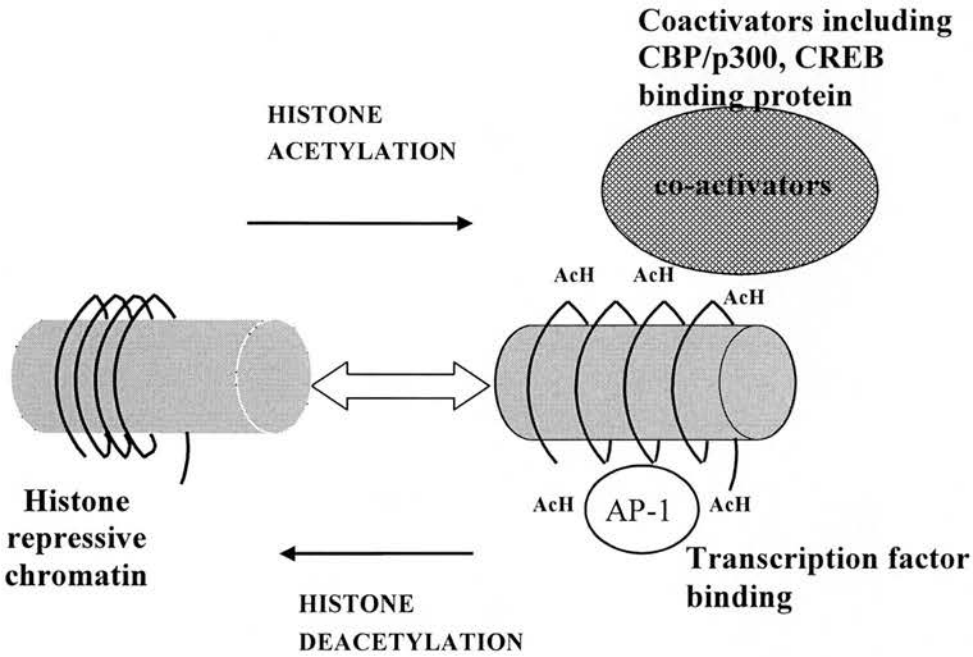


Figure 1.10 Histone acetylation and deacetylation

Acetylation of Transcription factors

Transcriptional coactivators such as CREB binding protein (CBP), and p300/CBP–associated factor (PCAF) have intrinsic HAT activity (Ogryzko *et al.*, 1998) and this acetylation activity is activated by binding of transcription factors such as AP-1 and NF-κB.

In unstimulated cells transcriptionally inactive nuclear NF-κB consists of homodimers of either p65 or p50 complexed with the histone deacetylase HDAC-1. The p50-HDAC-1 complexes bind to DNA and suppress NF-κB dependent gene expression. In the face of an appropriate stimulus there is nuclear localisation of NF-κB together with phophorylation of p65 that associates with CBP and displaces the p50-HDAC-1 complexes. Phosphorylation of p65 appears to be critical in determining whether p65 associates with CBP or HDAC-1, so that only p65 enters the nucleus (Zhong *et al.*, 2002)

Control of the duration of the NF-κB effect is influenced by histone deacetylation and its effect on inhibitory IκBα in the cytoplasm. Chen (Chen *et al.*, 2001) has shown that the acetylated RelA subunit of NF-κB becomes deacetylated through an interaction with HDAC-3 and this deacetylation promotes effective binding to IκBα and there is IκBα dependent nuclear export through a chromosomal region maintenance-1 (CRM-1)-dependent pathway. Deacetylation of RelA by HDAC3 acts as an intranuclear molecular switch that controls the duration of the NF-kappaB transcriptional response, and contributes to the replenishment of the depleted cytoplasmic pool of latent NF-κB-IκBα complexes.

Glucocorticoids and Histone acetylation/Deacetylation

Glucocorticoids are an effective treatment for inflammatory diseases such as asthma, and act by repressing the activation of transcription factors and induction of proinflammatory mediators. In addition corticosteroids stimulate the expression of some anti-inflammatory genes such as SLPI and IL-1β receptor antagonist (Levine *et al.*, 1996). However it has been postulated that repression of HAT activity, and consequent repression of inflammatory genes, is the predominant mode of action of corticosteroids in asthma. Ito et al (Ito *et al.*, 2002) have demonstrated more HDAC activity and less HAT activity in bronchial biopsies obtained from mild asthmatics

receiving inhaled corticosteroids. Reductions in HDAC activity and HDAC2 expression have also been detected in mild asthmatics compared with normal controls (Ito *et al.,* 2002). Similarly, mechanistic studies conducted in vitro have provided evidence that the corticosteroid, dexamethasone enhances HDAC activity and expression while decreasing HAT activity, responses that do not appear to be mediated through NF-κB (Newton *et al.,* 1998) Different patterns of histone acetylation are known to occur in response to dexamethasone and IL-1β stimulation (Ito *et al.,* 2000). In alveolar epithelial cells, IL-1β causes acetylation of histone H4 K8 and K12 and dexamethasone acetylates K5 and K16 with no effect on K8 and K12. These differences may occur because activated GR complex inhibits acetylation of K8 and K12 by acting both as a direct inhibitor of CBP-associated histone acetylation, and by recruiting HDAC2 to the p65-CBP HAT complex.

Role of Histone Acetylation/Deacetylation in Proinflammatory gene induction

The hydroxamic acid, trichostatin A, is an HDAC inhibitor that has been used to explore the role of histone deacetylases in vitro. Kagoshima (Kagoshima *et al.,* 2001) examined IL-1β stimulated GM-CSF release in A549 cells, following dexamethasone and trichostatin A. Incubation with dexamethasone resulted in inhibition of the GM-CSF release, and this inhibition was partially lost following incubation with trichostatin A. Over expression of CBP resulted in inhibition of the GM-CSF release. The implication being that the mechanism of glucocorticoid action in suppressing interleukin-1 beta-stimulated GM-CSF release in A549 cells may involve modulation of CBP-mediated histone-acetylase activity and DNA methylation.

Further evidence for the importance of chromatin remodeling in modulation of the inflammatory response in the lungs has come from examining IL-8 release in *in vitro* systems in response to HDAC inhibitors. IL-8 cytokine release is increased in A549 cells in response to particulate air pollution Pm_{10} (particles <10 microns in diameter)

and hydrogen peroxide stimulation. Co-incubation of these A549 cells with trichostatin A leads to an increase in IL-8 cytokine production (Gilmour *et al.,* 2003), increased HAT activity, and increased levels of acetylated histone 4. PM_{10} mediates IL-8 transcription via enhanced HAT activity and acetylation of H4 on the gene promoter. Pretreatment of epithelial cells with trichostatin A, followed by stimulation with LPS, TNF-α, and hydrogen peroxide leads to hyperacetylation and increased IL-8 (Iwata *et al.,* 2002; Rahman *et al., 2002).* Marwick et al (Marwick *et al.,* 2004) have also shown evidence of disruption of the histone acetylation/ deacetylation balance in response to cigarette smoke in vivo in the rat.

1.11 Early One Adenovirus Protein (E1A)

Childhood Respiratory Tract Infection and the Development of COPD

The critical question of, why only 15-20% of cigarette smokers go onto develop COPD (Tashkin *et al.,* 1984), remains unanswered. An environmental or genetic factor in these smokers may be important in the susceptibility to the development of COPD. It is likely that clinical airflow obstruction results from a lengthy and initial asymptomatic process, and a possible candidate for such an initial process or insult is an infectious agent.

In infants, children, adults, and animal models, acute viral respiratory infection leads to airway hyper-responsiveness (Sly and Hibbert 1989; Weiss *et al.,* 1985; Empey *et al.,* 1976; Laitinen *et al.,* 1991; McDonald *et al.,* 1988; Huang *et al.,* 1989; Robinson *et al.,* 1996; Piedemonte *et al.,* 1990). Viruses including respiratory syncytial virus (RSV), parainfluenza, rhinovirus and adenovirus are commonly identified in infants and young children with a lower respiratory tract infection (Avila *et al.,* 1989). Korppi and co-workers have shown that twenty years after children were hospitalised for RSV respiratory infections, lung function was found to be abnormal compared to a control group (Korppi *et al.,* 2004). Post-mortem lung tissue secretions obtained from fatal asthma, asthma suffers who died from non-asthma causes and non-asthma

controls all show a similar viral profile using the technique of PCR, and suggest that the lower respiratory tract acts as a reservoir for viruses (Macek *et al., 1999)*. Macek has also shown the presence of adenovirus capsid protein in lung tissue of children with steroid resistant asthma that developed after childhood bronchiolitis, and in some cases they were able to culture adenovirus (Macek *et al.,* 1994). Some epidemiological studies have implicated earlier childhood adenoviral infections as an independent factor in the development of adult asthma and COPD (Samet *et al.,* 1983). Also, there is a reduction in mean FEV_1 adjusted for age and height in men who had pneumonia in their first 2 years (Shaheen *et al.,* 1995). It may be that the timing of an insult (viral infection) with respect to the stage of child lung development is the critical factor to the final outcome (in terms of airflow obstruction development). Lower respiratory tract infection in childhood leads to impaired lung function as measured by FEV_1 in adulthood and it has been suggested that this reflects impaired lung growth. Low birth weight, and birth weight at 1 year is associated with worse lung function in adult life and death from COPD in adult life (Barker *et al.,* 1991; Edwards *et al.,* 2003).

There is evidence of chronic bacterial infection in patients with COPD (Möller *et al., 1998*: von Hertzen *et al.,* 1997). Chronic *Haemophilus influenzae* infection in explanted human lung is more common in those with underlying COPD (Möller *et al.,* 1998) and there is a higher presence of *Chlamydiae pneumoniae* in patients with COPD (von Hertzen *et al.,* 1997). Chronic colonization with *Chlamydiae pneumoniae* is also associated with increased frequency of COPD exacerbations (Blasi *et al.,* 2002).

Adenoviral Protein and cigarette smoke

A possible additive effect of adenoviral protein and cigarette smoke in lung inflammation is demonstrated by adeno 5 virus infected guinea pigs where there

were increased numbers of macrophages and T-lymphocyte helper cells in response to cigarette smoke (Vitalis *et al.,* 1998). Further, there is in vivo evidence in humans that latent adenoviral infection may be important in the pathogenesis of COPD as Matsuse showed that in resected lung tissue increased amounts of the E1A region of the viral genome is present in patients with COPD (Matsuse *et al.,* 1992). E1A viral protein has also been detected in human lung sections (Elliott *et al.,* 1995).

Adenoviral E1A Protein and susceptibility to COPD

Hogg has postulated that an earlier adenoviral infection may render the respiratory tract more susceptible to damage in the face of an oxidant stimulus such as cigarette smoke (Hogg *et al.,* 2001). Adenoviral DNA can integrate itself into the human genome and this can lead to the amplification of viral proteins (oncoptroteins). One such viral protein is the early one adenoviral protein (E1A). Human adenovirus type 5 (Ad5) produces early E1A messenger RNAs (mRNAs) of 13S and 12S that encode proteins of 289 and 243 residues (Perricaudet *et al.,* 1979). The major role of E1A is to induce cell entry into the S phase of the cell cycle so that the conditions are optimal for viral replication (Shenk *et al* 1996).

E1A and Proinflammatory Cytokines

The possible role of E1A in lung disease (inflammation) comes from the influence E1A can have on inflammatory cytokines and molecules. Metcalf (Metcalf *et al.,* 1996) found that stimulating E1A transfected cell lines (THP-1 and Jurkat) lead to increased production of TNF-α, compared to cells transfected with a control plasmid. E1A transfected A549 alveolar like epithelial cells when stimulated with endotoxin produce an increased amount of IL-8 (Keicho *et al.,* 1997). This group has also shown that the expression of the adhesion molecule ICAM-1 is increased in E1A transfected cells in response to LPS (Keicho *et al.,* 1997) and that E1A may modulate the activity of the ICAM-1 promoter in epithelial cells (Higashimoto *et al.,*

1999). Furthermore, it has been shown that LPS, and not TNF-α or PMA, stimulation of A459 E1A transfected cells leads to increased IL-8 expression (Keicho *et al.,* 1999). In addition E1A transfected cells, LPS stimulation fails to induce other inflammatory mediators, such as monocyte chemotactic and activating factor, TGF-β, IL-1β, IL-6, granulocyte-macrophage colony stimulating factor (GMCSF) and granulocyte colony-stimulating factor (Keicho *et al.,*1997). It is thus possible that the mechanism of E1A enhanced epithelial IL-8 cytokine release and ICAM-1 expression is exclusive for IL-8 and ICAM-1. It has also been demonstrated that the NF-κB binding nucleotide sequence of the IL-8 promoter region favours binding of the Rel-A containing homodimer rather than p50 homodimer (Keicho *et al.,* 1999; Brasier *et al.,* 1998). It is likely that the E1A protein does not bind directly to NF-κB and that its effect is through binding to co-activators. EIA transfected epithelial cells stimulated with PM_{10} release more IL-8 and ICAM-1 (both mRNA and protein) compared to non transfected, and such cells also show increased DNA binding of NF-κB (Gilmour *et al.,* 2001; Fujii *et al.,* 2003). However Fujii also showed that the monocyte chemoattractant MCP-1 levels were reduced in the cell supernatant of PM_{10} stimulated E1A transfected A549 cells.

E1A and Relationship with NF-κB and transcriptional co-activators

The E1A 13S splice variant binds and activates NF-κB (Paal *et al.,* 1997). E1A 13S mediated activation of NF-κB occurs via 2 separate pathways (Schmitz *et al.,* 1996). One pathway is the activation of NF-κB containing the p65 subunit from cytoplasmic NF-κB-IκB complex and involves reactive oxygen species, phosphorylation of IκB and IκB degradation. The other pathway involves E1A 13S stimulation of the transcriptional activity of the C-terminal 80 amino acids of p65 at a core promoter with either a TATA box or an initiator (INR) element. After NF-κB has been activated by E1A 13S there is upregulation of the transcription of pro-inflammatory genes such as TNF-α and IL-8 (Metcalf *et al.,* 1996; Keicho *et al.,* 1997).

Trancriptional coactivators p300 and CREB-binding protein (CBP) have common domains and functions, and are ubiquitously expressed and regulate a broad spectrum of biological activities such as cell differentiation. p300/CBP is able to recruit further trancriptional coactivators such as p300/CBP-associated protein (PCAF) (Yang *et al.,* 1996), NF-κB (Perkins *et al.,* 1997) and p53 (Avantaggiati *et al.,* 1997). The transcriptional coactivators p300/CBP and PCAF possess histone acetyltransferase activity (Bannister and Kouzarides, 1996). It is thought that CBP activates transcription by bridging the gap between DNA-bound transcription factors and components of the general transcriptional machinery. It is likely that CBP is central to different signaling pathways, and that sequestration of CBP by particular transcription factors results in a particular set of genes being expressed (Chakravarti *et al.,* 1996). There is evidence that E1A subverts cellular processes by displacing cellular transcription factors from CBP (Bannister and Kouzarides, 1995; Lee *et al.,* 1996).

E1A protein can interact with several different DNA binding domains and so can be recruited to different gene promoters and thus can regulate the transcription of numerous different genes that do not share the same promoter sequence (Liu and Green, 1994). The p53 tumour suppressor gene product can activate and repress transcription, depending upon which domain is bound (amino terminal domain and carboxy terminal domain). p53 binds to DNA sequences called p53 response elements, and these response elements may be present within or near the promoter of genes, and in these cases will affect transcription. p53 binds to the TATA-binding protein (TBP) and binding of the carboxy terminal domain of the p53 to TBP represses gene transcription. E1A may act by reducing transcriptional repression by displacing p53 from TBP, and in vitro EIA 13S can displace the carboxy terminal bound p53 and so release the gene from repression (Horikoshi *et al.,* 1995).

1.12 Summary and rationale of thesis

Cigarette smoking, in a proportion of susceptible individuals will lead to chronic obstructive pulmonary disease (COPD) and ultimately death from respiratory failure. Oxidative stress from cigarette smoke and endogenously produced oxidants by cellular processes contributes to the inflammation that occurs in the lungs in COPD. The glutathione redox system is one of the central antioxidant defence systems in the human lung. The altered transcription of pro-inflammatory cytokines and mediators also contributes to the inflammatory process. NF-κB and AP-1 are both redox sensitive transcription factors which are involved in regulation of gene transcription of many pro-inflammatory mediators

The general premise though out this thesis, has been that lung tissue obtained from subjects with airflow obstruction would show alterations in glutathione and related enzymes, and changes in the DNA binding of the redox sensitive transcription factors NF-κB and AP-1, and that early one adenoviral protein would be identifiable in the lung.

HYPOTHESIS

1. There is increased glutathione levels and increased γ-GCS gene transcription in the lungs of patients with COPD and a generalised oxidant/antioxidant imbalance in favour of oxidants.

2. The altered redox status associated with airflow obstruction leads to activation of redox regulated transcription factors in human lungs and there is increased DNA binding of the transcription factors AP-1 and NF-κB.

3. Latent adenoviral infection is present and can be detected in the lungs of patients with airflow obstruction.

AIMS

The studies described herein were designed to measure glutathione levels, the activity and expression of γ-GCS, activation of redox transcription factors (AP-1 and NF-κB) and to examine for the presence of EIA, and to illustrate their relationship to the presence of COPD *in vivo* using human lung tissue.

Specific objectives

1. To measure GSH levels in human lung tissue obtained from smokers with and without COPD
2. To measure the activity of γ-GCS activity in human lung tissue from smokers with and without COPD
3. To perform semi-quantitative analysis of γ-GCS expression by *in situ* hybridisation
4. To measure levels of lipid peroxidation products in human lung tissue from smokers with and without COPD
5. To measure levels of 4-HNE by Western blotting
6. To measure levels of antioxidant capacity in plasma obtained from smokers with and without COPD
7. To measure the DNA binding of NF-κB and AP-1 in human lung tissue from smokers with and without airflow obstruction
8. To identify the presence of E1A protein in human lung tissue

CHAPTER 2

THE ASSESSMENT OF ANTIOXIDANT STATUS IN HUMAN SMOKERS LUNGS

2.1 Introduction

The glutathione reductase system is probably the most important antioxidant defence system within the lung. In vitro studies using alveolar type II like cells have shown alterations in GSH and γ-GCS gene in response to an oxidant stimulus including cigarette smoke (Rahman *et al.,* 1995). In an attempt to understand the inflammatory process that is occurring in smoking related lung disease, we hypothesised that in lung tissue obtained from smokers with severe airflow obstruction there would be enhanced GSH levels and increased activity of the rate limiting enzyme γ-GCS by way of a protective response to smoking related oxidant attack. In this study systemic and local oxidative stress, glutathione, glutathione peroxidase activity, and γ-GCS activity were measured in lung tissue, and γ-GCS gene expression was examined, in patients with normal lung function, airflow obstruction and patients with severe airflow obstruction who had undergone lung volume reduction surgery.

2.2 Methods

2.2.1 Methods for lung tissue collection

Lung tissue was obtained from patients who were undergoing resection for suspected peripheral bronchial carcinoma, lung volume reduction surgery (LVRS) and lung biopsy on the Thoracic Surgical Unit, Royal Infirmary Edinburgh between May 1998 and June 2001. The study was approved by Lothian Research Ethics Committee and also had approval from the Research and Development Unit of the Edinburgh Hospital Trust. Patients gave written consent for the use of lung tissue and a blood sample in the research project. A smoking history was obtained from the patient the day before the operation, and on the morning of the operation a blood sample was taken in an EDTA coated tube and carried on ice back to the laboratory. On the morning of the operation the patient was questioned as to the timing of their last cigarette and an estimate made as to whether this was within 24 hours of the surgery.

Lung tissue was taken from the thoracic surgical theatres immediately after it was resected, and taken on ice to the pathology department where it was examined macroscopically by the pathologist who then removed a piece of macroscopically normal tissue from the resected lung. The tissue was placed in cold phosphate buffered saline (PBS) and taken to the laboratory on ice. The lung tissue was then washed several times in PBS, gently dried in sterile gauze, and then cut into approximately 0.5g pieces before being stored at -70°C. The first fifteen lung tissue samples collected were placed directly into the -70°C freezer, but the later samples were snap frozen in liquid nitrogen first and then placed in the -70°C freezer. The EDTA collected samples were centrifuged at 6000 rpm, 4°C for 6 minutes and the supernatant stored at -80°C.

The majority of patients had pre-operative spirometry performed in the lung function laboratory at the Royal Infirmary Edinburgh and this data was obtained. In those who had not had lung function performed in Edinburgh, the referring hospital was contacted to obtain spirometry. In a proportion of patients it was not possible to obtain

lung function data. The final histological diagnosis of the resected tissue was obtained from the pathology department of the University of Edinburgh.

A pool of lung tissue was created which consisted of four groups, and which included severe airflow obstruction (LVRS), moderate airflow obstruction, no airflow obstruction and never smokers. Twenty patients had undergone lung volume reduction surgery. Of these nineteen, two patients had undergone LVRS bilaterally, and two patients had LVRS trimmings taken from different lobes of the lung.

The definition of COPD used in this study was based on that of the global strategy for the diagnosis, management and prevention of chronic obstructive pulmonary disease (GOLD), (Pauwels *et al.,* 2001). Moderate airflow obstruction consisted of subjects with an FEV_1/FVC ratio of less than 70%, and a percent predicted FEV_1 of less than 80% and greater than or equal to 30%. The presence of no airflow obstruction was defined as normal spirometry. The non smoking group consisted of subjects with no smoking history and no previous lung disease besides the current problem necessitating lung resection. The fourth subject group were those with clinical and CT evidence of airflow obstruction and emphysema who were undergoing lung volume reduction surgery and were considered to have severe airflow obstruction, although technically a number of these subjects did not fulfil the GOLD criteria for severe COPD based on spirometric data.

141 patients had a histological diagnosis of bronchial carcinoma, although there was a wide range of histological diagnosis including TB, benign hamartoma and suppurative lung disease. Two patients who had undergone resection for suspected bronchial carcinoma were found to have histological evidence of tuberculosis, and four patients were suffering from a supurative lung disease. Two patients had a benign hamartoma, one patient had a chronic scaring process present, and one patient had chronic interstitial pneumonia. Eight patients had interstitial lung disease and one patient had bronchiolitis organising pneumonia.

2.2.2 Trolox Equivalent Antioxidant Capacity (TEAC)

All reagents used in this thesis were obtained from Sigma Aldrich Co Ltd., Poole, UK unless otherwise stated.

Blood samples were collected in EDTA coated tubes on the morning of the operation. Seventy two subjects were included and comprised patients undergoing thoracic surgery for suspected bronchial carcinoma or lung volume reduction surgery (LVRS) for severe emphysema. Plasma was obtained by centrifugation of the blood samples at 6000 rpm for 6 minutes. TEAC was measured by the method of Rice-Evans and Miller (Miller *et al.,* 1993). TEAC was calculated by defining the concentration of the vitamin E analogue Trolox that had the equivalent antioxidant capacity to 1mmol/L of the plasma sample under investigation. Solutions of 0.5mM 2,2' Azino-bis (3-ethylbenz-triazoline-6-sulfonic acid) diammonium salt (ABTS), 450μM hydrogen peroxide and metmyoglobin (70μM) were prepared. A solution of metmyoglobin was prepared by making a 740 μM solution of potassium ferricyanide (K_3 FE $(CN)_6$ in PBS. Myoglobin (7.5 mg/ml horse heart Sigma) was added to the ferricyanide solution to give a 400 μM solution of metmyoglobin. The ferricyanide and myoglobin were allowed to react for five minutes with occasional shaking, and the solution placed in a dialysis tube and dialysed in PBS in the cold room for 6 hours. The metmyoglobin concentration was calculated using the equation:

$$\text{Metmyoglobin conc} = 146 \times (\text{Abs}_{490}) - 108 \times \text{Abs}_{560} + 2.1 \times \text{Abs}_{580} - \text{Abs}_{700}$$

Absorbances were read in a spectrophotometer using 1ml plastic cuvette with 0.1 ml metmyoglobin and 0.9ml PBS and read against 1ml PBS blank.

A 70 μM working solution of metmyoglobin was used for the assay.

The reagents {497 μl (PBS + plasma/Trolox), 300 μl 0.5 mM ABTS, 36 μl 70 μM metmyoglobin, and 167 μl 450μM hydrogen peroxide} were added to a 1ml plastic cuvette, and the change in absorbance at 734 nm over 6 minutes was recorded. A

standard curve was produced by using increasing concentrations of trolox (0, 2.5 nmole-12.5 nmole) and measuring the absorbance at 734 nm after 6 minutes. The results reported were the mean ± SEM of one experiment performed in quadruple.

2.2.3 Lipid Peroxidation

Malondialdehyde was measured using a Calbiochem calorimetric assay kit, EMD Biosciences, Inc, an Affiliate of Merck K GaA, Darmstadt, Germany.

Frozen lung tissue was weighed and then homogenised on ice in 2 mls Tris Cl pH 7.4 in 15 ml size falcon tubes. The homogeniser probe was cleaned between each sample with 75% alcohol and distilled H_2O. Six hundred µl of lung homogenate was placed in 1.5 ml eppendorf and centrifuged at 13000 rpm, 4°C for 10 mins. Two hundred µl of supernatant was used in the assay.

The assay is based on the reaction of reagent R1(10.3 mM N –methyl-2-phenylindole in acetonitrile) with malondialdehyde (MDA) at 45°C, in which the reaction yields a stable chromophore with a maximal absorbance at 586 nm. The reagent R1 is diluted in methanol with 3 parts R1 to 1 part methanol to give R1 mix. The assay was carried out in clean glass tubes. MDA standards 0.5 µM, 1 µM, 1.5 µM, 2 µM, 4 µM and 6 µM (in the final volume of reaction of 1ml) were produced using 5, 10, 15, 20, 40, and 60 µl of the standard (standard concentration 0.1 mM). Reagent R1mix and the standard or sample were added together with an appropriate volume of 20 mM TrisCl pH 7.4 to bring volume to 850 µl and vortexed for 5 seconds. One hundred and fifty µl of 12 N HCL was added to the glass tube which was vortexed again. Plastic bungs were inserted into the tubes before incubation at 45°C for 1 hour in a water bath, and then cooled on ice for 10 minutes. The glass tubes were centrifuged at 3000 rpm for 10 mins at room temp. The supernatant was placed in 1ml plastic cuvettes and read against the standard blank at 586 nm. A standard curve was produced and values for the samples calculated using the standard curve. Lipid peroxidation was expressed in

μmoles per gram of lung tissue. The results reported were the mean ± SEM of one experiment performed in duplicate.

2.2.4 GSH and GSSG assays

Approximately 0.5 g of lung tissue was homogenized on ice in 0.1 M phosphate buffer pH 7.5 with 5 mM EDTA (KPE buffer) and the supernatant was used in the assay. Glutathione levels were measured using an assay described by Tietze (Tietze *et al.,* 1969). This assay detects the oxidation of GSH by the sulfhydryl reagent DTNB (5,5'-dithiobis[2-nitobenzoic acid]) in the presence of NADPH, following the conversion of GSSG to GSH by glutathione reductase to form the yellow derivative 2-nitro-5-thiobenzoic acid which can be detected spectrophotometrically. This assay measures total glutathione and does not distinguish between oxidized (GSSG) and reduced glutathione (GSH). 20 μl of supernatant, 680 μl of phosphate buffer with EDTA, 100 μl DTNB (5mM) and 100 μl glutathione reductase (1 unit/100μl) were added to a 1 ml plastic cuvette. 100 μl of 2.4 mM NADPH was added and the rate of change of the absorbance measured for 1 minute at 410 nm. The total glutathione concentration was obtained using linear regression from a standard curve using reduced GSH in the range of 0.33 to 1.35 nmol. The results were expressed in nmole of GSH per mg of total protein. To measure GSSG, the supernatant (100 μl) and standards were treated with 2-vinylpyridine (10 μl) for 30 minutes, followed by the addition of 30 μl triethanolamine and thereafter used in the assay for GSH as described above, but with GSSG standards (0.165 nM -1.65 nM). The results are expressed nmole/mg protein, and as mean ± SEM. The results reported were the mean ± SEM of one experiment performed in triplicate.

0.1 M Phosphate Buffer with 5mM EDTA, Ph 7.5 (KPE)

Solution A	6.8g KH_2PO_4 in 500 mls dH_2O stored at 4°C
Solution B	8.5g K_2HPO_4 in 500 mls dH_2O stored at 4°C

0.1M phosphate buffer made by adding 16mls of solution A to 84 mls solution B and diluting with 100 mls dH_2O, adjusting pH to 7.5 and adding 0.327g EDTA

2.2.5 γ-Glutamyl cysteine Synthetase (γ-GCS) Activity

γ-GCS activity was measured using the technique of Seelig and Meister (Seelig and Meister, 1985). The enzyme activity was measured in a reaction mixture containing L-glutamate, L-α-Aminobutyrate, and ATP by a coupled enzyme procedure in which the rate of formation of ADP, in the presence of pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, and NADH, is obtained from the decrease in absorbance of NADH at 340 nm. The determination of γ-GCS activity is based on the oxidation of NADH which is assumed to be equal to the formation of ADP following addition of all the necessary reagents.

The reactions are:~

$$\text{L-glut.} + \text{ATP} \xrightarrow[\text{α-aminobutyric acid}]{\text{γ-GCS}} \text{ADP} + \text{glutamylbutyrate} + \text{amino phosphate}$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$$

Approximately 100 mg lung tissue was homogenised on ice in 1ml of 0.1 M Tris HCL pH 8, and γ-GCS measured immediately after homogenisation without freezing the homogenate as the γ-GCS enzyme is very unstable.

Briefly Tris HCL (0.1M), pH 8 with 150 mM KCl, 10mM L-glutamate, 10mM L-aminobutyrate, 20 mM Mg Cl, and 2 mM sodium EDTA were prepared in advance and stored at 4°C. On the day of the experiment Na_2ATP, phosphoenolpyruvate and

NADH at concentrations of 5 mM, 2 mM, and 0.2 mM respectively were added to this buffer solution and this γ-GCS buffer was brought to room temperature. The reaction was carried out in 1ml glass cuvettes. γ-GCS buffer (880 µl), 100 µl lung supernatant, 17 µg of pyruvate kinase enzyme (in 10 µl) and 17 µg of lactate dehydrogenase enzyme (10µl) were added, mixed and the decrease in absorbance at 340nm recorded over 1 minute. A blank was created using 100 µl water was used for comparison. γ-GCS activity was measured using the molar coefficient of 6.22 in the following equation:

$$\frac{(\Delta\text{Abs of sample} - \Delta\text{Absorbance of blank})}{6.22 \times \text{sample volume (mls)}} \times \text{total cuvette volume(mls)} = \text{units/ml}$$

µmoles of NADPH oxidised per minute per ml of sample (units/ml).

All results were expressed per mg protein. The results reported were the mean ± SEM of one experiment performed in triplicate.

2.2.6 Glutathione Peroxidase (GPx) Activity Assay

This method is based on the assay described by Paglia and Valentine (Paglia and Valentine, 1967). The GPx reaction involves the reduction of hydroperoxides (ROOH) to water using glutathione (GSH) as a reducing substrate with the production of GSSG. GSSG is then recycled by glutathione reductase (GR) and NADPH.

The reactions are:~

$$2GSH + ROOH \xrightarrow{\textbf{GPx}} GSSG + ROH + H_2O$$

$$GSSG + 2NADPH \xrightarrow[\textbf{GR}]{} 2GSH + 2NADP^+$$

The assay measures the rate of disappearance of NADPH at 340 nm. Using cumene hydroperoxide as the hydroperoxide, results in the selenium dependent form of GPx activity being assessed.

For this assay approximately 100 mg lung tissue was homogenised in 1 ml 0.2 M potassium phosphate buffer, pH 7 (39 mls 0.2 M KH_2PO_4 and 61 mls 0.2M $K_2HPO_4 \cdot 3H_2O$) with 2mM Na_2EDTA (0.2 M KPE buffer) and supernatant used to measure GPx activity. The reaction buffer consisted of 35 mls KPE buffer with 57 mg reduced GSH and 10 mg NADPH. A solution of cumene hyroperoxide (70 µl cumene and 50 mls d H_2O) was prepared fresh and heated in water bath to 30°C. Nine hundred µl of the precombined buffer was mixed with 100 µl of sample and 1unit of glutathione reductase (in volume of 5 µl d H_2O, heated in a heating block to 30°) in a quartz cuvette and allowed to stand at room temperature for 5 minutes. Twenty five µl of cumene solution at 30°C was added and the decrease in absorbance at 340 nm recorded over 1 minute. The activity of each sample was determined using a bovine erythrocyte standard (Sigma). Glutathione peroxidase activity was expressed as the µmoles of NADPH oxidised per min per ml of sample and the results were expressed in µmoles/min/mg protein. The results obtained represented the mean ± SEM of one experiment performed in duplicate.

2.2.7 Protein Measurement

Protein was measured using the BCA protein assay reagent kit by Pierce, Rockford, USA. This technique utilizes bichinchoninic acid (BCA). Protein in an alkaline environment causes the reduction of Cu^{2+} to Cu^{+}, and the cuprous cation is detected calorimetrically using BCA. A purple coloured product is formed by chelation of two molecules of BCA with one cuprous ion and this soluble complex exhibits a linear absorbance at 562 nm with regard to protein concentration. Ten µl of appropriately diluted supernatant was added to each well of a 96 well plate, followed by the addition of 200 µl of a solution composed of 50 parts solution A and 1 part solution

B. A standard curve for the protein was generated using concentrations of bovine albumin 0.1 -1 mg diluted in PBS. The 96 well plate was incubated at 37°C and then read in a spectrophotometer at 570 nm

2.2.8 Statistical analysis

Results were analysed using the computer programe GraphPad Instat, and the results expressed as mean ± SEM. ANOVA was used for comparison between several groups, unpaired t test for comparisons between two groups and Pearson correlation for the assessment of correlation with lung function.

An assessment of the power of the study and determining the sample size was not undertaken prior to undertaking the study, and this will be addressed in the discussion section.

2.2.7 *In situ* Hybridisation for γ-Glutamylcysteine Synthetase (γ-GCS)

Hybridisation is the base pairing that takes place between complementary pieces of DNA or RNA. It allows the localisation of DNA or RNA sequences (genes) within a mixed population of cells within a tissue. This is achieved by the annealing of labelled complementary nucleic acid probes to DNA or RNA sequences within tissue sections.

Synthesis of γ-GCS-HS digoxigenin riboprobe

The γ-GCS-HS digoxigenin riboprobe was prepared by Dr Irfan Rahman. The 1.679 kilobase complementary DNA insert of human γ-GCS-HS in pBluescript 11 SK (ATCC 79023) was used to derive the complementary sense and anti sense probes. The plasmid containing γ-GCS-HS complementary DNA was digested using *Pst*1,

resulting in a 760 base pair fragment, and was recloned into pBlue-script II SK. The plasmid was linearised by restriction enzymes *Bam* H1 and *Hind* III. T7 and T3 RNA polymerases were used to make antisense and sense probes respectively. Digoxgenin-labelled cRNA probes were synthesised using digoxigenin-11-UTP (Boehringer Manheim).

