Effect of Activated Neutrophil Products on *Ex Vivo* Small Airway Structure and Function

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Abstract

In COPD, small airways are the main site of airflow obstruction. Neutrophil infiltration into small airways is significantly associated with lung function decline. This thesis investigated the effects of activated neutrophil products on *ex vivo* small airway structure and function using precision-cut lung slices (PCLS).

Peripheral blood neutrophils were isolated from non-smokers, smokers and COPD patients; stimulated with fMLP, and supernatants obtained. Supernatant protease concentration/activity was assessed. There were significant increases in protease concentration/activity upon fMLP stimulation, but no significant differences between subject groups or correlations with subject demographics.

Rat PCLS were incubated overnight with neutrophil supernatants, and small airway structure, function and PCLS viability assessed. Stimulated COPD neutrophil supernatants caused significant airway dysfunction. All neutrophil supernatants significantly reduced PCLS viability. There was a significant inverse correlation between supernatant active MMP-9 concentration and maximal airway closure. Incubation of rat PCLS with H_2O_2 or porcine pancreatic elastase did not fully reproduce the effects of neutrophil supernatants: both reduced maximal airway closure; whilst H_2O_2 alone significantly reduced PCLS viability.

Pre-incubation of COPD neutrophil supernatants with a NE inhibitor led to restoration of maximal airway closure only. Pre-incubation with neither an antioxidant, *N*-acetyl-cysteine (NAC), nor a MMP-9 inhibitor, had any effect on neutrophil supernatant-induced PCLS damage.

Finally, small airways in PCLS from COPD lung tissue had greater maximal airway closure, compared with smokers and non-smokers. There were no correlations between subject demographics and small airway function.

Overall, neutrophils from COPD patients induce small airway dysfunction and reduce tissue viability. Neither oxidative stress nor elastase reproduced these effects fully; and pre-

incubation with neither antioxidants nor protease inhibitors restored small airway function. Thus, there is greater complexity in neutrophil mediation of this damage. Finally, rat small airways do not fully reproduce the response of small airways in COPD lung tissue, which exhibit evidence of hypercontractility.

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Declaration of Originality

I confirm that I have performed all of the experiments and analysis reported in this thesis, unless otherwise stated.

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Abbreviations list

ACh	Acetylcholine
AChE	Acetylcholinesterase
AMPA	p- aminophenylmercuric acetate
BALF	Bronchoalveolar lavage fluid
BSA	Bovine serum albumin
BSA	Bovine serum albumin
Calcein AM/EthD-1	Calcein acetoxymethyl/ethidium homodimer-1
CAT	COPD assessment test
CCh	Carbachol
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CINC	Cytokine-induced neutrophil chemoattractant
COPD	Chronic Obstructive Pulmonary Disease
COX	Cyclooxygenase
CSE	Cigarette smoke extract
CXCL8	Interleukin-8
Cyt B	Cytochalasin B
DAG	Diacylglycerol

DMEM	Dubecco's minimum essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
EVG	Elastin van Giesons
FEV_1	Forced expiratory volume in one second
fMLP	Formyl-Methionyl-Leucyl-Phenylalanine
FPR	Formyl-peptide receptor
FPRL	Formyl-peptide receptor like
FVC	Forced vital capacity
GCSF	Granulocyte colony stimulating factor
GINA	Global Initiative for Asthma
GLUCOLD	Groningen Leiden Universities Corticosteroids in Obstructive Lung Disease
GOLD	Global Initiative for Lung Disease
GRO	Growth-regulated oncogene product
GSH	Glutathione

GSH-PX	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HBSS	Hanks Balanced Salt Solution
HCl	Hydrochloric acid
HDAC	Histone deacetylase
HNP	Human neutrophil peptide
HOCl	Hypochlorous acid
HRCT	High-resolution computed tomography
HRP	Horseradish peroxidase
ICS	Inhaled corticosteroids
IFN	Interferon
IL	Interleukin
IMS	Industrial methylated spirit
IP_3	Inositol triphosphate
LABA	Long-acting β-agonists
LAMA	Long-acting muscarinic antagonists
LPS	Liposaccharide
LTB	Leukotriene
MAP	Mitogen Activated Protein

MCP	Monocyte chemoattractant protein
MDA	Malondialdehyde
MMP	Matrix metalloproteases
mMRC	Modified Medical Research Council
MPO	Myeloperoxidase
MRI	Magnetic Resonance Imaging
MSD	Meso Scale Delivery
N-MeO-Suc-AAPV-pNA	N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide
NAC	*N*-acetyl cysteine
NaCl	Sodium Chloride
NaO_{AC}	Sodium acetate
NADP	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NF	Nuclear factor
NO	Nitric oxide
O_2^-	Superoxide
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCLS	Precision-cut lung slices

PDE_4	Phosphodiesterase-4
PG	Prostaglandin
PGP	Proline-glycine-proline
PI	Propidum idodide
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear leukocytes
PPE	Porcine Pancreatic Elastase
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
SAD	Small airways disease
SOD	Superoxide dismutase
TGF	Transforming growth factor
TLR	Toll-like receptors
TMB	5,5' tetramethylbenzidine
TNF	Tumor necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
α1-AT	α1-antitrypsin

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Chapter 1:

Introduction

1. Introduction

Breathing involves the passage of air into the lungs, followed by oxygen and carbon dioxide exchange at the alveoli, and, finally, passage of air out of the lungs. For this process to occur effectively, the airways leading from the nose and mouth to the alveoli need to be open and offer minimal resistance to airflow. In health, this is not even considered. In respiratory diseases, such as asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, and other fibrotic lung diseases, the airways show increased resistance to airflow, making breathing difficult. The resistance to airflow can be due to blockage of the airway lumen, for example, by mucus, or to physical narrowing of the airway by remodelling. This thesis addresses the issue of remodelling of parenchymal (lung) tissue and small airways, with particular reference to COPD. The following sections introduce the various aspects that lead to the execution of the experimental work.

1.1. The airways

The architecture of the airways, often referred to as the tracheobronchial tree, is organised into a series of dichotomous branches that direct inspired air from the trachea down to the gas exchange region of the lung (Florens *et al.*, 2011). Each division (branch) is referred to as a generation, and there are ~23-27 generations of airways in the human tracheobronchial tree. The branching begins at the distal end of the trachea, which splits into the two main bronchi. From here, branching proceeds through the lobar and segmental bronchi to the terminal bronchioles, which mark the end of the conducting zone (~generation 16). Beyond this region are the transitional and respiratory zones, which are the sites of gas exchange. Here, respiratory bronchioles open into a number of alveolar ducts, which then further divide into alveoli, of which there are ~300 million across both lungs, with an approximate cross-sectional surface area of 50-70m^2 (Weibel, 1963) (Figure 1.1). At each generation, the airway diameter decreases; however, due to the exponential increase in the number of airways, the cross-sectional area increases at each generation.

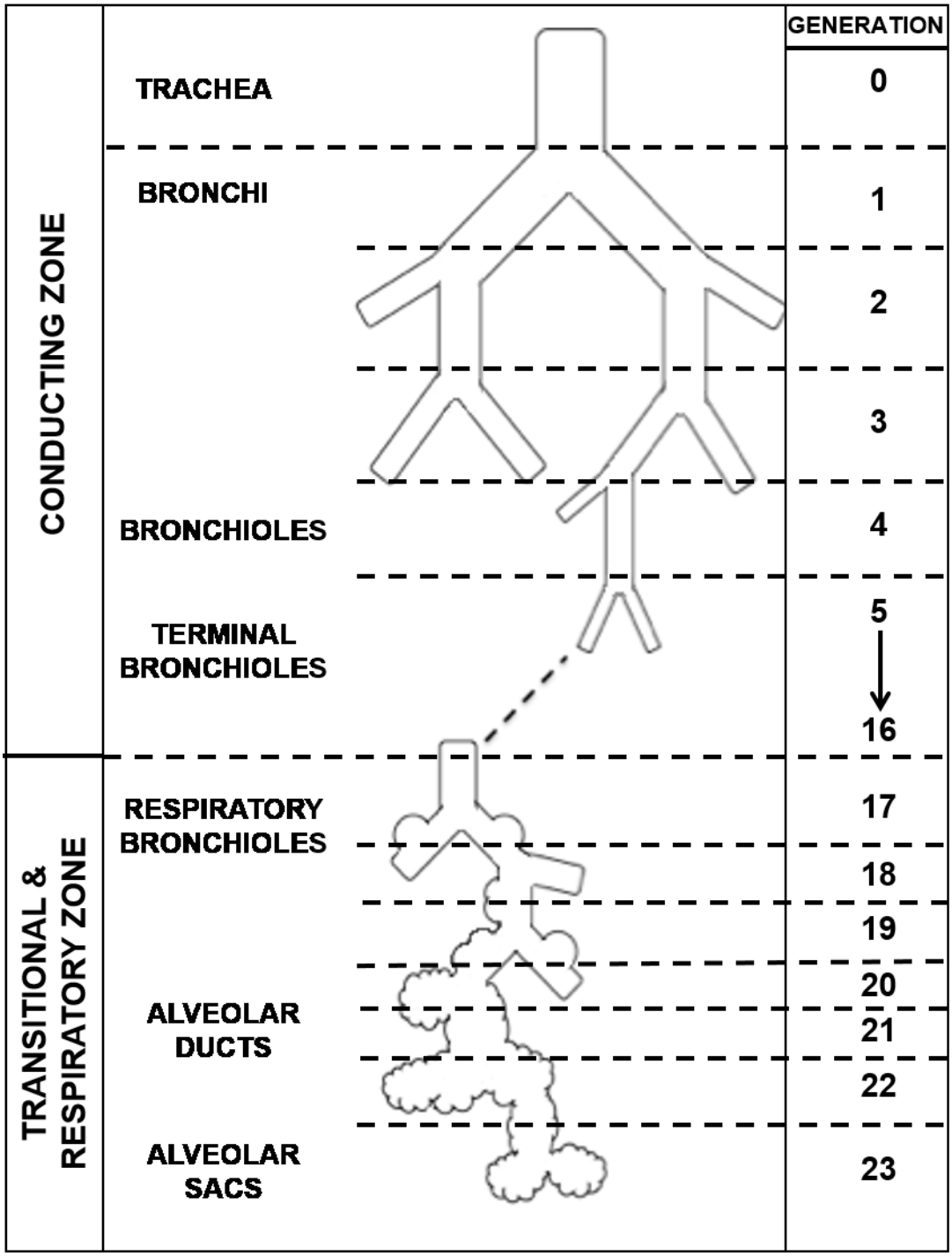


Figure 1.1. Diagram of the tracheobronchial tree. Airway size decreases but cross-sectional area increases with increasing generations. Transitional and respiratory zones are the sites of gas exchange. Adapted from (Weibel, 1963).

Hagen-Poiseuille's law describes airflow resistance, whereby resistance is inversely proportional to the airway radius raised to the fourth power, which means that halving the radius results in a 16-fold increase in airflow resistance (Bosse *et al.*, 2010). Airflow resistance is thought to be greatest between the fourth and eighth generations, and negligible beyond the 16th (Figure 1.2). The fourth to the 14th generations comprise the small, or peripheral, airways; which are defined as having a diameter of <2mm (Stewart *et al.*, 2013). Therefore, pathological narrowing of the small airways, as is the case in a number of

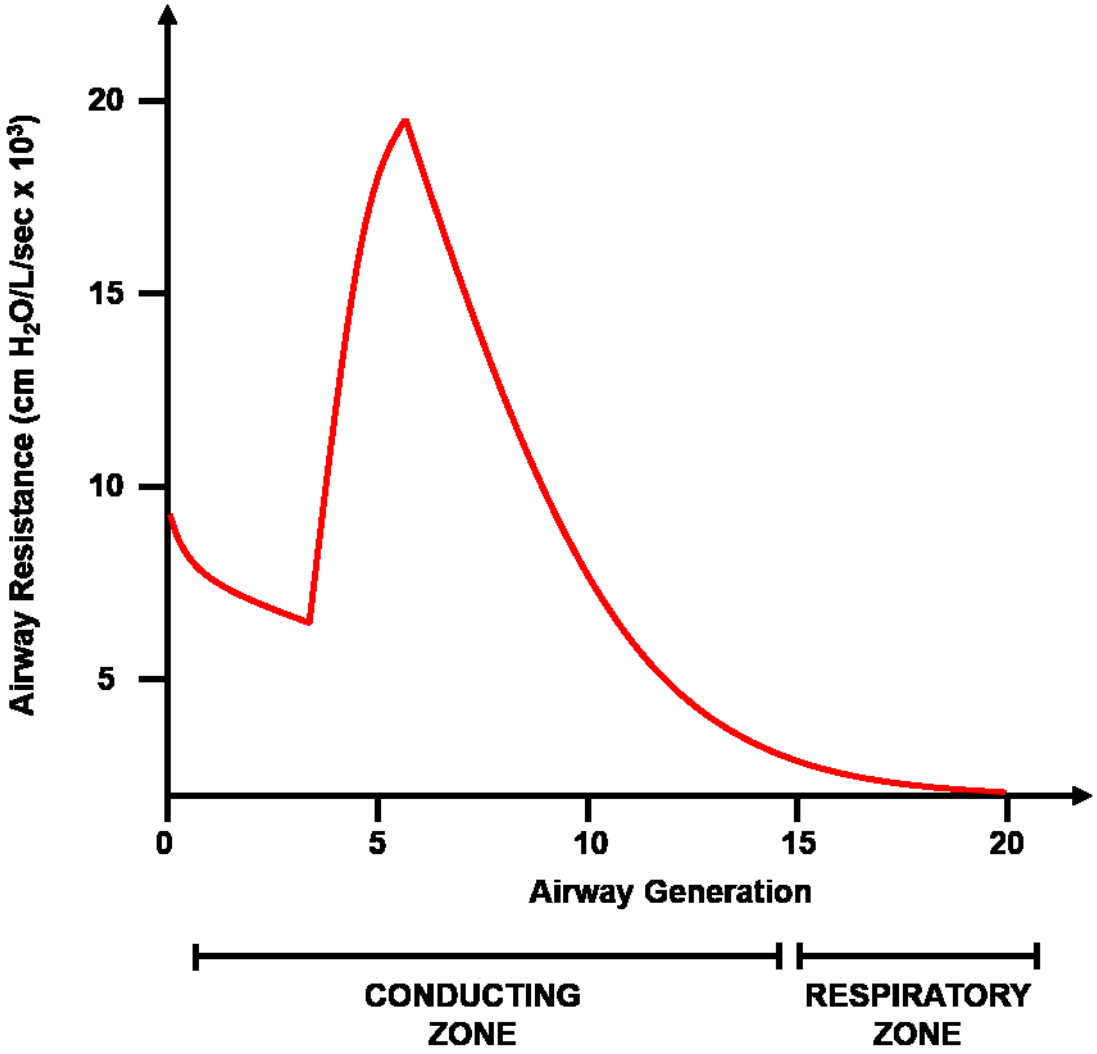


Figure 1.2. Relationship between airway generation and resistance to airflow. Airway resistance peaks between generations 4-8 (the small, or peripheral, airways), and is negligible beyond generation 16. Adapted from Bosse *et al*, 2010.

respiratory diseases (see Section 1.2), will result in a marked increase in airflow resistance, which may manifest as chronic cough, difficulty in breathing, and lung function decline.

1.2. Respiratory Diseases

In 2012, COPD, lower respiratory tract infections, and various lung cancers were, respectively, the third, fourth and fifth leading causes of death worldwide (WHO, 2014), demonstrating the increasing global burden of lung disease. Diseases of the respiratory system arise due to various genetic or environmental factors, which may be acute or chronic in nature, and can vary from mild to debilitating and/or life threatening.

1.2.1. Cystic Fibrosis

One example of a genetic disease with debilitating effects on the lung is cystic fibrosis (CF). CF is an autosomal recessive disorder, caused by a mutation in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. This causes dysregulated chloride ion transport, and associated sodium ion and water transport at the apical epithelial cell membrane; which results in dehydrated, thick, viscous mucus and, consequently, impaired mucocilliary clearance (Collins, 1992). Symptomatically, this leads to recurrent respiratory infections and bacterial colonisation of the lungs; as well as disorders of the pancreas, liver and reproductive organs (Ratjen *et al.*, 2003). CF is usually diagnosed at, or shortly after, birth, and the current life expectancy for patients is ~40 years (MacKenzie *et al.*, 2014). Despite the disease affecting multiple organ systems, over 90% of CF patients die of lung disease, which is characterised by mucus plugging and obstruction of the airways; heightened inflammatory responses; persistent infection and bacterial colonization; and progressive bronchiectasis (Davis PB *et al.*, 1996). The heightened inflammatory profile observed in CF is driven largely by neutrophils; however, there is a long-standing paradox that, despite the increased numbers of neutrophils, they cannot effectively control the colonisation of bacteria present in the CF lung. There is thought to be innate dysfunction of CF neutrophils linked to the mutation in the CFTR gene that leads to impaired intracellular

killing of bacteria, alongside enhanced reactive oxygen species (ROS) production, granule enzyme activity, cytokine output, and dysfunctional apoptosis (Gifford *et al.*, 2014).

Current treatment strategies for CF are predominantly symptomatic, including intravenous, inhaled and oral antibiotics to treat infections, which are often given prophylactically to help control bacterial colonisation; physiotherapy to aid expectoration of mucous secretions in the chest; and, ultimately, lung transplantation. However, more causative therapies are being explored: a clinical trial has investigated the efficacy of the CFTR potentiator, Ivacaftor (VX-770), which was found to be well-tolerated and caused significant improvements within patients in nasal potential difference (a measure of CFTR-mediated chloride transport) and lung function, and significant changes in sweat chloride levels *versus* the placebo group (Accurso FJ *et al.*, 2010). Research and clinical trials into gene therapy to correct the defective receptor are also underway, which may lead to even more causative therapies to treat the disease in the future (Armstrong *et al.*, 2014).

1.2.2. Asthma

Asthma is a heterogeneous, chronic inflammatory condition of the lungs; characterised symptomatically by wheezing, coughing, chest tightness, shortness of breath and airflow limitation that can vary in intensity and duration (GINA, 2014). The pathophysiology of asthma is complex and incompletely understood, but is thought to arise from a combination of genetic and environmental risk factors. For example, pre- and post-natal maternal smoking habits are associated with significantly increased risks of children developing asthma (Burke *et al.*, 2012). Attacks can be triggered by a number of factors, including allergens, environmental pollutants, exercise, or viral infections. Stable disease is managed with inhaled corticosteroids, which supress the inflammation; and short-acting and long-acting β_2-adrenergic agonists, which manage the bronchial hyperresponsiveness and constriction (GINA 2014). Severe asthma affects ~5% of patients, whereby their disease is difficult to manage with usual therapies.

In terms of pathophysiology, allergen inhalation activates mast cells, which release a number of mediators, such as prostaglandins, histamine and ROS. These act as bronchoconstrictors, and induce mucous hypersecretion and vasodilation. Mast cells and airway epithelial cells release a number of cytokines that recruit other inflammatory cells to the airways, in particular eosinophils and T_H2 cells, that further perpetuate the inflammatory response (Barnes, 2008). The inflammation observed in asthma is largely confined to the large airways; however peripheral airways have also been implicated in more severe phenotypes. Bronchial biopsies from asthmatic patients demonstrate structural changes, such as reticular basement-membrane thickening, which occurs as a result of collagen deposition underneath the epithelium; smooth muscle hypertrophy and hyperplasia; mucous hyperplasia; and an increased number of blood vessels (angiogenesis) (Barnes, 2008). Different treatment strategies that are currently being investigated, in particular aimed at severe asthmatics, include anti-IgE therapies, that prevent activation of inflammatory cells in response to allergens; drugs targeting inflammatory cytokines, such as T_H2 cytokines, interleukin (IL)-4 and IL-13; and broad spectrum anti-inflammatory drugs, such as kinase inhibitors (Barnes, 2011).

1.2.3. COPD

COPD is defined as “a preventable and treatable disease that is characterised by progressive and persistent airflow limitation, associated with enhanced chronic inflammatory responses in the airways and lung to noxious particles or gases” (GOLD, 2014). Whether, in fact, COPD can be described as ‘treatable’ is a moot point, as will be explored below. Risk and frequency of exacerbations, defined as an acute worsening of symptoms beyond normal day-to-day variation, as well as comorbidities, also contribute to the severity of disease in individual patients (GOLD, 2014). The progression of COPD is divided into four stages, based on spirometric measurements, as defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD). The main spirometric measurements used to determine the extent of airflow obstruction are the forced vital capacity (FVC), which is the volume of air

forcibly expired following maximal inspiration; and the volume of air forcibly expired in the first second following maximal inspiration (forced expiratory volume in one second (FEV_1)). All patients diagnosed with airflow obstruction have an FEV_1/FVC ratio of <0.7, and its severity defined as follows: GOLD stage 1 is considered mild, with an $FEV_1 \geq$ 80% predicted; GOLD stage 2 is moderate, with 50%≤ FEV_1<80% predicted; GOLD stage 3 is severe, with 30%≤ FEV_1<50% predicted; and GOLD stage 4 is very severe, with FEV_1< 30% predicted (GOLD, 2014).