Paraffin embedded sections of 4μm thickness were placed on Superfrost slides with two sections per slide, separated using a Dako paraffin pen. Sections were deparaffinised by heating at 60°C for 45 minutes, and washed in xylene, followed by a 100% ethanol wash. The slides were allowed to dry at room temperature, following which they were immersed in 0.2 N hydrochloric acid (HCL) for 20 minutes and then washed twice in a PBS/5 mM $MgCL_2$ solution for 10 minutes. Following this the slides were soaked in a detergent 0.3% Triton X-100 in PBS for 15 minutes. The tissue was made porous to the incoming probe by using proteinase K (5μg/1ml buffer) (buffer 20 mM Tris CL/5mM EDTA), and incubating at 37°C for 15 mins. The action of proteinase K was stopped by soaking the slides in 0.2% glycine/PBS for 10 mins. Sections were then fixed by washing in 4% formaldehyde in PBS. The non specific sites were then blocked using 10 mM DTT in PBS at 45°C for 10 minutes, followed by x2 washes in PBS for 1 min. The slides were soaked in triethanolamine with acetic anhydride, followed by washing with 2x SSC. Slides were then preheated to 62°C (the hybridisation temperature) for 30 minutes prior to hybridisation.

Hybridisation mix and slides were heated at 62°C for 10 minutes. 20ng of DIG-γ-GCS probe per 200 μl hybridisation mix were added and 200 μl of the probe/mix was placed on each section and hybridisation was allowed to take place overnight.

The following morning the slides were washed twice in 50% formamide in 2x SSC at 50°C, followed by a 30 minute wash at 50°C, followed by a 45 minute wash in 0.1 x SSC /20 mM β-mercaptoethanol at 62°C. The slides were incubated with 2 units RNAse T1 (Boehringer Mannheim) per ml in 1mM EDTA pH 7.8/2x SSC at 37°C

for 30 minutes. The slides were rinsed in buffer 1 at room temperature and then blocked using normal sheep serum 1:2000 in buffer 2, at room temperature for 30 minutes. The immunohistochemical detection of digoxigenin-labelled hybrids was performed using sheep antibody against digoxigenin (Boehringer Mannheim) at a concentration of 1:2000 in buffer 2 for 60 minutes. The slides were then rinsed in buffer 1 and then buffer 3. Colour development was achieved using nitroblue tetrazolium as a chromogen and bicholyl-indolyl phosphate as a coupling agent (Boehringer Mannheim).

Semiquantitative Analysis

Paraffin embedded lung tissue sections from 4 patients with airflow obstruction and 4 patients with no airflow obstruction were included.

The slides were reviewed by Dr Donald Salter (Consultant pathologist, University of Edinburgh) and cell types within the airways and alveoli were scored for the presence of γ-GCS-HS staining. The scoring system consisted of < 5% of cells staining score 0, 5-30% score 1, 30-90% score 2, > 90% score 3.

Hybridisation Mix

Reagents per 5ml of mix in 4x SSC

salmon sperm DNA	125 µl
t RNA 25ng/ml	200 µl
1 M DTT	49 µl
100x Denhardt's	50 µl
50% Dextrane sulphate	1000 µl
20x SSC	1000 µl
formamide	2500 µl
water	67 µl

100 ng/ml labelled probe

Buffers

PBS (X10)

7g $Na_2HPO_4 .2H_2O$
43.8 g NaCl
1.1g KH_2PO_4
made up to 500 mls with RNAse free autoclaved milliQ water

I M Tris Ph 7.4 and pH 9.5

60.6 g Tris ($NH_2C(CH_2OH)_3$)
made up to 500mls with RNAse free autoclaved milliQ water

20 x SSC

175.3 g NaCl
88.2 g sodium citrate ($HOC(COONa)(CH_2COONa)_2.2H_2O$)
made up to 1000 mls with RNAse free autoclaved milliQ water

Buffer 1 (2x buffer 1)

17.5g NaCl
23.2 g maleinezuur
made up to 800 mls RNAse free milliQ water
Bring pH to 7.5 using NaOH
Make up to 1000 mls with RNAse free autoclaved milliQ water

Buffer 2

5 g blocking-reagents in 500ml buffer 1

Buffer 3

10 ml Tris-buffer (IM Ph 9.5)
2 ml NaCl (5M)
5 ml $MgCl_2$ (1M)
made up to 100mls with RNAse free autoclaved milliQ water

2.3 Results Oxidant Imbalance

2.3.1 Markers of systemic Oxidative stress

Trolox Equivalent Antioxidant Capacity (TEAC)

System oxidative stress was assessed by measuring the plasma trolox equivalent antioxidant capacity (TEAC) in 72 patients. The characteristics of the patients are shown below in table 2.1, and the mean TEAC levels are shown in table 2.2. No significant difference in mean plasma TEAC was found in patients with normal lung function, airflow obstruction or those with severe airflow obstruction, p=0.093, ns. Normal lung function was defined as $FEV_1 \geq 80\%$ predicted and FEV_1/ FVC > 70%. The majority of plasma samples were obtained from patients undergoing surgery for a suspected malignant condition. Table 2.3 shows the levels of TEAC in current smokers and acute smokers, and those with malignant and non-malignant lung histological diagnoses.

Table 2.1 Patient Characteristics

	Normal Lung Function n=28	***COPD n=36***	***Severe COPD n=8***
Age	64(43-77)	63.5(21-73)	54(42-66)
Male:female	15:13	28:8	4:4
Smoking History pack yrs	45.4 (1-112)	45.3 (13-74)	67.7 (30-184)
FEV_1 litres (±SD)	2.57 ± 0.76	1.2 ± 0.44	0.79 ± 0.35
FEV_1 % pred	95 ± 10.5	65.3 ± 10.61	28.9 ± 16.7
FVC litres	3.47 ± 0.95	5.56 ± 0.67	2.53 ± 0.78
FEV_1/ FVC %	73.8 ± 8.08	61.2 ± 10.01	31.42 ± 10.4

Table 2.2 TEAC measurements in subjects with normal lung function, COPD and those with severe COPD who had undergone lung volume reduction surgery

	Normal Lung Function n=28	***COPD n=36***	***Severe COPD n=8***
Mean TEAC (mM) (±SD)	0.68 ± 0.12	0.75 ± 0.12	0.68 ± 0.11

	Mean TEAC ± SD (mmole)	
	smoking within 24 hrs (n=22)	no smoking within 24 hrs (n=16)
Current smokers n=38	0.73 ± 0.12	0.69 ± 0.13
Total samples n=83	Malignant n=64	non-malignant n=19
	0.73 ± 0.13	0.71 ± 0.11

Table 2.3 TEAC measurements in current smokers and acute smokers, and those with malignant and non-malignant lung histological diagnoses.

The severity of airflow obstruction assessed using percent predicted FEV_1 did not correlate with systemic oxidative stress as measured by TEAC, p=0.57, ns, figure 2.1.

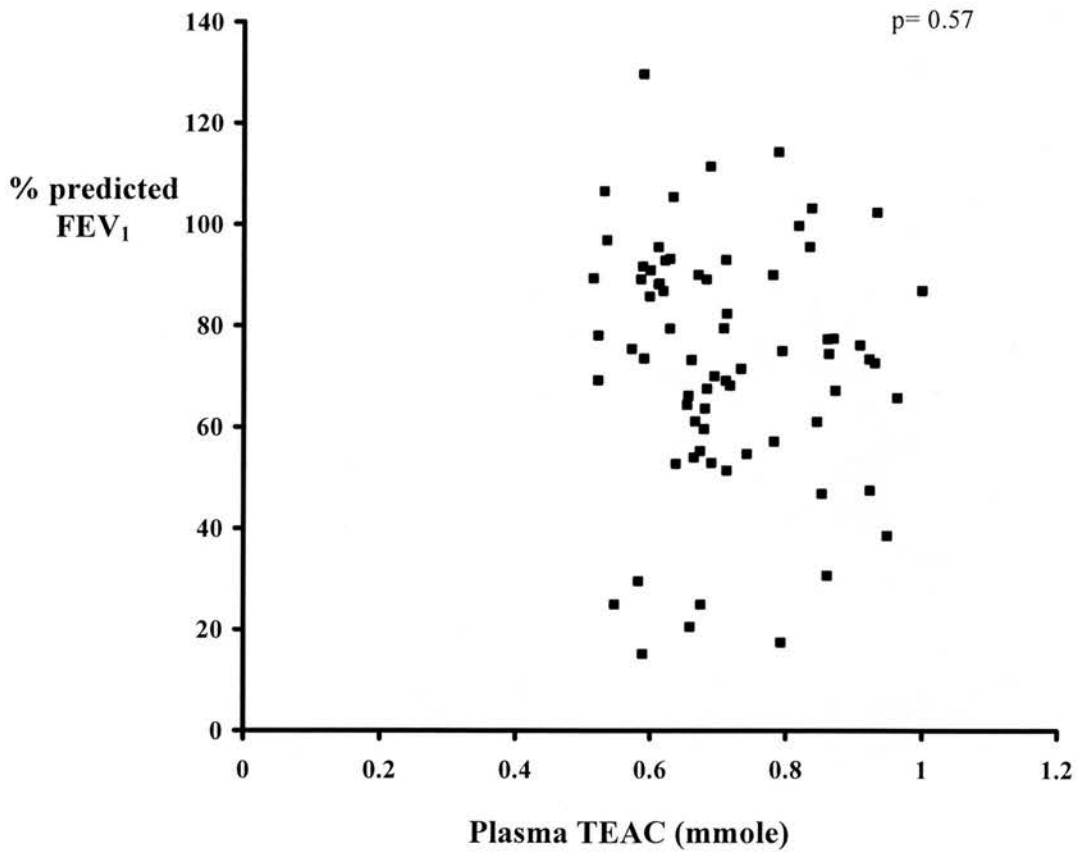


Figure 2.1 The relationship of serum TEAC, a marker of systemic oxidative stress with lung function as measured by % predicted FEV_1. There is no significant correlation between TEAC and % predicted FEV_1 (p=0.57, ns). Each point represents the mean values obtained from quadruple plasma samples.

To assess whether acute cigarette smoking had an effect on plasma TEAC, current smokers were divided into those who had smoked within 24 hours of the blood sample being obtained and those who had not. There were 38 current smokers and 22 of these had smoked a cigarette within 24 hours, 13 had not smoked in 24 hours, and smoking history regarding last cigarette smoked was unavailable in three patients. There was no difference in plasma TEAC levels in current smokers who had smoked acutely compared to current smokers who had not smoked acutely (mean TEAC in acute smokers 0.73 ± 0.12 nmole, and mean TEAC in current smokers with no cigarette smoking within 24 hours 0.69 ± 0.13 nmole). A higher level of plasma TEAC was not found in those who had smoked acutely, p=0.3, ns, figure 2.2.

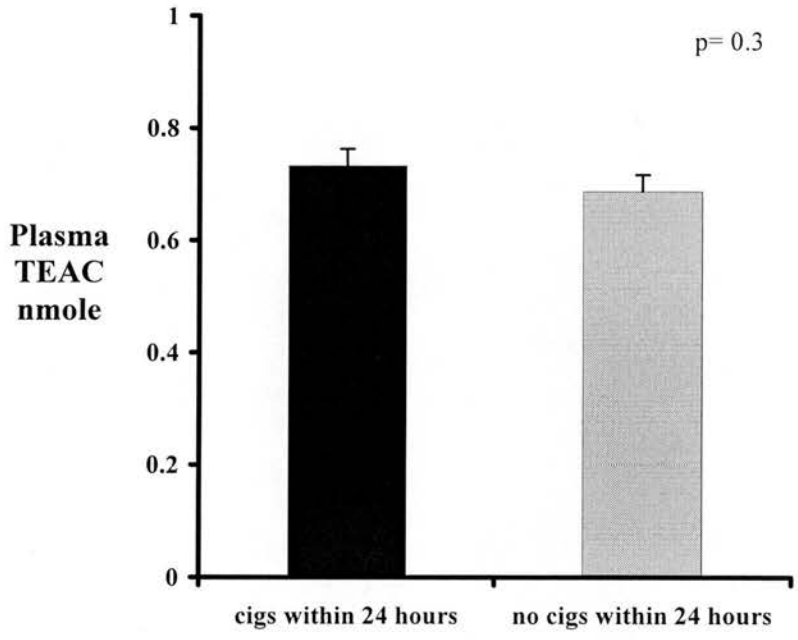


Figure 2.2 Comparison of serum TEAC levels in current smokers who had, or had not smoked within 24 hours of the sample being taken. The histograms represent the mean value and the bars the SEM. No difference in plasma TEAC level was demonstrated in these two populations (p=0.3, ns).

The effect of chronic cigarette smoking with respect to systemic oxidative stress was examined, and no correlation was found between pack years of smoking and serum TEAC, r=-0.13, p=0.32, ns, figure 2.3.

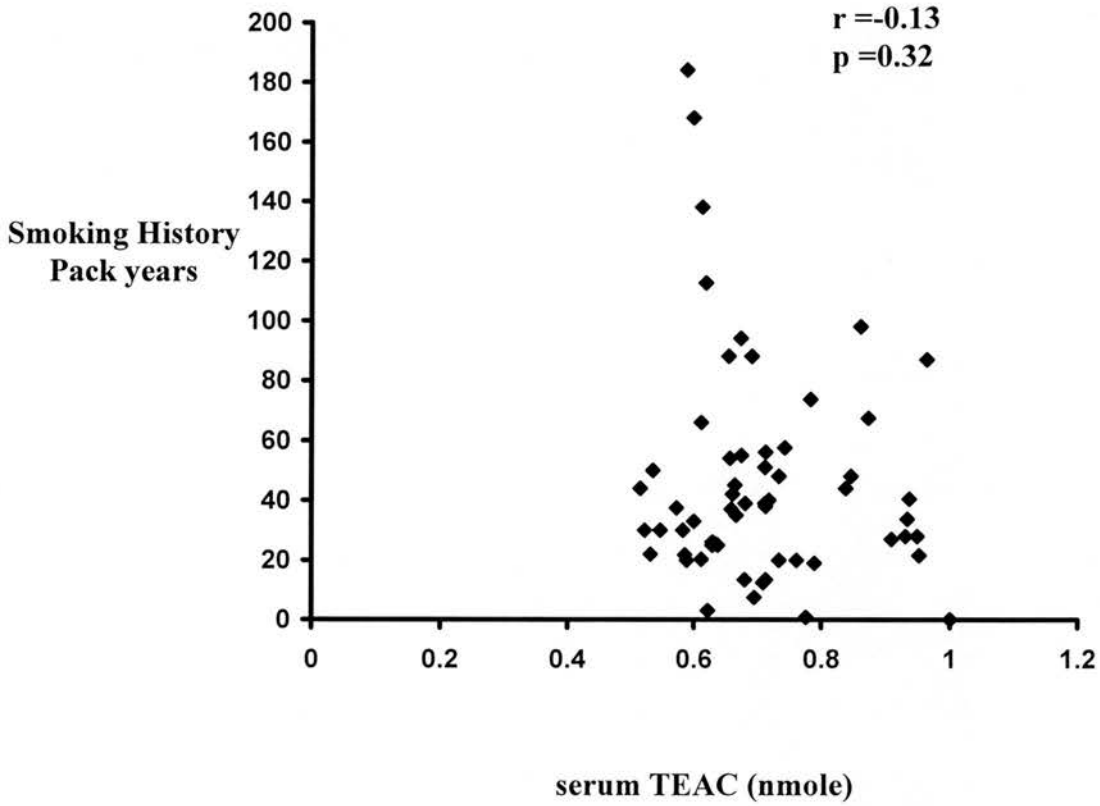


Figure 2.3 The relationship of serum TEAC levels with pack years of smoking. There is no significant correlation between TEAC and amount smoked as measured by pack years of smoking,(r=-0.13, p=0.32 ns).

2.3.2 Local Oxidative stress:Malondialdehyde in lung tissue

To investigate local lung oxidative stress, the lipid peroxidation product malondialdehyde (MDA) was measured in human lung tissue from 46 subjects (18 smokers with normal lung function, 20 with COPD, and 8 with severe COPD having undergone LVRS). Table 2.4 shows the characterstics of the patients in whom lung tissue was obtained to study lipid peroxidation. Table 2.5 shows the levels of lipid peroxidation products and figure 2.4 shows a graph of the mean MDA levels in lung tissue obtained from patients with no airflow obstruction, airflow obstruction and those with severe airflow obstruction who had undergone LVRS, and no significant difference in mean MDA levels within these three groups was found, p=0.6 ns.

Table 2.4 Patient characteristics for MDA (lipid peroxidation)

	Normal Lung Function n=18	***COPD n=20***	***Severe COPD n=8***
Age	62(43-77)	60(21-73)	54(42-66)
Male:female	9:9	13:7	3:5
Smoking History pack yrs	44.3 ± 12.6	37 ± 5.9	54 ± 26.9
FEV_1 litres (±SD)	2.9 ± 0.11	1.9 ± 0.08*	0.6 ± 0.09*
FEV_1 % pred	98 ± 3.4	67.5 ± 2.0*	22.3 ± 2.4*
FVC litres	3.9 ± 0.17	2.9 ± 0.15	2.4 ± 0.31
FEV_1/ FVC %	80.4 ± 2.3	62.9 ± 2.4*	26.5 ± 2.0*
MDA µmole/g tissue (mean ±SEM)	49.3 ± 8.28	40.4 ± 6.34	50.9 ± 9

* $p \leq 0.0001$ versus normal lung function group

Table 2.5 Levels of MDA (lipid peroxidation) in human lung tissue

	Normal Lung Function n=18	***COPD n=20***	***Severe COPD n=8***
MDA µmole/g tissue (mean ±SEM)	49.3 ± 8.28	40.4 ± 6.34	50.9 ± 9

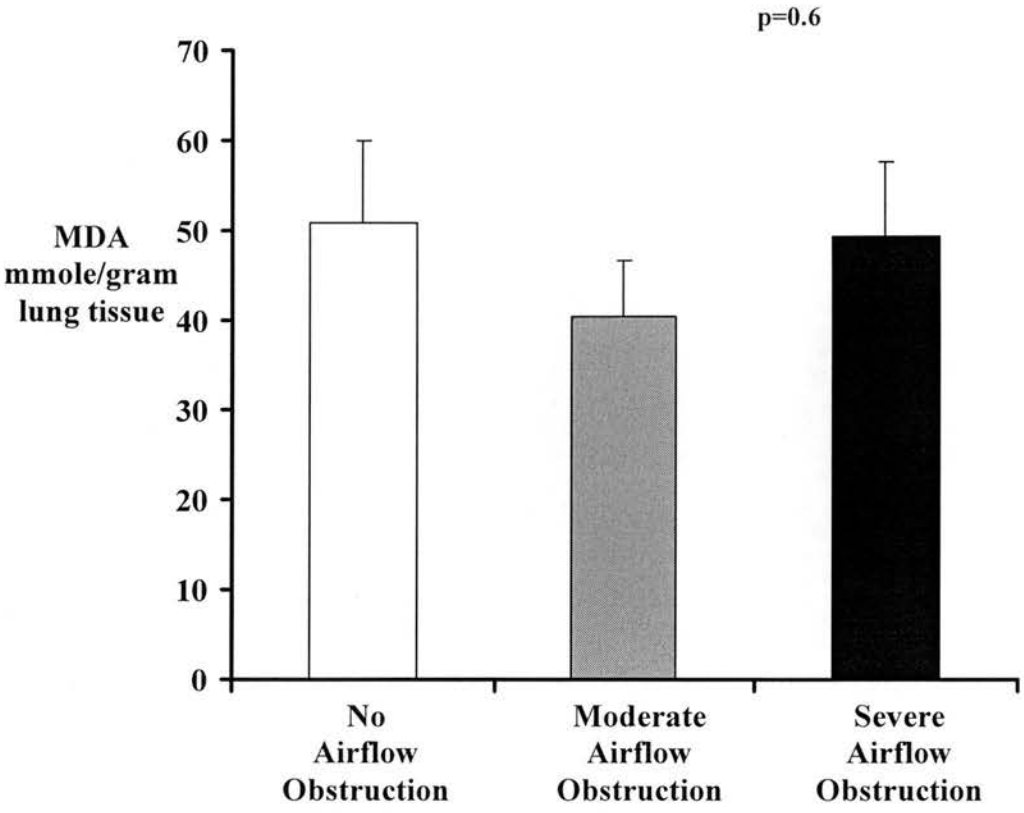


Figure 2.4 MDA (malondialdehyde) levels in lung tissue from patients with no airflow obstruction, moderate COPD, and severe COPD. The histograms represent mean values and the bars SEM. No significant difference in MDA levels in these three groups of patient is found, p=0.6 ns. Results are reported as the mean ± SEM of one experiment performed in duplicate.

2.3.3 Relationship of Glutathione levels, γ-Glutamylcysteine synthetase activity (glutamate cysteine ligase) and glutathione peroxidase with lung function in human lung tissue

Table 2.6 shows the characteristics of the 90 patients from whom lung tissue was obtained to make measurements of total GSH and γ-GCS (GCL) activity, and the levels of GSH are shown in table 2.7. Thirty five patients had normal lung function, thirty eight patients had airflow obstruction and seventeen patients had severe airflow obstruction and had undergone lung volume reduction surgery. Measurements were made on a total of 100 patients, but 10 were excluded from the final analysis as three

were found to have interstitial lung disease and so could not fulfill the criteria of normal lung tissue, one patient had tuberculosis and lung function data was incomplete in six patients. Ninety patients were therefore included in the final analysis.

Table 2.6 Patient characteristics for measurements of glutathione and γ-GCS activity

	Normal Lung Function ***n=35***	***COPD*** ***n=38***	***Severe COPD (lung volume reduction surgery)*** ***n=17***
GSH nmole/mg protein (mean ± SD)	9.57±5.67	8.08±5.51	9.84±5.57
Age (mean ± SD)	63.3±9.2	65.4± 7.6	55.9±6.5
Male:female	11:24	30:8	4:13
Smoking History (pack yrs)	38.8±30.9	44.8± 27.9	58±41.6
Current:Ex:Never:Unknown	20:10:3:2	19:16:3:0	6:10:0:1
FEV_1	2.28±0.67	2.0±0.46	0.68±0.32
FEV_1 % pred	96.5 ±13.1	62.9± 10.8	30.9±18.7
FVC	3.1±0.89	3.3 ±0.73	2.3±0.68
FEV_1/ FVC %	72.7± 6.5	60.7±10.7	33±14.2

Table 2.7 Levels of glutathione and γ-GCS activity

	Normal Lung Function ***n=35***	***COPD*** ***n=38***	***Severe COPD (lung volume reduction surgery)*** ***n=17***
GSH nmole/mg protein (mean ± SD)	9.57±5.67	8.08±5.51	9.84±5.57

Out of the 90 patients included in the study, 45 were current smokers, 36 were ex-smokers, 6 had never smoked and there was incomplete smoking history in 3 patients. Three of the current smokers had an inaccurate smoking history recorded

with regard to timing of their last cigarette. Of the current smokers, twenty two patients had smoked within 24 hours of the surgery and 20 patients had not smoked.

Mean total glutathione levels were not significantly different in lung tissue obtained from patients with severe airflow obstruction (9.57 ± 5.67 nmole/mg protein), airflow obstruction (8.08 ± 5.51 nmole/mg protein) and the non obstructed group (9.84 ± 5.57 nmole/mg protein), p=0.41, ns figure 2.5.

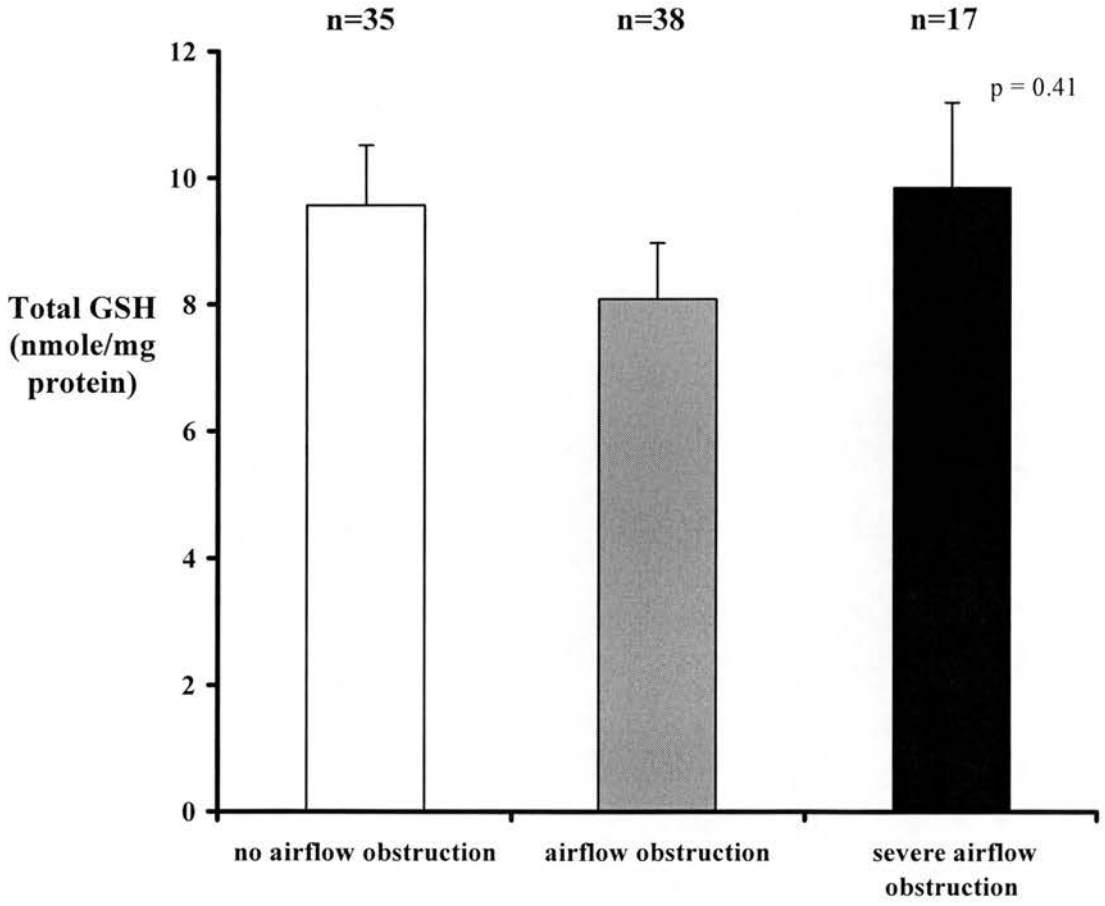


Figure 2.5 Mean total glutathione levels in human lung tissue in patients with no airflow obstruction, moderate airflow obstruction, and severe airflow obstruction (LVRS). Histograms represent the means and the bars the SEM. There is no significant difference in these groups p=0.41, ns. Results are reported as the mean ± SEM of one experiment performed in triplicate.

The level of total glutathione in current (mean GSH 9.61 ± 0.77 nmole/mg protein) and ex-smokers (mean GSH 8.36 ± 0.99 nmole/mg protein) was not significantly different p=0.22, ns, and similarly acute cigarette smoking (within 24 hours of surgery) did not influence GSH levels with similar levels being found in smokers who had (mean GSH 10.56 ± 1.15 nmole/mg protein) and who had not smoked (mean GSH 8.72 ± 1.17 nmole/mg protein) within 24 hours of surgery, p=0.24, ns, figures 2.6 and 2.7.

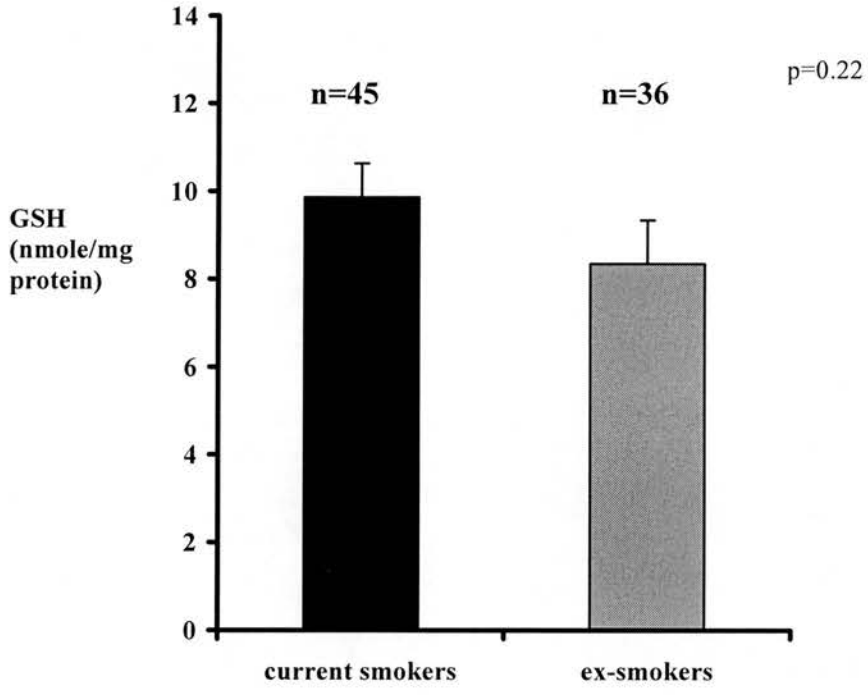


Figure 2.6 Glutathione levels in current and ex-smokers. Histograms mean and bars SEM. No significant difference in total GSH in current and ex-smokers, p=0. 22, ns.

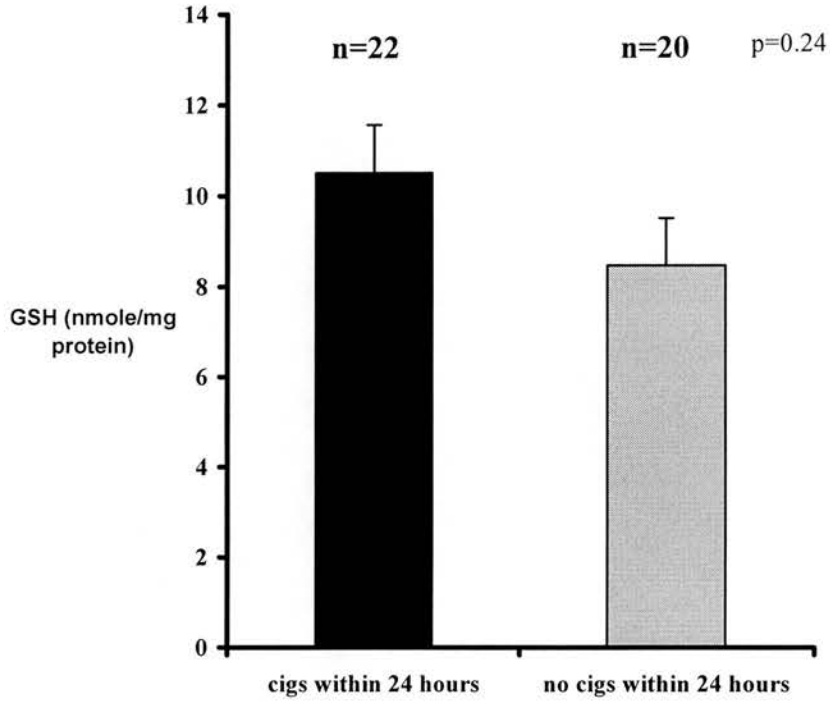
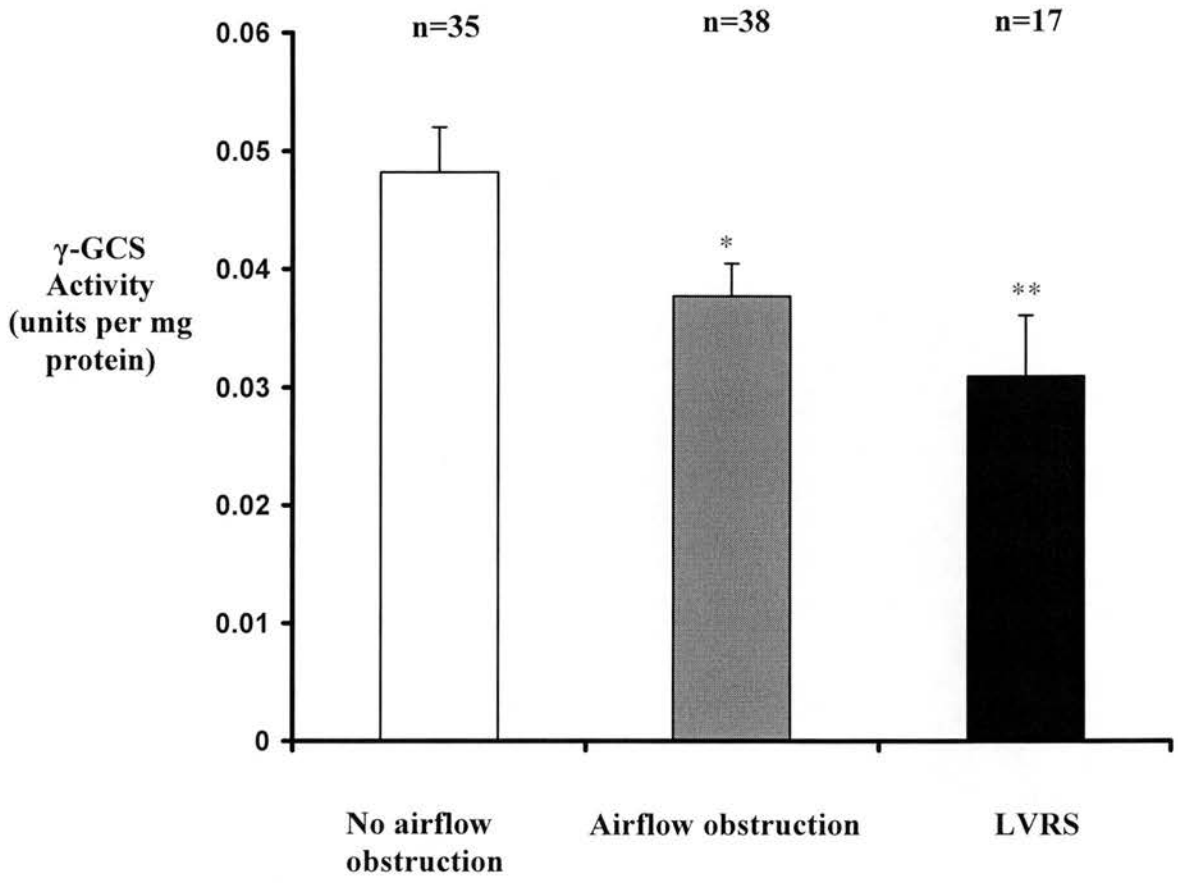


Figure 2.7 Glutathione levels in lung tissue of smokers who have smoked within 24 hours of surgery and those who have not. Histograms represent means and bars the SEM. No significant difference is demonstrated p=0.24, ns.

Measurements of reduced glutathione (GSSG) in lung tissue supernatants revealed negligible amounts of GSSG.

Although no difference in total GSH was found in patients with severe airflow obstruction, we were interested to know if there was a difference in the activity of the rate limiting enzyme in GSH production, γ-GCS (GCL). In contrast to the GSH data, γ-GCS (GCL) activity in lung tissue obtained from smokers with severe airflow obstruction was significantly lower that of smokers without airflow obstruction, $p<0.01$, figure 2.8.



*p< 0.05 airflow obstruction versus no airflow obstruction

** p< 0.001 LVRS versus no airflow obstruction

Figure 2.8 γ-GCS activity in human lung tissue from patients with no airflow obstruction, airflow obstruction, and severe airflow obstruction (LVRS). Histograms represent the means and the bars the SEM. There is a significant difference in γ-GCS activity in the group undergoing LVRS compared to the group with no airflow obstruction, p<0.01, and there is a significant difference in γ-GCS activity in the group with airflow obstruction compared to the group with no airflow obstruction p<0.05. Results are reported as the mean ± SEM of one experiment performed in triplicate.

The relationship of chronic cigarette smoking as measured by pack years of smoking did not correlate with the level of γ-GCS activity in human lung tissue, r=-0.1, p=0.18, figure 2.9.

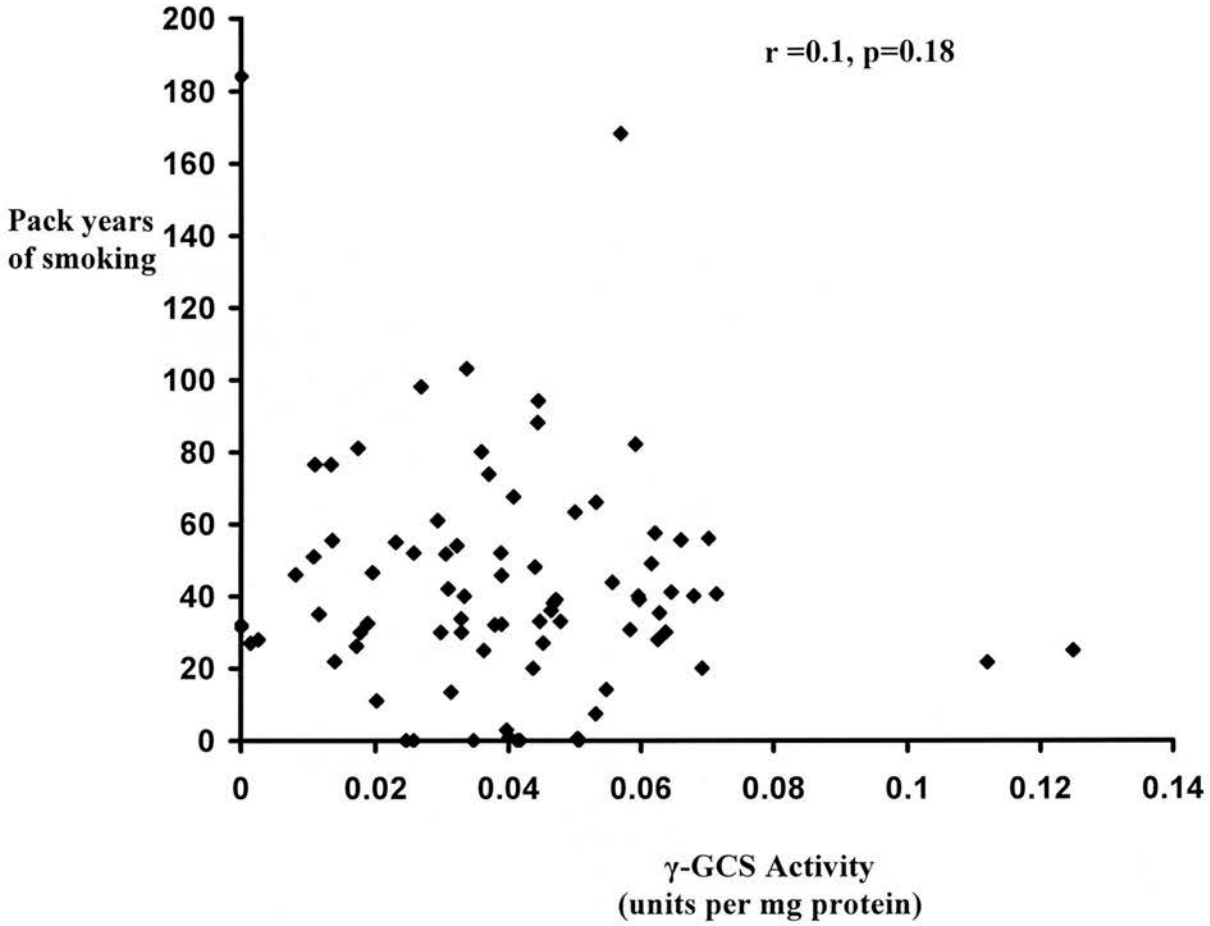


Figure 2.9 The relationship of g-GCS with smoking history (pack years). This graph shows that there is no significant correlation between pack years of smoking and levels of γ-GCS in human lung tissue, (r=0.1, p=0.18, ns).

Glutathione peroxidase activity was measured in a representative sample (46) of the 81 patients who had measurements of GSH made in lung tissue. There was no difference between this group of 46 patients and the group as a whole (81 patients). Table 2.7 shows the characteristics of the 46 patients, and the levels of glutathione peroxidase are shown in table 2.8.

Table 2.7 *Characteristics of the 46 patients in whom glutathione peroxidase activity was measured in human lung tissue.*

	Normal Lung Function n=18	***COPD n=21***	***Severe COPD n=7***
Age (range)	64.3 (37-75)	64.5 (21-73)	55.9 (42-66)
Male:female	4:14	13:7	3:4
Smoking History (pack yrs)	35.9 ± 4.8	45.1 ± 6.4	74.4 ± 23.9
FEV_1 litres (±SD)	2.1 ± 0.16	1.9 ± 0.09	0.61 ± 0.15
FEV_1 % pred	94.8 ± 3.2	64.6 ± 2.1	27.7 ± 0.9
FVC litres	2.9 ± 0.2	3.2 ± 0.18	1.7 ± 0.3
FEV_1/ FVC %	71.6 ± 5.9	61 ± 2.4	39 ± 5.9

Table 2.8 Levels of glutathione peroxidase in human lung tissue

	Normal Lung Function n=18	***COPD n=21***	***Severe COPD n=7***
Glutathione Peroxidase Activity (units/mg protein) Mean ± SD	0.076 ± 0.018	0.07 ± 0.018	0.077 ± 0.019

No significant difference in glutathione peroxidase activity level was found in smokers without obstruction (mean GPx 0.076 ± 0.018 units/mg protein), with airflow obstruction (mean GPx 0.07 ± 0.018 units/mg protein), and with severe

airflow obstruction (mean GPx 0.077 ± 0.019 units/mg protein), p=0.77, ns figure 2.10.

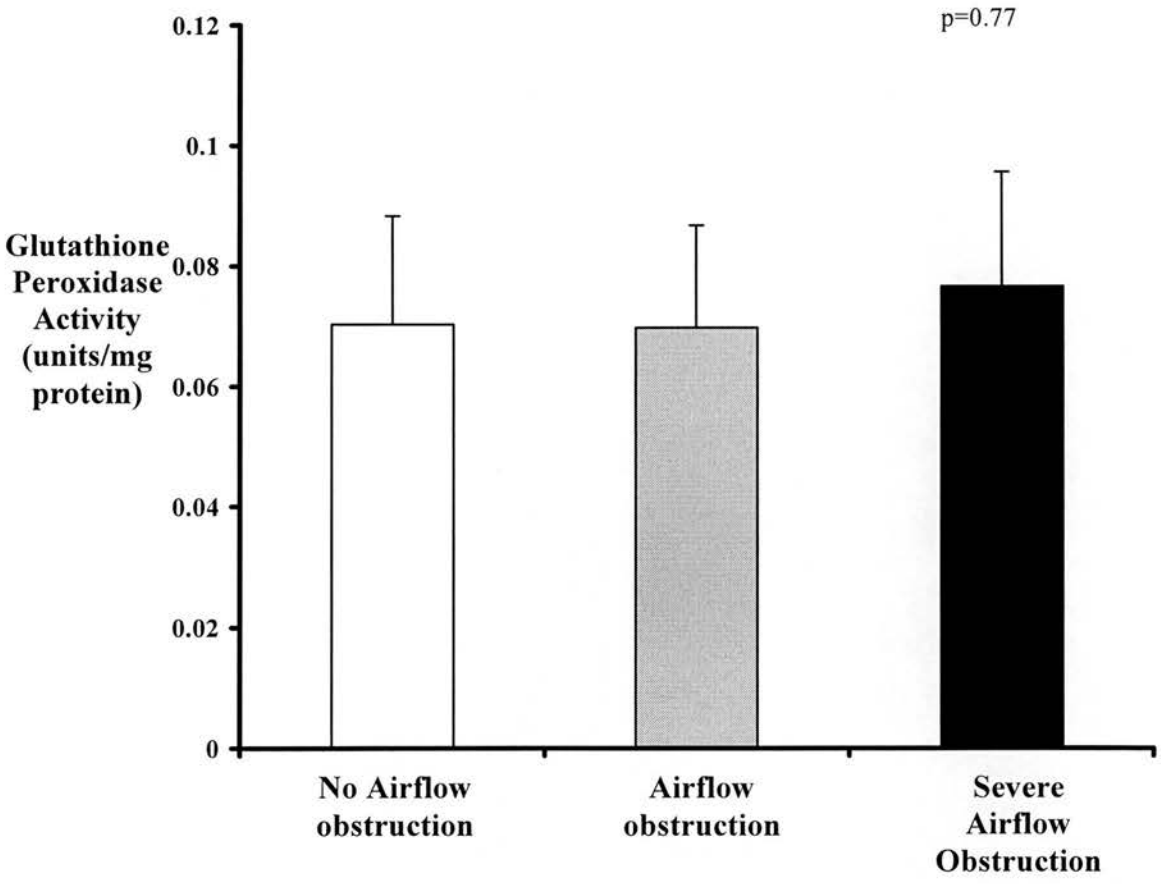


Figure 2.10 *Shows glutathione peroxidase activity in human lung tissue from the patient groups no airflow obstruction, moderate airflow obstruction, and severe airflow obstruction (LVRS). Histograms represent the means and the bars the SEM. No significant difference in glutathione peroxidase activity was found, p= 0.77, ns. Results are expressed as the mean ± SEM of one experiment performed in duplicate.*

Levels of glutathione peroxidase in human lung tissue did not correlate with pack years of cigarette smoking, r=0.01, p=0.95, ns, figure 2.11.

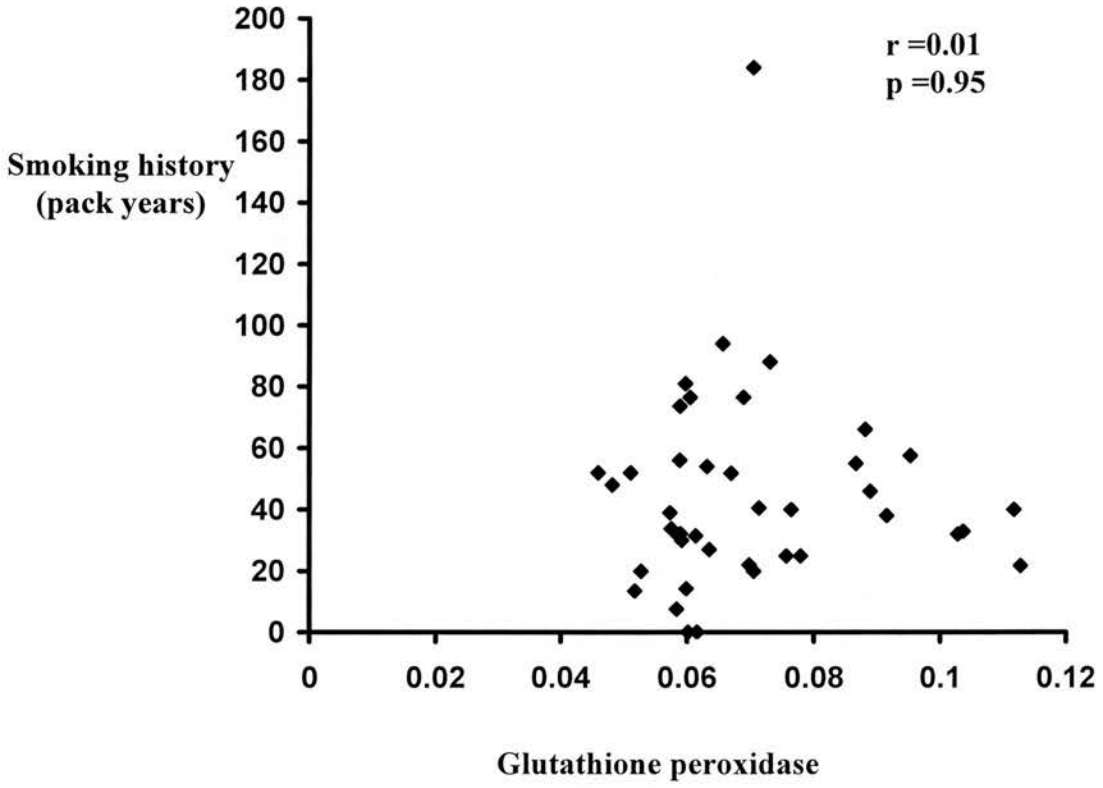


Figure 2.11 The relationship of lung glutathione peroxidase levels with pack years of smoking. There is no significant correlation between TEAC and amount smoked as measured by pack years of smoking, (r=0.01-, p=0.95 ns).

3.3.4 *In-situ* hybridization for γ-Glutamylcysteine Synthetase expression in human lung (GCL)

Expression of γ-GCS-HS was examined in human lung tissue sections from eight subjects with and without airflow obstruction using *in situ* hybridization. Table 2.9 summarises the data concerning age, lung function and smoking history for the two groups of patients examined. All patients were current smokers (having smoked regularly within the last 6 months). Only one patient in the no airflow obstruction

group had smoked within 24 hours of the lung surgery, whereas three of those with airflow obstruction had smoked within 24 hours of the surgery. Mean pack years of smoking was lower in the airflow obstruction group with a large standard deviation compared to the no airflow obstruction group.

Table 2.9 Clinical characteristics of subjects

	n	Age (years)	FEV_1 % predicted	FEV_1/ VC (%)	Pack years
No airflow obstruction	4	61.5± 5.3	89± 1.8	75.2± 3.5	61.5± 11.9
Airflow obstruction	4	63.3± 8.6	65.9±5.9	59.5± 5.4	48.7± 18.9

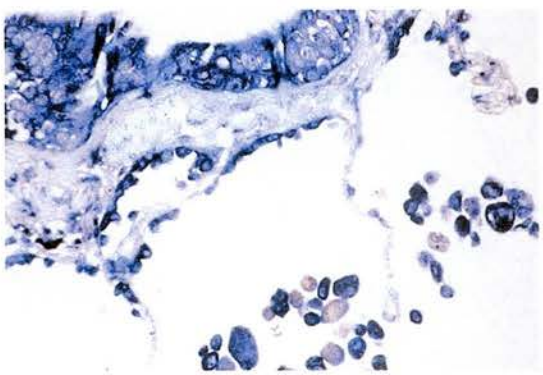


Figure 2.12

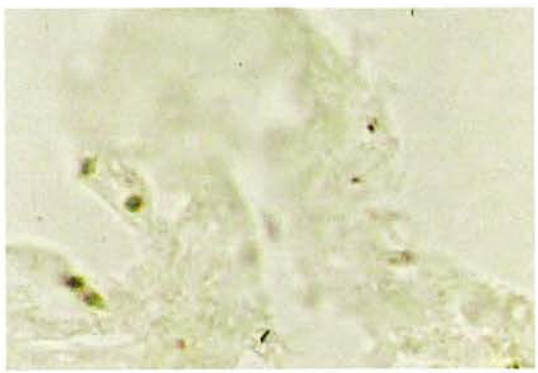


Figure 2.13

Figure 2.12 and 2.13 Photomicrograph examples of staining using antisense and sense m RNA probes for γ-GCS-HS in lung tissue from a patient with no airflow obstruction. In figure 2.12 there is strong dark blue staining in a cytoplasmic distribution in the bronchial epithelial cells and also the alveolar epithelial cells. The sense probe in figure 2.13 shows no staining.

Table 2.10 Staining scores for γ-GCS-HS expression in epithelial cells in lung tissue

	Bronchial Epithelium	type I Alveolar Epithelium	type II Alveolar epithelium
No airflow obstruction	3	3	3
	3	3	3
	3	1	3
	3	2	3
Airflow obstruction	3	3	3
	3	3	3
	3	1	3
	3	3	3

Table 2.11 Staining scores for γ-GCS-HS expression in smooth muscle cells in lung tissue

	Vascular smooth muscle		Airway smooth muscle	vascular endothelium	
	bronchial	alveolar		bronchial	alveolar
No airflow obstruction	3	0	2	1	0
	3	0	3	0	0
	2	0	2	2	0
	2	0	2	1	0
Airflow obstruction	3	0	3	1	0
	2	3	2	2	0
	0	1	2	2	1
	3	0	3	3	0

Tables 2.10 and 2.11 are the staining scores for γ-GCS-HS expression in epithelial cells and smooth muscle cells. The epithelial cells of all types in airflow obstruction and no airflow obstruction stain consistently for γ-GCS-HS expression, where as in smooth muscle this is more variable, with virtually no staining in alveolar smooth muscle and alveolar vascular endothelium.

The predominant cell types that expressed γ-GCS-HS mRNA in lung tissue from smokers with and without airflow obstruction were epithelial cells, including bronchial, and type I and type II alveolar epithelial cells. Figure 2.12 and 2.13 show photomicrographs of the staining obtained using anti-sense and sense RNA probes. Strong dark blue staining in a cytoplasmic distribution is shown with the antisense γ-GCS-HS probe. Tables 2.10 and 2.11 show the scores for the γ-GCS-HS gene expression in different cell types. No difference in γ-GCS-HS mRNA expression in epithelial cells is shown in the score for subjects with and without airflow obstruction. In both groups alveolar vascular smooth muscle, bronchial, and alveolar endothelium stained poorly suggesting that there is very little expression of γ-GCS-HS in these cell types.

2.4 Discussion Antioxidant imbalance

Chronic obstructive pulmonary disease is characterised by an abnormal inflammatory response in the lungs to inhaled particles and gases usually in the form of cigarette smoke (Pauwels *et al.,* 2001). The mechanism by which inflammation is 'abnormal' or enhanced in smokers who develop COPD is unknown but is the focus of intensive study. Enhanced oxidative stress is proposed as a mechanism by which inflammation is amplified in the lungs of COPD patients. Several studies have measured enhanced markers of oxidative stress in breath/breath condensate in COPD patients as an indication of increased airspace oxidative stress (Kharitonov and Barnes, 2001). The studies in this thesis have used fresh human lung tissue in an attempt to provide evidence for an altered overall oxidant/antioxidant imbalance in favour of oxidants in the lung tissue of smokers who have developed COPD compared to smokers with a similar smoking history, that have normal lung function.

Reactive oxygen species are generated in smoking related lung disease from inflammatory leucocytes, the normal process of aerobic metabolism and exogenous sources such as cigarette smoke. Protective antioxidant systems including the GSH/redox system, SOD and catalase operate to balance the oxidant attack. Free radical scavengers within the blood and tissues such as vitamins C and E, α-tocopherol, albumin and amino acids can help detoxification of these oxidants. Free radical activity can be assessed by measurements of lipid peroxidation, levels of plasma antioxidants such as albumin, and measurements of total antioxidant capacity in the systemic circulation. There are various methods of assessing free radical–scavenging activity in plasma including the total peroxyl radical-trapping antioxidant parameter (TRAP) assay (Wayner *et al.,* 1987) which is based on the constant production of water-soluble peroxyl radicals by the spontaneous decomposition of an initiator such as 2,2'-azobis(2-amidopropane) hydrochloric acid (ABAP), enhanced chemiluminescent (ECL) assay (Whitehead, 1992) and the use of the ABTS assay, of which TEAC is a variant. Measurements of total antioxidant activity are highly dependent on the assay used, with each assay having its own specific oxidative stress

and index marker of oxidation. Different assays tend to be more influenced by one particular antioxidant component than others. In the case of TEAC the major antioxidant component is plasma proteins (albumin 43%, urate 33%, ascorbate 9% and vitamin E 3%). Urate contributes 70% of the plasma antioxidant capacity by the ECL method and 58% in the TRAP assay.

In this study the trolox equivalent antioxidant capacity (TEAC), a marker of systemic oxidative stress, was measured in plasma obtained before patients underwent surgery for suspected bronchial carcinoma or LVRS. No significant difference in mean plasma TEAC level was found in the three groups, and levels of TEAC did not correlate with the degree of airflow obstruction. Rahman also failed to demonstrate a correlation between degree of airflow obstruction and systemic oxidative stress as measured by TEAC in the peripheral blood of smokers with and without airflow obstruction, and a healthy control population (Rahman *et al.,* 2000). However, Rahman has also shown that BALF TEAC is elevated in healthy smokers compared to healthy non-smokers, and that both smokers and non smokers with IPF have reduced BALF TEAC compared to healthy smokers and non smokers (Rahman *et al.,* 1999). Acute smoking and also exacerbations of COPD have been associated with a reduction in serum TEAC compared to controls (Morrison *et al.,* 1999; Rahman *et al.,* 1997). However, we found that in chronic smokers, smoking within 24 hours of collection of the sample did not affect TEAC levels, and the presence of a malignant or non malignant diagnosis did not influence the TEAC levels. It is likely that changes in TEAC in response to acute cigarette smoking occur earlier (than 24 hours) and that levels have returned to baseline by 24 hours. Tumour cell lines generate constitutively high levels of hydrogen peroxide (Szatrowski and Nathan, 1991), and reduced plasma peroxyl radical trapping capacity has been found in lung cancer patients compared to controls (Erhola *et al.,* 1997). It is therefore possible that reductions in serum TEAC in patients with severe airflow obstruction were masked as the comparison groups (no airflow obstruction and moderate airflow obstruction) may have had a reduced serum antioxidant capacity by virtue of the lung

cancer. However it has also been shown that patients undergoing thoracotomy for both malignant and non malignant processes have the same baseline TRAP levels (antioxidant capacity) before surgery, which rises several months after surgery (Erhola *et al.,* 1998). Our study aimed to determine if there was increased systemic oxidative stress in patients with severe airflow obstruction compared to those with normal lung function, and so a control group of normal healthy volunteers was not included. However previous data indicate higher levels of TEAC in a control group of healthy non smokers of 1.31 mmol/L (Rahman *et al.,* 2000).

The lipid peroxidation product MDA was measured in resected human lung tissue. The levels of MDA in human lung tissue, were not significantly different in the three patient groups. Others have demonstrated higher levels of lipid peroxidation products in the epithelial lining fluid of patients with COPD compared to controls (Morrison *et al.,* 1999). However in lung tissue there have been conflicting reports with regard to the level of lipid peroxidation products in smokers lungs compared to non smokers (Fahn *et al.,* 1998; Petruzzelli *et al.,* 1990).

The GSH redox system plays a critical role in maintaining the intra- and extra-cellular oxidant/antioxidant balance, particularly in the lungs. The purpose of the experiments reported in this chapter was to identify differences in GSH and its enzyme system in lung tissue from patients with airflow obstruction, compared to those without airflow obstruction. The hypothesis was that alterations in the GSH redox system may be related to the enhanced oxidative stress and hence the susceptibility to the effects of cigarette smoke, which may be a factor leading to the development of airflow obstruction in susceptible smokers.

We found no difference in lung total GSH levels in smokers with and without airflow obstruction, decreased levels of γ-GCS (GCL) activity in patients with severe airflow obstruction, and no alteration in GPx activity in the airflow obstruction group.

The response of the GSH redox system to cigarette smoke has been studied by Rahman et al (Rahman *et al.,* 1995) who showed in the A549 human alveolar

type II-like cell line that cigarette smoke condensate leads to an initial depletion of intracellular GSH, followed by a rebound increase in GSH synthesis. The in vivo corollary of these experiments show that epithelial lining fluid GSH is increased two fold in chronic smokers, but is decreased in the face of acute smoking (Morrison *et al.,* 1999). Linden found that airflow obstruction as measured by percent predicted FEV_1, showed a correlation with BALF GSH (Linden *et al.,* 1993). However, in an earlier study BALF GSH levels were found to be similar in chronic smokers with and without airflow obstruction (Linden *et al.,* 1989). In my study, the levels of total glutathione in whole lung tissue from smokers with severe COPD, moderate COPD and normal lung function were not significantly different between these three groups. Measurement of GSSG (oxidised GSH) were virtually undetectable in the vast majority of samples analysed using the method based on Tietze (Tietze, 1969). It is possible that if a different method with a greater sensitivity to detect GSSG had been employed then the GSH:GSSG ratio could have been calculated and this may have been altered indicative of increased oxidative stress. High performance liquid chromatography (HPLC) developed by Reed *et al* (Reed *et al,* 1980) has been used to measure GSH/GSSG in the nanomole range, and liquid chromatography/mass spectrometric methods are also able to detect levels of GSH and GSSG as low as 3.3 pmol (Guan *et al.,* 2003). Due to limitations in the amount of lung tissue available, it was not possible to undertake repeated measurements of glutathione in lung tissue using another method.

Human lung tissue consists of numerous cells types, connective tissue and blood vessels which contain red blood cells. Red blood cells contain GSH, and it was hoped that a correction for haemoglobin GSH could be included. We attempted to measure haemoglobin levels in the supernatant from the homogenised samples using an haemoglobin measuring kit from Sigma, however the levels in the samples were below the detection range of the kit suggesting haemoglobin contamination was not a problem. Moreover, the levels of GSH that we found were similar to previous reports (Cook *et al.,* 1991; Blair *et al.,* 1997). Both these previous studies included much

smaller numbers of lung tissue samples than the experiments presented in this thesis. Blair (Blair *et al.,* 1997) used a flourometric method using supernatant from lung tissue homogenised in 5% trichloroacetic acid, and did not use a correction for haemoglobin. Cook (Cook *et al.,* 1991) used the Tietze method (Tietze *et al.,* 1969) to determine GSH levels in cell suspensions obtained from enzymatic digested lung tissue, and removed red blood cells from the cell suspensions by lysis.

Degraded tumour biopsies show cell population variations in thiol stains, suggesting that subpopulations within tumour cells have different GSH levels (Cook *et al.,* 1991). Homogenised lung tissue, may not reflect more subtle changes that are occurring in individual cells such as macrophages and bronchial epithelial cells and may therefore give the false impression that there is no alteration in GPx, and GSH levels. Alternatively it could be argued that the initial assault on the oxidative/antioxidative balance from cigarette smoke occurs in the epithelial lining fluid, where the protective response is to increase ELF GPx and GSH to protect the valuable epithelial cells, and GPx and GSH is therefore not altered within the cells.
In cell lines acute exposure to cigarette smoke condensate decreases the levels of GSH (Rahman *et al.,* 1995), and so details of smoking history including the time of the last cigarette in relation to the surgery was recorded. We also examined whether there was a difference in GSH in smokers compared to ex-smokers, and also whether smoking a cigarette within 24 hours of obtaining the lung tissue had an effect on GSH levels. No difference in GSH levels was found in any of these groups.

In our work patients with severe airflow obstruction had undergone LVRS, a surgical procedure for a non malignant process, whereas the majority of the other pieces of lung tissue specimens were obtained from subjects with an underlying malignant process affecting the lungs. Melloni *et al* has examined GSH levels in ELF and found higher levels in the ELF from patients with non small cell lung cancer compared to non smokers, and smokers without lung cancer (Melloni *et al.,* 1996). To examine whether the presence of malignancy influenced GSH or γ-GCS levels, a

comparison was made between malignant and non malignant histological diagnoses, and no significant differences were identified.

Extracellular antioxidants are the initial defence against inhaled ROS, which subsequently become utilised in the epithelial lining fluid of the lungs. Glutathione peroxidase is an important enzyme and is found intracellularly (cGPx) as well as extracellularly (eGPx). The airway epithelium expresses and secretes GPx into the epithelial lining fluid. There is increased eGPx expression in bronchial epithelial cells and macrophages from chronic smokers compared to non smokers (Comhair *et al.,* 2000), and increased GPx acitivity is present the ELF of patients with chronic berrylium disease compared with healthy controls and healthy smokers (Comhair *et al.,* 1999). I found no significant increase in GPx activity in human lung tissue from patients with airflow obstruction, or current smokers versus ex-smokers, and nor did the timing of the last cigarette influence the GPx levels.

In epithelial cell lines cigarette smoke and also other oxidant stress leads to an initial decrease in GSH followed by a rebound increase in GSH, and these changes are mirrored by changes in γ-GCS mRNA (Rahman *et al.,* 1996) and also γ-GCS activity (Ray *et al.,* 2002). After examining GSH levels in lung tissue, we studied the activity of the rate limiting enzyme γ-GCS. Interestingly, there was a significant correlation between lung function as measured by percent predicted FEV_1 and γ-GCS activity, and more specifically that the lung tissue from patients with severe COPD undergoing LVRS had significantly lower levels of γ-GCS activity compared to smokers without airflow obstruction. These γ-GCS results in some respect appear to be in conflict with the GSH results in which no association with lung function was found. However, it could be that a real association of GSH with lung function was masked due to inaccuracies in the measurement of GSSG, or contamination in the tissue homogenate from red blood cell glutathione. γ-GCS is the rate limiting enzyme in the synthesis of GSH and so it would be expected that decreased γ-GCS activity would be associated with decreased levels of GSH. In our

studies the fact that γ-GCS activity is decreased in lung tissue of smokers with reduced FEV_1 and lower levels of γ-GCS activity did not result in lower lung GSH in COPD patients, suggests post transcriptional / translational modification of γ-GCS. The premise has been that a difference in GSH levels in smokers with and without airflow obstruction would support the theory of susceptibility to development of airflow obstruction in the severe airflow obstruction group. However the converse may be true, with no difference in GSH levels in patients with and without COPD, suggesting a failure to mount an appropriate response to an enhanced oxidant burden in smokers with COPD.

Rahman *et al* (Rahman *et al.,* 2000*)* examined human lung tissue using *in situ* hybridisation and found that there was increased expression of γ-GCS in the alveolar epithelium in smokers with COPD. Similarly animal work has also shown localisation of γ-GCS protein and message to the alveolar epithelium in response to an oxidant stimulus (Shukla *et al.,* 2000).

To investigate if there were more subtle changes in γ-GCS expression in individual cellular components of lung tissue, *in-situ hybridisation* was performed on lung sections from patients with and without airflow obstruction. Although small numbers were used, a consistent finding in both groups was that the predominant cell types expressing γ-GCS mRNA were epithelial cells, both bronchial and alveolar, and there was a general lack of staining in alveolar endothelium. My work, although on smaller numbers of tissue sections, is in agreement with others (Rahman *et al.,* 2000) and has shown localisation of γ-GCS expression to be predominantly in the epithelium of the lung.

Inaccuracies may have been present in this study. The use of resected lung tissue inevitably carries the risk of intraoperative factors influencing the results. Also the use of homogenised lung tissue, as is the case in this thesis, may not reflect more subtle changes that are occurring in individual cells such as macrophages and bronchial epithelial cells. The blood samples were collected in EDTA coated tubes

and then centrifuged to yield plasma. Collection in heparin coated tubes may have been better as heparin does not interfere with the TEAC assay, and EDTA coated tubes can give lower values. The mean results obtained for TEAC in our population of severe airflow obstruction and moderate airflow obstruction, 0.68 nmole and 0.75 nmole respectively, is lower than that reported by Rahman who collected blood samples in heparin coated tubes, were levels of 0.81 were found. Haem proteins will promote the formation of ABTS+ and give falsely elevated TEAC levels, and so careful separation of whole blood is important. This was not a problem in our study as the whole blood was collected in 4 separate EDTA coated tubes which were centrifuged, and samples with inadequate separation were discarded, without contamination of the remaining samples. The smoking history may not have been accurate as the majority of patients were undergoing surgery for a lung malignancy and so may not have disclosed their true smoking history or time of their last cigarette. Also we did not use an objective measurement of the smoking status such as urinary cotinin or carboxy haemoglobin to aid confirmation of ex-smokers. Drugs may influence systemic oxidative stress and inflammation and no details of the drugs the patients were receiving were included in this work.

Before embarking on the various studies documented in this thesis, an assessment of the power of the study should have been undertaken. This is particularly important as a large number of the studies included in this thesis failed to show sufficient evidence to reject the null hypothesis. One possible reason for this is that the sample size could have been too small to detect any statistically significant differences between the groups of interest.

A key component of the power of a research study is determining the sample size. The sample size will be influenced by the size of the clinically relevant expected difference in the measurement that is being investigated in the different groups. If the expected size of the difference in the measurement is small, and also if the measurements show a lot of variability then the sample size will need to be larger. The variability of the measurement under observation can be obtained from

previously published work or by performing a pilot study. Certainly there is previous published work (Cook *et al.,* 1991; Blair *et al.,* 1997) that has examined levels of GSH in human lung. However, the earlier work did not examine GSH levels in the specific patient groups that we were interested in and so details of the expected difference in the measurements were not readily available. However, data presented in this thesis could now be used to help determine the power of a future study.

In summary no difference in local or systemic oxidative stress, and no difference in lung total GSH or GPx levels were found in patients with or without airflow obstruction, although γ-GCS activity was reduced in patients with severe airflow obstruction. It is possible that the epithelial lining fluid is the site that manages oxidative stress inorder to minimise intracellular changes, and this may explain our findings in lung tissue.

CHAPTER 3

THE ASSESSMENT OF NF-κB AND AP-1 DNA BINDING IN HUMAN LUNG TISSUE IN PATIENTS WITH AND WITHOUT AIRFLOW OBSTRUCTION

3.1 Introduction Transcription factors

Increased inflammatory cytokine production is evident in the lungs of smokers with COPD, and this may initiate or contribute to the inflammation and damage that occurs, and the subsequent development of airflow obstruction. Cytokine expression is under the influence of transcription factors such as NF-κB and AP-1. NF-κB and AP-1 are both considered to be redox sensitive transcription factors, although there is evidence that the pathways for NF-κB activation may be different depending up on the stimulus, the presence of associated antioxidants in the local cellular environment, and also on the type of cell (Janssen-Heininger *et al., 2000;* Brennan *et al.,* 1995; Bonizzi *et al.,* 1999; Korn *et al., 2001).* Reactive oxygen species may also act as second messengers leading to NF-κB activation (Schreck *et al.,* 1991; Flohé *et al.,* 1997).

We hypothesized that lung tissue obtained from smokers with airflow obstruction would demonstrate increased DNA binding of the transcription factors NF-κB and AP-1 compared to lung tissue obtained from smokers without evidence of airflow obstruction. We also examined the subunits of NF-κB and AP-1 in lung tissue.

3.2 Methods

Transcription Factor Nuclear Binding in lung tissue

Lung collection and processing was as previously described in chapter 2 methods. The DNA binding of the transcription factors NF-κB and AP-1 was assessed in lung tissue obtained from thirty five patients who had undergone surgery for suspected bronchial carcinoma or who had undergone LVRS. In this group lung tissue was obtained from three individuals who had never smoked, four subjects who had severe airflow obstruction and who were undergoing LVRS for emphysema, and twenty eight smokers who were undergoing resection for suspected bronchial carcinoma (14 with airflow obstruction and 14 without airflow obstruction). In the case of surgery for suspected tumour, macroscopically normal tissue away from the tumour was used. Lung tissue was stored at -80˚C until the experiments were carried out.