Combined COPD assessments have recently been introduced to GOLD guidelines to reflect the heterogeneity of the disease between patients, and form the basis for more personalised treatment strategies. This assessment incorporates severity of symptoms, as determined by the COPD assessment test (CAT) or modified Medical Research Council (mMRC) dyspnea scale; spirometry data and GOLD stages, as described above; and exacerbation frequency. This assessment categorises patients into groups A-D: patient group A are low risk and have less symptoms; patient group B are low risk with more symptoms; patient group C are high risk with fewer symptoms; and patient group D are high risk with more symptoms (GOLD 2014, Figure 1.3).

Tobacco smoke has historically been considered the main risk factor pertaining to the development of COPD. A study by Fletcher and Peto (1977) was one of the landmark reports highlighting an association between cigarette smoking and lung function decline; however, their study cohort was limited to only men of working age (30-59 years). Kohansal and colleagues (2009) expanded on this by investigating the effects of lung function decline over time and the impact of continued cigarette smoking using the Framingham Offspring cohort (Kannel *et al.*, 1979), which incorporated both men and women across a much larger age range (13-80 years). Their results confirmed those of Fletcher and Peto (1977): continued cigarette smoking results in increased lung function decline. The progressive nature, heterogeneity of symptoms, and differences in diagnostic criteria mean that COPD is often under-diagnosed (Lindberg *et al.*, 2005). It has been previously reported that 15-20%

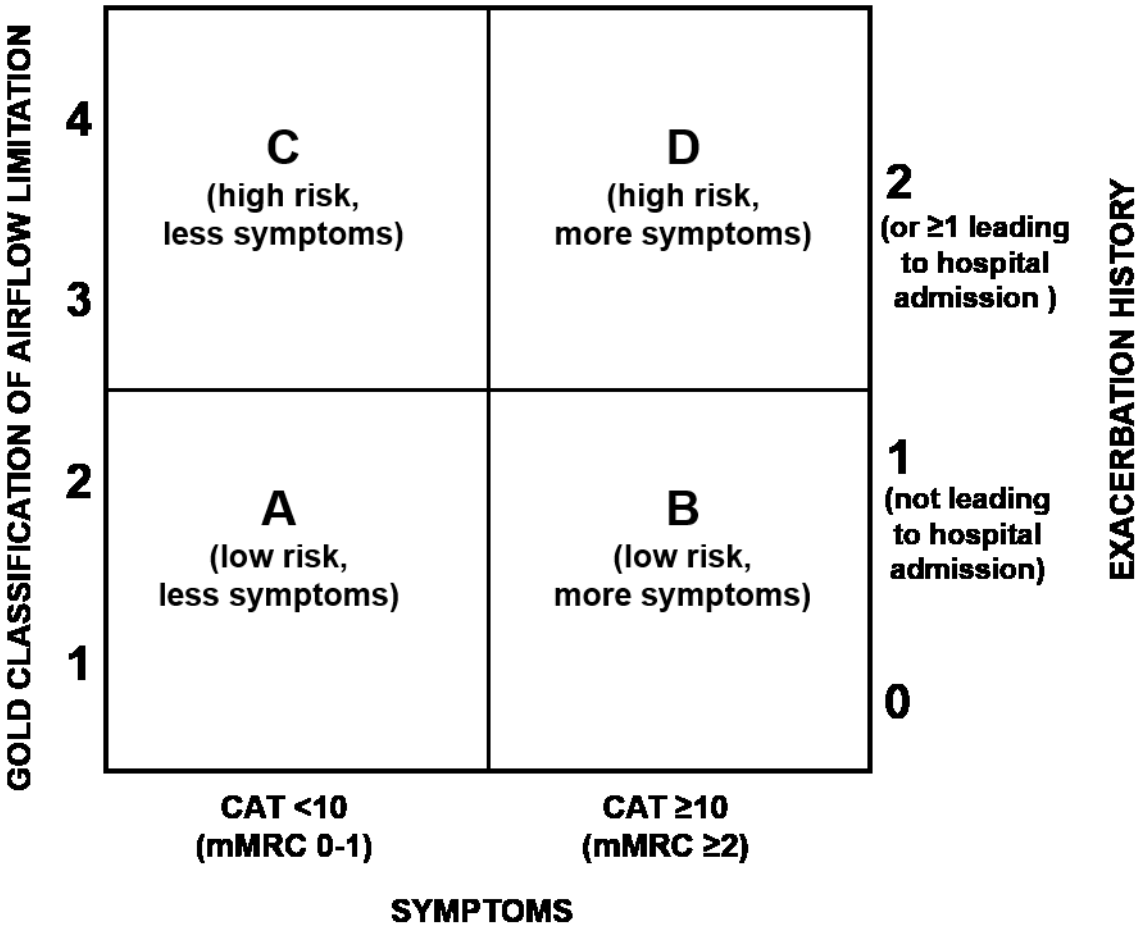


Figure 1.3. GOLD guidelines for COPD assessment. Patients are grouped according to spirometry and airflow limitation (left-hand side); symptoms, as determined by the CAT score, or mMRC dyspnea scale in its absence (bottom); and exacerbation frequency (right-hand side). Adapted from GOLD 2014 report.

of smokers develop COPD; however, a study by Lündback and colleagues (2003) suggests COPD is present in approximately 50% of smokers.

In addition, increasing evidence suggests that there are several other important risk factors for COPD aside from cigarette smoking. In particular, the use of biomass as the primary fuel source in ~50% of homes worldwide is thought to be as great a risk factor for development of COPD as cigarette smoking, and, in particular, is attributed to the prevalence of COPD in women in developing countries who have never smoked (Hu *et al.*, 2010, Salvi, 2010).

Symptoms of COPD can include chronic cough, sputum production and shortness of breath. Treatment guidelines include the use of short-acting and long-acting bronchodilators in mild-to-moderate stable COPD (although these are far less effective than in asthma); and a combination of inhaled corticosteroids (ICS), β_2-agonists and/or long-acting anticholinergics in patients at risk of exacerbations (Vestbo *et al.*, 2013). However, the majority of COPD patients respond poorly to ICS treatment (Barnes, 2013): a recent clinical trial demonstrated that gradual withdrawal of ICS treatment, when being used in combination with long-acting $beta_2$- agonists (LABA) and long-acting muscarinic antagonists (LAMA), did not result in increased risk of exacerbations (Magnussen *et al.*, 2014). This highlights the need for new (anti-inflammatory) therapeutic agents to effectively alleviate symptoms and, in particular, slow the progression of COPD.

1.3. Pathophysiology of COPD

COPD is an umbrella term that encompasses three main conditions: chronic bronchitis, emphysema and small airways disease; characteristics of which are exhibited heterogeneously between patients (Figure 1.4).

1.3.1. Chronic bronchitis

The definition of chronic bronchitis is “chronic cough and sputum production for at least three months per year for two consecutive years” (GOLD 2014). The pathophysiology of chronic bronchitis is overproduction and hypersecretion of mucus by goblet cells and submucosal glands, in response to a number of different stimuli; including cigarette smoke, viral and bacterial infection, and activation of mucin genes by inflammatory mediators (Kim *et al.*, 2013). Epithelial cells from brushings of large airways were found to have significantly higher mucin stores in healthy smokers and in smokers with airflow obstruction, compared with non-smokers, which was driven by both goblet cell hypertrophy and hyperplasia (Innes *et al.*, 2006). There is also a significantly higher number of goblet cells in peripheral airways of

smokers with COPD, compared with non-smokers (Saetta *et al.*, 2000), and this mucous metaplasia was significantly correlated with disease severity (Kim *et al.*, 2008).

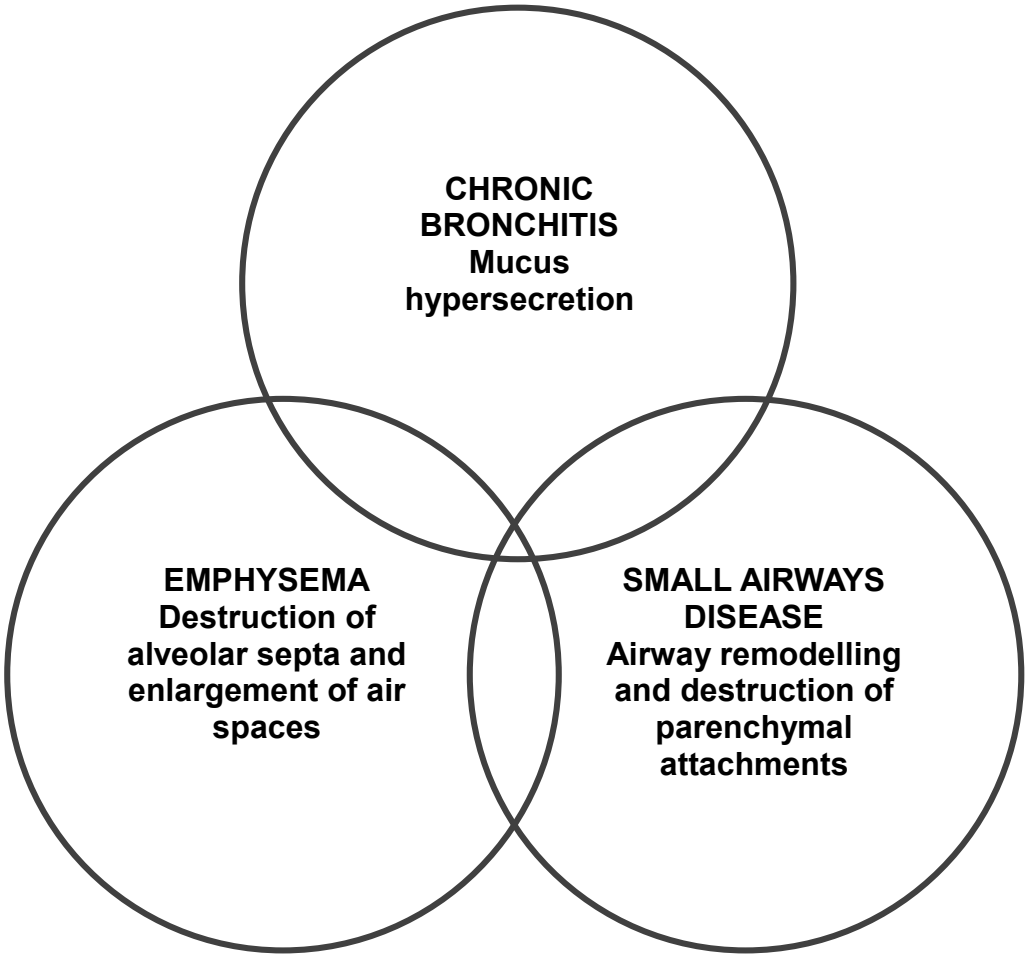


Figure 1.4. The three main pathological conditions encompassing COPD: chronic bronchitis, emphysema and small airways disease. Patients may exhibit one, two or all of these conditions to a greater or lesser extent, highlighting the heterogeneity of the condition. Adapted from Tubby et al, 2011.

1.3.2. Emphysema

Emphysema is characterised by enlargement on the air spaces, caused by destruction of the alveolar septa, which results in reduced surface area for gas exchange. There are two main types of emphysema based on the areas of lung affected: centrilobular, whereby parenchymal destruction is predominantly in the central regions of the lung and at the apices; and panlobular, where destruction is more homogenously distributed across the

entire lung (Anderson *et al.*, 1973). Generally, centrilobular emphysema has been associated with cigarette smoking, due to cigarette smoke reaching central areas first; whereas panlobular is thought to be more prevalent in non-smoking related disease, such as the genetic disease of alpha-1 antitrypsin (α1-AT) deficiency. As such, centrilobular emphysema is significantly associated with smoking history, symptoms such as dyspnea and shorter six-minute walk distances, and is significantly more prevalent in patients with severe COPD (Smith *et al.*, 2014). In a group of 131 patients with moderate to severe COPD, the presence of emphysema was a significant prognostic factor regarding rapid decline in FEV_1 (Cerveri *et al.*, 2013). It has been postulated that emphysematous destruction of the lung is mediated by excessive proteolytic action, as a result of heightened inflammatory responses in the lung. The first evidence of this came from the observation by Laurell and Eriksson (1963) that patients with a deficiency in α1-AT, the main inhibitor of neutrophil elastase (NE), developed early-onset emphysema. This has led to the hypothesis that a protease-antiprotease imbalance mediates the pathogenesis of COPD, which will be discussed in more detail in Section 1.4.2. Centrilobular emphysema is significantly associated with small airway abnormalities, including smooth muscle hypertrophy, fibrosis and inflammatory cell infiltration, compared with panlobular emphysema, suggesting this form of emphysema and small airway disease may be intrinsically linked (Saetta *et al.*, 1994).

1.3.3. Small airways disease

One of the prevailing theories regarding COPD pathophysiology is that the small (or peripheral) airways, defined as being non-cartilaginous and <2mm in diameter, are the main site of airflow obstruction (Hogg *et al.*, 1968). Small airways disease is characterised by remodelling of the small airways, which includes fibrosis, thickening of the airway wall layers and destruction of extracellular matrix proteins, all of which may contribute to aberrant airway function and airflow obstruction (Figure 1.5).

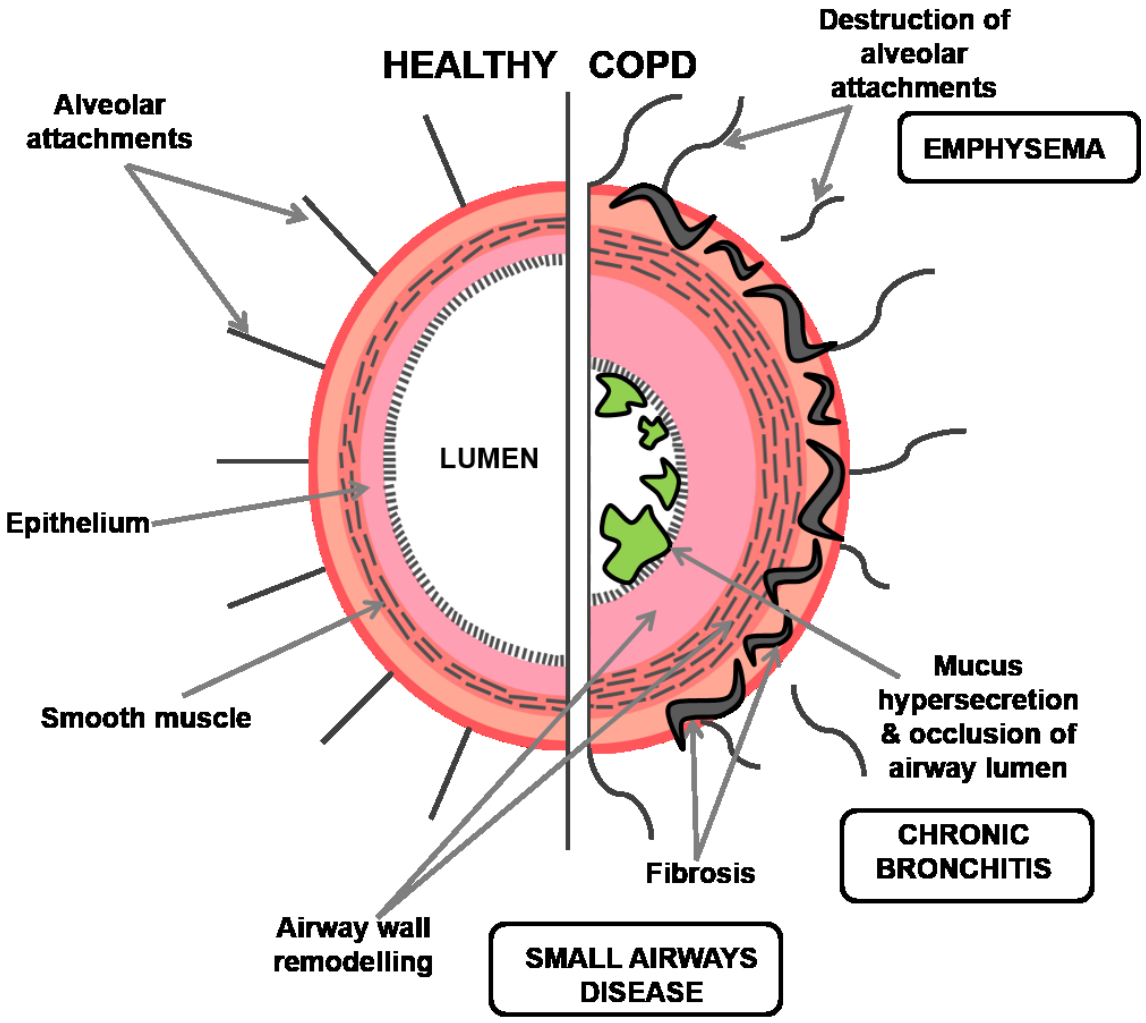


Figure 1.5. Small airway pathology in COPD. Diagram illustrates the pathophysiological changes in the small airways that lead to COPD and its associated symptoms. Airway narrowing will be a combination of increased wall thickness, due to fibrosis; and airway collapse, due to destruction of alveolar 'attachments' (emphysema). Adapted from (Barnes, 2004).

COPD progression is associated with increasing infiltration of inflammatory cells into the small airways, along with thickening of the airway walls and extent of luminal occlusion (Hogg *et al.*, 2004). Cosio and colleagues (1980) demonstrated significant abnormalities in the small airways of smokers, compared with non-smokers, with respect to goblet cell metaplasia, inflammatory infiltrate, smooth muscle hypertrophy, and respiratory bronchiolitis. However, as their cohort was made up of people who died suddenly, outside of hospital, from non-respiratory related disease, no lung function data was available, therefore making it

difficult to establish whether their observations are an effect of smoking, or disease, or both. Later studies have also found that airway wall thickness of both large and small airways is increased in COPD patients, compared with non-smokers and healthy smokers, and this was correlated positively with smoking history, and negatively with lung function (Deveci *et al.*, 2004). In addition to thickening of the airway wall, there is a significant reduction in the amount of elastin in the walls of small airways and alveoli, compared with non-obstructed smokers, and the amount of elastin was significantly correlated with predicted FEV_1 (FEV_1 % predicted) (Black *et al.*, 2008). Elastin degradation in the airway wall and surrounding parenchyma results in reduced elastic recoil of the airway, which may lead to partial, and sometimes complete, airway collapse.

1.4. Cells and mediators in COPD

1.4.1. Inflammatory cells

There are a number of inflammatory cells implicated in the development and progression of COPD, including macrophages, T-lymphocytes, and neutrophils (Figure 1.6). All three cell types are significantly increased in induced sputum of COPD patients, compared with non-smokers and asymptomatic smokers; and all are inversely correlated with lung function decline (Di Stefano *et al.*, 1998). In addition, epithelial cells and fibroblasts are also involved in COPD pathophysiology, but are not pertinent to the subject matter of this thesis and will not be discussed further.