3.2.1 Extraction of Nuclear Protein

Samples of approximately 0.5 g of frozen lung tissue was homogenised on ice (using a polytron homogeniser) in 1 ml ice cold buffer A (10 mM HEPES pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM NaF, 0.4 mM PMSF, leupeptin 1 μg/μl and 0.2mM Na orthovanadate). Pieces of fibrous material within the homogenate were discarded. The homogenised sample was left to stand on ice for 15 minutes, after which 200 μl of buffer B (10% Nonidet-P40 solution in distilled water) was added to the homogenate and vortexed hard for 15 seconds. The sample was centrifuged at 13000 rpm, 4°C for 20 seconds, the supernatant discarded and the pellet retained. Depending on the size of the pellet remaining then 50 μl or 100 μl of buffer C (50 mM Hepes, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM EDTA, 1 mM DTT, 0.4 mM PMSF, 10%glycerol, 0.2 mM NaF and 0.2 mM $NaVO_3$) was added and the mixture pipetted up and down using a wide bore pipett tip. The sample was agitated on ice for 30 minutes and centrifuged 13000 rpm 4°C for 5 mins. The supernatant fraction (nuclear protein extract) was stored at -80°C.

3.2.2 Total Protein Assay

Nuclear protein was measured using a BCA protein assay by Pierce, Rockford, USA. In brief standard concentrations of bovine serum albumin (BSA) were made up of 0.05 mg/ml - 1mg/ml. The nuclear protein samples were either used in their concentrated form or in an appropriate dilution. 10µl of sample and 10µl of the standards were added to a 96 well plate. Reagents A and B were mixed together in 1:50 ratio. 210µl of (A&B) mix was added to samples and standards and incubated for 30 minutes at 37°C, and then the absorbance was read at 570 nm using a spectrophotometer. Standard curves were drawn and sample protein concentrations calculated.

3.2.3 Electromobility Gel Shift Assay (EMSA)

This is a technique in which nuclear proteins are incubated with a radio labelled oligonucleotide sequence and then resolved on an acrylamide gel. In this technique proteins that bind specifically to an end-labelled DNA fragment (probe) retard the mobility of the fragment during electrophoresis and result in discrete bands that correspond to the protein-DNA complex. The density of the bands reflects the affinity of binding and gives a quantitative assessment of the DNA binding.

Preparation and radioactive labelling of lung tissue nuclear extracts

Ten µg of nuclear extract was incubated on ice for 20 minutes in a 500 µl eppendorf with 4 µl of loading buffer (50 mM Tris Cl pH 7.5, 500 mM KCl, 2.5 mM $MgCl_2$, in 40% glycerol and water solution) and 0.5 µl of poly dI-dC, Promega, Southampton, UK and made up to a final volume of 19 µl with distilled H_2O. One µl of labelled oligonucleotide probe was added to the mix and incubated for 20 minutes at room temperature.

Cold competitor: 2μl of the same unlabelled oligonucleotide sequence was added to the mix, together with the labelled probe.

Cold non-competitor: 2μl of a different oligonucleotide sequence was added to the mix, together with the labelled probe.

Negative Control: Distilled water replaced the sample volume.

Positive control:HeLa Extract (Promega)

Radioactive labelling of consensus oligonucleotide probe

(γ-^{32} P) ATP (Amersham Pharmacia Biotech, Buckinghamshire, UK) within the second half life of decay was used and 2μ l (1.75 pmol/μ l) of consensus oligonucleotide of interest was used.

Consensus sequence of NF-κB-DNA binding site:

5' AGT TGA GGG GAC TTT CCC AGG C-3' (Promega)

Consensus sequence of AP-1 -DNA binding site:

5'-CGC TTG ATG AGT CAG CCG GAA-3' (Promega)

Consensus sequence of Oct-1 (Promega)

5'-TGT CGA ATG CAA ATC ACTAGA A-3'

1μl 10x T4 polynucleotide kinase buffer, 1μ l T4 polynucleotide kinase (5010u/μl), 5μl d H_2O_2, and 1μ l (γ-^{32} P) ATP (3000Ci/mmol at 10mCi/ml) were added to a 500μl eppendorf tube and incubated at 37°C for 10 minutes. The reaction was stopped with 1μl 0.5 M EDTA and 89 μ l of 1x TE buffer was added. The labelled probe was stored at -20°C.

Loading buffer (DNA protein binding buffer)

50 mM Tris CL, 500 mM KCl, 5 mM DTT, 5 mM EDTA, 2.5 mM $MgCl_2$ and 40% glycerol.

Electromobility mobility shift assay (EMSA)

The protein-DNA complexes were resolved on a nondenaturing 6% acrylamide gel (10 mls 1x TBE buffer, 10mls acrylamide bis, 10 mls d H_2O_2 , 700 µl fresh 25% ammonium persulphate and 20 µl M,M,M',M'- tetramethylethylemediamine (TEMED) in 1x Tris-borate-ethylenediamine tetraacetic acid (TBE) running buffer for 4 hours at 100V. The polyacrylamide gel mixture was poured immediately into the prepared gel plates and the comb inserted for creation of the wells. The gel plate system was loaded into the tank with approx 500 mls 1x TBE buffer and the apparatus checked to identify leaks, before electrophoresis was commenced. Prior to loading the samples in the wells, the gel was pre run for 30 minutes at 150 volts. Samples were added to the wells and the gel continued to be run at 150 volts until the samples had passed through the wells, and the voltage was then reduced to 100 volts. The gels were removed from the glass plate apparatus, vacuum dried onto Whatman paper and autoradiographed at -70°C using Kodak film.

DNA binding was assessed by autoradiography and quantitative analysis was performed by densitometry measurements of the bands using UVP Grab and Gelplate program (UltraViolet Products, Limited, Cambridge, UK). The HeLa nuclear extract was used as the positive control and all other bands were measured relative to this. An arbitary value of 100 was assigned to the HeLa band and all other bands were graded relative to this. To combat any inaccuracies in gel loading, a normalization technique was used. The process of normalisation was achieved by using the universal transcription factor, Octamer-1 (Oct-1) and expressing the amount of transcription factor DNA binding as a ratio to Oct-1 binding.We were thus able to compare bands on different EMSA gels.

Data was expressed as mean ± SEM. GraphPad Instat statistical package was used to analyse the data. Spearman Rank correlation was used to analyse the data with regard to the correlation of the DNA binding with lung function, and Mann-Whitney test to compare DNA binding within the groups with and without airflow obstruction.

3.2.4 Supershift Assay

EMSAs for NF-κB and AP-1 gave more than one band. This is because NF-κB and AP-1 have several homo and heterodimers, all of which share a similar amino acid sequence. The classical activated form of NF-κB is a heterodimer consisting of a 65 k D heavy subunit and a 50 k D light unit. Using the same technique as an EMSA, but using an antibody to the NF-κB subunit to be identified, the supershift technique allows identification of the different subunits that are represented by particular bands on the gel shift. Antibodies are available for c-fos, c-jun, p50 and p65.

For the NF-κB supershift a mix consisting of 10μg nuclear protein, 4 μl of loading buffer, 0.5 μl poly dI/dC, 8μg p50 or p65 antibody (NF-κB p65 rabbit polyclonal 200μg/0.1ml Santa Cruz SC-109X, p50 rabbit polyclonal 200μg/0.1ml Santa Cruz SC-114X, Santa Cruz Biotechnology, CA, USA) was made up to 20μl with distilled water and incubated at room temperature for 1 hour. A control of non specific rabbit IgG , cold competitor and non competitors were included.

For the AP-1 supershift 2μg of c fos, c jun and Nr-f2 (rabbit polyclonal IgG S, mouse monoclonal IgG, and rabbit polyclonal IgG, Santa Cruz Biotechnology, CA, USA) were used and the mix was incubated overnight at 4°C. Non-specific rabbit IgG, and mouse IgG were included as controls and cold competitor and non competitors were also included. Labelled probe was added and allowed to incubate for 20 minutes at room temperature before the samples were loaded into the wells and the acrylamide gel resolved.

3.2.5 Western Blotting for c-jun

The Supershift technique for the subunits of AP-1, using antibodies against c-fos and c-jun failed to demonstrate retarded bands. We therefore used the technique of Western blotting to identify c-jun protein. Nuclear protein was extracted and quantified as previously described from four lung samples (two with, and two without airflow obstruction). Control proteins were included which consisted of

nuclear extract from A549 cells treated with TNF-α which had previously been shown to demonstrate c-jun on Western blotting. Bio-rad biotinylated high range SDS-PAGE standards were used, with 10μl (standard diluted 1:4 with sample loading buffer, heated to 98°C for 5 minutes) loaded on the gel. A standard Western procedure was performed (see methods section 4.2. for detailed Western protocol) using 10μg of nuclear protein, resolved at 200 volts for 40 minutes on (6% and 10% stacking and running) an acrylamide gel. Transfer of protein was as per the previously documented method using 100 volts for 1hour. The primary antibody used was c-jun (200μg/ml), Santa Cruz, CA, USA and this was diluted 1:500 in TBS/0.1%Tween/Marvel and incubated with the nitrocellulose membrane for one hour hour at room temperature. The secondary antibody was Sapu HRP antimouse IgG, SAPU, in 10mls TBS/0.1%Tween (without Marvel) with 6.6μl streptavidin and this was incubated for one hour hours at room temperature. Bands were detected using enhanced chemiluminescence (ECL Western blotting detection system, Amersham Biotech, Buckinghamshire, UK).

3.3 Results

Transcription factor DNA binding in human lung tissue

3.3.1 Patient Characteristics

Table 3.1 shows the characteristics of the 35 patients included in the study. Lung tissue was obtained from three subjects who had never smoked cigarettes, four subjects who had severe airflow obstruction and who were undergoing lung volume reduction surgery for emphysema, and twenty eight smokers who were undergoing resection for suspected bronchial carcinoma (14 with airflow obstruction and 14 without airflow obstruction). The mean percent predicted forced expiratory volume in one second was 67% in the never smokers, 39% in the LVRS group, and 60% and 94% respectively in the smokers with and without airflow obstruction. The severe airflow obstruction group and the airflow obstruction group had a mean smoking history of 73 pack years, and 55 pack years respectively which was significantly higher than that of the group with no airflow obstruction (mean pack years 35).

Table 3.1 Patient characteristics of the groups in which the DNA binding of transcription factors AP-1 and NF-κB were measured in lung tissue

		Smokers		
	Never Smokers	***Normal Lung Function***	***COPD***	***Severe COPD***
	n=3	***n=14***	***n=14***	***n=4***
Age	55 (21-73)	60 (37-73)	51(57-70)	57(49-59)
Male:female	0:3	2:12	11:3	2:2
Smoking History pack yrs	0	35 ± 3.2	55 ± 6.5*	73 ± 36.9***
FEV_1 litres (±SD)	1.5 ± 0.12	2.1 ± 0.2	2 ± 0.09	0.9 ± 0.21*
FEV_1 % pred	67 ± 6.7**	94 ± 2.1	60 ± 1.5***	39 ± 11.9***
FVC litres	2.1± 0.32	2.9 ± 0.27	3.5 ± 0.16	2.6 ± 0.42
FEV_1/ FVC %	73.3 ± 5.9	72.7 ± 1.2	56.6 ± 1.5***	35 ± 6.6***

Values are mean ± SEM

* $p \leq 0.01$ versus normal lung function group
** $p \leq 0.001$ versus normal lung function group
*** $p \leq 0.0001$ versus normal lung function group

Table 3.2 shows the normalised DNA binding for the transcription factors AP-1 and NF-κB in human lung tissue and this NF-κB data is also presented graphically in figure 3.2, and the AP-1 data presented graphically in figure 3.5. There is no significant difference in the mean normalised NF-κB DNA binding in never smokers (239 ± 40), those with normal lung function (153 ± 41.1), airflow obstruction (178 ± 25.6), and those with severe airflow obstruction who have undergone LVRS (265 ± 32.4), p=0.061.

Table 3.2 Normalised DNA binding for NF-κB and AP-1 in the four groups of subjects never smokers, smokers with normal lung function, smokers with airflow obstruction and patients with severe emphysema who had undergone lung volume reduction surgery

			Smokers		
		Never Smokers	***Normal Lung Function***	***COPD***	***Severe COPD***
		n=3	***n=14***	***n=14***	***n=4***
Normalised DNA binding	NF-κB	239 ± 40.6	153 ± 41.1	178 ± 25.6	265 ± 32.4
	AP-1	48.8 ± 15.3	30.4 ± 3	39.6 ± 4.8	24.9 ± 9.5

3.3.2 Negative correlation of NF-κB DNA binding with lung function

A significant negative correlation between the DNA binding of NF-κB and lung function (as measured by percent predicted FEV_1) was found using a non parametric Spearman Rank Correlation (r=-0.4, p=0.015), figure 3.1. A representative EMSA gel is shown in figure 3.3.

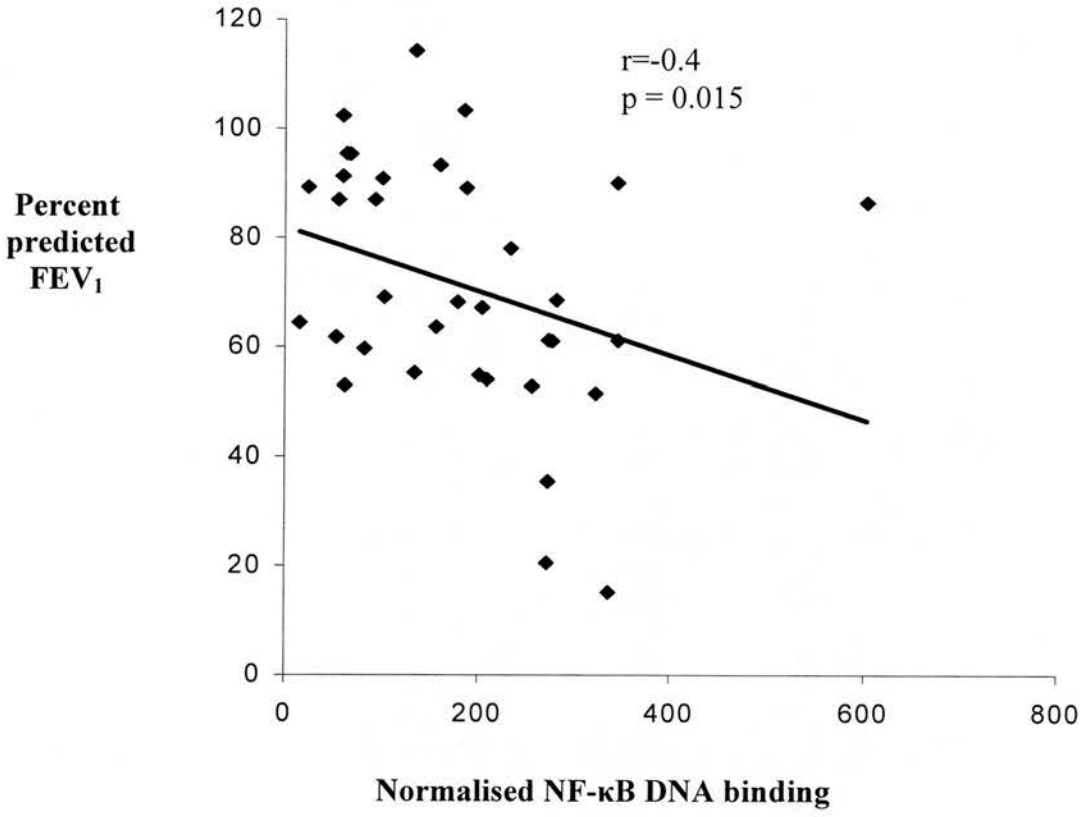


Figure 3.1 Relationship between normalised NF-κB DNA binding and lung function as measured by percent predicted FEV_1. A significant correlation is demonstrated p=0.015, r=-0.4.

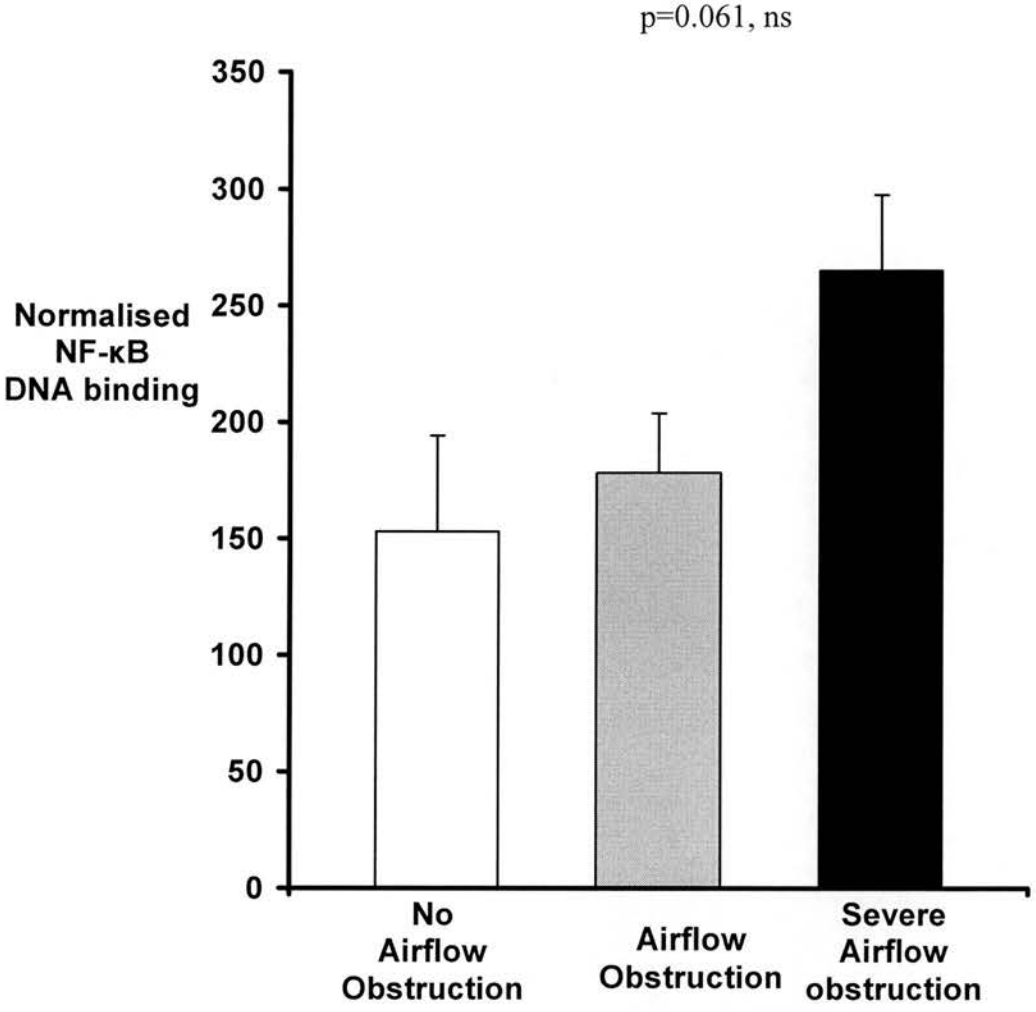


Figure 3.2 Normalised NF-κB DNA binding in human lung tissue from patients with severe airflow obstruction (LVRS), airflow obstruction, and no airflow obstruction. Histograms represent the means and the bars the SEM. The difference in normalized DNA binding of NF-κB in the group undergoing LVRS compared to the group with no airflow obstruction is not quite significant, p=0.061.

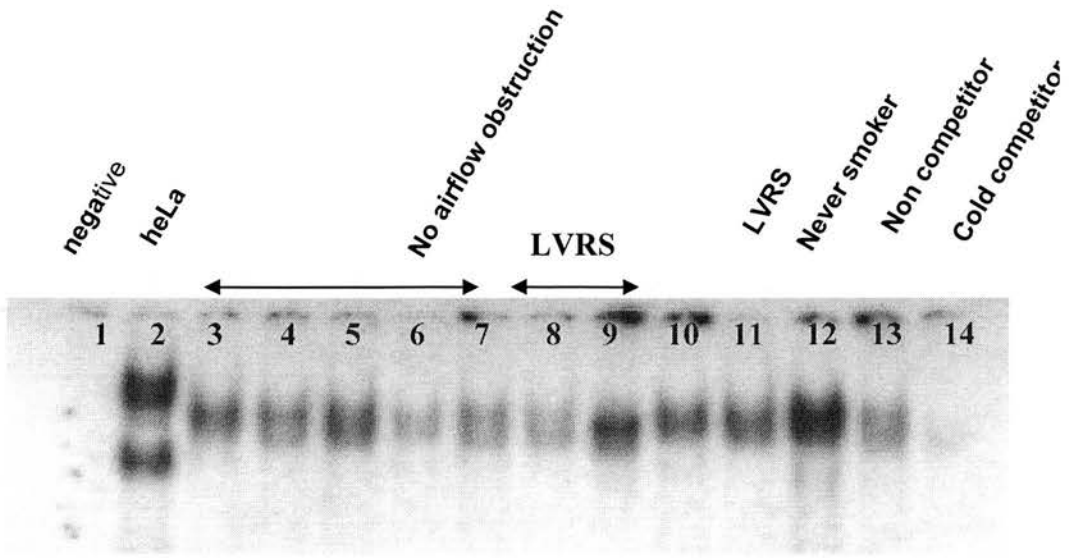


Figure 3.3 A representative electromobility shift assay for NF κB in different groups of patients is shown in figure 4. Lane 1 negative control, lane 2 positive HeLa, lanes 3-7 nuclear extract obtained from lung tissue from subjects without airflow obstruction, lanes 8,9 and 11 nuclear extract obtained from patients with severe airflow obstruction undergoing LVRS. Lane 10 and 12 nuclear extract from never smokers, and lanes 13 &14, nuclear extract incubated with a non competitor and cold competitor respectively. Bands were analysed using the UVP Grab A and Gelplate. Variability of the DNA binding is demonstrated between the patient groups.

To confirm that the band was NF-κB, a Supershift was performed to identify the subunits of the NF-κB. In figure 3.4 two retarded bands are identified in lane 6, and a single retarded band in lane 7. The upper retarded band is the heavier p65 subunit, and the lower retarded band is the p50 subunit.

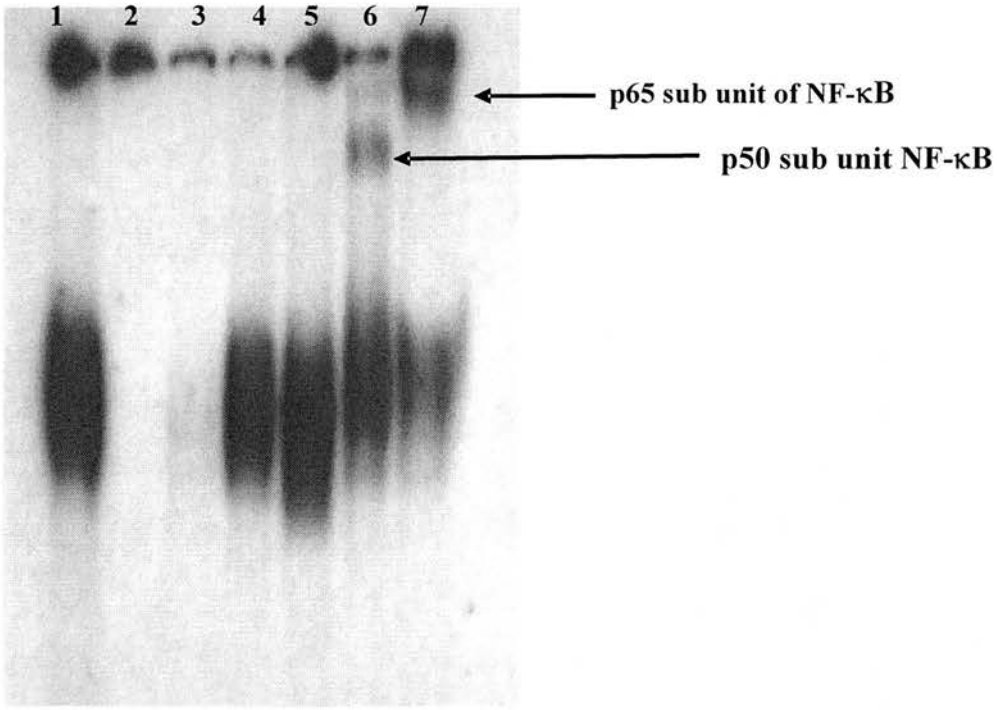


Figure 3.4 Supershift for NF-kB using nuclear protein extracted from human lung tissue. Lane1HeLa positive control, lane 2 negative (ddH_2O), lanes 3-7 nuclear protein from lung tissue samples incubated with the following: lane 3 cold competitor using excess of the oligonucleotide NF-κB, lane 4 non competitor, lane 5 a nonspecific antibody in the form of rabbit serum, lane 6 p50 antibody, lane 7 p65 antibody. There are two retarded bands in the upper part of the gel in lanes 6 and 7, which indicate the presence of the p50 and p65 subunits of the transcription factor NF-κB in nuclear extract from human lung tissue.

3.3.3 Relationship of AP-1 DNA binding and lung function

No significant correlation was found for AP-1 DNA binding and lung function as measured by percent predicted FEV_1 (r= -0.21, p=0.21, ns). Similarly there was no significant difference in the normalised DNA binding of the transcription factor AP-1 in the groups (never smokers 48.8 ± 15.3, normal lung function 30.4 ± 3, COPD 39.6 ± 4.8, and severe COPD 24.9 ± 9.5), p=0.15, ns. The relationship between AP-1 DNA binding and percent predicted FEV_1 is shown in figure 3.5.

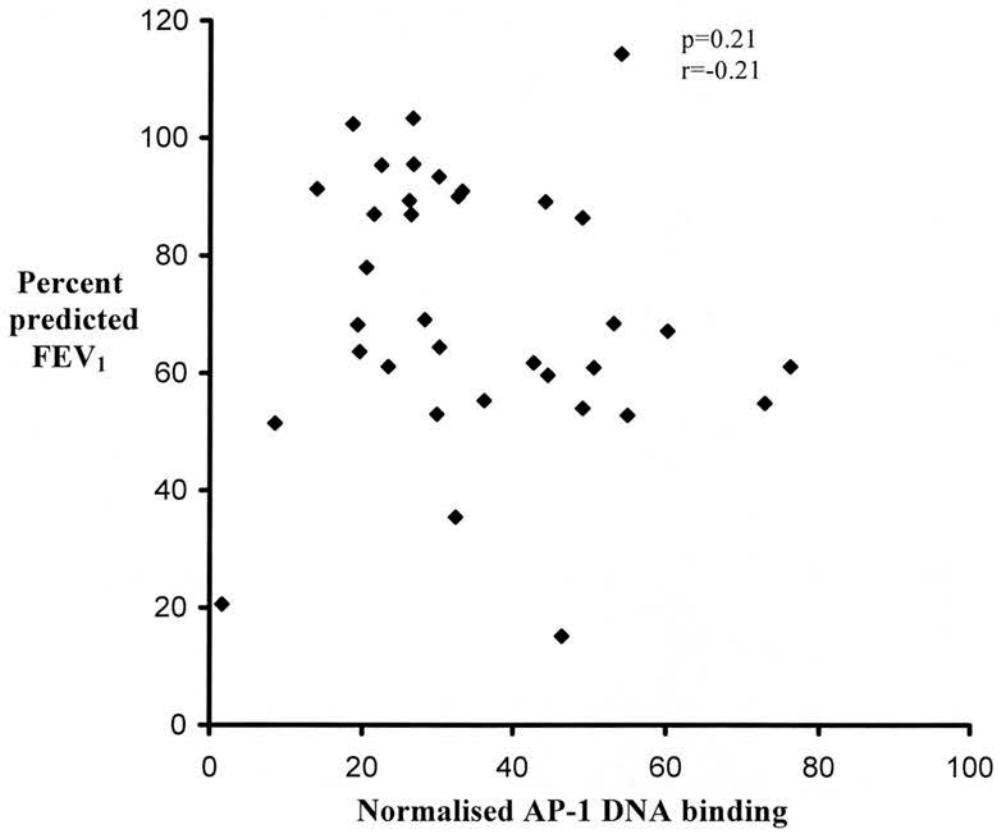


Figure 3.5 Relationship between normalised AP-1 DNA binding and lung function as measured by percent predicted FEV_1 r=-0.21, p=0.21 ns.

I was unable to demonstrate the c-fos and c-jun subunits of AP-1 using human lung nuclear extract using the Supershift technique. Western blot was therefore performed using a monoclonal antibody to c-jun, and a band in the expected region of approximately 97 kDa was seen. A representative Western blot is shown in figure 3.6.

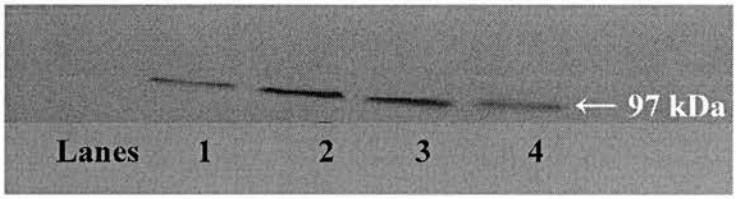


Figure 3.6 This shows a Western blot using antibody to c-jun and using nuclear extract from 2 patients with airflow obstruction (lanes 1 and 2) and two patients with no airflow obstruction (lanes 2 and 3). A protein band is visible at around 97kDa suggestive of c-jun.

3.3.4 DNA binding of the transcription factors NF-κB and AP-1 and smoking history

We examined whether the DNA binding of AP-1 and NF-κB in human lung tissue correlated with smoking history in terms of pack years of smoking. In human lung there was no significant correlation demonstrated between AP-1 DNA binding and pack years of smoking, p= 0.84, ns, or NF-κB and pack years of smoking, p=0.92, ns. Figure 3.7 and 3.8 show the relationship between pack years of smoking and the DNA binding of AP-1 and NF-κB respectively.

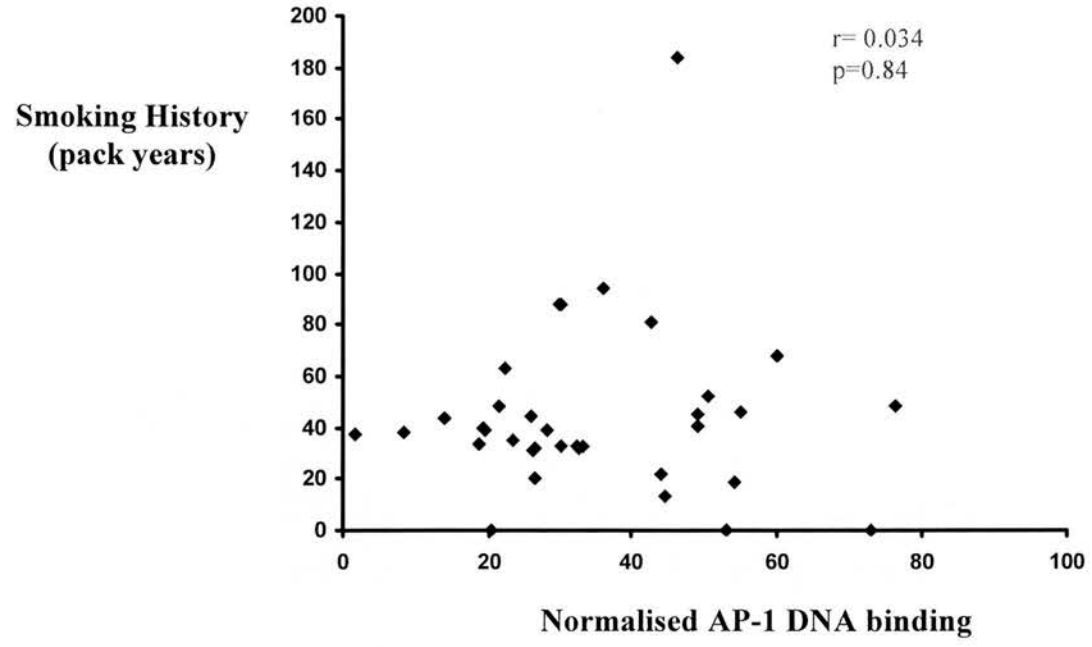


Figure 3.7 The relationship between pack years of smoking and the DNA binding of AP-1 in human lung tissue. This graph shows that there is no correlation between smoking history and the DNA binding of AP-1 in human lung tissue, p=0.84, ns.

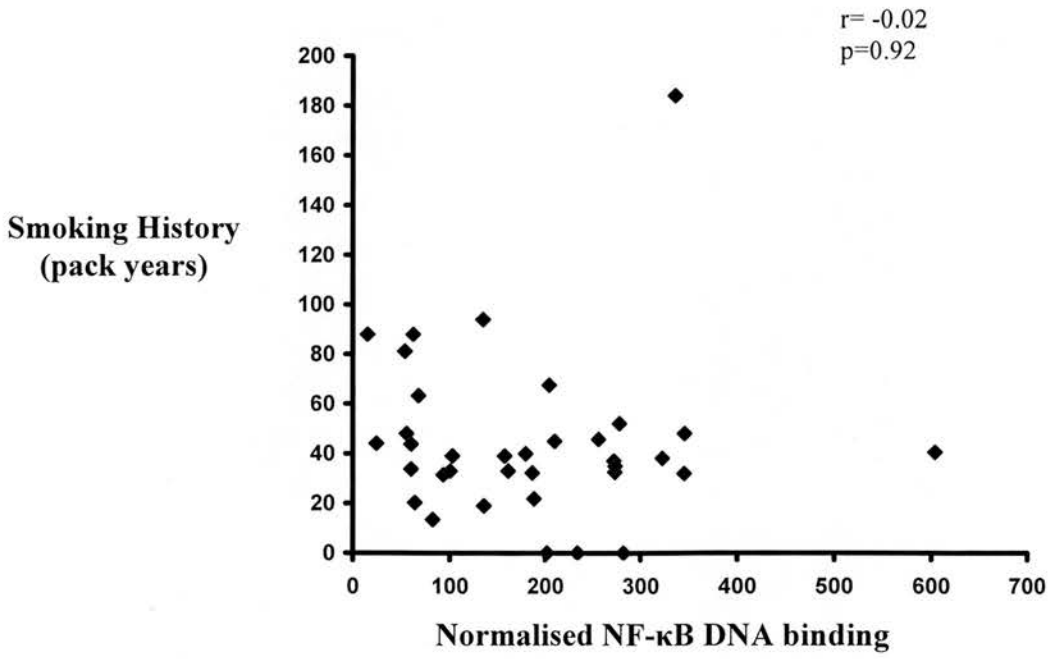


Figure 3.8 The relationship between pack years of smoking and the DNA binding of NF-κB in human lung tissue. This graph shows that there is no correlation between smoking history and the DNA binding of NF-κB in human lung tissue, p=0.92, ns.

3.4 Discussion

I was able to demonstrate a significant negative correlation between NF-κB DNA binding and the degree of airflow obstruction as measured by the percent predicted FEV_1, however no significant correlation between AP-1 DNA binding and lung function was found. The DNA binding of AP-1 and NF-κB did not correlate with pack years of smoking. I was also able to show that the NF-κB subunits represented in lung tissue are p50 and p65. Using supershift to identify the subunits of AP-1, no c-fos or c-jun subunits were found in lung homogenate, however c-jun was identified in nuclear extract by Western blotting.