Macrophages are 'professional' phagocytic cells that differentiate from circulating monocytes in response to inflammatory signals. Macrophages are activated by cigarette smoke, causing the release of pro-inflammatory mediators (Ito *et al.*, 2001) and, thus, recruit other inflammatory cells, including neutrophils, to the site of inflammation. COPD macrophages are inherently defective at phagocytosing bacteria (Taylor *et al.*, 2010), and appear to be skewed towards a pro-inflammatory phenotype: they release increased amounts of pro-inflammatory cytokines, e.g. tumor necrosis factor (TNF)-α and CXCL8, and demonstrate

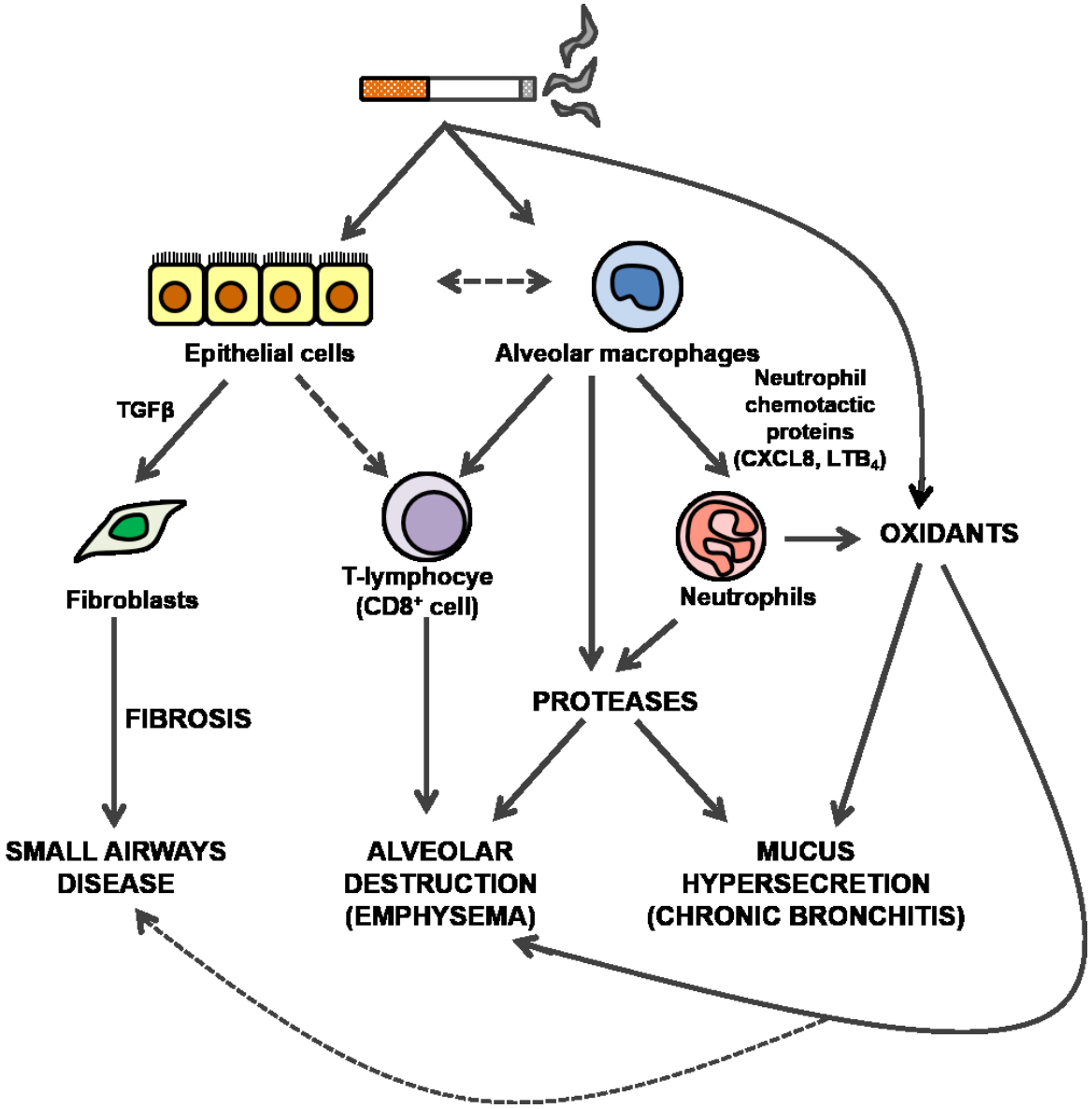


Figure 1.6. Heightened pro-inflammatory responses in COPD. Macrophage or epithelial cell activation by cigarette smoke triggers a cascade of pro-inflammatory responses that may manifest in the pathophysiogical conditions of COPD. CXCL8 = interleukin 8; LTB_4 = leukotriene B_4; TGFβ = transforming growth factor β. Solid lines = directly linked; dashed lines = indirectly linked. Adapted from Barnes, 2004.

reduced plasticity in differentiating towards an anti-inflammatory phenotype (Culpitt *et al.*, 2003, Chana *et al.*, 2014).

T-cells are a type of lymphocyte that is important in adaptive, rather than innate, immunity. There is a significant increase in number of $CD8^+$ T-cells in bronchial biopsies and lung

parenchyma of COPD patients, compared with non-smokers and asymptomatic smokers; and this was inversely correlated with FEV_1 % predicted (O'Shaughnessy *et al.*, 1997, Saetta *et al.*, 1999). In addition, over 90% of lung $CD8^+$ cells from COPD patients express toll-like receptors (TLR)- 4 and -9, both of which result in signal transduction pathways involving downstream pro-inflammatory pathways, compared with <20% in control subjects. In addition, cigarette smoke condensate appeared to perpetuate this pro-inflammatory phenotype by significantly increasing protein expression of these TLRs on peripheral blood $CD8^+$ cells, and their secretion of a number of pro-inflammatory cytokines, including TNF-α, IL-1β, and interferon (IFN)-γ (Nadigel *et al.*, 2011). There is substantial interplay between these inflammatory cells that propagates the heightened inflammatory response characteristic of COPD; however, the focus of the present work is on neutrophils.

1.4.1.2. Neutrophils

Neutrophils, otherwise referred to as polymorphonuclear leukocytes (PMNs), are an essential component of the innate immune system, and are considered to be one of the "first responders" of host defence (Figure 1.7). Upon activation, neutrophils tether and crawl along inflamed blood vessels prior to extravasation (Page *et al.*, 2013). This is accompanied by neutrophil polarisation and the localisation of the glycoprotein, PSGL-1, in the uropod (or hind edge). Neutrophils extend this PSGL-1-rich microdomain into the vessel lumen in order to interact with circulating activated P-selectin expressing platelets. This interaction appears to be essential for neutrophil crawling and diapedesis through the endothelium: neutrophils in mice transiently depleted of platelets, along with mice expressing defective PSGL-1 localisation or signalling, demonstrated virtually no crawling (Sreeramkumar *et al.*, 2014). Therefore, neutrophil-platelet interactions appear to be an essential step during neutrophil activation and recruitment to sites of inflammation.

Neutrophils, along with eosinophils and basophils, encompass the granulocyte fraction of white blood cells; which, as the name infers, pertains to the presence of granules in the cytoplasm (Amulic *et al.*, 2012). There are four distinct subtypes of granule: azurophil

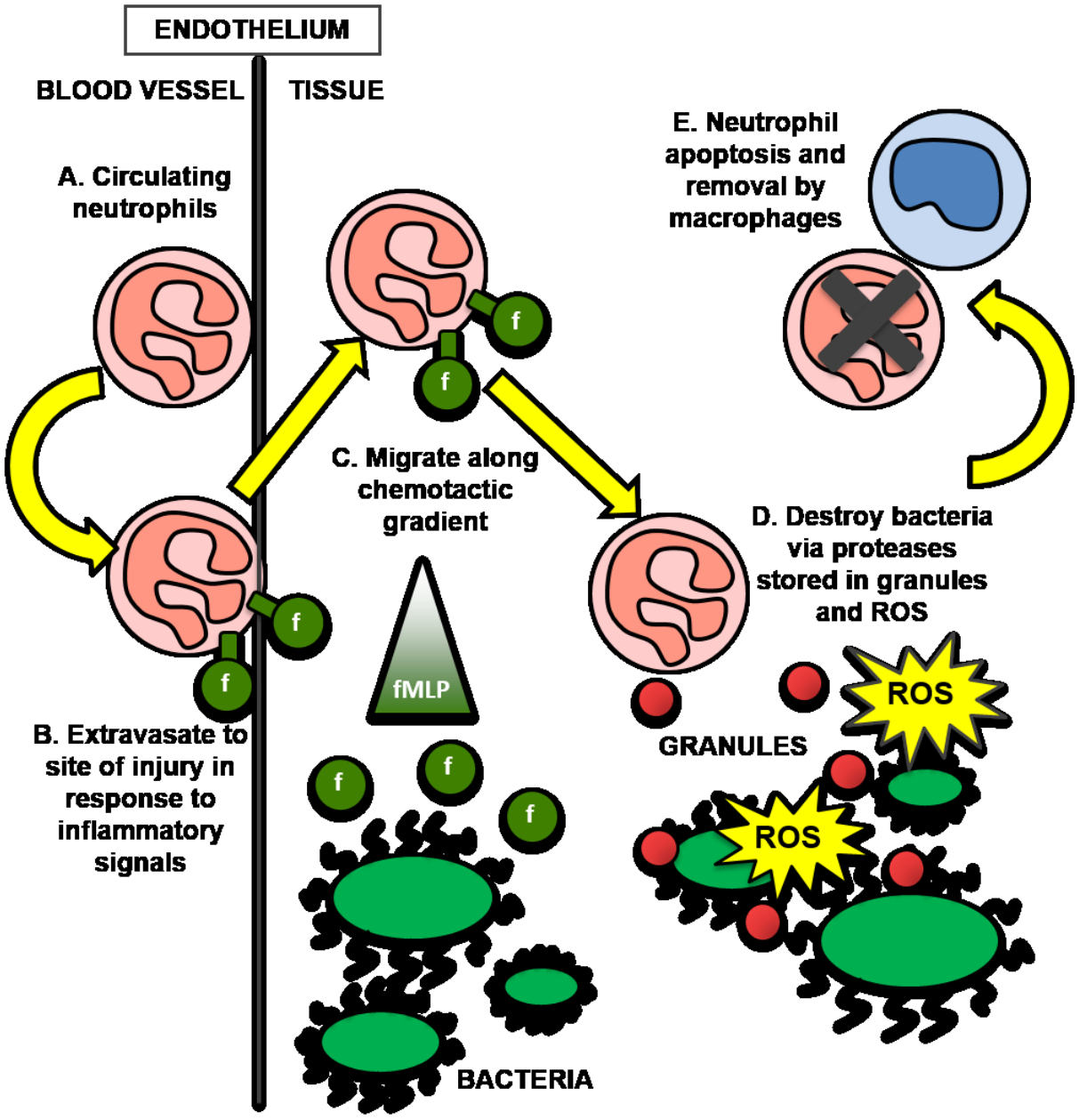


Figure 1.7. Summary of the role of neutrophils in the resolution of inflammation. (A) Quiescent neutrophils circulate in the blood stream. (B) In response to inflammatory signals e.g. Formyl-Methionyl-Leucyl-Phenylalanine (fMLP)) from bacteria acting on formyl peptide receptors (FPR) receptors on neutrophils, neutrophils extravasate through the endothelium and into the tissue, where they (C) migrate to the site of injury along chemotactic gradients. (D) Upon reaching the source of inflammatory signals, neutrophils destroy invading pathogens by degranulating, releasing granule proteins; and, via the respiratory burst, release reactive oxygen species (ROS). (E) Once inflammation has been resolved, neutrophils apoptose and are cleared by resident macrophages. F = fMLP. Original diagram by author.

(primary), specific (secondary), gelatinase (tertiary) and secretory vesicles (Figure 1.8). Historically, granules were defined as being either “peroxidase positive” or “peroxidase negative”, due to the presence of (myeloperoxidase) MPO in the former; however, further subdivision of granules has since been established (Borregaard *et al.*, 1997). Azurophil granules are MPO positive, whilst peroxidase negative granules are further subdivided into specific (secondary) and gelatinase (tertiary) granules. These granules are formed sequentially during neutrophil maturation in the bone marrow, and differ in both their contents and their propensity for exocytosis. Analysis of mRNA transcripts of 16 marker proteins from the four granule subsets revealed that there appears to be “blockwise” regulation of the synthesis of many of these proteins during neutrophil maturation, from myeloblasts to mature PMNs; i.e. proteins incorporated in the same granule subset are regulated together. These results led to the “targeting-by-timing” hypothesis: the point during neutrophil maturation at which granule proteins are synthesised dictates the granule subset in which they will reside (Cowland *et al.*, 1999) (Figure 1.9).

Azurophil granules are the first to be formed at the promyelocyte stage of neutrophil development in the bone marrow, and are identified by their MPO content. Azurophil granules can be further subdivided according to their expression of α-defensins (also known as human neutrophil peptides (HNP)), which are important antimicrobial peptides. These differ in expression depending on the timing of granule formation: those formed early in the promyelocyte stage have low α-defensin content, whereas those formed later, near the promyelocyte/myelocyte transition, are rich in α-defensins. Despite these differences, both subtypes of azurophil granule have the same propensity for exocytosis (Faurschou *et al.*, 2002). Specific granules are formed next at the myelocyte/metamyelocye stage, and contain high concentrations of lactoferrin; followed by gelatinase granules at the band cell stage that have a high gelatinase content (Faurschou *et al.*, 2003). Secretory vesicles are the last of the granules to be formed, but only contain plasma proteins and so are thought to be endocytic in origin. These vesicles have a particularly high density of membrane proteins

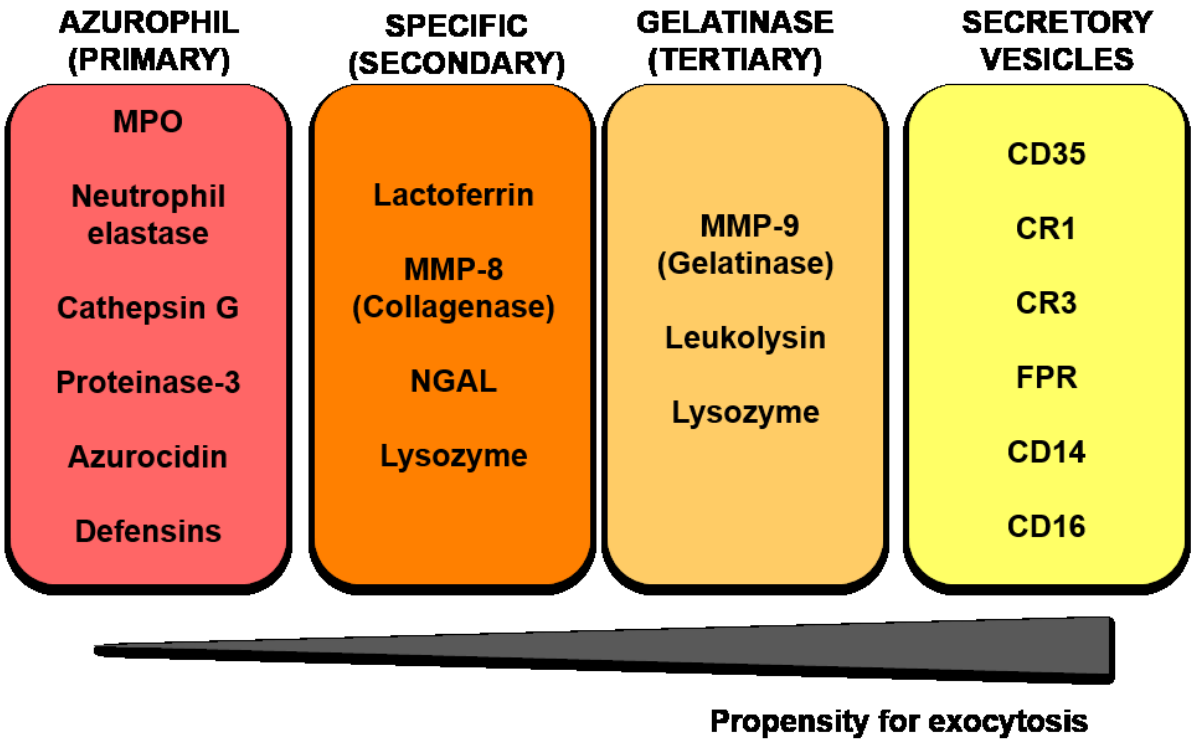


Figure 1.8. Granule subtypes in neutrophils. Neutrophils encompass four granule subsets, with different propensities for exocytosis depending on the level of stimulation/activation. This enables appropriate responses to inflammatory signals based on the concentration of agonist and/or chemokines. MPO= myeloperoxidase; MMP= matrix metalloprotease; NGAL= neutrophil gelatinase-associated lipocalin; CD= cluster of differentiation; CR= complement receptor; FPR= formyl peptide receptor. Adapted from Pham, 2002.

that are incorporated in the plasma membrane of the neutrophil when secretory vesicles are mobilised (Faurschou *et al.*, 2003). Granules are exocytosed in the reverse order to which they were formed: secretory vesicles are the first to be mobilised, followed by gelatinase, then specific and, finally, azurophil granules (Sengeløv *et al.*, 1993). This is thought to reflect the role of each granule subset in neutrophil diapedesis: secretory vesicles contain membrane receptors required for neutrophil rolling and firm adhesion to the endothelium, whereas azurophil granules contain a number of microbicidal proteases required for intracellular degradation of microorganisms (Pham, 2006). In addition, this ensures that neutrophils are activated at the right place and the right time to avoid damage to healthy host tissue.

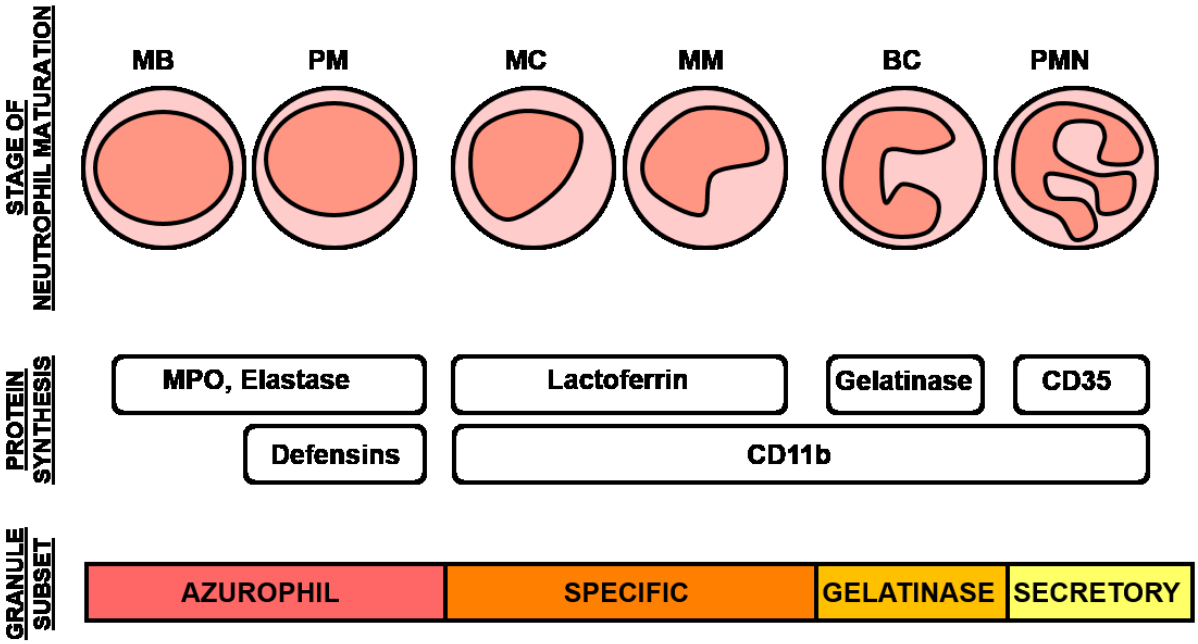


Figure 1.9. Granule subset formation during neutrophil maturation. Granule subsets are formed by the "targeted by timing" hypothesis, whereby granule contents are incorporated into different subsets based on the time during neutrophil maturation in which they are synthesised. MB= myeolblast; PM= promyelocyte; MC=myelocyte; MM= metamyelocyte; BC= band cell; PMN= polymorphonuclear neutrophil; MPO= myeloperoxidase

In addition to proteases, neutrophils also release ROS and a plethora of cytokines in response to stimulation, which are detailed in Tecchio et al (2014). These include, but are not limited to, a number of CXC chemokines, including CXCL1 and -8; CC chemokines, including CCL2, 3 and 4; TNF-α; TGF-β; and IL-1α, -1β, -6 and -7. Therefore, as well as having direct antimicrobial effects, neutrophils can also augment the proinflammatory environment through the release of cytokines and chemokines, which may further implicate them in COPD pathophysiology.

1.4.1.3. Neutrophils in COPD

Neutrophils have been heavily implicated in the pathogenesis of COPD. There are increased numbers of neutrophils in the sputum (Keatings *et al.*, 1996), bronchoalveolar lavage fluid (BALF) (Martin *et al.*, 1985), and airway tissue (See section 1.4.1.4) of COPD patients, and

this correlates with disease severity (Stănescu *et al.*, 1996, Hogg *et al.*, 2004, O'Donnell *et al.*, 2004). Burnett et al (1987) posited that neutrophils from patients with emphysema exhibit increased proteolysis and chemotaxis, compared with control subjects. Neutrophils from COPD patients also exhibited increased respiratory burst and increased expression of the adhesion molecule, Mac-1 (CD11b/CD18), both at baseline and after stimulation with TNF-α, compared with non-smokers and asymptomatic smokers (Noguera *et al.*, 2001). Neutrophils from patients suffering with early-onset COPD also demonstrated increased respiratory burst, and increased NE and CXCL8 release in response to fMLP stimulation, compared with non-smoker controls (Milara *et al.*, 2012). COPD neutrophils also demonstrate aberrant chemotaxis, having increased speed but reduced directionality and accuracy, compared with non-smokers, asymptomatic smokers or patients with α1-AT deficiency (Sapey *et al.*, 2011). Therefore, there is increasing evidence that the neutrophil contribution to pathophysiology of COPD is not only due to increased numbers, but also to inherent aberrant activity of these cells.