NF-κB is known to play an important role in the upregulation of a number of pro-inflammatory genes (Barnes and Karim, 1997; Rao *et al.,* 1997; Akira and Kishimoto, 1997), and may be the pivotal factor in the inflammatory process in COPD. I have demonstrated variability in the DNA binding of the transcription factors AP-1 and NF-κB in fresh human lung tissue obtained from smokers, and that increased DNA binding of NF-κB is associated with more severe airflow obstruction. Previous studies have shown evidence of increased DNA binding of NF-κB in pooled nuclear extracts from bronchial biopsies obtained from asthmatic patients (Hart *et al.,* 1998). The p65 subunit of NF-κB has also been examined in bronchial biopsies by Western blot and immunostaining techniques, and increased expression of p65 in bronchial epithelium of smokers with airflow obstruction has been demonstrated (Di Stefano *et al.,* 2002). In an animal model of lung reperfusion injury, Ross has demonstrated increased NF-κB activation in lung tissue which peaks at 30 minutes after reperfusion (Ross *et al.,* 2000). In vitro studies have examined the activation of NF-κB in cell lines in response to pro-inflammatory stimuli (Janny *et al.,* 2000, Karim and Ben-Neriah, 2000), however this study demonstrates changes in NF-κB nuclear binding in human lung tissue, and in particular in smokers with and without airflow obstruction. Although a relationship was shown between NF-κB DNA binding and lung function, as lung tissue contains numerous cells it is

impossible to know whether an individual cell or group of cells is responsible for this observation.

NF-κB is a member of the Rel family of proteins and can exist in a dimeric state, either as a homodimer or a heterodimer. In order to identify which components of the NF-κB complex were present in the lung nuclear extract supershift assay was performed. We have demonstrated by supershift that the retarded bands in nuclear extract from human lung are composed of both the p50 and p65 subunits of NF-κB. Our findings are in keeping with Hart et al who have shown p50 and p65 subunits of the NF-κB complex in bronchial biopsies of asthmatics (Hart *et al.,* 1998). The NF-κB complex may be species dependent as the NF-κB complex in pig lung with reperfusion injury has been shown to be a homodimer of p65 alone (Ross *et al.,* 2000).

It is possible that intra-operative factors or ex-vivo handling of the lung tissue affected the levels of DNA binding of the transcription factors, however these factors were present in all the patients. The time from removal of the resected lung from the thoracic cavity to being frozen in liquid nitrogen was not constant, and this may have affected transcription factor DNA binding. However Ross *et al* found no difference in NF-κB DNA binding in ex-vivo lung tissue which had undergone 6 hours of ischaemic organ storage (Ross *et al.,* 2000). Certainly my fresh lung samples were all carried on ice and processed well within six hours of being removed from the body. The lung tissue was removed from macroscopically normal tissue from one area only of the lung. Ideally lung tissue from multiple areas of the lung should have been examined to assess if there was variability of the DNA binding depending on the site within the lung in an individual. Although thirty five patients were included in this study of the transcription DNA binding, only three were non smokers and only four had severe airflow obstruction. The inclusion of more samples from these groups would have improved the results. Interestingly the group of never smokers had a mean FEV_1 % predicted of 67, demonstrating airflow obstruction, and so this group was not 'normal'. Included in this group was a 21 year old woman, who had airflow

obstruction demonstrated on lung function testing, and at surgery was noted to have a tumour that was behaving as a ball and valve effect, leading to airflow obstruction. The histology of this tumour was a low grade muco-epidermoid tumour, which is in contrast to all the other malignant pathologies included in this study, which were all the more standard lung cancers (10 squamous cell, 12 adenocarcinoma, 4 large cell, 2 small cell carcinoma, 1carcinoid, and 1 non small cell with neuroendocrine features).

A large number of lung samples (35) were assessed for transcription factor DNA binding and so a method of comparing samples from different gels had to be used. This was done by giving the HeLa positive control an arbitary value of 100 on each individual gel radiograph, and grading the samples relative to this. A normalisation process was also included so that errors in gel nuclear extract loading could be controlled for. The process of normalisation was achieved by using a universal transcription factor, Octamer-1 (Oct-1); a technique similar to using a housekeeping gene in RNA polymerase chain reaction. The transcription factor Oct-1 is a member of the POU homeodomain family and is ubiquitously expressed. This protein binds to the specific-octamer sequence ATGCAAAT, and plays a role in transcription of genes that contain an Oct-1 binding motif. This normalisation method has also been used by Hart who examined pooled nuclear protein DNA binding in asthmatics (Hart *et al.,* 1998).

AP-1 is a transcription factor and the promoters of many inflammatory response genes, especially those encoding cytokines and chemokines, have AP-1 binding sites (Kracht and Saklatvala, 2002). In this study we failed to show a correlation of AP-1 DNA binding with lung function. This is particularly interesting as AP-1 is intimately involved in the upregulation of the important antioxidant enzymes γ-Glutamylcysteine synthetase (γ-GCS) and hemoxygenase-1 (Oguro *et al.,* 1996), and chapter two of this thesis has shown that there is a significant reduction in γ-GCS activity in patients with severe airflow obstruction. It therefore may have been expected that increased AP-1 DNA binding would be associated with better lung function. Studies in human epithelial cell lines show that AP-1 is acutely sensitive to

oxidant stimuli such as cigarette smoke (Rahman *et al.,* 1996; Rahman *et al.,* 1996). There are also cell type specific responses to cigarette smoke with regard to AP-1 subunit expression. Cigarette smoke exposed A549 cells show enhanced AP-1 binding (Rahman *et al.,* 1996), but there is no increased AP-1 DNA binding in cigarette exposed U397 cells which are a premonocytic cell line (Favatier and Polla, 2001). It may be that a relationship between AP-1 DNA binding and lung function does exist in particular cells within lung tissue and the use of whole lung tissue may mask changes in individual cells.

Another explanation may be that in the human lung after a prolonged oxidant insult (cigarette smoke), there is loss of the acute cigarette-induced affects on cells. The timing of an acute oxidant stimulus may therefore be important in the DNA binding of these transcription factors.

AP-1 is a dimeric protein composed of members of the jun (c-jun. jun-B, and Jun-D) and fos (c-fos, fosB, fra-1 and fra-2) family that can bind to the promoter region of various genes. Using supershift, it was not possible to demonstrate c-jun or c-fos in whole lung nuclear extract, but using Western blot a band in the region of 97 kDa, highly suggestive of c-jun was found. The same c-jun antibody was used in the Supershift and Western experiments and this suggests that the conditions for the Supershift were not correct. It is possible that the c-jun and c-fos antibodies used in the Supershift were not immunoreactive to human antigen, however this seems unlikely given that the Western technique was successful for c-jun. In a rat model of acute inflammation conflicting results using Supershift and Western have been also been found (Guo *et al.,* 2002). Guo *et al* failed to demonstrate c-jun in whole lung nuclear extracts from rats by Supershift and identified c-fos, jun-B and jun-D. However, using Western identified a band suggestive of c-jun. The work by Guo also showed time related changes in levels of the AP-1 subunits in response to IgG related acute lung injury and also that there is increased c-jun protein expression in macophages compared to lung. This group has also reported that the AP-1 complex in nuclear extract from alveolar macrophages from these same animals does not

supershift with c-fos antibodies. It therefore appears likely that there are different heterodimeric patterns of AP-1 in alveolar macrophages and lung tissue. Maturational differences in AP-1 protein subunit predominance have been shown in rat lung in response to hyperoxia with a relative lack of c-jun and abundance of junB in neonatal rats compared to older rats (Yang *et al.,* 2000). Recent in vitro work has shown that there is upregulation of c-fos and fra-1 in IL-1 induced IL-8 production and that Fra-1 is a negative regulator of IL-8 (Hoffmann *et al.,* 2004).There is therefore a complex relationship between AP-1 and NF-κB in inflammation as fra-1 counteracts the positive regulatory effects of c-fos and p65 NF-κB on IL-8 gene expression. Our work concentrated on c-fos and c-jun and we should have extended this work to include Supershift and Western blotting to examine for other subunits including jun-B and jun-D and fra-1.

The expression of jun and fos proteins varies in different cell lines and also in malignantly transformed versus non transformed cell lines. For example high levels of c-jun, jun-B, jun-D and fra2 are found in the non-transformed mouse alveolar cell line C10 (Shukla *et al.,* 2001), but lower levels of junB, c-fos, and fra-1 are found in malignant transformed HBE cells compared to non tansformed HBE cells (Birrer *et al.,* 1992). Interestingly it has been reported that neoplastic lung tissue has higher expression of c-jun in atypical, hyperplastic and metaplastic epithelium, and in the surrounding normal epithelium expression is low or undetectable (Szabo *et al.,* 1996). This altered expression of jun and fos proteins may be relevant in my study as although the lung tissue used appeared macroscopically normal, (away from the site of the tumour), histological examination of this same area was not performed to confirm that this area was infact normal. Thus a relationship between AP-1 DNA binding and airflow obstruction may not exist in this population of patients examined and the division into groups with and without airflow obstruction may be a false division by virtue of the majority of the subjects examined having lung malignancy.

In summary a relationship between the DNA binding of NF-κB and lung function has been shown, but no such relationship was found with AP-1. Further work is required

to define whether the relationship of NF-κB DNA binding and airflow obstruction holds true in all parts of the lung and/or individual cell types, as this is likely to be pivotal to the understanding of NF-κB in smoking related lung disease.

CHAPTER 4

4-HYDROXY-2-NONENAL IN HUMAN LUNG

4.1 Introduction

Cell membrane lipid peroxidation of polyunsaturated fatty acids generates aldehyde end-products including malondialdehyde (MDA), 4-Hydroxy-2-nonenal (4-HNE) and 4-hydroxy-2,3-alkenals. 4-Hydroxy-2-nonenal is a specific product of lipid peroxidation and the adverse effects of 4-HNE result from its effect on signal transduction, gene expression, apoptosis, cell proliferation and cellular responses (Parola *et al.,* 1999). Animal models show elevated levels of 4-HNE in response to cigarette smoke and in human lung tissue there are increased 4-HNE adducts in airway epithelial cells of smokers with COPD (Aoshiba *et al.,* 2003, Rahman *et al.,* 2002). Higher levels of MDA have been reported in the lung tissue of smokers with a heavy smoking history compared to non-smokers (Fahn *et al.,* 1998).

The aim of this work was to identify 4-HNE in human lung tissue using the techniques of Western blotting and immunohistochemistry.

4.2 Methods

4.2.1 Immunohistochemistry for 4-Hydroxy-2-Nonenal in human lung tissue

This work was in collaboration with Dr Pim de Boer. Lung sections from 30 patients were examined for 4-HNE using the technique of immuohistochemistry. The lung tissue was obtained from patients who were undergoing resection for suspected bronchial carcinoma and patients who were undergoing LVRS for severe emphysema. Ten patients had severe emphysema, 10 patients had airflow obstruction and ten patients had no airflow obstruction.

Three micron thick lung sections were cut and deparaffinised, rehydrated and pretreated before the immunohistochemistry was performed. The lung sections were then refixed. The primary antibody used was a mouse monoclonal antibody specific for 4-HNE adducts (Japan Institute for the control of aging, Fukuroi City, Shizuoka Prefecture, Japan) at a dilution of 1:500 and incubated overnight at room temperature in a humidified chamber. The avidin-biotin-peroxidase complex (ABC) method was used, and novaRED (Vector Labs, Burlingame, CA) was used as the chromogen, and the slides were counterstained with haematoxylin. The immunohistochemical staining of the tissue sections from all thirty subjects was performed in a single batch.

The negative control consisted of the same procedure as above, but with the omission of the primary antibody. The positive control was a section of lung tissue from a patient with adult respiratory distress syndrome.

The staining was assessed semi-quantitatively using a scoring system of 0-3 for cell types including type I and type II alveolar epithelial cells, alveolar macrophages and neutrophils. The slides were examined by a single observer (subjective viewing and non automated) and all areas of the slide were viewed, using a grid which divided the slide into 8 areas. A score of 0 reflected no staining present, score 1 weak staining, score 2 moderate staining, and score 3 reflected intense staining.

Data are expressed as mean ± SEM. 4-HNE staining scores in the three subject groups were compared using Student's t test.

4.2.2 Western Blot to examine for 4-Hydroxy-2-Nonenal in human lung tissue

My previous work (chapter 2, section 2.3) had failed to demonstrate a relationship between levels of lipid peroxidation products (MDA) in whole lung and lung function. An attempt to examine 4-HNE, a specific product of lipid peroxidation was undertaken in human lung tissue using the technique of Western blotting in which proteins are separated by movement through a gel containing a denaturing detergent (SDS/polyacrylamide gel) and then blotted and detected using antibodies. Various modifications of the basic procedure were undertaken in an attempt to demonstrate 4-HNE, and are outlined below.

Standard Western Blot

Cytosolic protein was extracted and then used in the Western Blot, and various modifications of protein extraction, samples and antibodies were carried out, and these are outlined later in pages 119-125.

General method for Western

A 10% acrylamide separating gel was prepared with an upper stacking layer of 4% acrylamide. The 10% separating gel was poured and topped up with water-saturated isobutanol and left to polymerise, following which the isobutanol was removed and the plates washed with distilled water and dried using filter paper. The 4% stacking gel was then added to the top of the separating gel, combs inserted and allowed to polymerise.

The protein lysates were prepared and then denatured by heating with sample loading buffer for 3 minutes at 95°C. The protein separating gels were prepared and the

denatured protein was added to the wells (20µl), and molecular markers to the left hand side well, and the protein and molecular weight standards were electrophoresed at 200V for 35 – 40 minutes in x1 running buffer. The gels were removed from the glass plates and the protein was then transferred to nitrocelullulose paper (Hibond) using the transfer apparatus in which a sandwich of gel, nitrocellulose paper (soaked in transfer buffer) and Whatman paper (soaked in transfer buffer) was formed and placed in the transfer chamber and electrophoresed at 25 volts for 90 minutes in transfer buffer. Transfer of protein was confirmed using Ponceau S red dye, and the protein lanes on the nitrocellulose membrane were marked with pencil, and then Ponceau red washed off using TBS. Non specific binding was blocked by placing the membrane in a solution of 1g Marvel in 20mls TBS on a rocker at room temperature for 15 minutes, followed by rinsing in TBS/0.05% Tween. The membrane was incubated over night using primary antibody 4-HNE Michael adducts (rabbit), Calbiochem diluted in TBS/Tween. After 3 washes in TBS-Tween the membrane was incubated with secondary antibody (HRP anti-rabbit IgG Diagnostics Scotland) diluted 1:10,000 in TBS/Tween with Marvel. Bands were detected using enhanced chemiluminescence (ECL Western blotting detection system (Amersham Biotech, Buckinghamshire, UK).

SDS-polyacrylamide gels

10% Separating Gel	**volume**
dH_2O 4.05ml	
1.5M Tris-HCL, pH8.8	2.5 ml
10% (w/v SDS stock)	100 µl
acrylamide/Bis(30% stock)	3.3 ml
10% fresh ammonium persulfate	100µl
TEMED	10 µl

4% Stacking gel	
dH_2O	6.1 ml
1.5M Tris-HCL, pH8.8	2.5 ml
10% (w/v SDS stock)	100 µl
acrylamide/Bis(30% stock)	1.3 ml
10% fresh ammonium persulfate	100 µl
TEMED	20 µl

Western Modification 1

In this preliminary experiment one lung tissue sample from each of the three previous patient groups (no airflow obstruction, airflow obstruction and severe airflow obstruction) that had been examined for lipid peroxidation, were used (lung samples 99, 91, and 83 respectively). The characteristics of the three patients are shown in table 4.1 in the results section.

1a. Total cellular protein was obtained by homogenising 0.5 g lung tissue in 2 mls 1.5% potassium chloride, and quantified using the BCA protein kit as previously described, and 40 μg protein was made up to 25μl with sample loading buffer and heated at 98°C for 5 minutes before loading onto the polyacrylamide gel. The concentration of the primary antibody (4-HNE Michael adducts) was 1:10,000.

1b. The conditions were altered so that the final volume of protein and sample loading buffer was 20μl and the concentration of primary antibody was increased to 1:1000.

1c. Protein extraction modification: Using the protein extracted in 1a, which had been stored at -80°C, a further centrifugation step was performed (13000rpm at 4°C for 15 minutes) and the supernatant used. Primary antibody was incubated at room temperature for 1hour. The concentration of the primary antibody (4-HNE Michael adducts) was 1:10,000.

Antiproteases were not used in 1a-1c.

Western blot modification 2

The lysis buffer was changed as described below, and antiproteases were added. The cytosolic extract obtained was used in the immunoprecipitation reaction followed by Western blotting.

2a. Lysis buffer. The method for the lysis buffer was based on the method by Cucchiaro (Cucchiaro *et a.,* .1999) and Current protocols in Immunology vol 2, unit 8.3. Frozen lung was homogenised in 1.1 ml of lysis buffer with 20 µl of antiproteases, followed by centrifugation at 13000 rpm at 4°C for 15 minutes and the supernatant used. Protein was measured using the BCA method. The lysis buffer consisted of 10 mM Tris Cl pH 7.6, 150 mM NaCl, 1mM EDTA, 0.1% Deoxycholine, 1% TritonX-100 and 46.4 mls dH_2O.

2b. Immunoprecipitation

Antibody binds to an antigen or protein complex and this antigen-antibody complex is precipitated, followed by dissociation of the antigen from the antibody and the released antigen is then electrophoresed on a gel and a standard Western procedure is carried out.

To asses whether the technique of immuno-precipitation was working, we chose to examine for the presence of a protein within a sample that was likely to be positive. The protein chosen was EIA (early one adenoviral protein), and it was postulated that cell lysates from hydrogen peroxide treated E1A transfected A549 cells would demonstrate a protein band using immunoprecipitation and Western blotting. Immunoprecipitation was performed using stored cell lysates from hydrogen peroxide treated A549 cells, and A549 cells that had been transfected with E1A, and was immunoprecipitated using E1A mouse monoclonal IgG2a (Santa Cruz sc-25, conc 200µg/ml). Lung tissue lysate from a subject with severe airflow obstruction (sample 129), was immuno precipitated using anti-4-HNE Michael adducts. Non

immunoprecipitated tissue lysate from lung sample 129 was also included in the Western.

One hundred μg of cell lysate from:1. A549 cells, 2. hydrogen peroxide treated A549 cells, 3. E1A transfected A549 cells, 4. hydrogen peroxide treated A549 cells, and 500 μg lung sample 29 lysate was incubated with 2μg E1A mouse monoclonal IgG2a (Santa Cruz sc-25, conc 200μg/ml) at 4°C for 4 hours.

500μg of lung sample 129 lysate was incubated with 10 μl anti-4HNE Michael adducts (rabbit) at 4°C for 4 hours.

Protein G beads (Sigma) were washed twice in lysis buffer and made into a 50:50 mix with lysis buffer. 50 μl bead slurry mix was added to the protein lysate (500μg lung tissue lysate and 100μg for cell lysate) and allowed to incubate with agitation for 90 minutes. Following which the bead/protein mix was centrifuged at 13000 rpm at 4°C for 5 minutes and the supernatant discarded and the beads washed x2 in lysis buffer and the beads boiled in SDS page buffer.
For the non immunoprecipitated protein sample (129), then 40μg and 80 μg were made up to 20μl using SDS-page buffer and then boiled at 98°C for 5 minutes.

Primary antibody

E1A mouse monoclonal IgG2a	10 μl in 10mls TBS/0.05% Tween
anti-4-HNE Michael adducts	10 μl in 10mls TBS/0.05% Tween

Secondary Antibody

E1A	anti-mouse IgG HRP	2 μl in 20mls (1:10,000)

4-HNE anti-rabbit IgG HRP 2 µl in 20mls (1:10,000)

Western Modification 3

Human and rat lung tissue lysates were used in the immunoprecipitation technique. Rat lung tissue lysate that had been prepared earlier for a different Western experiment and stored at -70˚C and lung sample lysate (129) that had been stored from the previous experiment, and 4-HNE-myoglobin adduct were used. The 4-HNE-myoglobin was used as it was postulated that this may be one of the 4-HNE protein adducts that is present in the human lung and so if the lung tissue produced a band at the same level as the 4-HNE-myoglobin, then it would imply that there was a protein present that was myoglobin or a protein that had the same molecular weight as myoglobin.

Tissue lysates consisting of 500 µg of human tissue and 100 µg of rat lung tissue and 200 µg of 4-HNE-myoglobin adduct (2mg/ml) were made up to a final volume of 200µl in lysis buffer. Ten µl 4-HNE adduct was added to each protein/lysis mix and agitated for 4 hours at 4°C. The method for the rest of the immunoprecipitation was as previously described on page 126. Standard non immunoprecipitated tissue lysates were also used and made up to a final volume of 20 µl in sample loading buffer and heated for 5 minutes at 98°C. Twenty µl of the lysate/SDS reducing buffer mix was added to each well and the Western performed as previously described with the following modifications

1. Primary antibody 1:500 in TBS/Tween 0.05%/marvel. One hour incubation at room temperature

2. Addition of streptavidin in a dilution of 1:1500 with TBS/Tween 0.05% alone (without marvel) was added at the same time that the secondary antibody was incubated inorder to allow the molecular weight markers to develop.

Modification to Western procedure 4

It was not clear if cytosolic protein extraction had been achieved and so the method was altered. The lung tissue samples (127 and 129) which had been stored at -80°C, underwent cytoslolic and nuclear extractions as per the new protocol which is described below, and the protein used in the Western.

4.a Method for Extraction of cytosolic protein

Lung tissue was homogenised in 1ml soloution A, and then incubated on ice for 15 minutes, following which 125µl of solution A' was added and mixed by inversion and centrifuged at 3000rpm at 4°C for 4 minutes and the supernatant extracted.

Buffer A

10 mM HEPES
10 mM KCl
1.5 Mm $MgCl_2$

Solution A
10 mls buffer A with one antiprotease tablet

Solution A'
10 mls buffer A with 250 µl NP-40

40 µg of cytosolic and nuclear protein used for Western which was performed as per modification 3 without the immunoprecipitation step.

5. Western Modification 5

The previous Western blot experiments failed to demonstrate 4-HNE in human lung cytosolic extractions. It was possible that there was no 4-HNE in these samples, but the other possibility was that the 4-HNE was rapidly lost during the process of handling the lung tissue. Frozen lung tissue (lung tissue sample 132) was defrosted, finely chopped and approximately 200 mg was incubated with a 100 mM solution of 4-HNE in PBS (4-HNE 5mg/0.5 ml N-hexane, Alexis cat no. 270-245-M005) at

37°C, and 200 mg lung tissue incubated in PBS solution alone for 1hour at 37°C. Lung sample 132 was from a 45 year old male, current smoker with no airflow obstruction, who had undergone a pneumonectomy for squamous cell carcinoma. Cytosolic extraction was as per modification 4, and the amount of protein included in the Western was 20 μg and 40 μg. 20 μg myoglobin-4-HNE adduct was also used in the Western.

Gel 1: 20 μg cytosolic protein, or myoglobin-4-HNE adduct made up to 20 μl with SDS reducing buffer and heated at 98°C for 8 minutes.

Gel 2: 40 μg cytosolic protein and 20 μg myoglobin-4HNE adduct made up to 20μl with SDS reducing buffer.

The primary antibody concentration was 1:500, and the secondary antibody concentration was 1:10,000.

Western Modification 6

Same as modification 5, but increased amounts of the cytosolic protein from human lung sample 32 (4-HNE incubated and control that had been used in the previous experiment and stored at -80°C) was used, 20 μg, 40 μg and 100 μg. 4-HNE-myoglobin adduct was also used as in the previous modification. The primary antibody concentration was 1:500, and the secondary antibody concentration was 1:10,000.

Modification to Western 7

The product information sheet for the anti-Michael adducts stated that use of more than 0.01% Tween 20 can interfere with antibody binding in Westerns. Therefore the previous experiment was repeated using 0.01% Tween for the primary and secondary antibody incubations.

Lysis Buffer
10 mM Tris Cl Ph7.6
150 Mm NaCl
1 mM EDTA
0.1% Deoxycholine
1% TritonX-100
46.4 mls dH_2O

SDS Reducing agent

1 ml glycerol
0.5 ml β-mercaptoethanol
2ml 10& SDS
1.25 ml 1 M Tris HCL Ph 6.7
3ml 0.05% bromophenol blue

5x Running buffer
9g Tris
43.2 g Glycine
3g SDS
Dissolve in 400 ml dH_2O
Make up to 600 mls with dH_2O

Transfer Buffer
2.9g Glycine
5.8g Trizma Base
0.37g SDS
Dissolve in 500 ml dH_2O
Add 200 ml methanol
Make up to IL with dH_2O

10x Tris Buffered Saline (TBS)
24.2g Tris base
Dissolve in 800 ml dH_2O
Adjust pH to 7.4 with HCL and make up to IL with dH_2O

4.3 Results

4.3.1 Immunohistochemistry for 4-HNE in lung tissue

4-HNE a specific product of lipid peroxidation was examined in lung tissue in thirty patients undergoing resection for suspected peripheral bronchial carcinoma or LVRS. There were ten patients in each patient group consisting of patients with severe airflow obstruction, moderate airflow obstruction and no airflow obstruction. Table 4.1 shows the characteristics of the patients included in this study. Staining was assessed semi-quantitatively using a scoring system of 0-2.5 for cell types including type I and type II alveolar epithelial cells, alveolar macrophages and neutrophils. Table 4.2 shows the mean 4-HNE staining scores for the different cell types in the three patient groups. The histograms in figure 4.1, 4.2 and 4.3 demonstrate 4-HNE staining scores for bronchial epithelial cells, macrophages and neutrophils. There is a higher mean 4-HNE staining score in bronchial epithelial cells, macrophages and neutrophils in lung tissue obtained from subjects with severe airflow obstruction (LVRS), compared to those with no airflow obstruction.

	Normal Lung Function n=10	***COPD n=10***	***Severe COPD n=10***
Age	61.7(49-74)	57.3(21-72)	54.3 (49-64)
Male:female	5:5	6:4	2:8
Smoking History pack yrs	54.45 (3-168)	50.6 (30-57)	37.5 (25-76)
FEV_1 litres (±SD)	2.51 ± 0.73	1.92 ± 0.41	0.66 ± 0.22
FEV_1 % pred	94.6	62	25.8
FVC litres	3.34 ± 0.99	3.38 ± 0.94	2.32 ± 0.89
FEV_1/ FVC %	75.8 ± 6	58.7 ± 8.7	30.28 ± 10.17

Table 4.1 Patient characteristics and staining scores for 4-HNE immunostaining

	Normal Lung Function n=10	*COPD n=10*	*Severe COPD n=10*
4-HNE staining score (mean ± SEM)			
Bronchial epithelium	0.909 ± 0.15	1.071 ± 0.13	1.45± 0.15
Alveolar epithelium (type1)	0.363 ± 0.13	0.389 ± 0.13	0.75 ± 0.17
Alveolar epithelium (type II)	0.363 ± 0.09	0.667 ± 0.14	0.65 ± 0.15
Alveolar macrophages	1.227 ± 0.12	1.44 ± 0.15	1.7 ± 0.133
neutrophils	1.05 ± 0.19	0.88 ± 0.073	1.8 ± 0.133

Table 4.2 Mean 4-HNE staining scores in the three patient groups

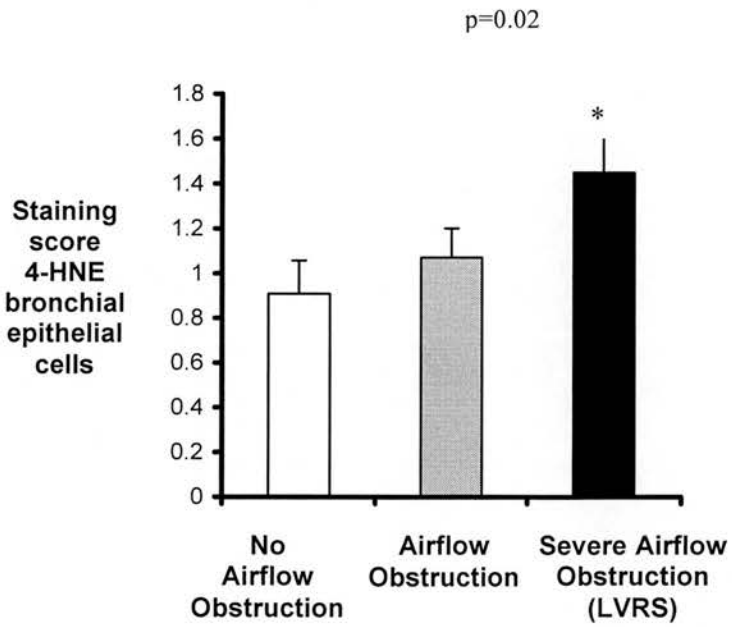


Figure 4.1 shows the 4-HNE staining score in bronchial epithelial cells in lung sections from patients with no airflow obstruction, airflow obstruction and severe airflow obstruction (LVRS). The histograms are mean values and the bars represent SEM. There is a significant increase in 4-HNE staining in the severe airflow obstruction group compared to no airflow obstruction p=0.02.

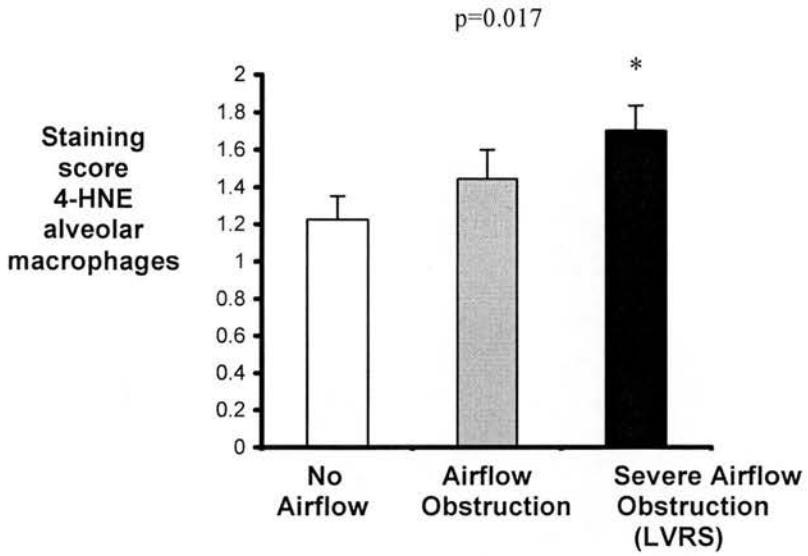


Figure 4.2 shows the 4-HNE staining score in alveolar macrophages in lung sections from patients with no airflow obstruction, airflow obstruction and severe airflow obstruction (LVRS). The histograms are mean values and the bars represent SEM. There is a significant increase in 4-HNE staining in the severe airflow obstruction group compared to no airflow obstruction p=0.017.

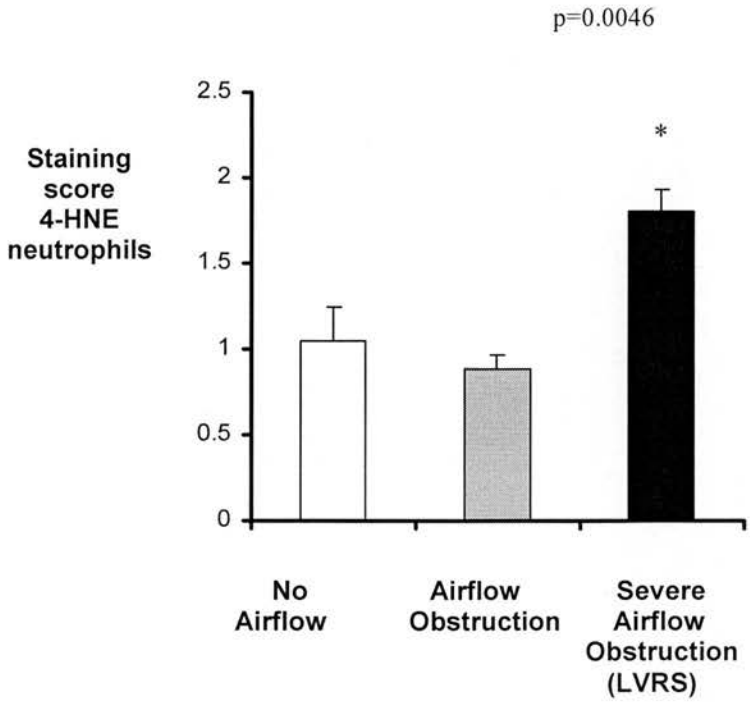


Figure 4.3 shows the 4-HNE staining score in neutrophils in lung sections from patients with no airflow obstruction, airflow obstruction and severe airflow obstruction (LVRS). The histograms are mean values and the bars represent SEM. There is a significant increase in 4-HNE staining in the severe airflow obstruction group compared to no airflow obstruction p=0.0046.

4.3.2 Western Blotting for 4-HNE

Lung tissue from three patients was used with patient characteristics shown below in table 4.3.

	Smokers		
	Normal Lung Function n=1	***COPD n=1***	***Severe COPD n=1***
Age (years)	63	69	52
Male/female	male	female	female
Smoking History pack yrs	168	32	not documented
FEV_1 litres (±SD)	3.0	1.06	0.42
FEV_1 % pred	85	66	17.5
FVC litres	3.79	2.02	2.1
FEV_1/ FVC %	76.9	52.4	20

Table 4.3 Characteristics of patients from whom lung tissue was used

The use of a 1.5 % potassium chloride solution lysis buffer to obtain cytosolic cellular protein failed to demonstrate protein bands on Western blotting. The introduction of a further centrifugation step also failed to yield anything on Western blotting of the cytosolic extract. (methods 1a-1c).

The alteration of the lysis buffer to that outlined on page 120 of the methods section also failed to yield any protein bands on Western when this protein extract was used.

4.3.3 Immunoprecipitation

The technique of immunoprecipitation was used to examine lung tissue for 4-HNE and E1A. Lung tissue was examined from a 58 year old woman with severe airflow obstruction (FEV_1 of 0.6 L and FEV_1/VC ratio of 26%), who had undergone LVRS.

Figure 4.4 shows a Western blot using immunoprecipitated protein from A549 cells and human lung tissue, using an E1A primary antibody. EIA transfected A549 cells were examined for E1A protein as these cells should be positive for EIA and would act as a control to determine if any bands were the result of the immunoprecipitation process or were truly positive for E1A. If bands were similar in the E1A immunoprecipitated and 4-HNE Michael adduct immunoprecipitated protein lysates then it would imply that the bands were the result of the extraction process.

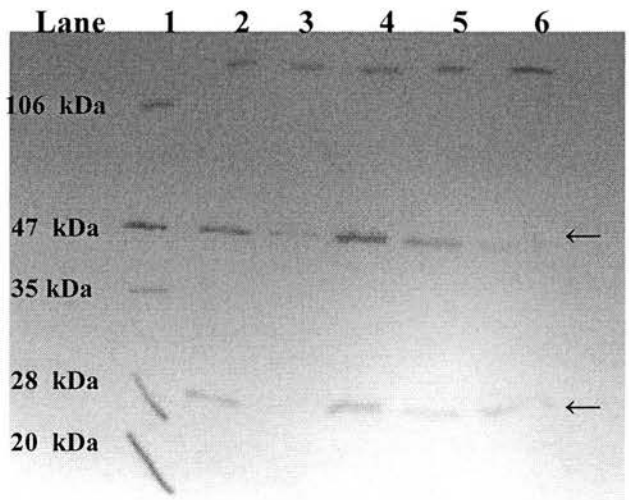


Figure 4.4 Western blot using immunoprecipitated protein and primary antibody to E1A. Lane 1 represents molecular weight markers, lane 2 cytosolic protein extracted from A549 cells, lane 3 cytosolic protein from A549 cells transfected with E1A, lane 4 cytosolic protein from A549 cells stimulated with hydrogen peroxide, lane 5 cytosolic protein from A549 cells transfected with E1A stimulated with hydrogen peroxide, and lane 6 cytosolic protein from human lung. All of the samples demonstrated two bands (as indicated by the arrows), a higher band in the region of 47 kDa and a lower band in the region of 20-28 kDa. It is unlikely that these bands represent E1A protein as the same pattern is achieved irrespective of whether the extract was obtained from E1A transfected A549 cells, or A549 epithelial cells that were not transfected with E1A.