1.4.1.4. Neutrophil infiltration of small airways in COPD

There are significantly increased numbers of neutrophils and macrophages in the subepithelium in bronchial biopsies from COPD patients, compared with healthy smokers, and this was negatively correlated with FEV_1 % predicted (Di Stefano *et al.*, 1998). There are significantly increased numbers of neutrophils in the smooth muscle of small airways in COPD patients and smokers with normal lung function, compared with non-smokers; and this was also significantly negatively correlated with FEV_1 % predicted and FEV_1/FVC (Baraldo *et al.*, 2004). The percentage of neutrophils in induced sputum was significantly increased in moderate to severe COPD patients, compared with non-smokers, smokers with normal lung function, and mild COPD patients; and this was also significantly correlated with evidence of small airway obstruction, as determined by the expiratory/inspiratory mean lung density ratio (O'Donnell *et al.*, 2004). In the same study, increasing small airway obstruction was also correlated with decline in FEV_1 % predicted, further validating the influence of small

airway obstruction on COPD progression. Isajevs *et a*l (2011) also demonstrated significant neutrophil infiltration in small airways of COPD patients, compared with asymptomatic smokers and non-smokers; and, in addition, revealed a significant increase in nuclear factor (NF)-κB positive cells and a significant decrease in histone deacetylase (HDAC)-2 positive cells in the subepithelium and epithelium of small airways in COPD patients, which indicates increased expression of pro-inflammatory genes in the small airways.

1.4.2. Proteases

As mentioned previously, neutrophils contain a number of different proteases that enable them to effectively destroy invading organisms. Of particular interest are the serine proteases, most notably NE, matrix metalloproteases (MMPs), and MPO.

1.4.2.1. Neutrophil elastase

NE, Cathepsin G and Proteinase-3 are a group of proteases known as serine proteases, and are stored in their active form in azurophil granules (Pham, 2006). In particular, NE has been heavily implicated in COPD pathophysiology (see section 1.4.2.4). The main role of NE is to destroy pathogens within the phagolysosome. NE has been shown to be important in the destruction of Gram-negative bacteria, as $NE^{-/-}$ knockout mice had increased mortality and inability to clear infection of *Klebsiella pneumoniae* and *Escherichia coli*, but not Gram positive *Staphylococcus aureus* (Belaaouaj *et al.*, 1998). In humans, mutations in the gene encoding NE, *ELA2*/*ELANE*, can lead to cyclic or severe congenital neutropenia (Horwitz *et al.*, 2007). NE is also important in the destruction of virulence factors on bacterial species such as *Shigella*, *Salmonella* and *Yersinia*, which otherwise cause severe enteric infections (Weinrauch *et al.*, 2002).

However, NE is also capable of destroying a number of extracellular matrix (ECM) proteins, including elastase, collagen, fibronectin and proteoglycans (Chua *et al.*, 2006), and this is thought to mediate some of the tissue destruction observed in COPD. Yoshioka *et al.*, (1995) showed that smokers with emphysema had significantly higher concentrations of NE in their

BALF, compared with non-smokers and asymptomatic smokers. In addition, NE significantly increased transforming growth factor (TGF)-β expression in airway smooth muscle cells *in vitro*, and this was downstream of NF-κB induction (Lee *et al.*, 2006). NE has also been shown to induce expression of a number of mucin genes, including MUC1 (Kuwahara *et al.*, 2005), MUC4 (Fischer *et al.*, 2003) and MUC5AC (Shao *et al.*, 2005); thereby implicating it in the mucus hypersecretion also observed in COPD. NE has additionally been shown, *in vitro,* to be involved in the induction of proteases, such as MMP-2 and Cathepsin B (Geraghty *et al.*, 2007); inflammatory cytokines, such as CXCL8 (Kuwahara *et al.*, 2006); and lipid mediators, such as prostaglandin (PG) E_2 (Perng *et al.*, 2003).

1.4.2.2. Matrix metalloproteases

Another group of proteins that have been of particular interest in COPD is the MMPs. The MMPs comprise a large family of 25 related proteins that have a conserved pro-domain and catalytic domain, which comprises a zinc ion in their active site (Parks *et al.*, 2004). Substrate specificity varies between MMPs, but they largely degrade components of the ECM. Neutrophils contain MMP-2 (Gelatinase A), MMP-8 (Collagenase-2), MMP-9 (Gelatinase B), and MMP-25 (Leukolysin), which are all contained in specific or gelatinase granules (Parks *et al.*, 2004). MMP-8 and MMP-9 are increased in sputum of COPD patients, compared with asymptomatic smokers and non-smokers (Vernooy *et al.*, 2004, Culpitt *et al.*, 2005); and this is evidenced even at very early stages of the disease (previously termed GOLD stage 0, whereby lung function is normal but clinical symptoms of COPD are present, i.e. symptomatic smokers) (Ilumets *et al.*, 2007). The sputum concentration of MMP-8 and MMP-9 was also significantly negatively correlated with airflow obstruction (Vernooy *et al.*, 2004). Patients with emphysema had increased concentrations of both MMP-8 and MMP-9 in their BALF, compared with current and ex-smokers without emphysema (Betsuyaku *et al.*, 1999). MMP-9 can also induce TGF-β1 expression in human airway epithelial cells *in vitro* (Perng *et al.*, 2011).

1.4.2.3. Myeloperoxidase

MPO is another protease implicated in COPD pathophysiology. MPO is contained in azurophil granules, and reacts with hydrogen peroxide (H_2O_2), formed by nicotinamide adenine dinucleotide phosphate (NADP)H oxidase (see Section 1.4.3) and chloride ions, to create hypochlorous acid (HOCl), an extremely powerful oxidant. In a longitudinal study of 68 Swedish males, serum levels of MPO were significantly increased in continual smokers, compared with never-smokers or smokers who had quit during the study period; although it is worth noting that, in this particular study, this was not related to lung function, and no subjects had clinically diagnosable COPD by the end of the study period (Andelid *et al.*, 2007). However, a recent meta-analysis published in the European Journal of Medical Research found that sputum MPO concentration is significantly higher in COPD patients, compared with controls; and is also significantly higher during exacerbations, compared with stable COPD, implicating MPO as a potential biomarker for COPD (Zhu *et al.*, 2014). There are also significantly higher levels of MPO in BALF from COPD patients, compared with non-smokers (Pesci *et al.*, 1998). As such, MPO may be a marker of the inflammatory effects of smoking, with associations with COPD progression.

1.4.2.4. Protease-antiprotease imbalance

One of the leading hypotheses regarding the development and progression of COPD is that of a protease-antiprotease imbalance. As discussed in Section 1.4.2, there is an increased proteolytic burden in the lungs of COPD patients, due to increased numbers and aberrant activity of inflammatory cells. The initial evidence for this hypothesis came from the observation by Laurell and Eriksson (1963) that patients with a deficiency in α1-AT, the main inhibitor of NE, developed early-onset emphysema. A number of animal models have also given this hypothesis further credence: hamsters (Senior *et al.*, 1977) and rats (Yokoyama *et al.*, 1987) given *in vivo* instillation of NE developed emphysematous lesions and abnormal lung function; and $NE^{-/-}$ knockout mice were protected against emphysema following long-

term cigarette smoke exposure, compared with wildtype littermates (Shapiro *et al.*, 2003). In addition, α1-AT can be oxidised by H_2O_2, which greatly reduces its ability to inhibit NE (Taggart *et al.*, 2000). In addition, this oxidised form of α1-AT can induce release of CXCL8 and monocyte chemoattractant protein (MCP)-1 from A549 cells and human bronchial epithelial cells *in vitro*, with both chemokines being potent chemoattractants for inflammatory cells, such as neutrophils and monocytes (Li *et al.*, 2009). Similarly, proline-glycine-proline (PGP) is a peptide formed from ECM breakdown, and is a potent neutrophil chemoattractant, suggesting that proteolytic degradation of the ECM can then further propagate the inflammatory response by recruiting more immune cells (Weathington *et al.*, 2006). Therefore, there is significant evidence that an imbalance of proteases, as a result of a heightened pro-inflammatory environment, cannot be adequately quenched by endogenous antiproteases, thus leading to tissue destruction and the progression of COPD.

1.4.3. Oxidative stress

ROS are unstable small molecules or free radicals derived from molecular oxygen that have the propensity to initiate oxidation. These can be formed endogenously by metabolic processes, such as the mitochondrial electron transport chain, or by inflammatory cells, such as neutrophils; or exogenously by inhalation of cigarette smoke. Oxidative stress is the term used to describe a scenario where there is an imbalance of oxidants versus antioxidants. ROS can have a number of deleterious physiological effects: they can directly damage cellular deoxyribose nucleic acid (DNA), leading to mutations and/or cell death (Ryter *et al.*, 2007); they are involved in cellular signalling and can activate pro-inflammatory transcription factors, such as NF-κB (Schreck *et al.*, 1991); and they can cause lipid peroxidation, leading to damage to the cell membrane and production of cytotoxic and genotoxic end-products (Esterbauer, 1993).

One of the main cellular sources of ROS is NADPH oxidase, an enzyme found in a number of inflammatory cells, including neutrophils, which catalyses the reaction between O_2 and

NADPH, to form superoxide ions (O_2^-). The enzyme is composed of five subunits: one is in the neutrophil cell membrane, and the other four in the cytoplasm. Upon 'sufficient' stimulation, the cytoplasmic components translocate to the membrane-bound subunit to form a functional enzyme, in a process often referred to as "respiratory burst" (Dahlgren *et al.*, 1999). As mentioned in Section 1.4.1.3, neutrophils from COPD patients demonstrate increased respiratory burst *in vitro*, which may perpetuate the oxidant imbalance in COPD if reproduced *in vivo* (Noguera *et al.*, 2001, Milara *et al.*, 2012).

Evidence of oxidative stress is a common observation in COPD patients. There is a significantly increased concentration of H_2O_2 in the exhaled breath condensate of stable COPD patients, compared with non-smokers; and this is further increased in exacerbations of COPD (Dekhuijzen *et al.*, 1996). A more recent study measured the concentrations of malondialdehyde (MDA), a lipid peroxide; the antioxidants, superoxide dismutase (SOD) and glutathione (GSH); and glutathione peroxidase (GSH-PX), an enzyme that reduces lipid hydroperoxides, in induced sputum of COPD patients and healthy controls. The authors found significant increases in MDA, and significant decreases in SOD, GSH and GSH-PX in stable and exacerbating COPD patients, compared with non-smokers and asymptomatic smokers; suggesting an imbalance of oxidants and antioxidants (Zeng *et al.*, 2013). Nadeem and colleagues (2005) demonstrated evidence of systemic oxidative stress in COPD patients, by observing significantly increased plasma levels of MDA, GSH, and GSH-PX, but significantly reduced total plasma antioxidant power, compared with non-smokers; and significant inverse correlations between FEV_1 % predicted and total blood GSH and total plasma antioxidant power. Therefore, there is considerable evidence to support the concept of an oxidant-antioxidant imbalance in COPD that may be involved in disease pathophysiology.

1.4.4. Inflammatory mediators

As mentioned in the above sections, immune cells are recruited to the site of tissue injury by inflammatory signals. These signals can be released from both inflammatory cells, such as macrophages, and also structural cells, such as airway epithelial cells. Cigarette smoke activates macrophages and epithelial cells: studies show cigarette smoke extract (CSE; media containing the soluble components of cigarette smoke) induces the release of CXCL8, MCP-1 (CCL-2), leukotriene (LTB) B_4 and granulocyte colony stimulating factor (G-CSF) from alveolar type II cells *in vitro*, all of which are potent neutrophil or monocyte chemoattractants (Masubuchi *et al.*, 1998). CSE can also stimulate release of CXCL8 and TNF-α in a monocytic cell line, via activation of NF-κB (Kode *et al.*, 2006); and BALF macrophages from smokers release significantly higher amounts of TNF-α and CXCL-8 in response to IL-1β stimulation, compared with non-smokers (Ito *et al.*, 2001).

A number of cells, including epithelial cells, macrophages, T-cells and airway smooth muscle cells, have the propensity to release TNF-α. TNF-α is stored in its membrane-bound form until appropriate stimulation (e.g. liposaccharide (LPS)) leads to conversion to its active form. Active TNF-α can activate NF-κB, a proinflammatory transcription factor that promotes the expression of over 150 target genes (Pahl, 1999); and p38 mitogen activated protein (MAP) kinase, which also has downstream proinflammatory effects (Barnes, 2004). There are increased concentrations of both TNF-α and CXCL8 in the sputum of COPD patients, compared with asymptomatic smokers and non-smokers, alluding to the heightened proinflammatory environment present in COPD lungs (Keatings *et al.*, 1996).

Cytokine-induced neutrophil chemoattractant (CINC)-1 (assessed in the present thesis) is the rat counterpart of human growth-regulated oncogene product (GRO); levels of which are elevated in sputum samples from COPD patients (Traves *et al.*, 2002). GROα is closely related to CXCL8; and CINC-1, like GRO and CXCL8, is a potent neutrophil chemoattractant (Handa *et al.*, 2006). CINC-1 can be induced by LPS, IL-1β, TNF-α, ischemia/reperfusion and H_2O_2, and this is thought to be mediated by NF-κB activation (Handa *et al.*, 2006). CINC-1 is released from a number of different rat cell types, including cardiac myocytes

(Seino *et al.*, 1995), gastric epithelial cells (Handa *et al.*, 2004) and alveolar epithelial cells (Riedemann *et al.*, 2002).

1.5. Investigating small airways function

1.5.1. Smooth muscle contraction

There are two predominant types of smooth muscle within the lung parenchyma: airway smooth muscle and vascular smooth muscle. As their names suggest, the former refers to smooth muscle surrounding the airways, and the latter refers to that surrounding the blood vessels. For contraction to occur, myosin light chains must be phosphorylated by myosin light chain kinase (MLCK), enabling interaction between actin and myosin filaments. Energy released by myosin ATPase enables cycling of actin-myosin cross-bridges, leading to functional shortening of the muscle fibers and, therefore, smooth muscle contraction.

Contraction is calcium (Ca^{2+})-dependent, and increases in cytosolic Ca^{2+} are initiated by release from intracellular stores, and through voltage- and receptor-gated Ca^{2+} channels. Agonists bind to specific membrane receptors and stimulate phospholipase C (PLC). This catalyzes the formation of the second messengers IP_3 and DAG. IP_3 stimulates the release of Ca^{2+} from intracellular stores in the sarcoplasmic reticulum; whilst DAG, in conjunction with Ca^{2+}, activates protein kinase C (PKC), which phosphorylates a number of downstream target proteins that are, generally, pro-contractile. Ca^{2+} combines with calmodulin, a serine-threonine kinase, which activates MLCK. MLCK phosphorylates myosin light chains and, therefore, initiates contraction of smooth muscle fibers. Phosphorylation of myosin fibers is also regulated by myosin phosphatase. This dephosphorylates myosin fibers, therefore inducing relaxation of the muscle fibers. Myosin phosphatase activity is regulated by Rho Kinase, a downstream target of RhoA. Rho kinase phosphorylates a subunit in myosin phosphatase, inhibiting its action and therefore promoting contraction. Therefore, the tonicity of smooth muscle is determined by the balance between these two enzymes: MLCK promoting contraction, and myosin phosphatase inducing relaxation (Sanderson et al. 2008).

G-protein coupled receptors (GPCR) are a superfamily of proteins that transduce extracellular signals through receptors in the plasma membrane, and initiate a plethora of intracellular signaling pathways vital for a diverse range of cellular functions. As such, GPCR are instrumental in mediating airway tone. For example, acetylcholine (or cholinergic mimetics, such as carbachol) acts through muscarinic receptors that initiate Gq-coupled receptor signaling and promote contraction; whereas β-agonists activate Gs-coupled β_2-adrenergic receptors and initiate bronchodilation (Billington & Penn, 2003).

There are a number of receptor/agonist combinations that can initiate smooth muscle contraction or relaxation, including acetylcholine (or cholinergic mimetics) or 5-Hydroxytryptamine (5-HT) acting through M_2 and M_3 muscarinic (see Section 1.) or 5-HT_{1A} or $_{2A}$ (Cazzola & Materra, 2000) receptors, respectively, on airway smooth muscle; or endothelin-1 and angiotensin II acting through ET_A and AT_1 receptors (Wynne et al, 2009), respectively, on vascular smooth muscle.

As described in section 1.4.1.2, neutrophils release a number of cytokines, proteases and ROS in response to stimulation, all of which can differentially affect both airway and vascular smooth muscle. The effects of some of these products are summarized in Table 1.1. It is clear that long-term exposure to neutrophil products may elicit many of the effects associated with COPD pathophysiology, including smooth muscle hypertrophy and hyperplasia, and airway hyperresponsiveness. With respect to the work in this thesis, the more chronic effects of exposure to these products, such as proliferation of the smooth muscle layer, may not be reflected due to the short time-frame imposed in the experiments. However, effects on contraction and relaxation of both airway and vascular smooth muscle may be pertinent in interpreting the results within this thesis.

1.5.2. Methods used to investigate small airway function

Tracheal and bronchial rings or strips, and parenchymal strips are used, depending of the generation of airway under investigation. Tracheal strips involve removing the trachealis

Table 1.1. Factors released by neutrophils that affect airway and vascular smooth muscle

	Airway smooth muscle	**Vascular smooth muscle**
Neutrophil elastase	Increases smooth muscle proliferation (Huang *et al.*, 2004) Increased TGF-β expression via NF-κB pathway (Lee *et al.*, 2006) Reduced CXCL8 mRNA expression (Ho *et al.*, 2009) Induces apoptosis (Oltmanns *et al.*, 2005)	No specific data found
Myeloperoxidase	No specific data found	Increases expression of endothelin receptor type B. Increases vasoconstriction (Lau *et al.*, 2014)
MMP-2, MMP-9	Increased proliferation (Johnson *et al.*, 1999)	Increased proliferation and migration (Chen *et al.*, 2013)
CXCL8	Increased contraction and migration (Govindaraju *et al.*, 2006)	Increases DNA synthesis, cell proliferation and cell migration. Induces PGE_2 release (Yue *et al.*, 1994)
CXCL1 (GROα)	Inhibits migration (Al-Alwan *et al.*, 2014)	No specific data found
TGFβ	Modulates proliferation (Cohen *et al.*, 1997) Potentiates responsiveness to bradykinin (Kim *et al.*, 2005)	Modulates proliferation (Tsai *et al.*, 2009)
TNFα	Increase DNA synthesis (Stewart *et al.*, 1995) Induces hyperresponsiveness (Hunter *et al.*, 2003)	Induces migration (Goetze *et al.*, 1999) Induces IL-1 and PGE_2 release (Warner *et al.*, 1989)
LTB_4	Increase proliferation and migration (Watanabe *et al.*, 2009)	Induces DNA synthesis (Palmberg *et al.*, 1987)
PGE_2	Bronchodilation (Benyahia *et al.*, 2012)	Vasodilation (Zhu *et al.*, 2002)
Reactive oxygen species	Modulates airway tone (see Chapter 5, section 5.4.1)	Hypertrophy, proliferation and migration (Lyle *et al.*, 2006) Modulates airway tone (Ardanaz *et al.*, 2006)

TGF= Transforming Growth Factor, TNF= Tumor Necrosis Factor, LTB_4= Leukotrine B_4, PGE= Prostaglandin E_2, IL= Interleukin, NF= Nuclear Factor

smooth muscle, which runs along the posterior of the trachea in the space between the edges of the C-rings of cartilage, from excised trachea, and cutting strips of this muscle. Bronchial strips are obtained in a similar way in larger animals where the bronchi are large enough to manipulate; however, in small animals (e.g. mice, guinea pig and rat), bronchial rings may be used instead. Parenchymal strips are isolated by taking strips of parenchyma from the periphery of the lung lobes (Cooper *et al.*, 2009).

In order to assess their function, strips are held in an organ bath, a chamber containing a physiological salt solution; whereby one end is fixed in the organ bath, and the other end is fixed to a transducer that measures mechanical responses. These can either measure changes in muscle length given a constant force (isotonic transducers), or measure changes in force generated whilst maintaining the strip at a constant length (isometric transducers) (Cooper *et al.*, 2009).

The advantages of these techniques are that the dynamics of larger airways can be investigated from a variety of different species, and multiple strips can be obtained from one trachea/bronchus, allowing several conditions to be investigated. However, one of their disadvantages are that, as the preparation of these strips requires its removal, the influences of load from surrounding structures (e.g. the parenchyma) on airway smooth muscle contraction is not taken into account. In addition, isotonic and isometric muscle contractions are not necessarily indicative of *in vivo* lung mechanics. With regards to investigating small airway function, one of the main disadvantages of parenchymal strips is the heterogeneity of the smooth muscle within the lung tissue, as it will include vascular smooth muscle as well as airway smooth muscle (Cooper *et al.*, 2009). Therefore, additional *ex vivo* preparations are required in order to address these drawbacks of the lung strip technique.

1.5.3. Precision-cut lung slices

Detailed examination of the function of specific airway generations is usually performed *ex vivo*. As mention previously, the small airways are thought to be the main site of airflow

obstruction in COPD (Hogg *et al.*, 1968), and different *ex vivo* models have been utilized to investigate the function of these airways. Precision-cut lung slices (PCLS) are an *ex vivo* preparation of lung tissue whereby small airways are maintained in their native microenvironment with intact parenchymal attachments. This allows for investigation of the macroscopic and microscopic architecture of the lung tissue in a similar environment to *in vivo* (Sanderson, 2011). The advantages and disadvantages of PCLS are outlined in Table 1.2. Throughout this thesis, PCLS are referred to as *ex vivo*, pertaining to the fact that the lungs, or sections of lung, are isolated and the PCLS generated outside of the host. The distinction between this and an *in vitro* preparation is subtle and possibly contentious. However, to remain concordant with the literature, whereby PCLS are often referred to as *ex vivo*, they will be considered and referred to as such henceforth.

Whole lungs (small laboratory animals) or lung tissue explants (human) are inflated with low-melting point agarose, which mimics the distension of the lung tissue and parenchymal loads at full inspiration (Cooper *et al.*, 2009). Inflation with agarose is essential, firstly to enable slicing of the lung tissue; and, secondly, as it provides a positive alveolar pressure (which would be equivalent to negative pleural pressure *in vivo*), and this prevents collapse of the lung slice (Sanderson, 2011). However, the physiological implications of agarose inflation are a potentially confounding issue. Agarose will always remain in the lung slice, as the low-melting point only refers to the temperature at which the agarose changes from fluid to gel, which is ~65°C compared with 87°C in standard agarose. Therefore, at 37°C, the temperature at which PCLS are maintained, the agarose will not melt and be washed from the slice. However, it is possible to ensure the removal of the agarose from the airway lumen through sequential washes following PCLS generation, therefore the agarose should not provide any resistance to airway closure in that respect. Different concentrations of agarose are used throughout the literature, from as low as 0.75% (Wohlsen *et al.*, 2001), compared with the 2-3% used herein. Agarose concentration, and therefore “stiffness”, will influence airway contraction, as it will have a determinant effect on the mechanical properties of the

Table 1.2. Advantages and disadvantages of the PCLS technique	
Advantages	**Disadvantages**
Multiple slices can be obtained from one animal, therefore substantial data can be generated with minimal animal usage. This will also provide enough PCLS for treatments and controls.	Inflation with agarose is essential, but this may impact on airway dynamics. This may be more or less pronounced at different concentrations of agarose, therefore intergroup comparisons may be difficult.
Accessible from a number of species, including rat, mouse, and human.	Analysis of airway function is potentially subjective, and should ideally be analyzed by a blinded investigator.
Airways maintained in native microenvironment, therefore impact of surrounding structures can be considered. Intercellular interactions and cellular communications will also be maintained, resulting in a more physiological model system.	With respect to human tissue, ability to obtain PCLS is largely dependent on quality of tissue; therefore investigation of severe disease states (e.g. emphysema) may not be possible.
Airways contract auxotonically, which is more indicative of *in vivo* airway function. In comparison, organ bath preparations measure either isometric or isotonic contraction, which are not as physiologically applicable.	Tissue can be damaged during the slicing procedure, particularly on the cut face of the slice. This is mostly applicable in thinner (100-200μm thick) PCLS, and this effect can be limited by a period of stabilization
A number of techniques can be employed using PCLS, including functional studies, mRNA and protein expression, and histology. These can often be utilized with the same PLCS, therefore enabling correlation between physiological responses and cellular data. This also maximizes the use of the tissue, again limiting animal/tissue usage.	Vast differences in PCLS preparation and maintenance between groups (e.g. slice thickness, agarose concentration, media composition, PCLS culture conditions), which may make comparisons and extrapolations from different groups within the literature difficult.
PCLS= precision-cut lung slices.	

lung tissue. In particular, relaxation of airways is impacted when using low concentrations of agarose (Sanderson, 2011, Schlepütz *et al.*, 2011). However, provided that agarose concentration is kept consistent throughout different experiments, comparisons between treatments will remain valid. To the author's knowledge, alternative methods of inflation have not been utilized with the PCLS technique; therefore the implications of agarose inflation will remain a potential drawback of the PCLS technique.

As airways in PCLS are maintained with all parenchymal attachments intact, these airways contract auxotonically, i.e. parenchymal load increases as airway smooth muscle length decreases, which is identical to *in vivo* airway dynamics. In contrast, bronchial/parenchymal strips contract either isometrically or isotonically, depending on how individual experiments are set up (Cooper *et al.*, 2009). An additional advantage to the PCLS system is that the effect of other structures within the parenchyma, for example blood vessels, can also be investigated, both independently and with respect to airway contraction.

PCLS have a range of applications, including investigating the effects of *in vivo* insults, e.g. cigarette smoke exposure (Cooper *et al.*, 2010) and elastase instillation (Khan *et al.*, 2010); pharmacological testing of potential therapeutic targets, e.g. LABA or LAMA (Sturton *et al.*, 2008); identifying mechanisms of small airway dynamics (Brown *et al.*, 2013); and, as in the present study, investigating *ex vivo* manipulation of the PCLS environment. PCLS also lend themselves to a wide range of laboratory techniques: small airway and vascular physiology can be assessed using time-lapse imaging, microscopic architecture of the tissue can be revealed by histology and by using immunohistochemistry, cytokine and mediator output can be measured in PCLS supernatant, and PCLS can be homogenized and protein expression detected through western blotting. Therefore, functional and cellular mechanisms can both be investigated in one preparation. In addition, large numbers of uniformly sized PCLS can be generated from one piece of tissue. To illustrate this point, in the present thesis ~50 PCLS can be generated from one set of rat lungs, which allows for ~10 different conditions to be assessed. Therefore, a large amount of data can be extracted from the use of just one

animal, overall minimising the number of animals required. Therefore, PCLS are an exceptionally useful adjunct to existing *in vivo* and *ex vivo* lung preparations, in order to investigate small airway physiology.

1.6. Drugs and compounds used in this thesis

A number of different drug compounds are used in this thesis, and these are introduced below.

1.6.1. Carbachol

Carbamoylcholine chloride, or carbachol (CCh), is a cholinergic agonist that acts on both muscarinic and nicotinic receptors. Airways are innervated by both sympathetic (adrenergic) and parasympathetic (cholinergic) branches of the nervous system; however, sympathetic innervation of the airways is sparse, compared with the parasympathetic supply (Barnes *et al.*, 1984). Adrenergic stimulation causes bronchodilation, therefore, β_2-adrenergic receptors, the predominant adrenoreceptor subtype on airway smooth muscle, are common therapeutic targets in diseases with bronchial hyperreactivity, such as asthma. By contrast, cholinergic stimulation causes bronchoconstriction through the action of acetylcholine (ACh) on muscarinic receptors. There are five subtypes of muscarinic receptors, but only M_1, M_2 and M_3 subtypes have been identified in human lungs. Despite the ratio of M_2 to M_3 receptors being ~ 4:1 in the lung, bronchoconstriction is mediated primarily through M_3 receptors on airway smooth muscle in both rats and human peripheral small airways (Brown *et al.*, 2013). M_3 receptors activate phospholipase C (PLC) via pertussis-insensitive G-proteins (G_q), which in turn increase concentrations of inositol triphosphate (IP_3) and diacylglycerol (DAG), leading to contraction of airway smooth muscle (Fryer *et al.*, 1998). Neuronal M_2 receptors are thought to be autoregulatory and provide a negative feedback mechanism: ACh released by (vagal) cholinergic nerves acts on M_2 receptors found on postganglionic nerves, which then inhibit further ACh release, thereby limiting the degree of airway constriction magnitude (Fryer *et al.*, 1998). However, airway smooth muscle cell M_2 receptors indirectly

mediate bronchoconstriction by inhibiting relaxation pathways of small airways in both rat and humans (Brown *et al.*, 2013).

1.6.2. fMLP

fMLP is a bacterial peptide that activates neutrophils, initiating chemotaxis, degranulation and respiratory burst (Showell *et al.*, 1976, Panaro *et al.*, 1999). There are two types of fMLP receptor found on human cells that are activated by formyl peptides: formyl-peptide receptor (FPR) and formyl-peptide receptor like-1 (FPRL-1). The former is a high affinity receptor, activated at pico- to nanomolar concentrations of fMLP; whereas the latter is lower affinity and requires micromolar concentrations (Le *et al.*, 2002). A difference in fMLP receptors on neutrophils has been posited as an explanation for the aberrant activity of neutrophils in COPD; however, there have been conflicting results. Stockley *et al* (1984) found significantly higher numbers of FPR receptors on neutrophils from patients with emphysema, compared with healthy smokers. Conversely, Matheson *et al* (2003) compared the numbers of fMLP receptors on neutrophils in healthy non-smokers and smokers, and COPD smokers and ex-smokers, and found that current healthy smokers had the highest number of fMLP receptors, but that there were no significant differences in receptor number between healthy smokers and COPD smokers. This suggests that an increase in fMLP number may be a function of smoking, rather than disease state. Conversely, Sapey and colleagues (2011) found no difference in FPR1 receptor expression on neutrophils from COPD patients and healthy non-smoking and smoking controls. The disparity between these results may be due to differences in methodology: the Stockley (1984) and Matheson (2003) papers used bound labeled-fMLP to quantify fMLP receptor number, whereas Sapey *et al* (2011) used flow cytometry to analyse receptor expression. It is also difficult to compare the size and characteristics of the subject groups: Matheson *et al* had only four COPD smokers, whilst Stockley *et al* had six; meanwhile, Sapey *et al* only had ex-smokers in their COPD group. Therefore, a difference in fMLP receptor expression in COPD neutrophils remains unclear.

1.7. Thesis hypothesis

To briefly summarise the aforementioned background to the experimental work of this thesis, neutrophils have been implicated in development and progression of COPD. There is evidence of increased numbers of neutrophils in the lungs of COPD patients, and some indication that COPD neutrophils behave abnormally, compared with healthy subjects. Small airways are thought to be the main site of airflow obstruction in COPD, and PCLS is an *ex vivo* technique that enables investigation of the structure and function of these airways. However, it should be noted that the small airways in the rat PCLS of the present thesis do not exhibit the fibrotic characteristics of human SAD. Consequently, the present investigation focuses on the effect, in isolation, of neutrophil products on parenchymal structure and small airway function.

As such, the hypotheses of this thesis are:

"Released products from activated human neutrophils (hereafter referred to as neutrophil supernatants) will significantly affect PCLS structure and small airway function in rat PCLS, and the effects of neutrophils from COPD patients will be more pronounced."

"In addition, these effects will be mediated by neutrophil proteases and/or oxidative stress, and addition of exogenous inhibitors or antioxidants will attenuate these effects."

"Finally, small airways in human PCLS from COPD lung tissue will behave differently, compared with those from asymptomatic smokers and non-smokers."

1.8. Aims

In order to investigate the above hypothesis and related sub-hypotheses, the aims of the present thesis are:

1. Collect supernatants from peripheral blood neutrophils from non-smokers, smokers and COPD patients, either unstimulated or stimulated with fMLP, and store at -80°C until ready for use.

2. Measure the concentration of NE, MMP-8, MMP-9 and MPO in the neutrophil supernatants, and investigate any correlations with subject demographics (Chapter 3).
3. Incubate rat PCLS overnight with neutrophil supernatants, and investigate the effects on small airway closure and reopening, PCLS viability and histological appearance of the airways; and investigate any differences between subject groups (Chapter 4).
4. Incubate rat PCLS in either porcine pancreatic elastase (PPE) or H_2O_2, to investigate the effects of elastolytic and oxidative stress on small airway closure, PCLS viability and histology; in order to determine if either/both mimic the effects observed after incubation with neutrophil supernatants (Chapter 5).
5. Pre-incubate neutrophil supernatants either with an antioxidant or with protease inhibitors, prior to incubation on rat PCLS, in order to determine whether these compounds can attenuate the effects of neutrophil supernatants, and potentially delineate the source of the tissue destruction (Chapter 6).
6. Investigate airway closure and reopening of small airways in PCLS from resected human lung tissue from COPD patients, asymptomatic smokers and non-smokers, and identify any differences between the subject groups and any correlations with subject demographics (Chapter 7).

Chapter 2:

Materials & Methods

2. Materials & Methods

2.1. Materials

Amphotericin-B	Gibco, Invitrogen, Paisley, UK
Brunel BMS Video Microscope	Brunel Microscopes Ltd., Wiltshire, UK
Carbamoylcholine Chloride	Sigma-Aldrich, Dorset, UK
Cell Proliferation Reagent (WST-1)	Roche Diagnostics Ltd., West Sussex, UK
Cytochalasin B	Sigma-Aldrich, Dorset, UK
Dextran	Sigma-Aldrich, Dorset, UK
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich, Dorset, UK
Dulbecco's Modified Eagles Medium (DMEM)	Gibco, Invitrogen, Paisley, UK
Dulbecco's Phosphate Buffered Saline (DPBS)	Sigma-Aldrich, Dorset, UK
Elastase Inhibtor III (324745)	Merck Millipore, Watford, UK
Eosin	VWR International, Leicestershire, UK
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Dorset, UK
Euthatal®	Merial Animal Health Ltd., Essex, UK
FLUOstar Optima plate reader	BMG Labtech Ltd., Bucks, UK
Formaldehyde (10% $^{v}/_{v}$)	Sigma-Aldrich, Dorset, UK
Gentamycin	Sigma-Aldrich, Dorset, UK
Glycerol	Sigma-Aldrich, Dorset, UK
GraphPad Prism v 5.0	GraphPad Software Inc., CA, USA
Hank's Balanced Salt Solution (HBSS)	Sigma-Aldrich, Dorset, UK
Harris Haemotoxylin	VWR International, Leicestershire, UK
HEPES	Sigma-Aldrich, Dorset, UK
Histo-clear	National Diagnostics, Yorkshire, UK
Human Active MMP-9 Fluorokine E® kit	R&D Systems, Abingdon, UK
Human Leukocyte Elastase	Sigma-Aldrich, Dorset, UK

Human MMP-8 (Total) Quantikine® kit	R&D Systems, Abingdon, UK
Human MPO (Total) Quantikine® kit	R&D Systems, Abingdon, UK
Hydrogen Peroxide	Sigma-Aldrich, Dorset, UK
Krumdieck Tissue Slicer **(Model MD1100-A2)**	Alabama Specialty Products Ltd., AL, USA
L-Glutamine	Gibco, Invitrogen, Paisley, UK
Low-melting point Type IX-A Agarose	Sigma-Aldrich, Dorset, UK
MMP-9 inhibitor I (444278)	Merck Millipore, Watford, UK
N-Acetyl- Cysteine	Sigma-Aldrich, Dorset, UK
***N*-Formyl-Met-Leu-Phe (fMLP)**	Sigma-Aldrich, Dorset, UK
N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide	Sigma-Aldrich, Dorset, UK
NaO_{AC}	Sigma-Aldrich, Dorset, UK
Neolus Needle 23Gx16mm	Scientific Laboratory Supplies Ltd., Yorkshire, UK
Penicillin/ Streptomycin	Gibco, Invitrogen, Paisley, UK
Percoll	GE Healthcare Life Sciences, Buckinghamshire, UK
Porcine Pancreatic Elastase	Merck Millipore, Watford, UK
Prostaglandin E2 Express EIA kit	Cayman Chemicals, Tallinn, Estonia
Rat CINC-1 Duoset	R&D Systems, Abingdon, UK
Rat TNF-α Duoset	R&D Systems, Abingdon, UK
RPMI	Sigma-Aldrich, Dorset, UK
Shandon™ Finesse™ 325 Manual Microtome	Thermo Fisher Scientific Inc., MA, USA
Sodium Chloride (0.9% $^{w}/_{v}$)	Baxter, Norfolk, UK
Sodium Chloride (9% $^{w}/_{v}$)	Sigma-Aldrich, Dorset, UK
Softmax Pro v 6.2	Molecular Devices Ltd., Berkshire, UK
Spectramax Plus 384 plate reader	Molecular Devices Ltd., Berkshire, UK

Tissue-Tek Vaccuum Infiltration Processor	Sakura Finetek Europe B.V., The Netherlands
Triton® X-100	Sigma-Aldrich, Dorset, UK
Zeiss Axiovision KS300 v 3.0	Carl Zeiss AG, Oberkochen, Germany

2.2. Methods

2.2.1. Rat precision-cut lung slices

The general protocol for PCLS generation is outlined in Figure 2.1.

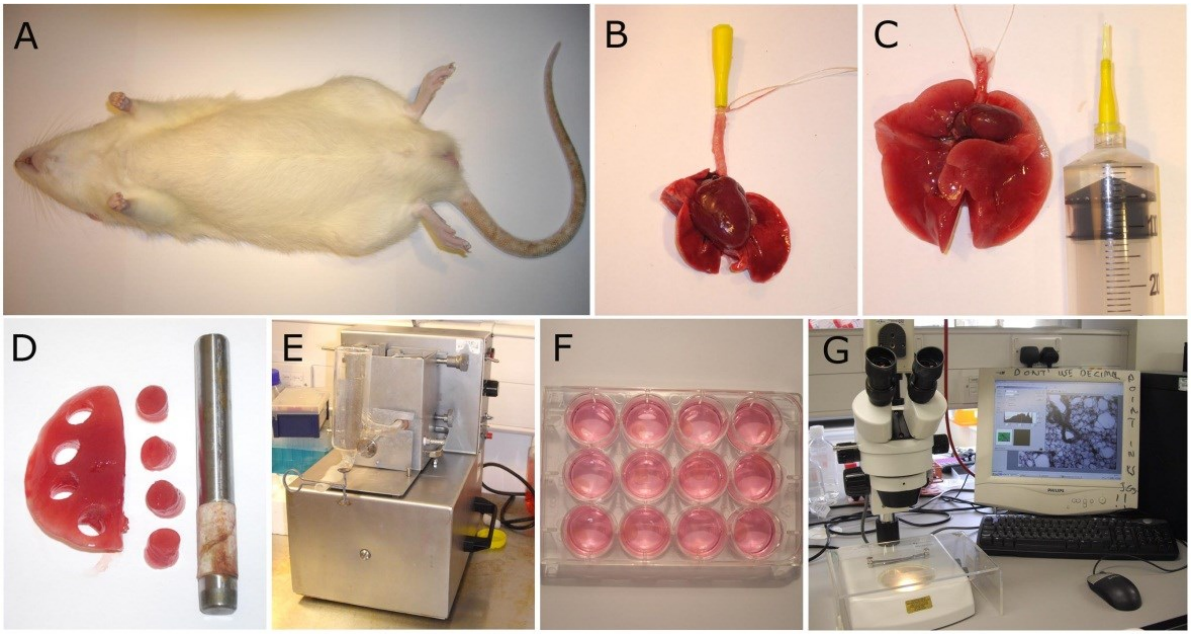


Figure 2.1. **Outline of precision cut lung slices protocol.** (A) Male Sprague-Dawley rats were euthanized. (B) Heart and lungs with trachea were excised, and (C) inflated with 2% ($^{w}/_{v}$) agarose. (D) When the agarose has set, cores were cut through the lobes of the lung (left lung shown). (E) PCLS were cut using a Krumdieck tissue slicer. (F) Slices were transferred to individual wells of a 12-well plate in 1ml incubation buffer. (G) PCLS observed by microscopy and Zeiss software was used to capture images and analyse contractility of the airways.

2.2.1.1. Lung excision

Rat incubation buffer (rat IB) comprised Dulbecco's Modified Eagle's Media (DMEM) supplemented with 100µg/ml (1% $^{v}/_{v}$) penicillin/streptomycin, 2.5µg/ml (1% $^{v}/_{v}$) amphotericin-B, 4mM (2% $^{v}/_{v}$) L-glutamine, and 50µg/ml (0.5% $^{v}/_{v}$) gentamycin. Slicing buffer comprised Hank's Balanced Salt Solution (HBSS) supplemented with 100µg/ml (1% $^{v}/_{v}$) penicillin/streptomycin. To make 2% ($^{w}/_{v}$) agarose, 0.6g agarose Type IX-A was dissolved in 30ml slicing buffer, with gentle heating for 30s.

The studies reported here conform to the UK Animals (Scientific Procedures) Act 1986. Male Sprague-Dawley rats, aged between 12 weeks and 1 year and weighing 400-700g, were euthanized via intraperitoneal injection of 200-300mg sodium pentobarbital (Euthatal®). Using dissecting scissors, an incision was made from the region of the diaphragm up to the jaw to remove the skin from underlying muscle. The skin was then pulled back to allow easier access to the body cavity. Using forceps, the salivary glands and sternohyoid muscle were separated to expose the trachea. The forceps were eased underneath the trachea and, using plain thread, a loose knot was tied around the trachea. Around a third of the way down the trachea, a small incision was made between two rings of cartilage and a cannula inserted. The knot was then tied tightly to hold the cannula secure. To open up the thoracic cavity, the manubrium and sternum were dissected apart and the ribcage removed to expose the heart and lungs. The trachea was then cut above the cannula, and the heart, lungs and trachea carefully dissected out (Fig 2.1 (B)). A 50ml syringe was then filled with 30ml agarose, attached to the cannula and the lungs inflated. When the lungs were adequately inflated (5ml per 100g body weight, up to 30ml) the cannula was removed and the trachea tied off securely to prevent leakage (Fig 2.1 (C)). The lungs were immediately placed on ice for 30-45min, to allow the agarose to solidify.