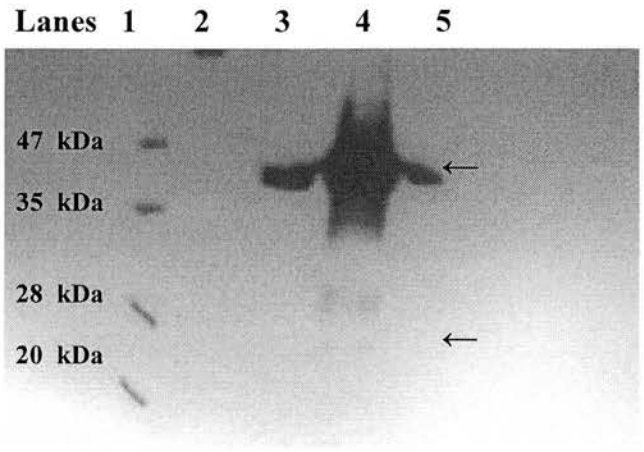


Figure 4.5 Western blot using primary antibody anti-4-HNE Michael adduct. Lane 1 represents molecular weight markers, lane 2 40μg lung tissue lysate, lane 3 80 μg lung tissue lysate, and lane 4 immunoprecipitated protein from human lung. Lane 5 represents spill over from lane 4. There is also some spill over into lane 3 from lane 4.

Figures 4.4 and 4.5 show two bands in the immunoprecipitated protein in all cases, irrespective of which primary antibody was used. The expected weight of E1A is 43,000 kDa. The higher band is at the approximate levels of 40 kDa and the lower band is between 20 kDa and 28 kDa. It is likely that the upper band represents heavy chain and that the lower band represents light chains from the immunoprecipitation reaction. The upper bands cannot represent E1A as these bands are present when E1A and 4-HNE primary antibodies are used. Similarly figure 4.6 shows similar bands when rat lung protein, human lung and 4-HNE-myoglobin adduct is immunoprecipitated. In figure 4.6, lane 3 which represents non immunoprecipitated lung tissue protein has no bands. Lane 5 which represents the 4-HNE-myoglobin adduct (non immunoprecipitated) shows 2 clear bands approximately at 66 kDa and 45 kDa. It is unclear whether the bands in lane 5 represent 4-HNE protein adduct.

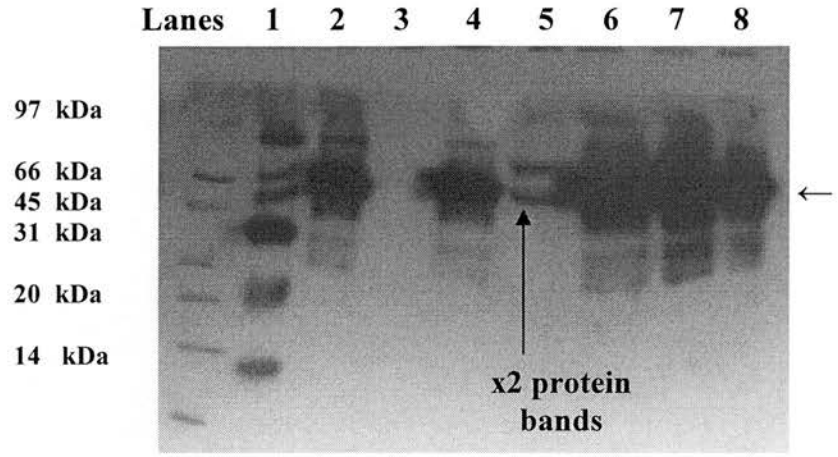


Figure 4.6 Western blot using a primary antibody to 4-HNE (anti-Michael adducts). Lane 1 molecular weight markers, lane 2 human lung sample 129 immunoprecipitated, lane 3 human lung cytosolic extract non immunoprecipitated, lane 4 4-HNE-myoglobin immunoprecipitated, lane, lane 5 4-HNE-myoglobin non immunopreciptated (with x2 protein bands), lanes 6, 7 and 8 rat lung cytosolic extract that has been immunoprecipitated. In all the immunoprecipitated samples, multiple bands are seen with dense bands in the region of 45,000 kDa to 66,000 kDa (see right arrow).

Using the modification 4a to obtain cytosolic extraction failed to yield any bands on Western blotting using anti 4-HNE-Michael adduct primary antibody.

Lung tissue was incubated with 100 mM solution of 4-HNE in PBS solution for 1 hour and then the cytosolic extract as per methods 4a was obtained and 20 μg and 40 μg used in the standard Western procedure. Twenty micrograms of myoglobin-4-HNE adduct was used as a control. Figure 4.7 shows the Western blot, and this shows that the lung samples that were incubated with 4-HNE and PBS all produce two bands, an upper stronger band at approximately 81 kDa and a weaker lower band at approximately 47 kDa. No band is demonstrated in the myoglobin-4-HNE adduct sample.

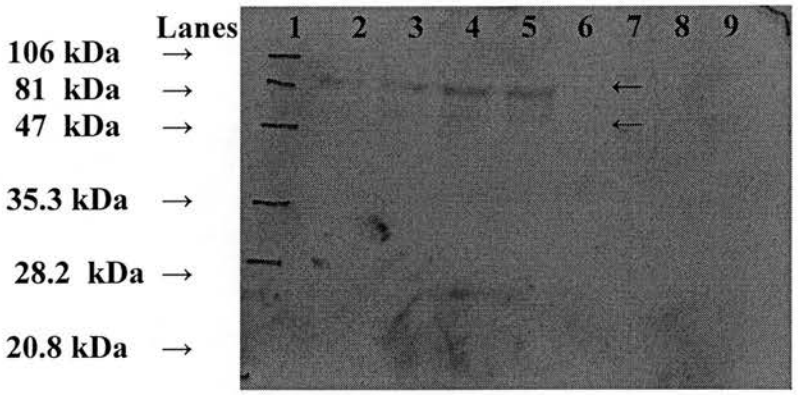


Figure 4.7 Western blot using primary antibody to 4-HNE (anti-Michael adduct). Lane 1 molecular weight markers, lane 2 and 3 human lung (40 μg), lanes 4 and 5 human lung incubated in 4-HNE (100μM), 40 μg, lane 6 empty, lanes 7 and 8 myoglobin-4HNE (20μg), lane 9 empty. Two faint bands (in the region of 47,000-81,000 kDa) are demonstrated in lanes 2,3,4 and 5 (human lung incubated with 4-HNE and control lung in PBS), indicated by the two arrows. No band is demonstrated in the myoglobin-4-HNE adduct.

Increasing concentrations of cytosolic extract (20 μg, 40 μg, and 100 μg) from 4HNE incubated lung tissue and control PBS incubated were used in the Western blot to determine if a more definite band could be identified on the gel using an increased concentration of cytosolic protein. The polyacrylamide gel in figure 4.8 is a poor quality gel, but similar bands at 81 kDa and 47 kDa can be seen. These bands become denser with increasing amounts of protein.

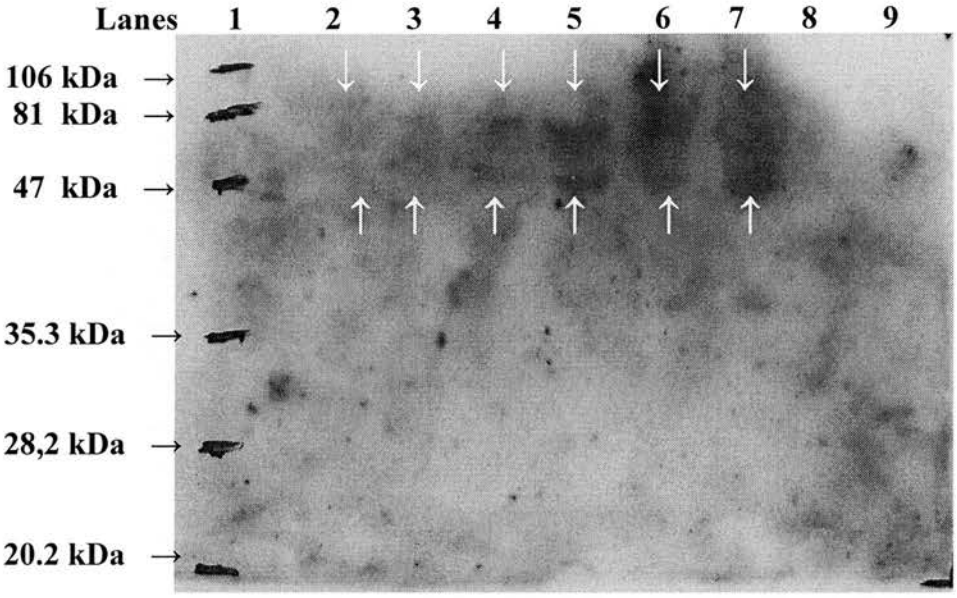


Figure 4.8 Western blot using primary antibody to 4-HNE (anti-michael adduct). Lane molecular weight markers, lanes 2 and 3 20 μg of control lung cytosolic extraction and 20 μg 4HNE incubated cytosolic extraction respectively, lanes 4 and 5 40 μg 4HNE incubated cytosolic extraction respectively, lanes 6 and 7 100 μg 4HNE incubated cytosolic extraction respectively, lanes 8 and 9 4-HNE-myoglobin adduct. Lanes 3, 4, 5, 6, 7 all show two faint bands in the region of 47,000-81,000 kDa (the bands are indicated by the arrows).

Changing the concentration of the Tween to 0.01% Tween 20 from 0.05% Tween 20 resulted in no bands being seen.

In summary Western blotting using 4-HNE-Micheal antibody and human lung tissue lysate has demonstrated bands which may represent 4-HNE protein adducts.

4.4 Discussion 4-hydroxy-2-nonenal in human lung

The lipid peroxidation product 4-HNE is highly reactive and capable of diffusion away from the original site of production (Esterbauer *et al.,* 1991), and can affect both intracellular and extracellular biological targets. The mechanism of 4-HNE lung related damage is not known, however it is well described that 4-HNE forms Michael-type nucleophilic protein adducts via amino acid residues of histidine, lysine, serine and cysteine (Esterbauer *et al.,* 1991).

4-HNE, a specific product of lipid peroxidation was detected and localised in human lung tissue using immunohistochemistry (IHC). The distribution and levels within patients with severe, moderate and no airflow obstruction was also examined. The mean 4-HNE staining score is higher in bronchial epithelial cells, macrophages and neutrophils in lung tissue sections, in subjects with severe airflow obstruction compared to those without airflow obstruction.

For the 4-HNE immunohistochemical analysis a manual method of subjective viewing of the slide was adopted using a single observer. The use of two observers would have been more accurate. Image analysis systems are increasingly being used in an attempt to remove observer and intra-observer bias and inaccuracies in immunohistochemical analysis. Automated image analysis can examine large areas from multiple biopsies and can also perform densitometry measurements on the stain. Sont (Sont *et al,* 2003) have used a fully automated technique for assessing inflammatory cell counts and cytokine expression in bronchial tissue, and have compared this technique to a semi-quantitative method and found that it compared well and that the automated method gave optimal repeatability of the analysis of cell counts and cytokine expression. One of the weaknesses of using a semi-quantitative method is that the cell staining will diminish over time and re-analysis at a later time can be problematic.

The ability to adequately quantify 4-HNE in tissue is limited using IHC, which best indicates the localisation of a protein. The technique of Western blotting was used in an attempt to provide more quantitative 4-HNE-protein adduct data in the tested human lung tissue. The aim of the 4-HNE Western experiments was to identify 4-HNE immuno proteins in lung tissue.

We found that combining immunoprecipitation with Western blotting using 4-HNE Michael antibody yielded several bands when using human and cellular cytosolic proteins. Non immunoprecipitated 4-HNE-myoglobin adduct produced two discrete bands on Western blotting and may represent a 4-HNE-protein adduct, however it was not of the expected molecular weight of 17 kDa. Lung tissue that was incubated at 37°C, both with and without 4-HNE, produced two faint bands when the tissue lysates were run on a Western using 4-HNE Michael antibody. Again these bands may represent 4-HNE immunoprotein.

The major problem with the Western blot experiments was the lack of a true positive control. We postulated that 4-HNE would form a protein adduct with myoglobin and created a 4-HNE-myoglobin protein adduct to act as a positive control. Immunoprecipitated samples gave a dense band at the level of 45-66 kDa that was likely to represent the IgG carried through from the immunoprecipitation. To confirm that this band did infact represent IgG, controls of primary antibody alone and sample buffer alone should have been included in the Western. A limitation of the immunoprecipitation technique is that immunoreactive proteins of 50-60 kDa, which have similar molecular weights to IgG cannot be detected. However the presence of such IgG bands does act as an internal control with regards to protein loading and successful immunoblotting.

After immunoprecipitation there is an antigen-antibody complex, and this complex is also complexed to the protein G beads. The addition of the reducing buffer (gel

loading buffer) to the immunoprecipitated lysate should release the antigen, however it can also have the effect of releasing the heavy and light chains from the E1A IgG by virtue of breaking the disulphide bonds of the heavy and light chains. The same antibody and therefore the same species of antibody had been used for the immunoprecipitation and the primary antibody for the Western. Ideally different species antibodies should be used for the immunoprecipitation step and as the primary antibody.

Optimising the tissue lysis buffer is critical in Western blotting, and may need to be tailored to the individual protein. Using 1.5% potassium chloride as the lysis buffer may have released protein as there was evidence of staining with ponceau red on the membrane, but no bands were detected on Western blotting. The use of antiproteases is vital and these were not included in the 1.5% potassium chloride lysis buffer. The lysis buffer was modified to a solution containing nonidet-P, and also a solution containing 0.1% deoxycholine/1% triton X-100, with both of these lysis buffers containing antiproteases. Altering the salt concentration in the lysis buffer can affect the cytosolic protein yield. In this work lysis buffer containing 10 mM potassium chloride, 1.5 mM magnesium chloride and also150 mM sodium chloride was used.

Tissue lysates obtained from lung tissue that had been incubated in 4-HNE/control PBS at 37°C, produced two bands, but the 4-HNE-myoglobin protein adduct that was run on the same Western did not give any bands. This differed from earlier Westerns in which two clear bands at 45-66kDa were found for the 4-HNE-myoglobin adduct. It is possible that the 4-HNE-myoglobin-protein adduct was incorrectly prepared in these later experiments. However the molecular weight of myoglobin is 17 kDa and so it would not be expected that the 4-HNE-myoglobin adduct would be seen at 45-66 kDa. It is possible that the 4-HNE-myglobin adduct is very unstable and degrades quickly and so may be very difficult to measure and impossible to use as a positive control. Another possibility is that the 4-HNE-myoglobin adduct is not present in

human lung tissue. As stated earlier we did postulate that myoglobin may be one such protein, but our data does not support this.

The 4-HNE primary antibody that was used in the Western experiments was a polyclonal rabbit immunoglobulin which recognised 1:1 cysteine-, histidine- and lysine-4-HNE 'Michael' adducts, and this differed from that used in the immunostaining work, which was a monoclonal antibody (Japan; Institute for Ageing Research).

A 32 kDa protein in human and murine alveolar macrophages in response to ozone has been identified using a 4-HNE antibody that recognises HNE-Cys, HNE-Lys, HNE-His adducts (Hamilton *et al.,* 1998, Kirichenko *et al.,* 1996). In our work whole lung tissue was lysed rather than using a pellet of macrophage cells. Like our work, Hamilton's group also did not have a 'true' positive. Usatyuk has used a Calbiochem anti HNE Michael adduct antibody to examine for 4-HNE in bovine lung endothelial cells by Western blotting and demonstrated several protein bands in the region of 40-203 kDa (Usatyuk *et al.,* 2004), and this group was similarly unable to identify what each protein band represented. The Usatyuk group also showed increased 4-HNE protein adducts when the endothelial cells were exposed to increasing concentrations of 4-HNE, and that adduct formation peaked at 45 minutes.

4-HNE- and MDA- adducted proteins have been examined in liver (Paradis *et al.,* 1997), and it has been postulated that lysine rich epitopes of 4-HNE adducts are less likely to be formed under physiological conditions, and that 4-HNE under normal physiological conditions would react most rapidly with sulfhydryl residues of proteins (Hartley *et al.,* 1997). The functional groups histidine, lysine and glycine react rapidly with 4-HNE under non physiological conditions of pH 8.8 and 10 mM concentrations. The experiments contained in this thesis have only examined for the

lysine, histidine and glycine epitopes of 4-HNE and have not examined for a sulfhydryl epitope. In studies analysing liver tissue sections by immunohistochemistry, 4-HNE protein adducts have been successfully detected by immuno-staining using antibodies against 4-HNE-amine epitopes of lysine-rich LDL (Houglum *et al.,* 1990; Benedetti *et al.,* 1982), but these antibodies have not been successful in detecting or identifying cellular protein targets by Western blot. Similarly Hartley et al (Hartley *et al.,* 1999) has demonstrated immunopositive proteins of 80, 150, and 205 kDa in liver when using immunoprecipitation techniques with anti-MDA-amine serum and 4-HNE sufhydryl serum, but not with 4-HNE amine epitopes. Bedossa et al, (Bedossa *et al.,* 1994) used antibodies directed to 4-HNE-amine epitopes, and like ourselves, also failed to demonstrate 4-HNE adducts in liver sections from carbon tetrachloride treated rats.

Formal identification of the protein that corresponded to the protein bands on Western blotting using the anti 4-HNE Michael adduct antibody should have been performed. Instead of using a denaturing gel alone which separates the proteins based on molecular size, a two dimensional gel system separating initially by their isolelectric point followed by SDS-polyacrylamide gel electrophoresis would yield the best resolution of the proteins. The bands of interest could be cut from the gel and the protein treated with either acid hydrolysis, or tryptic digest, or high pressure liquid chromatography could be used to release the free amino acids and identify the amino acids in the protein. Determination of the amino acid sequence of the protein can be performed by mass spectrometry using peptide mass fingerprinting. In the technique of mass fingerprinting the protein is cleaved into peptides giving a pattern for the 4-HNE protein adduct and this fingerprint is then compared to a database of proteins that have been cleaved electronically by computer simulation.

In summary protein bands in the region of 45 kDa and 66 kDa, and 47-81 kDa have been demonstrated on Western blotting using anti HNE Michael adduct antibody, and these may represent 4-HNE protein adducts, although no formal identification of these proteins has been performed.

CHAPTER 5

EARLY ONE ADENOVIRAL PROTEIN IN HUMAN LUNG TISSUE FROM A SCOTTISH POPULATION OF PATIENTS WITH AIRFLOW OBSTRUCTION

5.1 Introduction E1A

It is unknown why only a proportion of smokers go on to develop chronic obstructive pulmonary disease (COPD). An environmental factor may be important in the development of the enhanced inflammation associated with COPD. Epidemiological studies show that an earlier viral infection confers an increased risk of respiratory disease from asthma and COPD (Schroeckenstein *et al.,* 1988, Samet *et al.,* 1983), and it has been suggested that an earlier adenoviral infection may render the respiratory tract more susceptible to damage in the face of an oxidant stimulus such as cigarette smoke (Hogg, 1999). After adenoviral infection, the adenovirus can integrate into the human genome, and a crucial step in this process is the expression of early DNA proteins, A and B (E1A and E1B). E1A protein may enhance the inflammatory reaction by modulation of the transcription of inflammatory mediators (Higashimoto *et al.,* 2002), effects on cell signal transduction pathways (Shen *et al.,* 1998; Barbeau *et al.,* 1994), and displacement of transcription factors from the transcription machinery (O'Connnor *et al.,* 1999).

In vitro systems using E1A transfected cell lines (Higashimoto e*t al.,* 2002, Gilmour *et al.,* 2001, Keicho *et al.,* 1997, Keicho *et al.,* 1997) and also animal models (Vitalis *et al.,* 1996 and 1998) have demonstrated an enhanced inflammatory reaction in response to stimuli. In a Canadian population, genomic DNA extracted from resected lung tissue has shown increased amounts of the E1A region of the viral genome in patients with COPD, (Matsuse *et al.,* 1992), and E1A has also been demonstrated in lung tissue from patients with sarcoidosis (Kuwano *et al.,* 1997).

We therefore wished to establish whether such adenoviral protein DNA (E1A) was also present in a Scottish population of smokers with COPD. Human lung tissue was examined for the presence of E1A protein using the techniques of polymerase chain reaction, nested polymerase chain reaction, Southern hybridisation and immuno-histochemistry. We failed to conclusively demonstrate the presence of E1A in human lung tissue obtained from a Scottish population, and during the course of the work experienced problems with adenoviral contamination.

5.2 Methods E1A

A full summary of the patient characteristics for the lung tissue that was used in this series of E1A experiments is shown in the results section 5.3.

5.2.1 Genomic DNA Extraction from human lung tissue

Frozen lung tissue was sliced using a sterile scalpel, and the tissue was lysed by incubating in lysis buffer (100 mM NaCl, 10 mM Tris-Cl pH 8, 25 mM EDTA pH 8 and 0.5% SDS) and proteinase K, Sigma Aldrich Co Ltd. (Poole, UK) at 55°C. The volume of lysis buffer and proteinase K was varied depending on the lung weight (250 mg tissue then 1ml lysis buffer and 5µl of proteinase K (0.1mg proteinase K) was added). Further proteinase K diluted in lysis buffer was added at 12 and 24 hrs if the tissue had not lysed. The lysis buffer/tissue mix was mixed with an equal volume of saturated phenol/chloroform/isoamyl alcohol, vortexed gently and then centrifuged at 13000 rpm, 4°C. A further phenol chloroform extraction was performed. The aqueous top layer containing the DNA was transferred to another eppendorf and the DNA was precipitated using cold 100% alcohol and 3M sodium acetate solution. The DNA in the alcohol/acetate mix was stored at -20° for 48 hrs, or longer if not required for use. After storage at -20° the DNA was washed in 500µl of 70% alcohol and left to air dry for 20-30 minutes, and the genomic DNA was dissolved in 1x TE buffer (10 Mm Tris-Cl pH 7.5, 1 mM EDTA). This dissolving process took several days and often a week, and during this stage the DNA solution was stored at 4°C. The concentration and purity of the DNA was estimated by measuring the absorbance at wavelengths 260 nm and 280 nm in a glass cuvette in a spectrophotometer. A ratio of 260/280 of 1.8 was aimed for.

5.2.2 Assessment of E1A gene by Reverse transcriptase polymerase chain reaction using genomic DNA

Aliquots of genomic DNA were PCR amplified using a thermal cycler (Hybaid, Ashford, UK). Human genomic DNA was added to 50 µl of PCR mixture (a solution containing 2.5 mM $MgCl_2$, 1x PCR Buffer (Promega), 0.2 mM dNTPs and 10µl of each primer) with Taq polymerase (1U/µl), for human glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and early one adenoviral protein (E1A).

Conditions for the PCR:

E1A: 40 cycles of denaturation (93°C for 1 minute), annealing (63°C for 1 minute), and extension (72°C for 2 minutes), and final extension of 7 minutes.

GAPDH: 35 cycles denaturation (94°C for 45 seconds), annealing (60°C for 45 seconds), and extension (72°C for 90 seconds), and final extension of 10 minutes at 72 °C.

Primers were sequenced by MWG-Biotech (Milton Keynes, UK).

The primers chosen for GAPDH were as per previous published sequences (Maier *et al.,* 1990) and were as follows (sense 5' -CCA CCC ATG GCA AAT TCC ATG GCA-3') and (anti-sense 5'-TCT AGA CGG CAG GTC AGG TCA ACC-3')

The primers chosen for E1A were according to Vitalis and Matsuse who had previously performed PCR for E1A in lung tissue (Vitalis *et al.,* 1998; Matsuse *et al.*, 1992). The size of the amplified product using Matsue primers was 675 base pairs and the sequences were as follows (sense 5' -CTC AGG TTC AGA CAC AGG ACC T-3') and (antisense 5' -CTG CCA CGG AGG TGT TAT TAC C-3').

The size of the amplified product using primers from Vitalis was 486 base pairs and the sequences were as follows (sense 5'- TCA-GGC-TCA-GGT-TCA-GAC-NCA-G-3') and (antisense 5' -TAA-TGT-TGG-CGG-TGC-AGG-AAG-G-3').

PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide and the gel was scanned using a white/UV transilluminator (Ultra Violet Products, Cambridge UK).

The effect of the concentration of genomic DNA on PCR reaction

Previous reports examining E1A in human lung tissue by PCR had demonstrated a clear band when the PCR products had been electrophoresed on an agarose gel (Matsuse *et al.,* 1992). Our preliminary work using 10 µl (approximately 5µg) of genomic DNA in the PCR mix failed to show clear bands on PCR for either the GAPDH or E1A, however the positive controls demonstrated clear bands in the expected place. The effect of using 5 µl of 500 ng/µl, and 5 µl of 50 ng/µl of template genomic DNA in the PCR reaction was therefore examined for both E1A primers and GAPDH primers. The positive control was genomic DNA extracted from Graham 293 cells, and this was provided by Dr Shizu Hayashi, Vancouver. Graham 293 (G293) cells are human embryonic kidney cells that have been transformed by exposing the cells to sheared fragments of adenovirus type 5 DNA (Graham *et al.,* 1977).

For E1A primers the positive control was 1 µl G293 genomic DNA (concentration 10 µg/500µl in TE buffer; 20 ng), and the negative control was 5 µl dd H_2O.

For the GAPDH primers the positive control was 1 µl cDNA obtained from the RNA of A549 cells, and the negative control was 5 µl dd H_2O.

5.2.3 Dot Blot Southern Hybridisation of genomic DNA from human lung tissue

This technique allows the immobilisation of unfractionated DNA on a nylon membrane which is then hybridised with a probe.

The nylon membrane (Hybond non charged) was placed in the manifold apparatus. Five hundred µl of 6 x SSC (0.15 M NaCl, 0.015 M Na_3 citrate, pH 7) was applied to each individual dot chamber, followed by suction to pull the solution through the nylon. The genomic DNA from 24 human lungs was denatured by heating 20 µl of DNA with 10 µl 20 X SSC at 100°C for 10 minutes, followed by rapid cooling on ice. Thirty µl DNA/SSC aliquots were added to each dot chamber, followed by a further 500 µl 6x SSC and allowed to pass through the membrane. The membrane

was removed and placed on top of Whatman paper that had been soaked in denaturing solution (1.5M NaCl/0.5 M NaOH) for 10 minutes, followed by the membrane being placed on top of neutralising solution (1 M NaCl/0.5M Tris-Cl pH 7) for 10 minutes. The membrane was allowed to dry and the DNA samples were cross linked by placing the membrane under UV light (high strength) for 3 minutes.

Samples: 24 samples of genomic DNA from human lung tissue (concentrations 0.5 μg/μl, and 0.05 μg/μl). 20 μl of genomic DNA was used for each dot blot well chamber.

Positive controls: 1. genomic DNA from G293 cells

2. c DNA from E1A transfected A549 cells

Negative controls: 1. Water

2. c DNA from A549 cells

Preparation of EIA Probe used in dot blot Southern

The PCR product from an earlier E1A positive control using 675 bp E1A primers was cut from an agarose gel, and purified using a DNA purification kit (Promega, Madison, USA). Four μl of this purified DNA was added to 21 μl 1x TE buffer and this mix was heated to 100° C for 3 minutes and then cooled on ice for 2 minutes and then pulse centrifuged for 15 seconds. The 'ready to go' Pharmacia Biotech (Buckinghamshire, England) labelling kit ampoule was reconstituted using 20 μl dd H_2O and allowed to stand on ice for 60 minutes. The 25 μl of purified PCR product mix was added to the ready to go ampoule, together with 5μl α ^{32}P-dCTP (Redivue, Amersham Pharmacia Biotech UK, Buckinghamshire England)(50μCiu), mixed by pipetting and then incubated at 37 °C for 15 minutes and then placed on ice until used.

Hybridisation

The membrane was rolled in nylon mesh and prehybridised in 20 mls preheated (65°C) Church and Gilberts solution for 2 hours in the hybridisation chamber.

Hybridisation was achieved by adding the labelled probe to 20 mls fresh pre-heated (65°C) Church and Gilberts solution and the membrane was incubated overnight at 65°C. The membrane was then rinsed in 10 mls 2x SSC/0.1% SDS (65°C), followed by 20 minute rinse in fresh 20 mls 2x SSC/0.1% SDS (65°C), then 20 minute wash in 20 mls 0.5x SSC/0.1% SDS (65°C), and then two further washes for 20 minutes in 20 mls 0.1x SSC/0.1% SDS (65°C).

The membrane was developed using Kodak film.

Church and Gilberts solution

$Na_2HPO_4 \cdot 2H_2O$	51.34g
$NaH_2PO_4 \cdot H_2O$	29g

Dissolved in 800mls H_2O

70 g SDS added

Make up to 1L

5.2.4 Nested Polymerase chain reaction

In vitro deoxyribonucleic acid (DNA) amplification by the polymerase chain reaction allows the detection of rare DNA sequences, and can detect the presence of infectious agents that are present in low copy numbers. Nested PCR is a two step amplification process where outer primers, followed by inner primers are used in an attempt to enhance the sensitivity and specificity of the PCR. Kuwano (Kuwano *et al.,* 1997) has used nested PCR to detect E1A in the lungs of patients with sarcoidosis.

The outer primers were those that gave a PCR product at 675 base pairs and were (sense 5'-CTC AGG TTC AGA CAC AGG ACC T-3') and (antisense 5'-CTG CCA CGG AGG TGT TAT TAC C-3'). The inner primers were those that gave a PCR product at 261 base pairs and were (sense 5'-GAA CCA CCT ACC CTT CAC GAA CTG-3') and (antisense 5'-GTG GCA GGT AAG ATC GAT CAC CTC-3').

The conditions for the PCR using the outer (675bp) E1A primers were the same as outlined earlier. Four µl of the PCR product obtained from this PCR reaction using 675 bp primers was used in a second PCR reaction using the outer 261 bp primers. The reaction solution was the same as for the 675 bp PCR with the exception that the primers were different. 35 cycles of denaturation (93°C for 1 minute), annealing (63°C for 1 minute), and extension (72°C for 2 minutes), and final extension of 7 minutes were used.

5.2.5 Southern Hybridisation

Twenty samples of human genomic DNA were used in the PCR using E1A and GAPDH primers (details of the patient characteristics are detailed in section 5.3). The concentration of genomic DNA was reduced to 250 ng in 5 µl. The samples were run in triplicate on each gel. The PCR mixture consisted of 100 µl of 10x PCR buffer, Promega, 100 µl $MgCl_2$ 25mM, Promega, 20 µl dNTP mix, Pharmacia Biotech, 10 µl of each E1A 486 bp primers, MWG-Biotech, and 760 µl dd H_2O. The reaction mixture consisted of 45 µl of PCR mix, with 5 µl lung genomic DNA (250 ng), and 1 µl Taq polymerasere (2.5 units/ µl). The conditions of the PCR for the E1A primers were hot start at 90°C for 5 minutes, then 40 cycles of denaturation (93°C for 1 minute), annealing (63°C for 1 minute), and extension (72°C for 1 minute), and final extension of 6 minutes. Positive control was G293 genomic DNA (3ng), and negative control was PCR mix alone.

For the GAPDH PCR, the PCR mix was the same as for the E1A, with the exception of the primers. The volume of PCR mix was 45 µl, together with 5 µl lung genomic DNA (250 ng) and 1 µl Taq polymerase (0.5 units/ µl). The conditions of the PCR for the GAPDH primers were a hot start at 94°C for 10 minutes, then 35 cycles of denaturation (94°C for 45 seconds), annealing (60°C for 45 seconds), and extension

(72°C for 90 seconds), and final extension of 10 minutes. The positive control was 2 µl of cDNA from A 549 cells, and the negative control was PCR mix alone.

The PCR products were run on 1% agarose gels, and then transferred to nylon membranes, and then probed using the E1A probe, or the GAPDH probe.

E1A Probe

The E1A probe was provided by Dr Shizu Hayashi. The probe consisted of a fragment of DNA 756 base pairs long. The 742 bp Alu 1 fragment was cut from the E1A DNA adeno virus 5. This Alu 1 fragment was cloned in the *Hinc* II site of pUC13. This DNA was then digested using the restriction enzymes *Pst*1 and *Bam*H1. The DNA was run on a 1% agarose gel and the BamH1/Pst1 fragment was cut out of the gel and the DNA purified and 0.5 ng of DNA used for the probe. The probe was labelled with 5 µl α ^{32}P-dCTP (50µ Ciu) using the ready to go labelling kit (Pharmacia Biotech, Buckinghamshire, England), as previously described.

GAPDH Probe

This was prepared by cutting out the PCR product from an agarose gel in which 5 µl cDNA from A549 cells had been used in a PCR reaction for GAPDH. This PCR product was purified using DNA purification kit and then 0.5ng was used as the probe. The GAPDH probe was labelled with 5µl α ^{32}P d CTP (50µ Ciu) using the ready to go labelling kit (Pharmacia Biotech).

The PCR gels were denatured by soaking twice in 1.5 M NaOH/0.5 M NaOH for 10 minutes, and then neutralised in 1.5 M NaCl/0.5 M Tris-Cl pH 7.2,1 mM EDTA. The PCR products were transferred to the nylon membrane using a sandwich consisting of a weight, paper towels, Whatman paper, membrane, gel with PCR products facing upwards, and Whatman paper, that was placed on top of a wick of whatman paper that was soaking in 1x SSC solution and the transfer was allowed to

take place overnight. The membrane was allowed to dry at room temperature and the DNA crosslinked using UV cross linker for 30 seconds.

Prehybridisation Mix

6 x SSC

5 x Denhardts (Sigma D-2532)

0.5% SDS

20 μg/ml salmon sperm DNA (Sigma D-9156)

The membrane was prehybridised for 6 hours in prehybridisation mix at 65°C. Hybridisation was carried out by adding the labelled probe to 20 mls of prehybridsation solution and incubating this with the membrane over night at 65°C. The membrane was then rinsed in 2x SSC/0.1% SDS twice for 10 minutes at 65°C, followed by two washes in 2x SSC/0.1% SDS at room temperature. The membrane was then washed in 0.1x SSC/0.1% SDS for 30 minutes at 65°C and then rinsed in 2x SSC at 65°C. The membrane was then autoradiographed using Kodak developing film. The phosphoimager was used for developing and imaging for selected Southern experiments (Southern hybridisation using positive controls of plasmid E1A DNA).

Southern Hybridisation using Positive controls of plasmid E1A DNA and G293 genomic DNA, and a 'positive' lung sample

Dr Hayashi provided a sample of lung genomic DNA from the laboratory in Vancouver, which she had previously found to be positive for E1A. She also provided further G293 genomic DNA, and a sample of the plasmid DNA (5μg/20μl water) which the E1A probe had originally been constructed from (vector). Ten

nanograms of this vector DNA was also used as a further positive control in the PCR reaction.

Five μg of pUC EIA (plasmid DNA) was dissolved in 20 μl distilled water, and 10 μl was used in a double digest using 2 μl of *Pst*1 and *Bam*H1 restriction enzymes and enzyme buffer, Promega (Madison, USA), incubating overnight for 10 hours at 37°C. The cut DNA was run on a 1% agarose gel and then the 0.76 Kb insert was cut out of the agarose gel, weighed and the concentration of EIA DNA calculated, and distilled water added to give a concentration of 1ng/μl. It was heated to 65° for 20 minutes. Twenty five μl (25 ng) of DNA was used for the probe but was first denatured by heating at 95° C for 20 minutes, followed by 10 minutes at 37 °C, and room temperature for 10 minutes. The probe was labelled with 5μl α ^{32}P-dCTP (50μ Ciu) using the ready to go labelling kit (Pharmacia Biotech, Buckinghamshire, England), as previously described.

Template DNA: 7 samples of Scottish lung genomic DNA (5 μg)

1 sample of Vancouver lung genomic DNA (previously found to be positive in Vancouver) (5 μg)

genomic DNA from G293 cells (which had been in use for 5 months) positive control (3 ng)

genomic DNA from G293 cells (recently brought to UK by Dr Hayashi) positive control (3ng)

Vector DNA 10 ng

Negative controls were 15 μl dd H_2O_2.

A volume of 32.5 μl of PCR mix (1x PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM d NTP, 0.5 μM primers), 5 μg genomic DNA in final volume of 15 μl dd H_2O, and 5 μl Taq polymerase 500 units/100μl, Promega (Madison, USA). The E1A primers were 486 bp primers as described earlier, and the GAPDH primers were as described earlier. The PCR programme for E1A consisted of 35 cycles of denaturation (93°C for 1 minute), annealing (63°C for 1 minute), and extension (72°C for 1 minutes), and

final extension of 6 minutes, with an initial hot start of 90°C for 5 minutes. GAPDH PCR programme and conditions as previously described, using 1 μg genomic DNA, and 50 μl PCR mix.

5.2.6 Restriction enzymes to cut and then probe genomic DNA for E1A DNA sequences

The PCR amplification reaction may not have been very efficient using total genomic DNA, and the genomic DNA was cut using restriction enzymes. The restriction enzymes used were Alu-1, Boehringer Manheim, *Pst*1 and H*ind* III, Promega (Madison, USA). The template genomic DNA for these restriction enzymes was 2.5 μg G293 genomic DNA, and Scottish human lung sample 91 genomic DNA. Controls of DNA with buffer but without the restriction enzymes were included. Due to an error Pst-1 enzyme was added to the controls for the G293 genomic DNA and lung sample 91, and there were no controls for the Pst-1 enzyme. The DNA, buffer and enzymes were incubated for 24 hours at 37 ° C, and then were electrophoresed on a 1% agarose gel. The agarose gel was transferred to a nylon membrane and the membrane was hybridised using the 756 bp E1A probe as previously described.

5.2.7 Polymerase chain reaction and Southern hybridisation for E1A in collaboration with Dr Shizu Hayashi

81 samples of fresh frozen human lung tissue from well characterised patients were taken on dry ice to Vancouver, and genomic DNA extractions were performed in the laboratory at St Pauls Hospital Vancouver as per the previously documented protocol above. DNA from human placental tissue that had been stored at -80° in the St Pauls laboratory freezer was also extracted at the same time. The concentration of genomic DNA was calculated by measuring the absorbance at 260 nm, and lung and placental DNA was dissolved in water to a concentration of 0.1 μg/μl. Five μl of genomic DNA was used in the PCR reaction. Samples were run in duplicate and the PCR

reaction mix consisted of 1x PCR buffer, 2 mM $MgCl_2$,, 0.2 mM dNTPs and 10 µl of each primer (0.5 µM). Forty µl of this mix together with 5 µl of genomic DNA and 5 µl Taq polymerase (2.5 units/5µl) was used in the PCR reaction. A 3 minute hot start at 94°C was used prior to addition of the Taq polymerase. The size of the amplified product was 486 base pairs and the sequences were as follows (sense 5'- TCA-GGC-TCA-GGT-TCA-GAC-NCA-G-3') and (antisense 5' -TAA-TGT-TGG-CGG-TGC-AGG-AAG-G-3').

The conditions for the PCR were E1A: 40 cycles of denaturation (94°C for 1 minute), annealing (63°C for 1 minute), and extension (72°C for 2 minutes), and final extension of 7 minutes. The PCR products were allowed to stand overnight at 4°C in the PCR machine. Human genomic DNA of concentration 1mg/ml contained 2.7×10^5 copies of E1A per µg genomic DNA. Positive controls of 10, 100 and 1000 copy numbers were included by dissolving G293 in human placental DNA (6 pg/µl, 60 pg/µl, and 600 pg/µl). Negative controls of PCR mix alone, and 5µl of genomic DNA in the PCR reaction were used. The PCR products were transferred to a nylon membrane and hybridised as described earlier.

The HLA DQα primers produced an amplified product of 242 base pairs and the sequences were as follows (sense 5'-GTGCTGCAGGTGTAACTTGTACCAG-3') and (antisence 3'-GTTGAGATGGCGACGATGGCCTAGGCAC-5'). The conditions of the PCR reaction were as for the EIA and the programme consisted 40 cycles of 1 minute denaturation at 93°C, 2 minutes at 50°C annealing, and 2 minutes at 72° extension.

5.2.8 Immunostaining for E1A protein

This work was carried out in collaboration with Shizu Hayashi and Mark Elliot, UBC, Vancouver. Paraffin embedded sections from a population of Scottish patients (43) who had undergone LVRS or lung resection for suspected bronchial carcinoma

were examined. Full details of the patient characteristics are included in the results section, table 5.3.

Three micron thick paraffin embedded sections were cut and placed on Superfrost slides. Sections were deparaffinised by two 10 minute soakings in Hemo-DE solution. The sections were rehydrated using alcohol, initially 100% ethanol for 10 minutes (twice) and then placed in diethyl alcohol ether for 60 seconds, and then 0.4% nitrocellulose solution for 60 seconds, and then allowed to dry. After which the slides were placed in 70% alcohol for 3 minutes to allow to harden, and then they were placed in water until ready to use. The slides were autoclaved for 7 minutes in 6 M urea using a plastic Coplin jar and the slides were rinsed in water. The slides were then incubated with 10µl TBS and then the immunostaining started. The sections were incubated with 5% normal rabbit serum for 20 minutes at room temperature, and then poured off. One hundred µl of primary antibody (mouse monoclonal E1A Calbiochem DP11-100µG diluted 1:100 in 1% bovine serum albumin was incubated for 30 minutes at room temperature, followed by three 5 minute TBS washes. The secondary antibody was applied (rabbit antimouse Dako Z0259, 1:40 in TBS/1% BSA solution pH 7.6) for 30 minutes at room temperature, followed by three 5 minute washes in TBS buffer. 600 µl (APAAP-mouse monoclonal Dako D0651 diluted 1:100) was added to each slide for 30 minutes followed by washing in TBS buffer. The substrate was prepared fresh and the solutions A and B were mixed and filtered directly onto the slides.

Solution A

250 µl 4% sodium nitrite solution was placed in an Ehrlenmeyer flask and 100 µl of 5% New Fuschin (in 2M hydrochloric acid) was added and the solutions mixed. 50 µl of TBS buffer pH 8.7 was added to the solution and 50 µl 1 M levamisole was added.

Solution B

25 ng naphthol-AS-BI-phosphate was added to dimethyl formamide.

Three batches of lung sections were examined for E1A by immunohistochemistry. After the first batch, the positive control consisting of sections of HA 35-I cells was changed to A549 cells infected with adenovirus, as this gave a better positive result.

Positive controls: Batch 1 HA 35 –I (Human bronchial epithelial cell line which has been transfected with E1A)

Negative control: HA-35-II

Run 2 and 3: Positive controls:A549 cells infected with adenovirus

5.3 Early One Adenoviral Results

5.3.1 Amplification of genomic DNA using PCR

Standard amplification of 500 ng and 50 ng of template human genomic DNA from 24 patients who had undergone lung resection was performed using PCR with 675 bp and 486 bp E1A primers, and GAPDH primers. Nine patients had normal lung function (mean FEV_1 2.5 ± 0.77 litres), thirteen patients had airflow obstruction (mean FEV_1 1.8 ± 0.35 litres) and 2 patients had severe airflow obstruction having undergone LVRS (mean FEV_1 0.45 ± 0.21 litres). All patients were either current or ex-smokers except for three patients who had never smoked. Two patients who had never smoked were included in the airflow obstruction group, one of which had mild airflow obstruction with a percent predicted FEV_1 of 68%, and the other non smoker had a tumour that was acting as a ball and valve and resulting in airflow obstruction. One never smoker was included in the normal lung function group. The characteristics of the patients included in these PCR experiments are shown in table 5.1.

Table 5.1 Patient characteristics

	Normal Lung Function ***n=9***	***COPD*** ***n=13***	***Severe COPD (lung volume reduction surgery)*** ***n=2***
Age years(mean & range)	62.9 (58-72)	59.4 (21-77)	62 (49-55)
Male:female	4:5	7:6	0:2
Smoking History (pack yrs)	52.75±33.4	45.4± 25.3	34.6±28.6
FEV_1 (litres)	2.5±0.77	1.8±0.35 *	0.45±0.21**
FEV_1 % pred	96.2 ±10.3	62.6± 8.4**	20±7.1**
FVC (litres)	3.2±1.1	3.2 ±0.8	1.65±0.6
FEV_1/ FVC %	77.2± 3.65	58.6±7.7**	26±3.7 **

Data expressed as mean ± SD

* $p < 0.05$ versus normal lung function group

** $p < 0.001$ versus normal lung function group

The use of E1A primers and 500 ng of template DNA failed to show any single bands in the expected area when PCR products were electrophoresed on an ethidium bromide stained agarose gel and smears were observed. Reduction in the quantity of genomic DNA lead to multiple bands, but the strongest band was above the level of the positive control template DNA, figure 5.1. Similarly GAPDH primers produced smears when using 500 ng of genomic DNA but using 50 ng produced single clean bands on the ethidium bromide stained agarose gel. Figure 5.1 shows representative ethidium bromide stained gel of the PCR products using 486 bp primers and GAPDH primers with 50 ng genomic DNA from lung tissue.

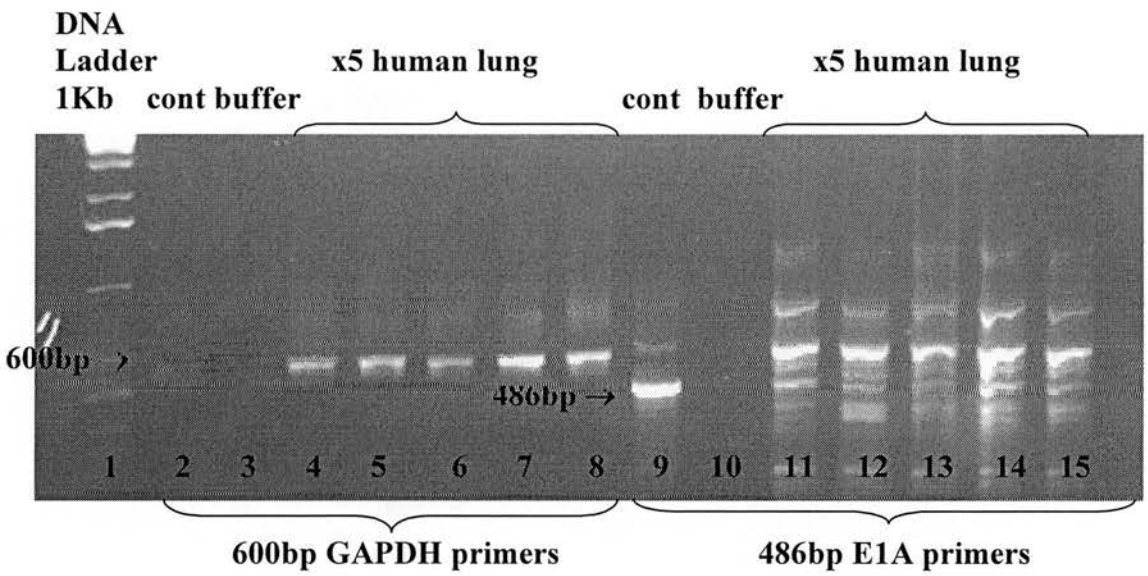


Figure 5.1 The amplification of genomic DNA from human lung tissue using GAPDH primers and 486 bp E1A primers. Lane 1 1Kb DNA ladder, lane 2 cDNA from A549 cells used as positive control, lane 3 buffer with no template DNA, lanes 4-8 human lung genomic DNA as template DNA. Lane 9 template DNA positive control genomic DNA from G293 cells, lane 10 buffer with no template DNA and lanes 11-15 human lung genomic DNA as template DNA.

5.3.2 Dot Blot Southern Hybridisation using 675bp Probe

PCR with E1A primers and electrophoresis on agarose gel had failed to demonstrate single bands in the expected area. The technique of dot blot Southern which allows unfractionated DNA to be immobilised on a nylon membrane, followed by hybridisation was used in an attempt to identify E1A in non amplified DNA.

Genomic DNA from 24 human lungs was used in the dot blot. The patient characteristics are shown in table 5.1, and DNA is from the same group as that used in section 5.3.1. The probe used was the positive control PCR product from the PCR reaction using 675 bp primers which had been run on agarose gel and cut out and purified and labelled with α ^{32}P dCTP. The positive controls included G293 genomic DNA and cDNA from A549 cells transfected with E1A. No lung genomic DNA was positive on dot blotting, and only the cDNA from E1A transfected cells was positive. The G293 cell genomic DNA was negative on dot blotting. This is shown in figure 5.2.

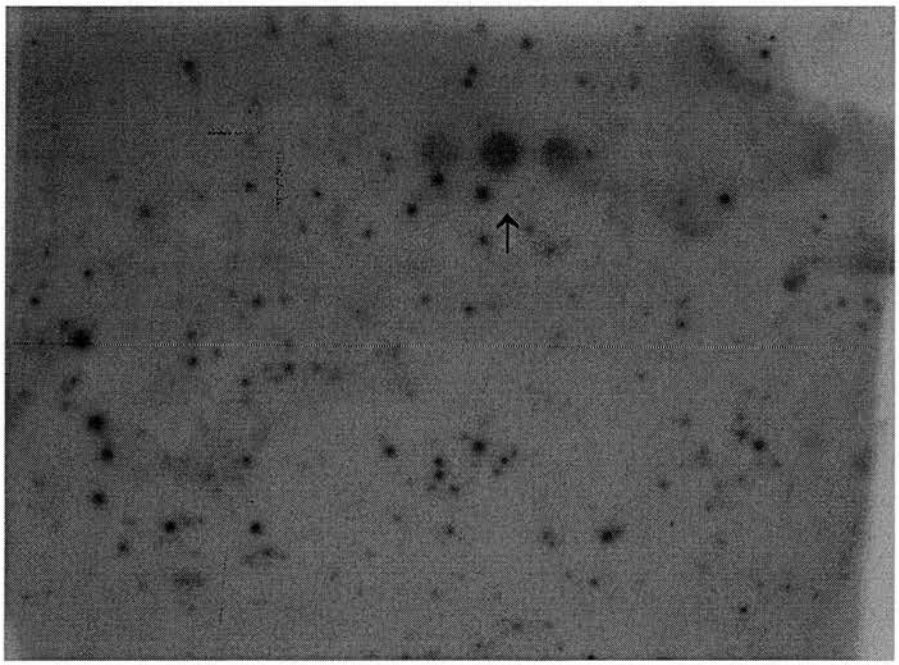


Figure 5.2 Dot blot Southern hybridisation of genomic DNA from 24 human lung samples. The three round dots, indicated by the arrow are the positive control of E1A transfected cell cDNA. No human lung genomic DNA was positive and nor was the genomic DNA from G293 cells. The probe consisted of the labelled positive control (G293 genomic DNA) PCR product (675bp) that was cut out of an agarose gel.

5.3.3 Nested PCR

Nested PCR with 675 bp outer primers and 261 bp inner primers for E1A was used to examine for the presence of E1A protein in 30 samples of human lung genomic DNA. The characteristics of the 30 patients are shown in table 5.2.

	Normal Lung Function	***COPD***	***Severe COPD (lung volume reduction surgery)***
	n=9	***n=17***	***n=4***
Age (mean ± SD)	63.3±7.2	62.9± 7.5	54.8±8
Male:female	5:4	11:6	1:3
Smoking History (pack yrs)	59.2±42.2	44.6± 22.6	32.7±17.5
FEV_1	2.41±0.73	1.93±0.47	0.62±0.23**
FEV_1 % pred	91.4 ±9.7	65.4± 8.1**	23.7±8.1**
FVC	3.2±1	3.2 ±0.85	2.29±0.93
FEV_1/ FVC %	75.8± 3.15	61±10.49**	27.3±6.1**

Data expressed as mean ± SD

** $p < 0.001$ versus normal lung function group

Table 5.2 Patient characteristics

On one occasion a positive result was obtained for lung sample number 64 (figure 5.3.). We were unable to reproduce this result. There were repeated problems with contamination in the experiments using nested PCR that followed this positive result and so it is highly likely that this positive result was the result of contamination.

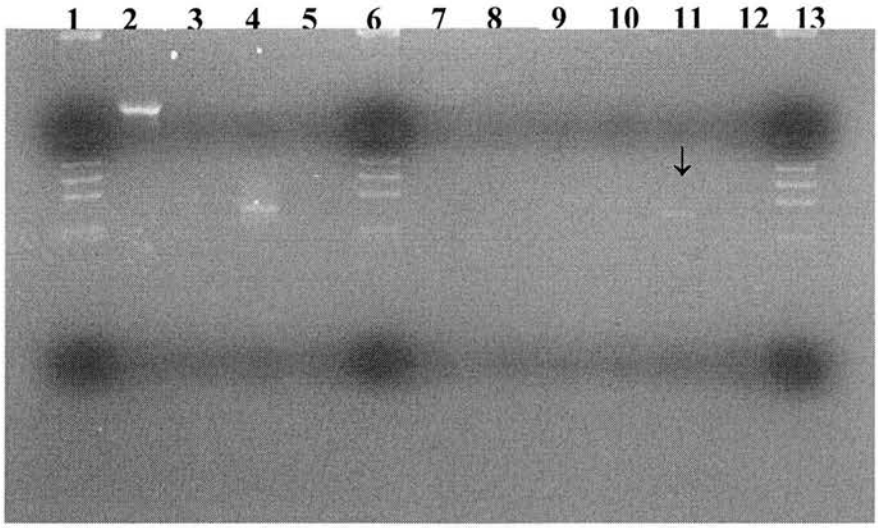


Figure 5.3. An ethidium bromide stained agarose gel with IKb DNA ladders demonstrated in lanes 1, 6 and 13. Lane 2 G293 genomic DNA PCR product using outer primers, 675bp. Lane 3 is a negative control with PCR mix alone. Lane 4 is a positive control for the nested PCR using inner and outer primers. Lanes 7-12 represent PCR products from human lung tissue genomic DNA which have undergone nested PCR using 675bp and 261bp primers. A faint positive band is present in lane 11, see arrow.

5.3.4 Southern Hybridisation

Genomic DNA from 20 human lung samples was amplified using E1A 486 bp primers and also using 600 bp GAPDH primers. These samples were obtained from patients with the characteristics outlined in table 5.3. Lung function was classified as obstructed if the percent predicted FEV_1 was less than 80 %. There were six subjects with normal lung function (mean percent predicted FEV_1 91.9), eight with airflow obstruction with mean percent predicted FEV_1 73.1, and six with severe airflow obstruction who had undergone lung volume reduction surgery for severe

emphysema, with percent predicted FEV_1 24.9. Two samples in the severe airflow obstruction group were from the same patient but were from different lobes of the lung. The airflow obstruction group included in this work contained patients with very mild disease rather than in previous experiments were subjects had more severe disease. Only the severe obstruction group had complete smoking histories recorded, and within the normal and airflow obstruction groups smoking history data was incomplete in 5 patients.

The PCR products were transferred to a membrane and probed with E1A probes and GAPDH probes. These 20 samples were all negative for E1A. All samples however were positive on agarose gel for GAPDH and also on probing using GAPDH probe.

	Normal Lung Function	***COPD***	***Severe COPD (lung volume reduction surgery)***
	n=6	***n=8***	***n=6***
Age years (mean & range)	63.8 (43-75)	67 (57-77)	62 (42-59)
Male:female	4:2	6:2	4:2
Smoking History (pack yrs)	90.25(22-168)	41.1(26-51.75)	61.1(25-184)
FEV_1 (litres)	2.57±0.59	2.18±0.56	0.75±0.2 *
FEV_1 % pred	91.9 ±7.4	73.14± 8.4*	24.9±7.44*
FVC (litres)	3.53±1.1	3.19 ±0.98	2.67±0.95
FEV_1/ FVC %	73.5± 3.65	69.5±12.01	29.3±5.4 *

Data expressed as mean ± SD

* $p < 0.001$ versus normal lung function group

Table 5.3. Patient characteristics

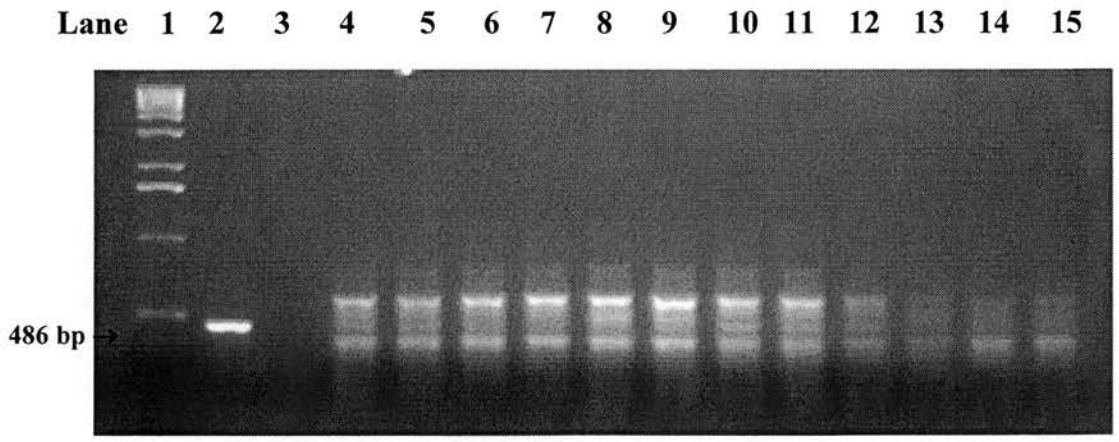


Figure 5.4.a

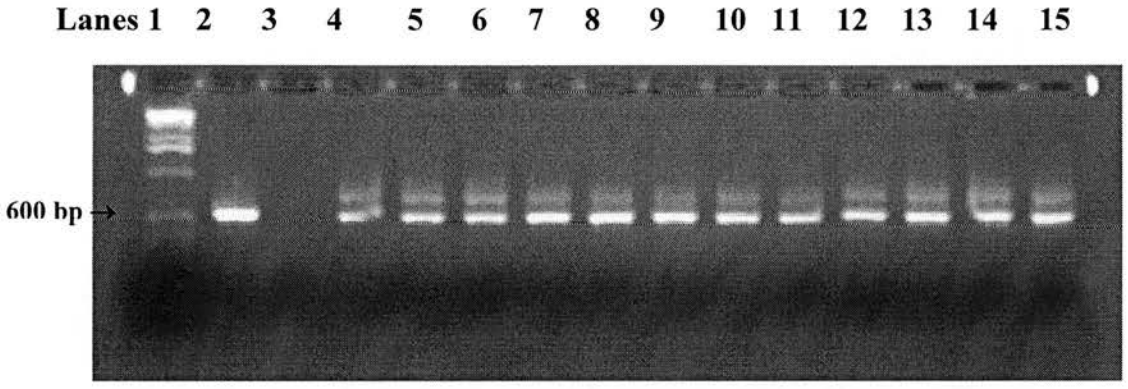


Figure 5.4.b

Figure 5.4.a is a representative ethidium bromide stained agarose gel of human lung genomic DNA amplified using 486 bp primers (in triplicate) lanes 4-15. Lane 1, 1Kb DNA ladder, lane 2 positive control of G293 genomic DNA, and negative control in lane 3 consisting of PCR mix alone. Amplification of lung genomic DNA gives multiple bands as previously shown in figure 5.1.

Figure 5.4.b is an ethidium bromide stained agarose gel of the same lung sample genomic DNA (lanes 4-15) as in figure 5.4a using GAPDH primers for amplification and the positive control of A549 cell cDNA. A single clean band is demonstrated for the GAPDH at 600bp.

To determine if any of the PCR product bands represented the 486 bp DNA of the E1A region, Southern blots of these agarose gels were probed using a DNA probe corresponding to the E1A region of the adenovirus genome. The human lung PCR amplification products obtained using E1A primers were negative on probing, but clear positives were obtained on probing the G293 positive controls, figures 5.4.a and 5.5. The human lung PCR amplification products obtained using GAPDH 600 bp primers were all positive on probing, and clear positives were obtained on probing the cDNA positive controls (figures 5.6 and 5.4.b).

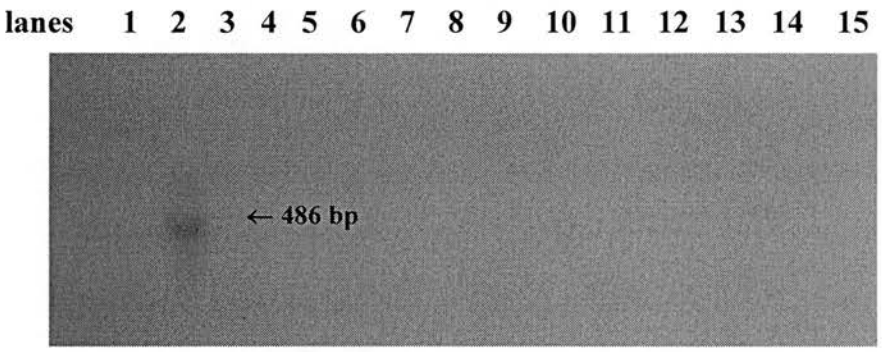


Figure 5.5. is the Southern transfer of the agarose gel in figure 5.4.a (ethidium bromide stained agarose gel of human lung genomic DNA amplified using 486 bp primers (in triplicate) lanes 4-12. Lane 1, 1KB DNA ladder, lane 2 positive control (template of G293 genomic DNA), and negative control in lane 3 consisting of PCR mix alone, which has been probed using E1A DNA probe. The previously noted amplification gave multiple bands in the lung DNA, but no signal is demonstrated on probing the Southern transfer of the gel and PCR products.

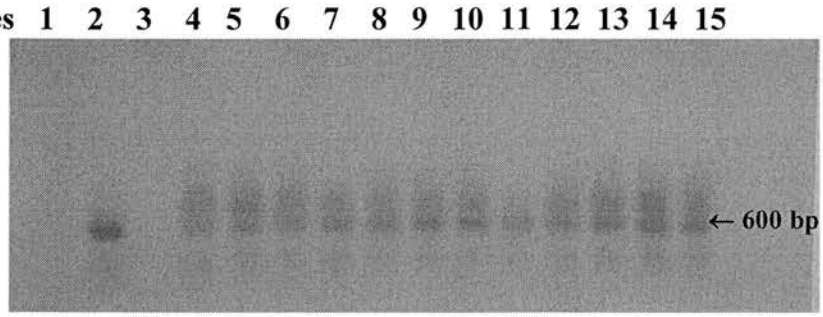


Figure 5.6. is an autoradiograph of the Southern transfer of the agarose gel in figure 5.4.b which has been probed using GAPDH DNA probe. All of the 12 lung samples lanes 4-15 (4 different genomic DNA performed in triplicate) demonstrate a similar signal on the autoradiograph. Lane 1 1Kb DNA ladder, lane 2 positive control of c DNA, and lane 3 negative control of PCR mix alone.

5.3.5 Southern Hybridisation using Positive controls of plasmid E1A DNA and G293 genomic DNA, and 'positive' lung sample from Vancouver

PCR amplification with 486 bp primers was carried out using 5 μg lung genomic DNA {from 8 lung samples including lung sample 64 (lane 9) which had a single unrepeatable positive using nested PCR, and 'positive' Vancouver lung sample (lane 10)} in the reaction mixture and plasmid DNA as control together with G293 genomic DNA. The PCR products were transferred to a nylon membrane and probed using the pUC E1A which had been cut using restriction enzymes *Bam*H1 and *Pst*1. The lung samples were all negative on PCR amplification and similarly they were all negative on probing (figure 5.7). No positive signal was found in lane 10 which represents the PCR product from genomic DNA that had been found to be positive in Vancouver, and no positive signal was found in lane 9 which had been found to be positive on one occasion using nested PCR. The nylon membrane was cut inorder to hybridise the positive samples (G293 PCR products) separately to the lung samples to ensure that the 'positive controls' were not preferentially binding the probe.

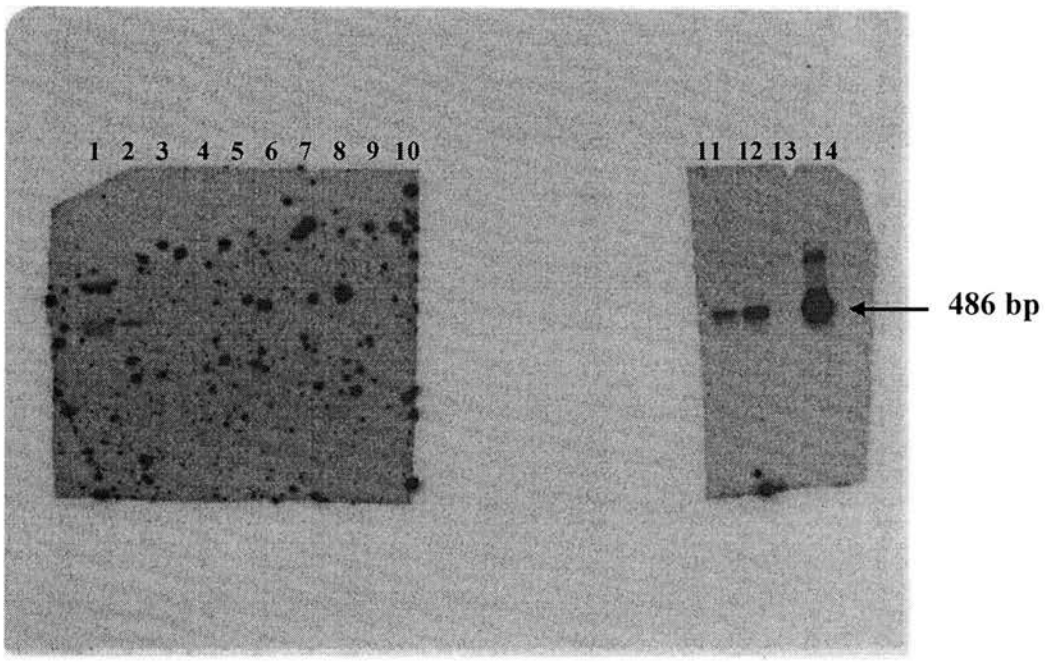


Figure 5.7 shows the Southern hybridisation of the transferred PCR products that have been probed using E1A probe. Lane 1 DNA ladder, lane 2 negative control with PCR mix alone, lanes 3-9 templates of Scottish lung genomic DNA, lane 10 template of Vancouver 'positive' lung genomic DNA, lane 11 G293 PCR products, lane 12 G293 PCR products, lane13 empty, and lane 14 Vector E1A DNA. Binding of the probe to the DNA ladder is also noted in lane 1. There is no positive signal in the area of lane 10, which is the genomic DNA from Vancouver which was reportedly positive on testing in Vancouver for E1A.

5.3.6 Restriction enzymes to cut and then probe genomic DNA for E1A DNA sequences

The PCR amplification reaction may not have been very efficient using total genomic DNA as unwanted DNA is included in the PCR reaction.

Genomic DNA from human lung and G293 cells was cut using the restriction enzymes Alu1, *Pst*1 and *Hin*d III, electrophoresed on an agarose gel and then probed using the same E1A probe as in the previous experiments. AluI failed to cut human lung genomic DNA, as did *Hin*d III, but *Pst*1 cut both human lung genomic DNA and G293 genomic DNA. Probing of these cut DNA sequences failed to yield any positive signals even for the cut G293 using DNA 756 bp E1A probe.

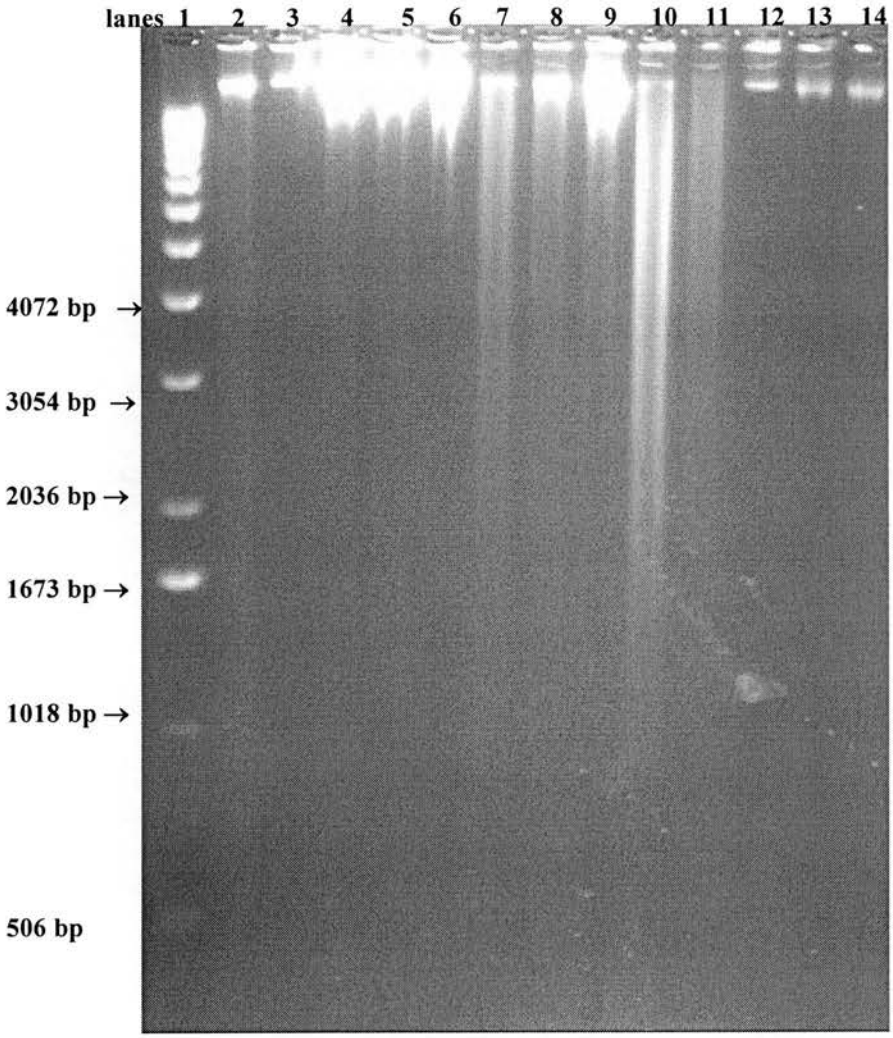


Figure 5.8. This shows an agarose gel with genomic DNA that has been cut using the restriction enzymes Alu-1, Pst-1 and Hind-III, which has been electrophoresed. Lane 1 1 Kb DNA ladder, lane 2 G293 genomic DNA cut with Alu-1, lane 3 G293 no enzyme control, lanes 4 and 5 human lung genomic DNA with Alu-1, lane 6 human lung genomic DNA no enzyme, lanes 7 and 8 G293 genomic DNA cut with Pst-1, lanes 9 and 10 human lung genomic DNA cut with Pst-1, lane 11 G293 genomic DNA cut with Hind-III, lane 12 G293 genomic DNA without enzyme, lane 13 human lung genomic DNA cut with Hind-III, and lane 14 with no enzyme.