2.2.1.2. Production of PCLS

Once the agarose had set, 8mm cores were cut transversely through the left lung, and the middle lobe of the right lung (Fig 2.1 (D)). These cores were placed in slicing buffer prior to slicing. A Krumdieck Tissue Slicer was used to generate rat PLCS (Fig 2.1 (D)). All tissue slicing was performed at 4°C. The machine comprises three main components: the microtome assembly, the reservoir assembly and the electrical housing. The microtome assembly contains one motor that controls a rapidly oscillating disposable blade, and a second motor that moves the core of tissue backwards and forwards across the blade. For this protocol, the conditions were set so that the blade only oscillates whilst the tissue is moved across the blade in one direction, and remains still whilst the tissue moves back to its

original position. This reduces damage to the cut surface of the tissue core, compared with a continuously oscillating blade. The speed at which the tissue core is moved across the blade was set at ~40 slices/minute. The reservoir assembly was filled with ~450ml cold slicing buffer, and slices cut between 400-420μm thick.

Three to 4 cores per rat lung were used, and ~12 PCLS cut per core of lung tissue to maximise the variation in lung tissue used for PCLS. This is to better represent a cross-section of the lung, to ultimately limit the impact of any variability of the lung tissue itself on experiments. Single PCLS were placed in individual wells of a 24-well tissue culture plate containing 1ml rat IB (Fig 2.1 (F)). PCLS were examined using an inverted microscope and selected based on the following criteria: 1) slice was fully circular in shape with no major tears or defects, 2) full transverse face of the airway was visible, and 3) airways had intact walls and parenchymal attachments. Once a sufficient number of PCLS had been generated, they were incubated at 37°C (95% air/ 5% ($^{v}/_{v}$) CO_2) for 1h. PCLS were then washed every 30 min for 2.5 h to remove any residual agarose from the airway lumen and inflammatory mediators that may have been generated during the slicing process. PCLS were then incubated overnight at 37°C.

2.2.1.3. Investigating airway function in PCLS

Airways were imaged using a Brunel BMS Video microscope (magnification x4.5) connected to a JVC TK-128-OE colour video camera and Zeiss Axiovision KS300 software (Fig 2.1 (G)). Two custom-made macros were used. The first enabled sequential imaging of the PCLS after inputting the name of the experiment and the number of PCLS to be imaged, and the images generated were thus named and numbered according to the input instructions. The second macro calculated the number of pixels in the airway lumen after it was manually selected from the images. Airway closure was then determined by calculating the percentage difference in airway lumen size, with respect to baseline measurements (see Section 2.2.1.4).

One airway was selected on each PCLS based on fulfilment of the criteria detailed in Section 2.2.1.2. The selected airway was imaged, and a diagram drawn so that the same airway could be identified throughout the experiment. PCLS were numbered and airway images taken sequentially.

2.2.1.4. Protocol for measurement of airway contractility

A summary of this protocol is shown in Figure 2.2. The cholinergic agonist, CCh (section 1.6.1), was used to induce airway closure. CCh was dissolved in rat IB at a stock concentration of 10mM, and aliquots were stored at -20°C until use.

An initial assessment of airway closure was performed to select airways for further study. For this initial assessment, two baseline images of the airways were taken at 5 min intervals before addition of 10μM CCh, and PCLS were incubated for 5 min at 37°C. Images were then captured of the airways following CCh addition. PCLS were washed 3 times in 1ml rat IB to remove CCh, and incubated for at least 1h at 37°C to allow airways to reopen.

Airways were selected for further use based on the following criteria: 1) after addition of 10μM CCh, airway closure was >50% with respect to baseline patency; and 2)

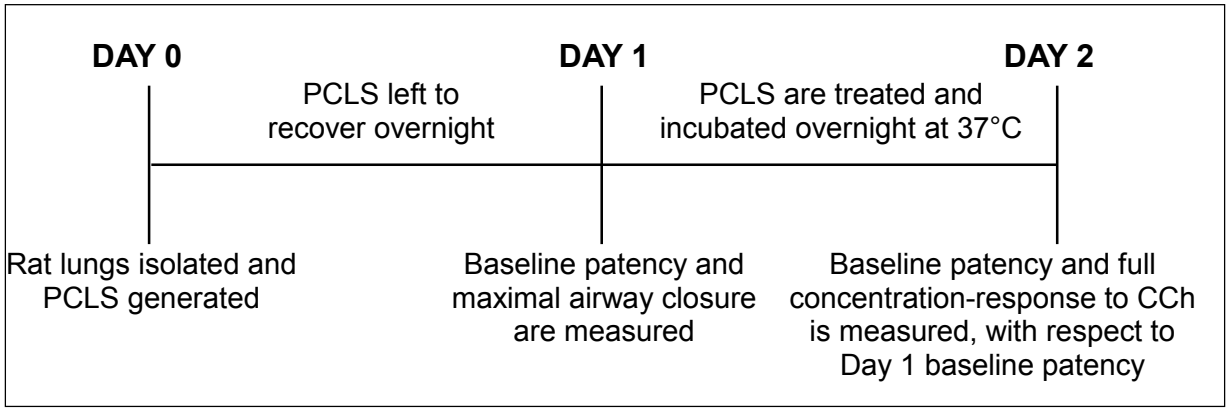


Figure 2.2. **Summary of protocol for treatment of PCLS.** PCLS were generated on Day 0 and left to equilibrate overnight. Baseline measurements were then taken on Day 1, and used to group PCLS for treatments. PCLS were then incubated overnight under selected treatment conditions. Baseline airway patency and closure were then assessed on Day 2, following treatment, compared with baseline measurements taken on Day 1.

PCLS/airways still conform to criteria outlined in Section 2.2.1.2. Airways that met these criteria were then grouped according to the following criteria: 1) a minimum of four PCLS per condition, 2) approximately the same number of PCLS in each group, and 3) the average maximal airway closure of all airways in each group is approximately equal across all groups.

PCLS were then treated as below and in subsequent chapters. Following treatment, PCLS were incubated overnight at 37°C. The next day, PCLS were washed once in 1ml rat IB in preparation for full CCh concentration-response analysis. Example images and graphs are shown in Figure 2.3.

For CCh concentration-response analysis, two baseline images of airways were taken at 5 min intervals, followed by cumulative additions of CCh at the following concentrations: 10nM, 30nM, 100nM, 300nM, 1μM, 3μM and 10μM. PCLS were incubated for 5 min at 37°C between each addition of CCh (Figure 2.3 (A)).

Concentration-response curves were constructed by plotting log [CCh] on the x-axis, against the percentage airway closure on the y-axis. Non-linear regression curves were then constructed, a representative of which is shown in Figure 2.3 (C). From these curves, three parameters can be determined: maximal airway closure (% closure at 10μM CCh), EC_{50} to CCh ([CCh] that produces 50% maximal closure), and baseline airway patency (% closure of airways at baseline, prior to addition of CCh).

Baseline airway patency measurements from before treatment (day 1) were used when calculating airway closure following treatments (day 2). This was to ensure that any differences in baseline airway patency that may have occurred in response to treatment could be identified.

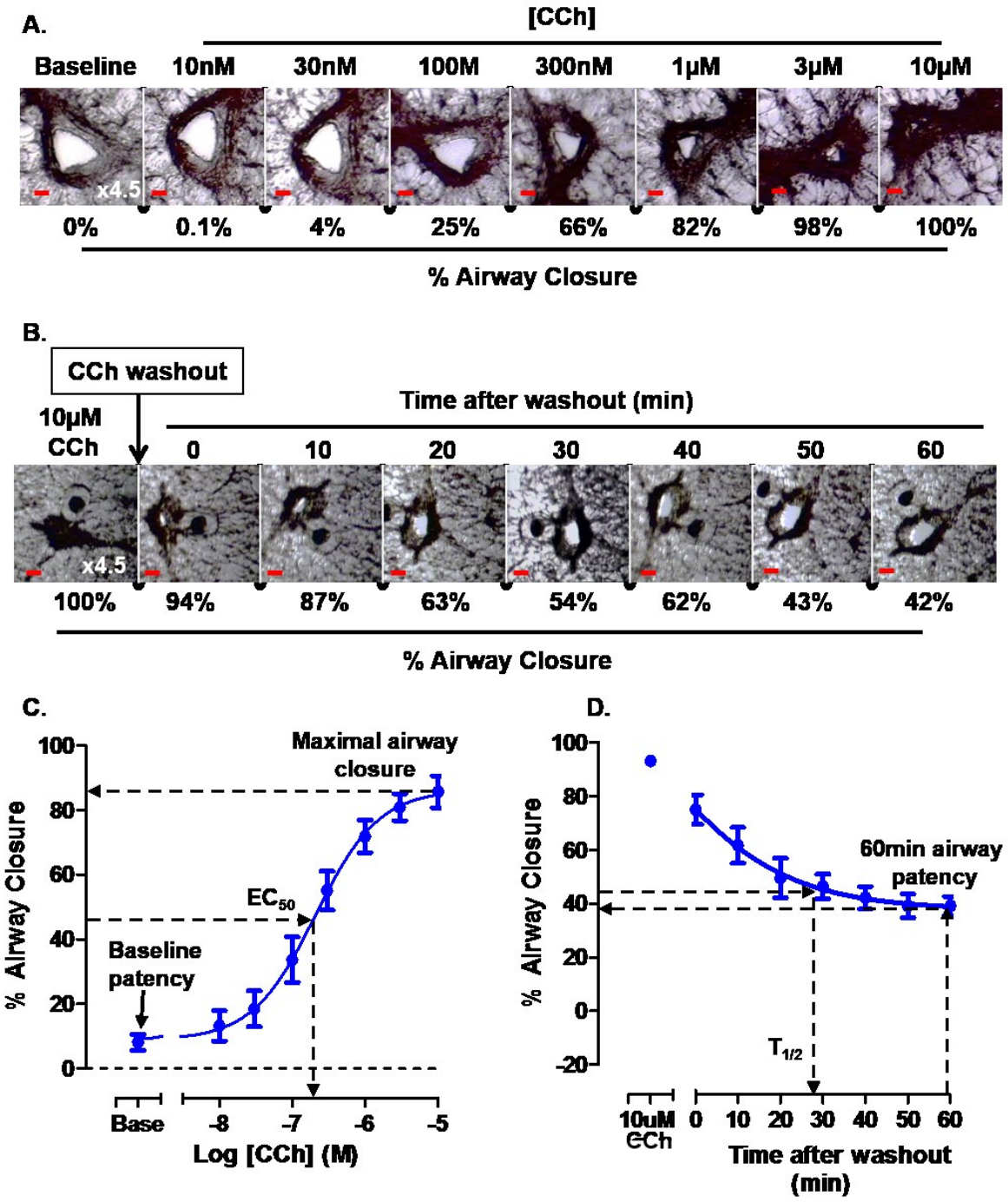


Figure 2.3. **Airway functional analyses.** Representative images of (A) closure of small airways with cumulative additions of CCh, and (B) reopening of airways following CCh washout, over a 60min time-course. (C) Representative concentration-response curve demonstrating measurable parameters: maximal airway closure, EC_{50} to CCh, and baseline airway patency. (D) Representative relaxation curve demonstrating measurable parameters: $t_{1/2}$ and 60min airway patency. Scale bar (red) = 100µm

2.2.1.5. Airway reopening

To investigate the effects of treatment on airway reopening, PCLS were washed 3 times in 1ml rat IB. Following the last wash, airways were immediately imaged, and this time was taken as 0min. Airways were then imaged every 10min for 1h (Figure 2.3 (B)). Due to the time constraints imposed by this experiment, a maximum of 4 airways were investigated per condition, up to 24 airways. Relaxation curves were constructed by plotting time after washout on the x-axis, and % airway closure on the y-axis. A representative curve is shown in Figure 2.3 (D). From this curve, a number of parameters can be determined: $t_{1/2}$ (time taken to reopen to 50% maximal airway closure) and 60 min airway patency (% closure of airways at 60 min).

2.2.2. PCLS viability

2.2.2.1 WST-1 assay

Viability of PCLS was determined using a Cell Proliferation Reagent WST-1. WST-1 is a tetrazolium salt that is cleaved to a soluble formazan product by metabolically active cells. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. The stock WST-1 solution from the kit was aliquoted and stored at -20°C until ready for use.

PCLS were transferred to a 48-well plate and 125µL 10% ($^{v}/_{v}$) WST-1 in rat IB was added to each PCLS. PCLS were then incubated at 37°C for 1 h. After this time, 50µL solution was pipetted, in duplicate, on to a 96-well microplate. This was then read at λ450nm, with reference λ690nm, on a Spectramax Plus 384 plate reader.

Viability of PCLS is expressed as % untreated PCLS viability: WST-1 O.D of treated PCLS is calculated as a percentage of the WST-1 O.D of untreated PCLS. To establish the assay output for non-viable PCLS, as a comparison with PCLS from the experimental treatments, 3-4 PCLS were lysed by macerating in 1ml 1% ($^{v}/_{v}$) Triton™ X-100 in rat IB for 15 min. These were then assayed alongside the rest of the experiment.

2.2.3. PCLS histology

2.2.3.1. Generation of paraffin-embedded sections for histology

Histological sections were generated by Miss. Lorraine Lawrence or Dr. Jie Zhu via the Imperial College Histology Service.

PCLS were fixed in 1ml 4% ($^{v}/_{v}$) formaldehyde in phosphate buffered saline (PBS) for 24h prior to sectioning. Fixed samples were then transferred to 1ml 100% ethanol for transportation to the histologist. Samples were transferred to embedding cassettes between 2 pieces of insert paper. The cassette was then processed using a Tissue-Tek Vaccuum Infiltration Processor, which gradually dehydrates the tissue through a series of ethanol and xylene washes. The tissue was then embedded in paraffin, with the surface of the PCLS facing the surface of the paraffin block. Sections were then cut at 5µm thickness using a Shandon™ Finesse™ 325 Manual Microtome. The paraffin 'ribbon' containing the sections was floated in a 45°C water bath, and then mounted on to slides. These were left to air dry for 30 min, and then baked overnight in a 45-50°C oven.

2.2.3.2. Elastin Van Giesons staining

Elastin Van Giesons (EVG) was used to stain histological sections, to allow investigation of the architecture of the PCLS following various treatments. Staining was performed by Dr. Jie Zhu.

EVG staining was performed as described in (Drury *et al.*, 1967). Briefly, sections were washed twice for 5 min in Histo-clear, to remove paraffin wax from the section. Sections were then gradually rehydrated through 2 washes in 100% industrial methylated spirit (IMS), followed by a wash in 70% ($^{v}/_{v}$) IMS. After rinsing in tap water to remove residual IMS, sections were bleached in Oxalic acid for 5 min, before rinsing in tap water. Sections were briefly rinsed in 95% ethanol, before staining in Elastin stain for 8-24 h. To differentiate the staining, sections were rinsed in 1% ($^{v}/_{v}$) hydrochloric acid (HCl), followed by multiple washes in tap water. Sections were then counterstained in Van Giesons (VG) solution for 1 min, and then differentiated in 95% ethanol for several seconds. Stained sections were then

dehydrated gradually by 30 s rinses in 70, 90 and 100% ($^{v}/_{v}$) IMS, before 3x5 min washes in Histo-clear. Sections were then mounted with coverslips using DPX mountant.

2.2.4. PCLS pre-treatments

PCLS were pretreated according to experimental procedures with human neutrophil supernatants, PPE and H_2O_2, and a number of inhibitor drugs, as described in Chapters 4, 5 and 6, respectively.

2.2.4.1. Neutrophil isolation & stimulation

2.2.4.1.1. Subject selection

Whole blood was obtained from non-smokers, smokers and COPD patients recruited by the National Heart & Lung Institute, London and the Royal Brompton Hospital, London. All subjects gave informed, written consent as approved by the Royal Brompton, Harefield and NHLI Research Ethics Committee.

2.2.4.1.1.1. Non-smokers

Non-smokers were aged between 48-76, with normal baseline lung function (section 2.2.4.1.2), as predicted by age, gender and height. Subjects did not present with a history of respiratory disease or smoking history, and were not on any regular medications.

2.2.4.1.1.2. Smokers

Smokers were aged between 44-69 with normal baseline lung function, as predicted by age, gender and height. Subjects did not present with a history of respiratory disease and were not on any regular medications. Subjects presented with a smoking history of >10pack years, as defined by one pack year being 20 cigarettes per day for one year.

2.2.4.1.1.3. COPD patients

COPD subjects were between 55-89 years old. They presented with an FEV_1: FVC ratio of <0.7, an FEV1 % predicted of <85%, a smoking history of >10pack years, and had not exacerbated for at least 6 weeks prior to giving blood.

2.2.4.1.2. Lung function assessment

Lung function was measured by nurses based in the Asthma Laboratory, Royal Brompton Hospital. Spirometric measurements used to assess lung function were FEV_1 and FVC. To obtain these measurements, subjects were required to inhale deeply before forcibly exhaling as quickly and for as long as possible in to a spirometer (Vitalograph). Values were expressed either as absolute values, or as a percentage of a predicted value based on age, gender and height, as determined by Vitalograph Spirotrac® software and ERS (1993) criteria (Quanjer *et al.*, 1993).

2.2.4.1.3. Leukocyte separation

Blood was processed by members of Professor Louise Donnelly's group as outlined below:

Neutrophils were isolated from whole blood from non-smoker, smoker or COPD patient volunteers. Sixty ml blood was collected via venepuncture at the antecubital fossa in to 3x20ml syringes containing 2.5% ($^{v}/_{v}$) 0.5M of the chelating agent ethylene-diamine-tetra-acetic acid (EDTA). The 3x20ml syringes of blood were transferred to 3x50ml Falcon tubes, and each topped-up to 50ml with 20ml HBSS and 10ml 6% ($^{w}/_{v}$) dextran solution in 0.9% ($^{w}/_{v}$) NaCl. Red blood cells were left to sediment at room temperature for 20min. Following this, the white cell-rich supernatant was transferred to clean 50ml Falcon tubes, topped up with to 50ml with HBSS and centrifuged at 350*g* for 10 min at room temperature.

Different subtypes of leukocytes were separated using a discontinuous Percoll gradient. A 100% Percoll solution was prepared using 10% ($^{v}/_{v}$) 9% ($^{w}/_{v}$) NaCl, to make an isotonic solution. This was then diluted in 0.9% ($^{w}/_{v}$) NaCl to make three different stock solutions: 81, 68 and 55% ($^{v}/_{v}$) Percoll. To create the gradient, 4ml 81% ($^{v}/_{v}$) Percoll was added to 2x15ml

Falcon tubes, and 4ml of the 68% ($^{v}/_{v}$) Percoll was layered over the top. The cell pellet from above was resuspended in 3ml 55% ($^{v}/_{v}$) Percoll, and layered on the top of the pre-prepared gradient. The tubes were then centrifuged at 750*g* for 25 min at room temperature, with reduced acceleration and deceleration.

The different subtypes of leukocytes were distributed along the Percoll gradient based on their density: peripheral blood mononuclear cells (PBMC) collect at the 55/68% ($^{v}/_{v}$) interphase, and PMN cells at the 68/81% ($^{v}/_{v}$) interphase. Once the Percoll gradients have been centrifuged, PMN were isolated from their respective layer and topped-up to 50ml with PBS and centrifuged at 350*g* for 5 min. The supernatant was aspirated and the PMN pellet resuspended in 2ml distilled water for 30 s to lyse any remaining erythrocytes. The cell suspension was then topped-up to 50ml with HBSS. To count neutrophils, the cell suspension was diluted 50% ($^{v}/_{v}$) in Kimura stain (Kimura *et al.*, 1973), a differential cell stain that stains nuclei. A haemocytometer was used to count neutrophils, which were then resuspended in rat IB.