5.3.7 Results from work carried out in collaboration with Vancouver laboratory

81 lung samples were taken on dry ice to the laboratory in Vancouver where genomic DNA extractions were undertaken, and PCR amplification of duplicate samples using 486 bp E1A primers and the PCR products were transferred to nylon membranes and probed using 756 bp E1A probe. The duplicate PCR samples were electrophoresed on separate agarose gels, giving a total of ten agarose gels.

Table 5.4 shows the patient characteristics for the 75 genomic DNA samples obtained from lung tissue that were included in the final analysis for the presence of E1A by Southern hybridisation. Six samples were excluded from the final analysis as they were negative for HLA DQα. DNA was obtained from 29 smokers with no airflow obstruction, 31 patients with mild to moderate airflow obstruction (FEV_1 < 70% predicted FEV_1/FVC ratio <70%), and 15 patients with severe emphysema who had undergone lung volume reduction surgery for emphysema.

	Normal Lung Function n=29	***COPD n=31***	***Severe COPD n=15***
Age (range)	63.9 (37-75)	63.2 (21-73)	55.7 (42-66)
Male:female	9:20	7:24	3:12
Smoking History (pack yrs)	43.4 ± 7.6	43.4 ± 5.1	47.7 ± 6.8
FEV_1 litres (± SD)	2.3 ± 0.13	1.9 ± 0.08	0.63 ± 0.05
FEV_1 % pred	93.9 ± 3.61	61.1 ± 1.8	25.5 ± 1.4
FVC litres	3.1 ± 0.18	3.3 ± 0.13	2.2 ± 0.21
FEV_1/ FVC %	72.7 ± .3	59 ± 1.78	3 0.9 ± 3
E1A positivity % patients	44.8	45.2	66.7

Data expressed as mean ± SD

Table 5.4 Characteristics of the 75 patients included in the final analysis for E1A by Southern hybridisation

Increased copy numbers of E1A in the positive controls clearly show an increase in density of the signal on Southern hybridisation, see figure 5.9.

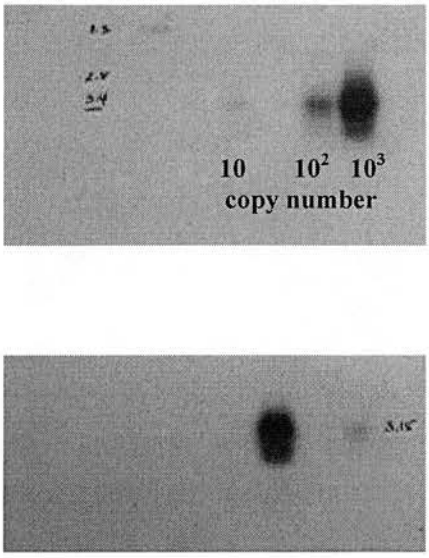


Figure 5.9 Autoradiograph of the Southern transfer of the two positive control areas from 2 separate gels. The upper autoradiograph shows increasing copy numbers of E1A (increasing concentrations of G293 dissolved in placental DNA) with increasing signal following hybridisation using E1A probe. In the lower autoradiograph the same copy numbers of E1A are present (10^2), but the stronger signal comes from a template of G293 dissolved in water and the weaker signal from G293 template dissolved in human placental DNA.

A representative agarose gel containing samples from 15 patients is shown in Figure figure 5.10a, and the corresponding autoradiograph of the transferred PCR products that has been probed using the radiolabelled E1A probe is shown in figure 5.10b. Lanes 1-2 represent negative controls in the form of PCR mix with distilled water and PCR mix with human placental genomic DNA respectively. Lanes 3 to 17 show the resolved PCR products from 15 patients (includes samples from patients both with and without airflow obstruction). Lane 18 is a 1Kb DNA ladder and lanes 19, 20 and 21 are resolved PCR products using increasing concentrations of G293 genomic

DNA diluted in human placental DNA. The level of the expected E1A bands on the autoradiograph is indicated by the arrow. The autoradiograph shows two bands an upper (predicted for E1A) and a lower band in 10 of the cases, a lower band alone in 4 cases and no bands in 3 cases. Samples in lanes 2, 3, 4, 7, 10, 12, 14, 15 and 17 are positive for E1A, however the negative control (human placental genomic DNA) is also positive for E1A, figure 5.10b.

Southern hybridisation of the PCR products from the ten gels (x5 that had been run in duplicate) showed that the negative control of human placental DNA was positive on at least one of each of the duplicate gels. The other negative using dd H_2O was consistently negative for E1A on probing. No PCR product from human lung genomic DNA gave a single band on ethidium bromide staining and all gave multiple bands similar to earlier work see figure 5.1. The positive controls for E1A that consisted of G293 dissolved in genomic DNA gave multiple bands on the ethidium bromide stained gel, where as those dissolved in water gave a single band.

Although human lung samples appear to be positive in a proportion of cases, the positivity of the negative control indicates that contamination has occurred in the process.

Figure 5.10a

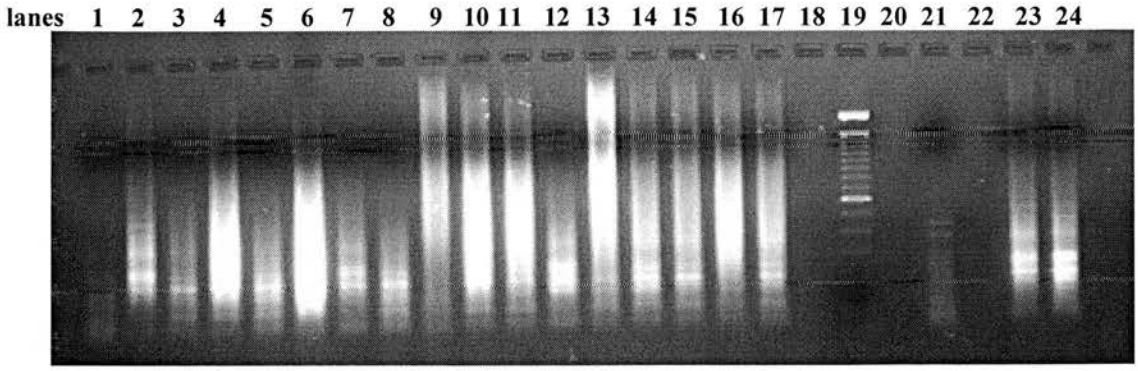


Figure 5.10b

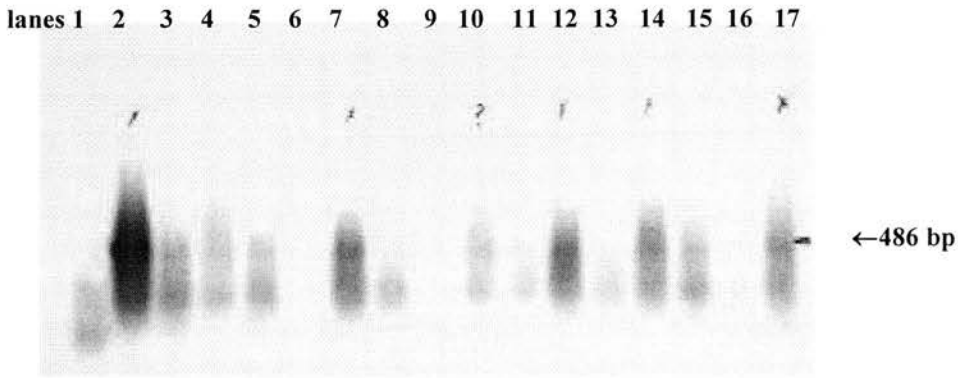


Figure 5.10a Polymerase chain reaction amplification of adenovirus DNA using genomic DNA from human lung tissue. Figure 5.10a represents the ethidium bromide stained agarose gel with the PCR products. The template DNA used in the polymerase chain reactions were as follows:lane 1 PCR mix alone, lane 2 human placental genomic DNA, lanes 3-17 genomic DNA from frozen human lung tissue. Lanes 18, 20, 22 empty, lane 19 1kB DNA ladder, lanes 21, 23 and 24 increasing concentrations of G293 template DNA.

Figure 5.10b is the autoradiograph of the transferred PCR products of the gel in a above probed with E1A DNA. In this case the positive controls have been cut from the membrane and have been hybridised separately.

5.3.8 Diluting G293 genomic DNA in human lung genomic DNA

To test if it was easy to contaminate human lung genomic DNA with G293 genomic DNA, and also to assess whether contamination produced a single clean band on ethidium bromide stained agarose gel electrophoresis, G293 genomic DNA was

diluted in human lung genomic DNA and PCR amplification performed. The agarose gel is shown in figure 5.10, and dilution of G293 genomic DNA in water up to 0.0002 μg/μl gave a single clean band. No single clean bands were obtained when G293 genomic DNA was diluted in human lung genomic DNA, however smears and two bands were seen using a concentration up to 20pg/μl, figure 5.12. Dilutions below this concentration failed to show any bands.

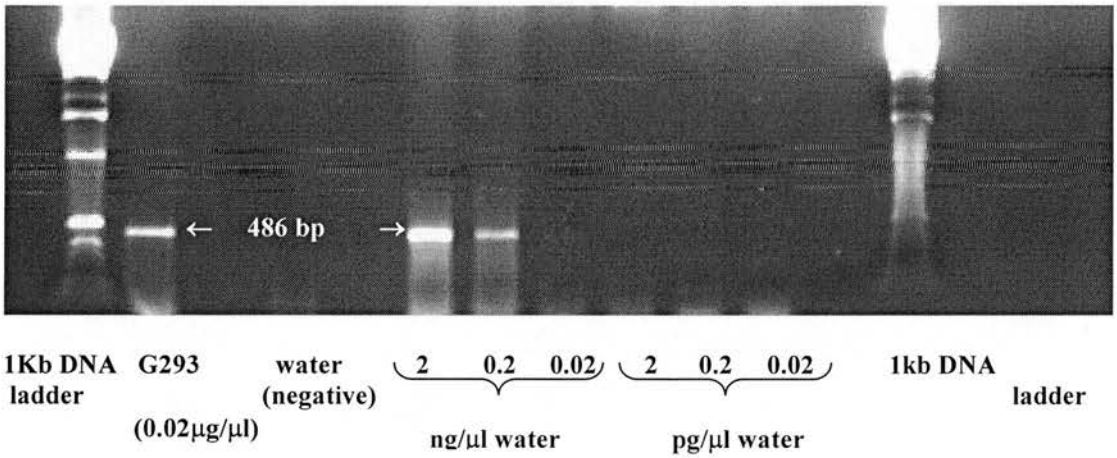


Figure 5.11 An ethidium bromide stained agarose gel with the PCR products from a PCR using various concentrations of G293 dissolved in water, in the PCR mix and 486 bp primers. A faint band is visible down to a concentration of 0.02ng of G293 genomic DNA/μl.

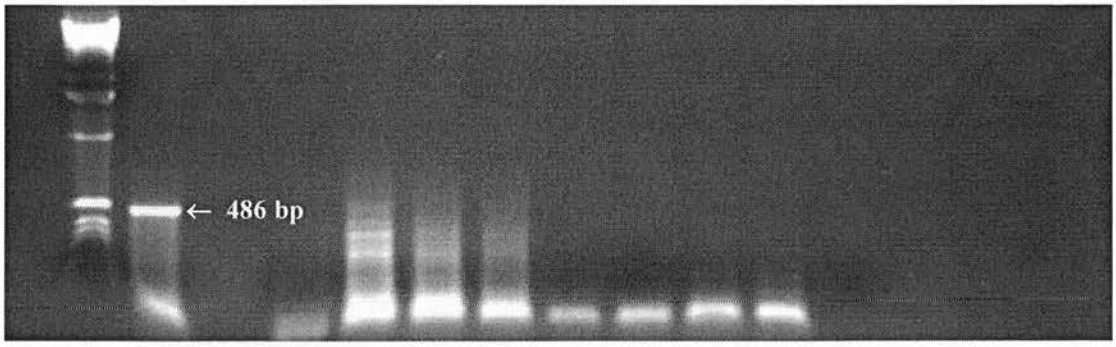


Figure 5.12 shows an agarose gel in which PCR products from a PCR reaction using 486 bp primers and using reducing concentrations of G293 genomic DNA diluted in human lung genomic DNA from lung sample 101 in the PCR mix. With reducing concentrations smears of DNA are no longer present. Of note, however is the fact that single clean bands are never produced when G293 genomic DNA is diluted in human lung DNA and used in the PCR.

G293 genomic DNA that is diluted in water and then used in the PCR reaction produces a single clean band in the expected area, however G293 genomic DNA that is diluted in lung genomic DNA and then used in the PCR reaction produces a smear and several faint multiple bands, none of which correspond to the expected 486 bp area.

5.3.9 Immuohistochemical Staining for E1A protein in human lung tissue

The PCR work for E1A had been complicated by contamination problems and we were unable to confirm the presence of E1A DNA sequences in human lung by this method and so immunohistochemistry for E1A was carried out. Forty three patients were included in the final analysis. The characteristics of the fourty three patients are shown in table 5.5.

		Smokers		
	Never Smokers	***Normal Lung Function***	***COPD***	***Severe COPD***
	n=4	***n=18***	***n=13***	***n=8***
Age years(range)	55.5 (21-70)	61 (43-730)	63.7 (57-72)	51.6 (42-58)
Male:female	0:4	8:10	9:4	1:7
Smoking History pack yrs	0	53.2 ± 11	50.5 ± 5.6	31.8 ± 5.5
FEV_1 litres (±SD)	1.75 ± 0.25	2.4 ± 0.18	1.8 ± 0.11	0.7 ± 0.08
FEV_1 % pred	69.1 ± 11.8	91.6 ± 1.96	59.6 ± 1.8	26.5 ± 2.4
FVC litres	2.32 ± 0.3	3.3 ± 0.23	3.3 ± 0.23	2.3 ± 0.25
FEV_1/ FVC %	76 ± 5	73.2 ± 1.3	54.9 ± 1.2	28.2 ± 1.6
E1A positivity %	0	5.5	0	0

Data expressed as mean ± SD

Table 5.5 Characteristics of patients

Only one section was positive for E1A by immunostaining. This positive section was from an exsmoker, with no airflow obstruction.

5.4 Discussion Early One Adenoviral Protein (E1A)

It is well described that smokers develop lung inflammation (Neiwoehner *et al.,* 1974). However the mechanism of the enhanced inflammatory response in the lungs of susceptible smokers who develop COPD is unknown. It has been postulated that the persistence of the adenoviral genome even without viral replication (latent infection), and the subsequent expression of the protein E1A in lung cells renders the cells susceptible to the effects of cigarette smoke (Matsuse *et al.,* 1992; Hogg, 2001). After human adenoviral infection, expression of part of the adenoviral genome can take place, and such expression occurs first with the Early Region 1A, and this is a crucial step for the integration of the adeno viral DNA into the human genome.

E1A protein has been implicated in enhancing lung inflammation by virtue of its inhibitory effect on the TGF-β signalling pathway (Tarakanova and Wold, 2003), modulation of inflammatory mediators including ICAM-1 and IL-8 (Keicho *et al.,* 1997; Higashimoto *et al.,* 1999, Gilmour *et al.,* 2001; Keicho *et al.,* 1997), its ability to subvert cellular processes by displacing cellular transcription factors from CBP and related p300 proteins (O'Connnor *et al.,* 1999; Lundblad *et al.,* 1995; Arany *et al.,* 1995) and the subsequent over expression of host inflammatory genes. E1A also exerts oncogene effects by the binding and inactivation of tumour supressor gene product Rb (Whyte *et al.,* 1988).

The presence of E1A has been demonstrated in human lung tissue using PCR (Kuwano *et al.,* 1997; Vitalis *et al.,* 1998), and its presence has been associated with the development of COPD in a Canadian population of patients undergoing lung resection. The aim of this part of the thesis was to assess for the presence of E1A DNA sequences in lung tissue, and E1A viral protein expression by immuno histochemistry in another population.

We found that in our patient population (in work carried out in UK), standard PCR using lung genomic DNA as template DNA produced smears and multiple bands, and Southern hybridisation failed to demonstrate the presence of E1A DNA sequences in human lung tissue. The use of lung tissue genomic DNA as template

DNA for the PCR for GAPDH demonstrated bands and similarly the Southern showed good signals for all the samples examined. Using nested PCR, bands suggestive of E1A were demonstrated on one occasion, but this could not be repeated. PCR performed in Vancouver for E1A produced multiple bands and Southern hybridisation demonstrated signals suggestive of positive E1A DNA sequences, however there were problems with the negative samples also showing these signals on Southern, suggesting contamination had occurred in the experiments.

It is likely that GAPDH primers worked well because greater copy numbers of the GAPDH nucleic acid sequence were present, unlike E1A in which the copy number is likely to be low. It is surprising that single bands were not obtained using the 486 and 675 bp primers as Matsuse and Vitalis (Matsuse *et al.,* 1992; Vitalis *et al.,* 1998) demonstrated single clean bands in their papers when genomic DNA from human lung tissue and rats had been used. Our work differed from that of Vitalis as we extracted genomic DNA from fresh frozen tissue and the Vitalis group extracted the DNA from paraffin embedded sections and lung tissue that had been inflated with Optimal cutting agent (OCT).

On one occasion, using the technique of nested PCR, a single band corresponding to the expected band was found in a lung sample from a patient with small cell lung carcinoma. Despite repeated attempts we failed to demonstrate this again, and following on from this initial positive there were problems with contamination as various lung samples and negative controls were intermittently positive. However once these contamination problems were rectified, then no further lung tissue genomic DNA samples were found to be positive for E1A. Kuwano (Kuwano *et al.,* 1997) who used the same nested PCR primers, examined lung tissue from patients with lung cancer and reported 30% of those with small cell lung cancer had E1A but was unable to detect E1A sequences in the non small cell group. Again our work differed from that of Kuwano (Kuwano *et al.,* 1997) who extracted DNA from paraffin embedded samples where as we used frozen lung tissue for the extraction.

In case there was a problem with my technique, I took 81 human lung samples on dry ice to the laboratory in Vancouver and carried out genomic DNA extractions, PCR, and Southern hybridisation under the supervision of Dr Hayashi and Ted Sedgewick. Genomic DNA from the Graham 293 cell line was used as the positive control, and there were two positive controls run on each gel consisting of G293 diluted in water, and also G293 diluted in human placental DNA. Once again no single bands were found on electrophoresis of the PCR products from human lungs. Southern hybridisation of the PCR products with the E1A probe did yield positives, however the results from the duplicate samples were not always consistent. Also, one of each of the duplicate PCR products' negative controls (human placental DNA) was positive on Southern hybridisation. This suggests that there was a contamination problem and the likely source was the placental DNA or the extraction process rather than the PCR mix, as the negative that consisted of water was consistently negative. In Matsuse's original report clear bands were demonstrated on electrophoresis of the PCR products from human lung (Matsuse *et al.,* 1992), however as stated earlier we were unable to show clear bands. Also Matsuse did not use human placental DNA in which to dilute the G293 genomic DNA positives in. The human placental DNA had been stored in the -80° C freezer in Vancouver. Another possibility is that human placental DNA contains E1A. It is possible that the lack of consistency with regard to positive signals on Southern hybridisation results from the low copy number of the E1A virus.

Due to the ubiquitous nature of the adenovirus, contamination is a well described problem with this type of work. In fact Fernandez-Soria had to discard tissue from sixty out of a total of ninety lymphoma samples being examined for E1A as there was a contamination problem (Fernandez-Soria *et al.,* 2002). Extractions, PCR and Southern were performed on the bench top in the ELEGI laboratory, but this laboratory had not previously been involved in E1A work. In Canada even though we tried to minimise the risk of contamination by performing the DNA extractions

and the running of the agarose gels in different laboratories, our results suggested that there was a contamination problem.

Contamination is a well described problem in PCR work, and sources of contamination within the laboratory include the PCR product and the target template. To minimise contamination then pre and post PCR events should be performed in separate laboratory areas using separate equipment. As stated previously the pre and post PCR work performed in Vancouver was performed in separate laboratories, although this was not the case in Scotland. The generation of aerosols of PCR amplicons can be minimised by the use of barrier tipped pipettes. Barrier tipped pipettes were not used routinely used for the PCR work performed in Edinburgh. Chemical methods such as UV photolinking can be used on the PCR equipment to inactivate amplicons by virtue of the pyrimidines on DNA strands being cross linked by UV light at 254 nm. We did not use this technique in the laboratory in Vancouver. Real time PCR systems can provide direct measurement of amplicon accumulation during the reaction without the need to handle the sample/open the sealed tubes, and this method would have been a good way to minimise contamination.

Contamination is one explanation as to why the PCR and Southern experiments performed in the laboratory in Scotland failed to yield positives, where as those performed in Vancouver did. However it could be argued that the smaller number of genomic lung DNA samples used in Scotland (20 compared to 81) were truly negative and that if a larger sample had been used, then positives would have been identified. This seems unlikely as 10 samples were common to both sets of experiments.

We were given a sample of lung genomic DNA that had been found to be positive on PCR testing in Vancouver, however when this was included in a PCR in Scotland, no positive bands were identified either on the agarose gel or by Southern hybridisation. It is possible that a low copy number DNA may not be present in every aliquot of genomic DNA sampled and this may explain the inconsistency in the results.

It is possible that the probe used in the E1A Southern hybridisation was not detecting the E1A DNA sequence. This seems unlikely as the positive controls for E1A (G293 cells) were always positive. We did see faint signals in the DNA ladder when the Southerns were performed in the UK, although this was not found in the Southerns in Vancouver.

To confirm that contamination from the G293 positive was possible, serial dilutions of G293 genomic DNA in water, and also serial dilutions of G293 genomic DNA in the genomic DNA from human lung sample 101 were performed. This showed that concentrations below 0.0002 μg/μl in water failed to give a band and that G293 genomic DNA diluted in the genomic DNA from human lung sample 101 gave smears and two bands up to G293 concentration of 20pg/μl. These PCR gels were not transferred or probed using the E1A probe. These findings suggest that human lung genomic DNA interferes with the amplification process for 675bp and 486bp primers, as a single band is repeatedly found when G293 genomic DNA is diluted in water, but not when it is dissolved in human genomic DNA.

As stated previously the striking feature of all the PCR experiments performed using lung genomic DNA and the positive controls of G293 dissolved in human placental DNA was the presence of smears of DNA, without a single clear positive band. One explanation for this could be failure of the PCR to work. This question could have been addressed by performing PCR using templates consisting of G293 genomic DNA alone, G293 genomic DNA diluted in placental DNA, genomic DNA from a positive lung sample by Southern, and an apparent negative lung sample on Southern, using PCR mixes with and without primers. If the later three templates all gave the same appearance (using mixes with and without primers) of smears without clear single bands then the implication would be that the PCR has not worked.

The fact that G293 genomic DNA can be diluted in lung genomic DNA without the production of a clean single band on PCR amplification suggests that examination using PCR products on agarose ethidium bromide gels is not a very sensitive test for

E1A. The other alternative explanation is that the presence of lung genomic DNA with G293 genomic DNA adversely affects the PCR reaction.

The probe used for Southern hybridisation was a 756 bp probe and the DNA sequence being sought was actually smaller than the probe at 486 bp.This combination is less efficient than using a small probe to seek a larger sequence. Also a large probe may lead to problems with non-specific binding. It may have been better to cut the genomic DNA using restriction enzymes and probe the cut fragments with a new smaller probe. To confirm that the bands noted on the agarose gel after running the PCR products (for the G293 positive controls), are the E1A sequence of interest, then the G293 genomic DNA could be cut using restriction enzymes that cut through the probe sequence, and also cut using restriction enzymes that cut around the E1A sequence. If the DNA enzyme mix from the restriction enzyme that cuts through the E1A sequence is run on an agarose gel and produces a band, then this would suggest that the band seen on the agarose gels is not E1A.

The dot blot technique using genomic DNA and positive controls consisting of cDNA from E1A transfected cells and genomic DNA from G293 cells, and genomic DNA from 24 lung samples gave a surprising result in that the only positive was the cDNA from E1A transfected cells. The G293 genomic DNA was negative, as were all of the lung genomic DNA samples. This result is surprising as the genomic DNA from G293 should have been positive on dot blot. The likely explanation for this is that the probe will bind more easily to single stranded DNA, compared to genomic DNA. Fernandez-Soria (Fernandez-Soria *et al.,* 2002) also attempted to use dot blot Southern using genomic DNA from lymphoma tissue samples and they were unable to detect E1A sequence by this method.

Imuunohistochemistry showed only one lung sample to stain positively for E1A protein, and this was localised to a macrophage. The positive controls used were cell lines rather than lung tissue from E1A transfected or adeno viral infected animals. It is thus possible that the technique was not optimised for lung tissue.

Our data have merely confirmed the great difficulty in performing experimental work identifying adenoviral related protein and DNA as contamination in the laboratory is an enormous problem. Although we have been unable to confirm the presence of E1A by PCR in a Scottish population, there is supporting in-vitro data to suggest that the presence of E1A leads to increased inflammation (Keicho *et al.,* 1997, Gilmour *et al.,* 2001). Animal models have also been used to mimic the E1A situation within the lung (Vitalis *et al.,* 1996; Vitalis *et al.,* 1998; Yang *et al.,* 2002). However, in vitro work using E1A transfected cell models have high copy numbers of EIA gene and protein, and although they provide an insight into how the inflammatory process may be altered in the presence of E1A, it is difficult to extrapolate this to the human lung where it is postulated that there is a low copy number.

Summary and future studies

The aims of this thesis were to investigate *in vivo* the protective glutathione antioxidant system and measure local and systemic oxidative stress in the lungs of smokers with airflow obstruction. The DNA binding of the redox sensitive transcription factors AP-1 and NF-κB was also measured, and human lung was assessed for the presence of E1A protein.

Glutathione synthesis has been shown to be sensitive to cigarette smoke *in vitro* and this may be important in the human lung with regard to the development of smoking related chronic obstructive pulmonary disease. Alterations in the glutathione enzyme system may render the lungs more susceptible to the effects of oxidants, with resultant inflammation and damage to airways. The present study has shown that the level of total glutathione is the same in the lungs of patients with severe airflow obstruction and those with no airflow obstruction. However, the activity of γ-GCS (GCL), the rate limiting enzyme in the synthesis of glutathione was found to correlate with lung function, and we found that the activity of γ-GCS (GCL) is significantly lower in the lungs of smokers with airflow obstruction and those with severe emphysema who have undergone lung volume reduction surgery, compared to those with normal lung function.

Cells expressing γ-GCS-HS were identified using *in situ* hybridisation, and the predominant cell types that expressed γ-GCS-HS in the lung were epithelial cells, including bronchial, type I and II alveolar cells, irrespective of the presence of airflow obstruction.

The finding in our study that γ-GCS activity is reduced in the lungs of subjects with severe airflow obstruction was unexpected, and given that there was no difference in total GSH in the groups with and without airflow obstruction, suggests that there may be post translational modification of γ-GCS. However before exploring post translational modification of γ-GCS, the measurement of GSH and γ-GCS activity within lung homogenate should be repeated to confirm this finding. Multiple areas of

the lung should also be included to ensure that the γ-GCS activity data was not a spurious finding. Also the measurement of GSH related enzyme levels and γ-GCS (GCL) message in primary culture of epithelial cells obtained from the different subject groups, including a group with potentially early disease would help clarify if the alveolar epithelial cell is a source of altered GSH homeostasis.

In the studies presented here, the emphasis and grouping of patients has been on the basis of the presence/degree of airflow obstruction. This may not have been ideal as cigarette smoking is the major risk factor for the development of COPD. In future studies it would be better to group subjects in terms of smoking history and airflow obstruction, such as smokers with and without airflow obstruction, non smokers, and ex-smokers with and without airflow obstruction. Grouping in this way will produce better characterised and more specific groups. In future work a detailed drug history including oral theophyllines and oral corticosteroids should be obtained as drugs such as these can affect histone acetylation. Lung tissue sampling needs to be more homogeneous across the subject groups, rather than using a non-specified area that was dependent on the pathologist. Similarly sampling from more than one area of the lung is needed to ensure that the results obtained are not just a reflection of the area of the lung sampled. A weakness of my studies has been the lack of any true 'normal' lung tissue, and the use of an animal model would allow the examination of the mechanisms of lung inflammation in normal and damaged lung tissue. Future studies would require a power calculation and determination of sample size, and the data presented in this thesis could be used to help determine the power of any future studies.

My work has examined total NF-kB DNA binding in lung tissue and has shown that the DNA binding of NF-κB in human lung tissue is inversely correlated with the degree of airflow obstruction, and the subunits p65 and p50 were identified using Supershift. No such correlation with lung function was found for AP-1 DNA binding. The DNA binding of NF-κB to specific genes/promoters, and examination

of these processes in specific cell types was not performed in my studies. Primary cell culture to isolate macrophages and type I and II bronchial epithelial cells from lung tissue from well characterised patients would allow DNA binding of NF-κB to be examined in a broad array of cells to determine if more subtle changes are present in individual cells.

The mode of activation of NF-κB in human lung needs to be characterised further. The investigation of pathways other than the IκB dependent NF-κB activation should be undertaken as these may be important in smoking related lung inflammation. Activation of the p38 MAPK pathway in-vitro by oxidative stress leads to activation of NF-κB. Using the techniques of Western blotting, levels of IκB and p38 could be measured in lung tissue. This work could be expanded to determine if there are cell specific differences in the p38 and IκB pathways of NF-κB activation by the use of immunohistochemistry.

Histone acetylation and deacetylation are important in allowing access of genes to the transcription machinery and subsequent gene transcription and repression. Understanding the role of these processes in lung inflammation is critical. Certainly recent work performed in the ELEGI laboratory has attempted to clarify histone acetylation and deacetylation in smokers with COPD, with the findings that there was decreased IκB-α levels in healthy smokers and current and ex smokers with COPD compared with non smokers, and that an increase in acetylated H4 was found in current smokers. Future work should include a group of healthy exsmokers, ie patients without evidence of airflow obstruction as this group may show the presence of a protective mechanism that prevents the development of symptomatic COPD.

The use of an animal model may provide further insight into the mechanism of inflammation and GSH regulation in the lung, as the molecular assessment of human lung tissues obtained at surgery is limited by the presence of only a single time point. In smoking related lung disease, examination of a single time point may not be

relevant as the disease often follows a long and initially clinically asymptomatic course.

The three major model types used to investigate COPD include an inhalation model (of noxious stimuli), a model based on the intracheal instillation of tissue degrading enzymes to induce emphysema like lesions within the lung, and gene targeting approaches which employ gene modifying techniques such as gene depletion and over-expression. There are limitations to animal models as such models do not necessarily mimic the entire COPD phenotype, or the different disease stages found in COPD, and the systemic effects of COPD are difficult to replicate in animal models. Most animal models tend to have the morphological features of COPD, but are lacking in the mucus hyper-secretion element of COPD disease. Also in the case of NF-κB it is recognised that there are both cell specific and species specific effects. In animal models it can be difficult to reproduce the small airways pathology present in COPD. It can also be difficult to obtain measurements of lung function in small animals such as mice. Ex-vivo precision cut lung slices combined with videomorphometry can be used in animal models re lung function assessment. The advantages of an animal model system include an abundant source of material, repeatability of the work, and experiments are not restricted to a single time point.

Lung inflammation is the result of increased pro-inflammatory mediators and cytokine production, and NF-κB regulates the expression of many pro-inflammatory cyokines and adhesion molecules, including IL-8 and IL-1β. Similarly the promoters of many inflammatory response genes have AP-1 binding sites. The DNA binding of the transcription factor NF-κB in human lung tissue was found to inversely correlate with the degree of airflow obstruction, and the subunits p65 and p50 were identified using Supershift. No such correlation with lung function was found for AP-1 DNA binding. Identification of AP-1 subunits using Supershift and antibodies to c-jun and c-fos failed, however a band suggestive of c-jun was identified by Western blotting. Further work is required to identify if the negative correlation of NF-κB DNA

binding with lung function is present in all areas of the lung and whether a particular cell or group of cells is responsible for this finding in lung tissue homogenate. There is also evidence to suggest that expression of jun and fos proteins is dependent on the cell type and the presence or absence of malignant transformation, and extending this work to include other AP-1 subunits would be useful.

Systemic and local oxidative stress as measured by trolox equivalent antioxidant capacity and lung MDA, were not related to lung function. The lipid peroxidation product 4-hydroxy-2-nonenal was identified in human lung using immunohistochemistry, with increased 4-HNE staining scores in bronchial epithelial cells, macrophages and neutrophils in lung tissue obtained from smokers with severe airflow obstruction compared to those with no airflow obstruction. The use of Western blotting to identify 4-HNE protein adducts yielded protein bands, however we were unable to confirm if these bands represented 4-HNE protein adducts. The antibody used in this work recognised cystine, histidine and lysine 4-HNE adducts, and a different antibody may yield better results. Future work to identify the protein bands identified by Western should be undertaken. This could be achieved by using a two dimensional gel system electrophoresis to resolve the proteins, followed by a tryptic digest or high pressure liquid chromatography, and then the use of mass spectrometry to determine the amino acid sequence.

Susceptibility to the effects of cigarette smoke is central to understanding the pathogenesis of COPD. *In vitro* there is a heightened inflammatory response in E1A transfected cells in response to inflammatory stimuli. Amplification of genomic DNA using polymerase chain reaction and Southern blotting was used to identify early one adenoviral protein in the lung. Work carried out in Scotland failed to identify E1A in human lung tissue. Using similar techniques in Vancouver, bands suggestive of E1A were identified on Southern, however the negative controls also showed bands suggesting contamination had occurred.

COPD is asymptomatic in the early stages of the disease, and study of the initial disease process in vivo is very difficult. Whole lung tissue contains multiple cell types, and potential significant results in individual cell types may be masked in whole lung tissue. In addition some of the techniques used in this thesis may require refining.

In conclusion the work presented in this thesis has examined *in vivo* glutathione and γ-GCS, NF-κB and AP-1 transcription factor binding, systemic and local oxidative stress and the relationship with airflow obstruction, and assessed for the presence of EIA. The activity of γ-GCS is reduced in the lung tissue of patients with airflow obstruction compared to those with no airflow obstruction.

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