2.2.4.1.4. Neutrophil stimulation

Neutrophils were resuspended at $10x10^{6}$ cells/ml in rat IB, and cell suspension divided into an appropriate number of Falcon tubes based on the number of stimuli that were to be administered (non-stimulated, 10 or 100μM fMLP). In all experiments, at least 1ml of the cell suspension was kept as a non-stimulated control sample, for comparative purposes in future experiments. Cells may become agitated during the isolation procedure and so, to reduce any priming effects that this may have, neutrophils were 'rested' for ~1h prior to stimulation: 30min at 4°C and 30min at room temperature. Cytochalasin B, an actin depolymerisation inhibitor that aids degranulation (Honeycutt *et al.*, 1986), was added to neutrophils at a final concentration of 10μM, and neutrophils were incubated for 10min in a 37°C water bath. The cell suspension was then stimulated with either 10 or 100μM fMLP. The cell suspension was incubated for 10 min in a 37°C water bath. Following this, neutrophils were centrifuged for 5

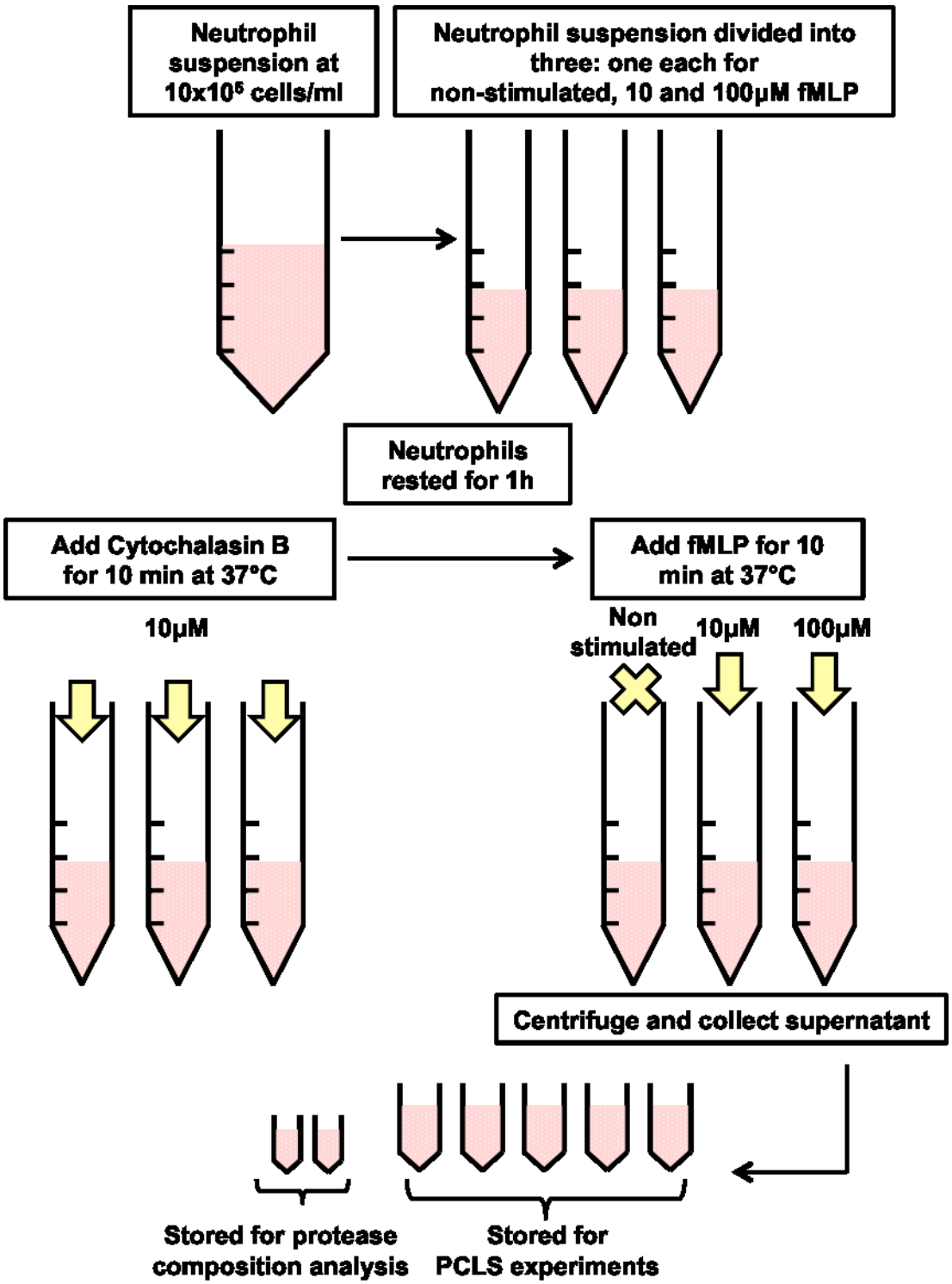


Figure 2.4. Summary of protocol for stimulation of neutrophils and collecting supernatants.

min at 350*g*. One ml of the supernatant was split into 2x500µL aliquots, for use in characterisation of the supernatants; whilst the remainder of the supernatant was divided into 1ml aliquots. Neutrophil supernatants were stored at -80°C until further use. This is summarised in Figure 2.4.

2.2.4.2. PPE

Protocols and procedures for pre-treatment of PCLS with PPE are detailed in Chapter 4.

2.2.4.3. H_2O_2

Protocols and procedures for pre-treatment of PCLS with H_2O_2 are detailed in Chapter 4.

2.2.5. Neutrophil Protease Assays

2.2.5.1. Neutrophil elastase activity assay

Assay was performed in HBSS supplemented with 0.1M HEPES and 0.5M NaCl, and adjusted to pH 7.4. HLE/NE was reconstituted in 50% ($^{v}/_{v}$) 0.02M NaO_{AC}/glycerol at 0.0072U/µl. Aliquots of 10µl (0.072U) were stored at -20°C. Before use, 90µl of buffer was added to aliquots, to make a stock of 0.0072U. N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (N-MeO-Suc-AAPV-pNA) was reconstituted at 200mM in dimethyl sulfoxide (DMSO), and stored at -20°C in 10µl aliquots.

NE dilutions, from 7.2 x 10^{-4} U/µl to 1.1 x 10^{-5} U/µl, were prepared from stock in assay buffer and 50µl added, in duplicate, to a 96-well plate. Neutrophil supernatants were diluted in assay buffer to either 50% ($^{v}/_{v}$), for non-stimulated samples, or 10% ($^{v}/_{v}$), for samples stimulated with fMLP, in order to ensure NE activity levels would fit on the standard curve. Fifty µl was then added, in duplicate, to a 96-well plate. N-MeO-Suc-AAPV-pNA was diluted to 1mM in assay buffer, and 50µl added to all samples/standards, at a final concentration of 0.5mM, immediately before the plate was read on a Spectramax Plus 384 plate reader. Absorbance at λ405nm was measured every 10 s for 30 min, and an activity curve was generated using Softmax Pro software. Initial rate of reaction (Vmax) was calculated from the curve, and plotted against known NE concentrations to generate a standard curve

(Figure 2.5 (A)), and lower limit of detection determined ($1.1x10^{-5}$ U/µl). Sample NE concentrations were then interpolated from the standard curve.

2.2.5.2. MMP-9 activity assay

Fluorokine E® Human Active MMP-9 kit was used to quantify the enzymatic activity of human MMP-9 in neutrophil supernatants. MMP-9 is secreted as a 92kDa zymogen (pro-MMP-9), and this is converted into an 82kDa active form by proteolytic removal of the *N*-terminal pro-region. Both forms are quantified by the kit. Supernatants were thawed and all reagents brought to room temperature prior to use. The protocol was performed as per manufacturer's instructions.

As the assay quantifies both pro-MMP-9 and active MMP-9 activity, neutrophil supernatants were duplicated in each assay: half to quantify endogenous active MMP-9 in the supernatant, and half to quantify total active MMP-9 in the supernatant. Pro-MMP-9 concentration can then be deduced by subtracting endogenous active MMP-9 from total active MMP-9. Briefly, neutrophil supernatants, diluted 1:500 in order to fit within the linear portion of the standard curve, and pro-MMP-9 standards serially-diluted from 16 to 0.25ng/ml, were added to a black 96-well plate, pre-coated with anti-MMP-9 antibody. Anti-MMP-9 antibody was specific for natural and recombinant MMP-9, and there was no significant cross-reactivity observed with any factors tested by the manufacturer. These were incubated for 2h at room temperature. The plate was then washed to remove unbound sample. An activation reagent, p-aminophenylmercuric acetate (AMPA), which converts pro-MMP-9 into its active form, was added to the MMP-9 standards, and half of the duplicated neutrophil supernatant samples, to measure total MMP-9 in samples. The plate was incubated for a further 2h at 37°C in a humidified environment, protected from light. Following additional washes, a fluorogenic substrate linked to a quencher molecule was then added to all wells and incubated overnight at 37°C in a humidified environment, protected from light. Active MMP-9 cleaves the fluorophore from the quencher molecule, allowing a fluorescence signal to be emitted that is directly proportional to the amount of active MMP-9

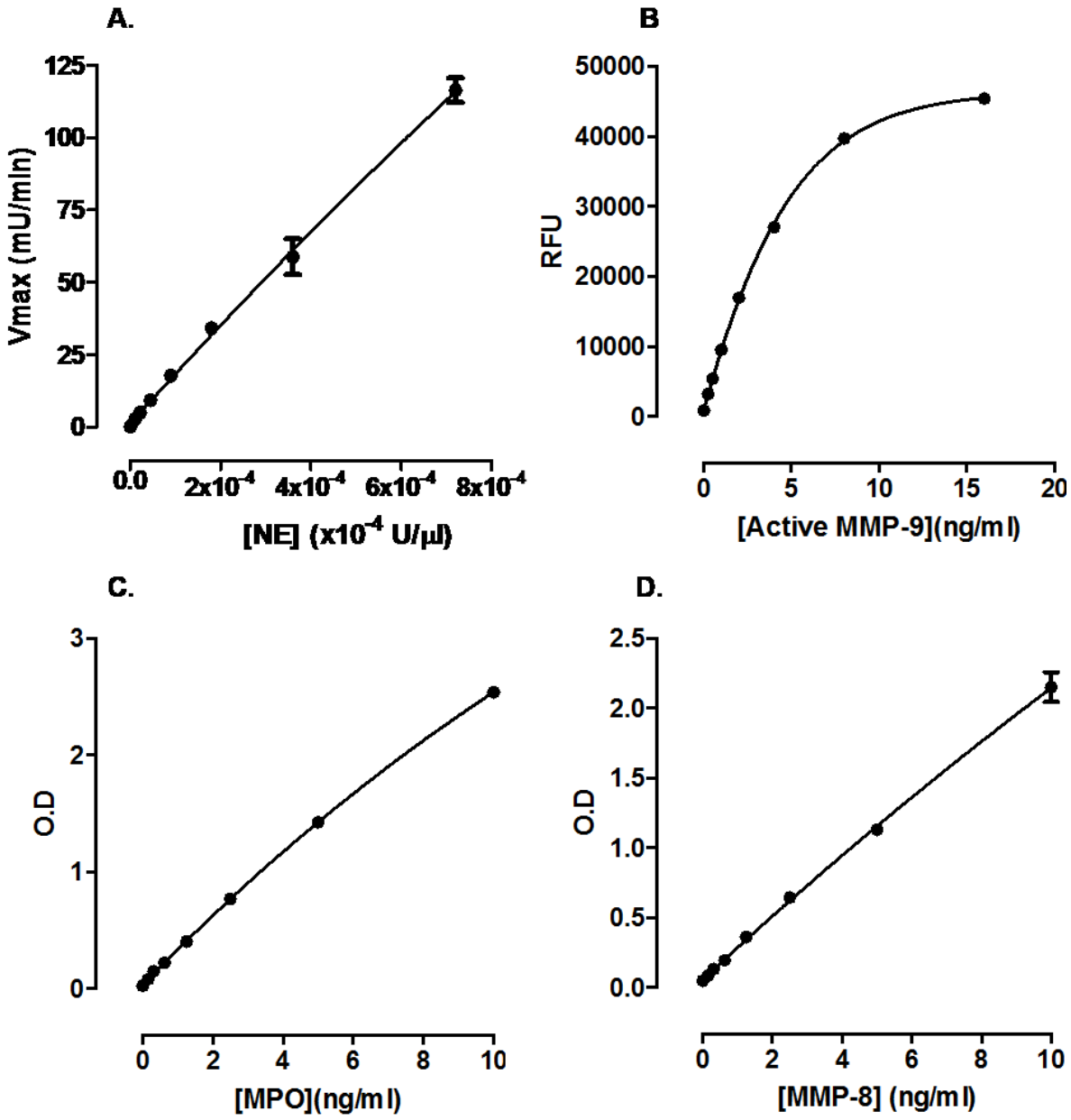


Figure 2.5. **Standard curves of protease assays.** (A) NE concentration against Vmax (rate of reaction, mU/min), lower limit of detection $1.1x10^{-5}$ U/µl. (B) Active MMP-9 concentration against fluorescence at excitation λ320, emission λ405nm, lower limit of detection 0.005ng/ml. RFU= relative fluorescent units. (C) MPO concentration against optical density (O.D), lower limit of detection 0.062ng/ml. (D) MMP-8 concentration against O.D at λ450-540nm, lower limit of detection 0.06ng/ml. Data are mean ± SEM of duplicate readings.

present in the sample. This was read the following day using a FLUOstar Optima fluorometer at excitation λ320nm and emission λ405nm. A standard curve was generated relating fluorescence to amount of active MMP-9, and sample MMP-9 concentrations were then interpolated from this standard curve (Figure 2.5 (B)), and lower limit of detection determined (0.005ng/ml).

2.2.5.3. MPO and MMP-8 immunoassay

Total MPO and MMP-8 were measured in neutrophil supernatants using Quantikine® immunoassay kits, as per manufacturer's instructions. For MPO, supernatants were diluted 1:100 for non-stimulated samples, and 1:2000 for fMLP-stimulated samples; and for MMP-8, supernatants were diluted 1:100 for non-stimulated samples, and 1:500 for fMLP-stimulated samples, in order to ensure that all sample MMP-8/MPO concentrations fit within the linear portions of the standard curve. These, along with serially-diluted recombinant MPO or MMP-8 standards (0.156-10ng/ml), were added to a 96-well plate pre-coated with either anti-MPO or anti-MMP-8 antibodies. MPO antibody was specific for human MPO protein, and cross-reacts ~1.8% with human eosinophil peroxidase protein (as determined by the manufactuer); and MMP-8 antibody was specific to human natural and recombinant MMP-8 and did not cross-react with any factors tested by the manufacturer. These were then incubated for 2h at room temperature. Unbound sample was washed off and either MPO or MMP-8 conjugates, which were enzyme-linked polyclonal antibodies specific for either protease, were added to the wells and incubated for a further 2 h at room temperature. Following further washes, substrate solution was added to the wells and incubated for 30 min at room temperature, protected from light. During this time, colour develops that is proportional to the amount of protease in the wells. Stop solution was added after 30min, and absorbance read at λ450nm, with a reference λ540nm. A standard curve was generated relating optical density (O.D) to amount of MPO/MMP-8, and sample MPO/MMP-8 concentrations were then interpolated from this standard curve (Figures 2.5 (C & D)), and lower limit of detections determined (MPO: 0.062ng/ml; MMP-8: 0.06ng/ml).

2.2.6. Enzyme-linked Immunosorbant assays (ELISA)

Measurement of CINC-1, TNF-α, and PGE_2 were performed as described in the Materials and Methods of Chapter 5 - section 5.2.6.

2.2.7. Human PCLS

2.2.7.1. Subject selection

Subject selection for human tissue samples was undertaken as described in the Materials and Methods of Chapter 7 - section 7.2.1.

2.2.7.2. Human tissue inflation and PCLS generation

Tissue inflation and PCLS generation were performed as described in the Materials and Methods of Chapter 7 - section 7.2.2.

2.2.8. Data Analysis

Comparisons between groups (subject groups, PCLS treatments, protease concentrations, etc) were conducted using Mann-Whitney t-tests, or Kruskall-Wallis (ANOVA) tests followed by Dunn's post-correctional tests. For all statistical tests, $p<0.05$ was considered significant. All statistical analysis was performed using GraphPad Prism v5.0 software.

Chapter 3.

Protease Composition of Neutrophil Supernatants

3. Protease Composition of Neutrophil Supernatants

3.1. Introduction

In response to inflammatory signals, circulating neutrophils are activated and migrate to the site of injury, where they degranulate and release a plethora of proteases and ROS (Amulic *et al.*, 2012). The contents of this antimicrobial armamentarium are held within four distinct granule subsets in the neutrophil cytoplasm, each containing different antimicrobial products (see Introduction, section 1.4.1.2). The formation and mobilisation of these granules is a tightly-regulated process: granules are formed at different stages of neutrophil maturation, and the timing of their formation denotes their propensity for exocytosis (Borregaard *et al.*, 1997). For example, secretory vesicles, the last of the granule subsets to be formed, are readily exocytosed upon relatively mild stimulation; whereas azurophil granules, which are the first to be formed, are the last granules to exocytose, and only do so upon maximal stimulation. This organisation of granule contents and mobilisation, firstly, prevents contents that may react with each other coming in to contact; and, secondly, enables neutrophils to elicit an appropriate response based on the level of stimulation (Borregaard *et al.*, 1997).

One of the leading hypotheses regarding COPD onset is that of a protease-antiprotease imbalance (see Introduction, Section 1.4.2.4): increased numbers and aberrant activity of neutrophils in the lungs results in an excess of protease activity that cannot be adequately quenched by endogenous antiproteases. Evidence to support this hypothesis came from the observation that patients with a deficiency in α1-AT, the main inhibitor of NE, develop COPD-like symptoms (Laurell *et al.*, 1963). In addition, hamsters instilled with both NE (Senior *et al.*, 1977) and proteinase-3 (Kao *et al.*, 1988) developed emphysematous lesions in the lungs.

In addition to *in vivo* evidence, there are increased concentrations of a number of neutrophil proteases in BALF and sputum of COPD patients, including NE (Yoshioka *et al.*, 1995), MPO (Yamamoto *et al.*, 1999), and MMP-8 and -9 (Betsuyaku *et al.*, 1999, Vernooy *et al.*, 2004). NE has also been implicated in having a number of roles in development of COPD

pathophysiology: it potentiates mucus hypersecretion by upregulating a number of mucin genes (Fischer *et al.*, 2003, Kuwahara *et al.*, 2005, Zhou *et al.*, 2013); it enhances the inflammatory response by inducing transcription and release of CXCL8 (Kuwahara *et al.*, 2006); and contributes to airway hyperresponsiveness and remodelling by inducing release of PGE_2 (Perng *et al.*, 2003), and TGF-β1 (Lee *et al.*, 2006). The activity and concentrations of neutrophil proteases can, therefore, determine the balance between a supportive, antimicrobial attack against invading pathogens, and a detrimental assault on healthy host tissue that is indicative of COPD pathophysiology.

FPRs are G-protein coupled receptors that are tightly associated with the cell membrane (Panaro *et al.*, 1999). They are present on all mature neutrophils (Allen *et al.*, 1992), and there are estimated to be ~53,000 ± 13,000 on resting cells (Sklar *et al.*, 1984). fMLP binds to FPRs on neutrophils, and triggers a cascade of biochemical events, such as cell polarisation, mobilisation, respiratory burst and degranulation (Panaro *et al.*, 1999). Wittmann *et al.*, (2002) reported that stimulation with 100nM fMLP activates ~50% of neutrophils, and this is calcium-dependent. A number of compounds are thought to augment neutrophil responses to fMLP: pre-incubation with LPS results in a four-fold increase in FPR expression (Allen *et al.*, 1992), and increased sensitivity and maximal responses, as determined by measurement of vitamin B_{12} binding protein, which is released by specific (secondary) granules (Chadwick *et al.*, 1992). Despite some conflicting reports, there is little evidence of increased numbers of FPRs on neutrophils in COPD; however this line of investigation is often confounded by differences in time-course, level of stimulation, methodology and receptor internalization (see section 1.6.2).

3.1.2. Chapter Hypothesis

From the above introductory observations, the hypothesis for this chapter was, therefore, that:

“Stimulation of neutrophils with fMLP will induce release of proteases, and there will be differences in protease composition between neutrophil supernatants from non-smokers, smokers and COPD patients”.

3.1.3. Aims

In order to investigate the above hypothesis, the aims for this chapter were to:

1. Obtain supernatants from peripheral blood neutrophils from non-smokers, smokers and COPD patients, both non-stimulated and stimulated with the bacterial peptide fMLP.
2. Measure the activity/concentration of NE, MPO, MMP-8 and -9 in the supernatants, and compare levels between subject groups.
3. Examine the relationship between protease activity/concentration and clinical parameters of the subjects.

3.2. Methods

3.2.1. Neutrophil isolation

Non-smokers, smokers and COPD patients were selected based on criteria outlined in 2.2.4.1.1. Sixty ml blood was taken, and neutrophils were isolated and stimulated as described in section 2.2.4.1. Briefly, red blood cells were sedimented and the leukocyte suspension centrifuged down a discontinuous Percoll gradient. PMN were isolated from the 68/81% ($^{v}/_{v}$) interface, and neutrophils were counted and resuspended in rat incubation buffer.

3.2.2. Collection of neutrophil supernatants for PCLS experiments

Neutrophil supernatants were isolated as described in Materials & Methods, section 2.2.4.1.4. Aliquots (500µL) were thawed to room temperature (or 37°C for NE activity assay) prior to assessing protease composition.

3.2.3. fMLP concentration-response

Neutrophils were resuspended at $20x10^6$ cells/ml, and rested for ~1h. Neutrophils were then stimulated with cytochalasin B (final concentration 10µM) for 10 min at 37°C. To make fMLP concentrations, stock fMLP (10mM) was diluted in 500µl rat incubation buffer to 2x the desired concentration for the concentration response in 1.5ml Eppendorfs. Following addition of cytochalasin B, 500µl neutrophil suspension was then added to the Eppendorfs. The concentration of fMLP was 1x the desired concentration, and the concentration of neutrophils was $10x10^6$ cells/ml. Neutrophils were incubated for 10min at 37°C. Neutrophils were then centrifuged for 5 min at 350*g* and supernatant collected and stored at -80ºC. This protocol is outlined in Figure 3.1.

3.2.4. Assessment of NE activity of neutrophil supernatants

NE activity was measured as described in Chapter 2, Section 2.2.5.1.

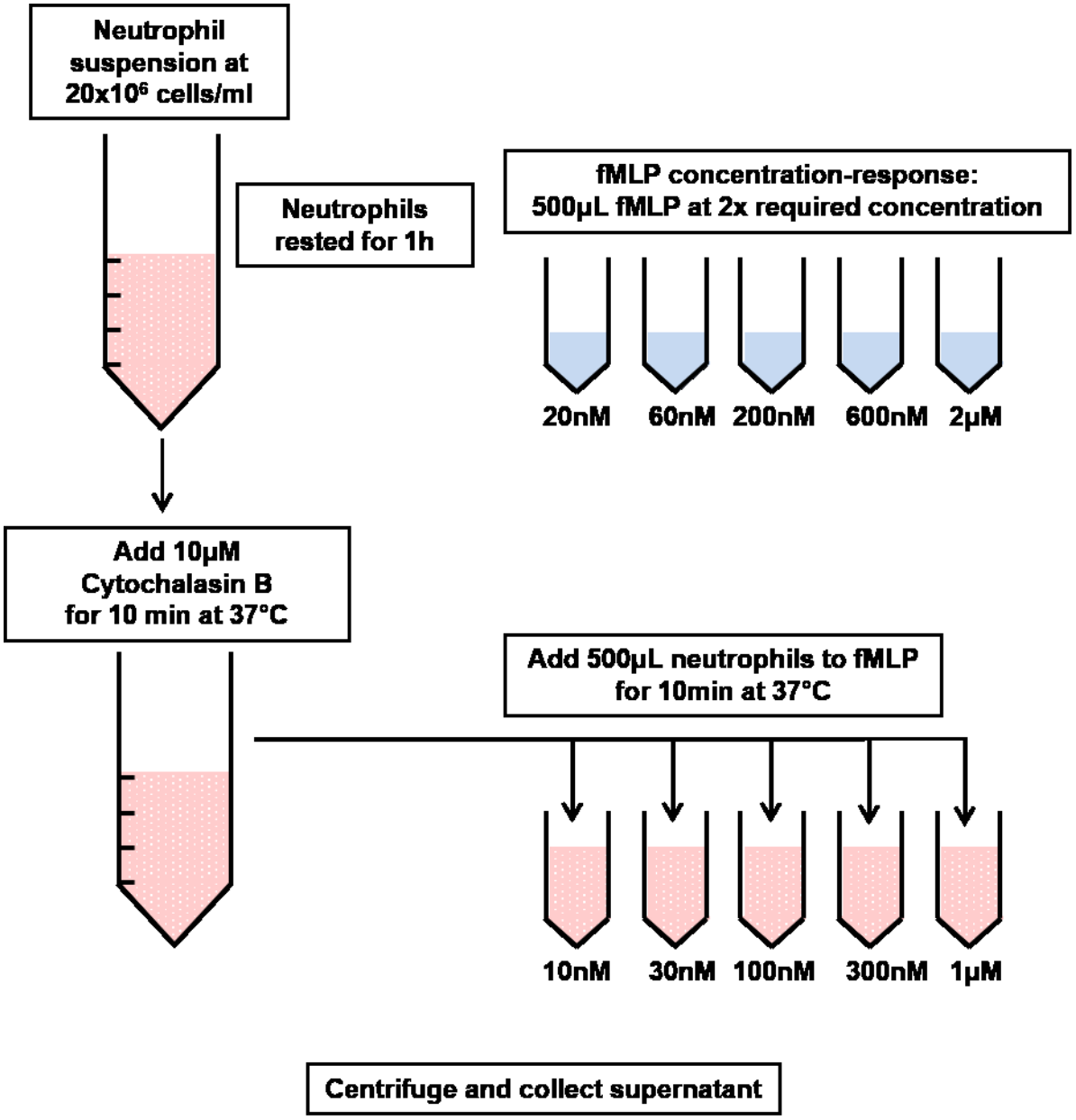


Figure 3.1. Protocol outlining treatment of neutrophils for fMLP concentration-response.

3.2.5. Assessment of MMP-9 concentration of neutrophil supernatants

MMP-9 concentrations were measured as described in Chapter 2, Section 2.2.5.2.

3.2.6. Assessment of MMP-8 and MPO concentration of neutrophil supernatants

MMP-8 and MPO concentrations were measured as described in Chapter 2, Section 2.2.5.3.

3.2.7. Data Analysis

In this chapter, patient demographics were compared using a Kruskal-Wallis with Dunn's post-hoc tests. Protease concentration/activity was determined by interpolation from appropriate standard curves (Figure 2.5). Concentration/activity data were not all normally distributed, as determined by the D'Agostino-Pearson omnibus test. Consequently, the Kruskal-Wallis test was used, with Dunn's post-hoc tests. For familiarity of presentation, data are displayed as mean ± SEM. The null hypothesis of no significant differences between groups was rejected at $p<0.05$.

3.3. Results

3.3.1. NE activity

3.3.1.1. Subject demographics

NE activity was measured in peripheral blood neutrophil supernatants from 21 non-smokers, 18 healthy smokers and 19 COPD patients (Table 3.1). COPD patients were significantly older (by ~8years) and had significantly worse lung function, compared with non-smokers and smokers, as determined by FEV_1, FEV_1 % predicted and FEV_1/FVC, which were reduced by about 30-50%. COPD patients also had significantly greater smoking history, compared with healthy smokers.

Table 3.1. Subject demographics for neutrophil supernatants used to measure NE activity.

	Non-smokers	Smokers	COPD
N	21	18	19
Age (years)	60 ± 2	59 ± 1	68 ± 2$^{*/\#\#}$
Gender (m/f)	8/13	9/9	9/9
Smoking history (pack years)	N/A	34.0 ± 4.7	45.6 ± 3.6$^{\#}$
FEV_1 (L)	2.8 ± 0.2	2.7 ± 0.2	1.3 ± 0.1$^{\$\$\$}$
FEV_1 predicted (%)	102.2 ± 3.9	94.7 ± 3.1	51.4 ± 4.5$^{\$\$\$}$
FVC (L)	3.8 ± 0.2	3.8 ± 0.3	2.7 ± 0.2$^{**/\#}$
FEV_1/FVC (L)	0.75 ± 0.02	0.73 ± 0.02	0.48 ± 0.04$^{\$\$\$}$

Data are mean ± SEM. $^{*/\#}p<0.05$ non-smokers/smokers *vs*. COPD; $^{**/\#\#}p<0.01$ non-smokers/smokers *vs*. COPD; $^{\$\$\$}p<0.001$ non-smokers and smokers *vs*. COPD.

One pack year is defined as smoking 20 cigarettes per day for one year. NE= Neutrophil elastase; FEV= Forced expiratory volume; FVC= Forced vital capacity

3.3.1.2. fMLP concentration-response

In order to identify the appropriate concentration to stimulate neutrophils and elicit a maximal response, an fMLP concentration-response was performed and NE activity measured. NE activity was chosen for two reasons: firstly, the assay is fast, high-throughput and inexpensive; and, secondly, NE is stored in azurophil granules, which are the last to degranulate; therefore NE activity in neutrophil supernatants will denote maximal neutrophil responses.

Neutrophils from each subject group were stimulated with increasing concentrations of fMLP (1nM-10μM) for 10 min, prior to supernatants being isolated and assayed (Figure 3.2). NE activity increased in a concentration-dependent manner, and plateaued at around 1μM fMLP, with an overall increase of ~20-fold above non-stimulated. There were no significant differences in the maximal NE activity between subject groups (non-smoker 1.6 ± 0.2, n=9; smoker 1.6 ± 0.2, n=7; COPD 1.7 ± 0.2 x 10^{-3} U/μl, n=11). There was a reduction in EC_{50} to

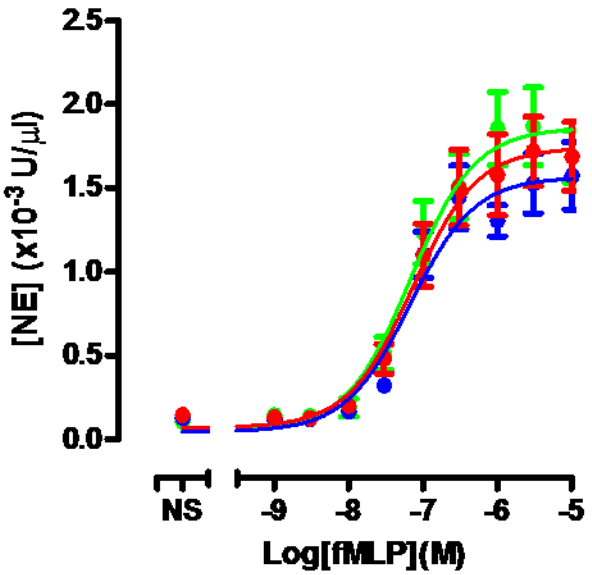


Figure 3.2. Effect of fMLP on NE activity in peripheral blood neutrophils in the three subject groups (concentration-response). Non-smoker (●), n=9; smoker (●), n=7; COPD patients (●), n=11. NS = non-stimulated. Data are mean ± SEM. All data points above 100nM fMLP were significantly increased from NS (p<0.05-0.0001).

fMLP in neutrophils from smokers and COPD patients, compared with non-smokers; however this was not significant (non-smoker 193 ± 123, n=9; smoker 78 ± 21, n=7; COPD 97 ± 17nM, n=11 fMLP).

The fMLP concentration-response above suggests that there was maximal NE released from neutrophils following stimulation with 10μM fMLP. However, when incubated with rat PCLS, supernatants from neutrophils stimulated with 10μM fMLP had only minimal effects on small airway function (see Chapter 4). As such, neutrophils were also stimulated with 100μM fMLP to investigate whether the effects on rat small airway function could be augmented when incubated with these supernatants, and whether protease release in these supernatants was further elevated, compared with supernatants from neutrophils stimulated with 10μM fMLP. Therefore, proteases were measured in supernatants from non-stimulated neutrophils, and those from neutrophils stimulated with either 10 or 100μM fMLP.

3.3.1.3. Neutrophil supernatants

In non-smoker neutrophil supernatants, there was a significant increase in NE activity with both 10 and 100μM fMLP stimulation, compared with non-stimulated supernatants, with increases in activity approximately 17-fold above non-stimulated values (Figure 3.3 (A)). There was similar NE activity in supernatants from neutrophils stimulated with 10 and 100μM fMLP, therefore this was not a concentration-dependent response. A similar pattern was observed for smokers and COPD patients. There were, however, no significant differences in NE activity between 10 and 100μM fMLP stimulation in any of the subject groups. There were also no significant differences in NE activity between subject groups, either in non-stimulated supernatants, or after either 10 or 100μM fMLP stimulation.

3.3.2. MMP-8

3.3.2.1. Subject demographics

Compared with non-smokers and smokers, COPD patients were significantly older, by ~8years, and had significantly worse lung function, as determined by FEV_1, FEV_1 %

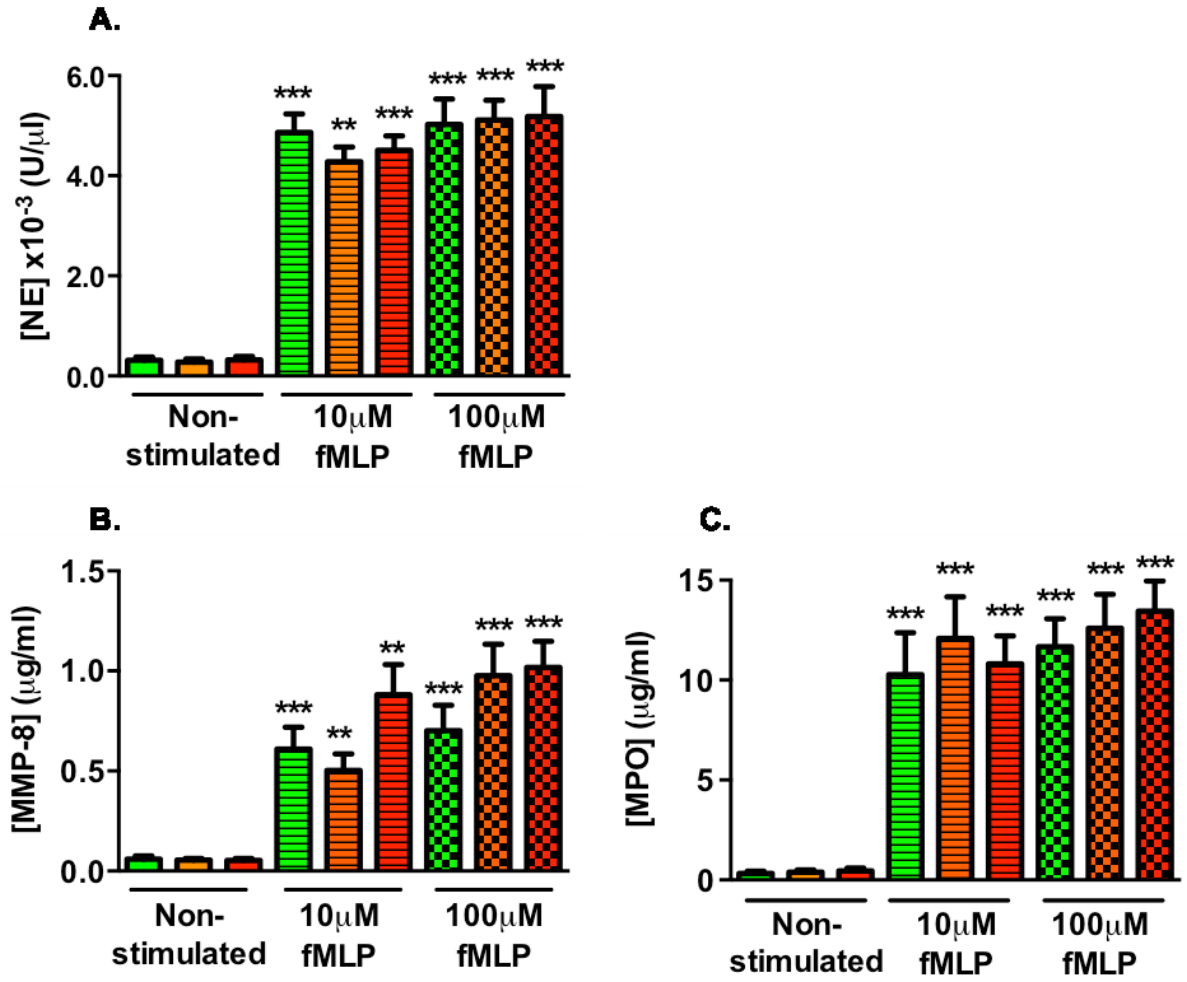


Figure 3.3. Effect of fMLP on protease composition of neutrophil supernatants. Non-smoker (●), smoker (●), COPD (●). (A) neutrophil elastase (NE), non-smoker: n=12-16; smoker: n=13-14; COPD: n=13-17. (B) MMP-8, non-smoker: n=10-12; smoker: n=12; COPD: n=10-12. (C) MPO, non-smoker: n=9-12; smoker: n=12; COPD n=12. Data are mean ± SEM. **/*** p<0.01/p<0.001 *vs.* non-stimulated, as determined by Kruskal-Wallis and Dunn's post-hoc tests.

predicted and FEV_1/FVC, with an ~40-70% reduction in parameters (Table 3.2). COPD patients also had significantly greater smoking history, compared with healthy smokers, with an ~37% greater pack year history.

3.3.2.2. Neutrophil supernatants

In non-smokers, there was a significant increase in MMP-8 concentration after both 10 and 100μM fMLP stimulation, compared with non-stimulated samples (Figure 3.3 (B)). A step-

wise increase between 10 and 100μM fMLP was observed in smokers and COPD patients, with an ~12-20 fold increase compared with non-stimulated values. Between subject groups, there was a trend for higher MMP-8 concentrations in COPD neutrophil supernatants after 10μM fMLP stimulation (~1.5 fold increase), compared with non-smokers and smokers; however this did not reach significance. Similarly, there is a trend indicating higher MMP-8 concentrations after 100μM fMLP stimulation in smoker and COPD neutrophil supernatants, compared with non-smoker supernatants; however, this was also not significant.

Table 3.2. Subject demographics for neutrophil supernatants used to measure MPO and MMP-8 concentrations.

	Non-smokers	Smokers	COPD
N	21	18	18
Age (years)	62 ± 2	60 ± 2	68 ± 2$^{\#}$
Gender (m/f)	8/13	9/9	9/9
Smoking history (Pack years)	N/A	32.5 ± 4.7	44.7 ± 3.6$^{\#}$
FEV_1 (L)	2.8 ± 0.2	2.7 ± 0.2	1.2 ± 0.1$^{\$\$\$}$
FEV_1 predicted (%)	102.3 ± 4.0	94.8 ± 3.1	48.9 ± 4.0$^{\$\$\$}$
FVC (L)	3.8 ± 0.2	3.8 ± 0.3	2.6 ± 0.2$^{**/\#}$
FEV_1/FVC (L)	0.74 ± 0.01	0.72 ± 0.01	0.53 ± 0.05$^{***/\#}$

Data are mean ± SEM. $^{*/\#}p<0.05$ non-smokers/smokers *vs*. COPD; $^{**/\#\#}p<0.01$ non-smokers/smokers *vs*. COPD; $^{\$\$\$}p<0.001$ non-smokers and smokers *vs*. COPD.

One pack year is defined as smoking 20 cigarettes per day for one year. MMP= Matrix metalloprotease; MPO= Myeloperoxidase; FEV= Forced expiratory volume; FVC= Forced vital capacity

3.3.3. Myeloperoxidase

3.3.3.1. Subject demographics

Subject demographics were the same as for MMP-8 (Table 3.2).

3.3.3.2. Neutrophil supernatants

In non-smokers, there was a significant increase in MPO concentration in neutrophil supernatants after both 10 and 100μM fMLP stimulation, compared with non-stimulated supernatants (Figure 3.3 (C)). There was a similar pattern observed for both smokers and COPD patients, with an approximately 30-35 fold increase compared with non-stimulated values. In all subject groups, the supernatant concentration of MPO was quite similar after 10 and 100μM fMLP stimulation. There were also no significant differences in MPO concentration between the three subject groups, either in non-stimulated supernatants, or after either 10 or 100μM fMLP stimulation.

3.3.4. MMP-9

3.3.4.1. Subject demographics

Compared with non-smokers and smokers, COPD patients were significantly older, by ~8years, and had significantly worse lung function, as determined by FEV_1, FEV_1 % predicted and FEV_1/FVC, with an ~50% decrease in parameters (Table 3.3). COPD patients also had significantly worse smoking history, by ~20 pack years, compared with healthy smokers.

3.3.4.2. Pro MMP-9

In non-smokers, there was a significant increase in pro-MMP-9 concentrations after 10μM fMLP stimulation, compared with non-stimulated supernatants (Figure 3.4 (A)). There was a similar pattern observed in smokers and COPD patients, with an approximately 3.5-7 fold increase, compared with non-stimulated values. When stimulated with 100μM fMLP, there was an increase in pro MMP-9 compared with non-stimulated supernatants; however a 35-

Table 3.3. Subject demographics for neutrophil supernatants used to measure pro- and active- MMP-9 concentrations.

	Non-smokers	Smokers	COPD
N	19	17	18
Age (years)	61 ± 2	59 ± 1	67 ± 2$^{\#}$
Gender (m/f)	7/12	9/8	7/11
Smoking history (Pack years)	N/A	33.2 ± 5.1	51.5 ± 5.7$^{\#\#}$
FEV_1 (L)	2.8 ± 0.2	2.7 ± 0.2	1.2 ± 0.1$^{\$\$\$}$
FEV_1 predicted (%)	102.6 ± 4.3	95.1 ± 3.2	50.8 ± 4.3$^{\$\$\$}$
FVC (L)	3.8 ± 0.3	3.8 ± 0.3	2.6 ± 0.2$^{**/\#\#}$
FEV_1/FVC (L)	0.74 ± 0.02	0.72 ± 0.01	0.49 ± 0.04$^{\$\$\$}$

Data are mean ± SEM. $^{*/\#}p<0.05$ non-smokers/smokers *vs*. COPD; $^{**/\#\#}p<0.01$ non-smokers/smokers *vs*. COPD; $^{\$\$\$}p<0.001$ non-smokers and smokers *vs*. COPD.

One pack year is defined as smoking 20 cigarettes per day for one year. MMP= Matrix metalloprotease; FEV= Forced expiratory volume; FVC= Forced vital capacity

55% decrease compared with 10μM fMLP values. There were no significant differences in pro-MMP-9 concentration between the three subject groups, either in non-stimulated supernatants, or after either 10 or 100μM fMLP stimulation.

3.3.4.3. Active MMP-9

In contrast to pro-MMP-9, in non-smokers, there was a stepwise, significant increase in active MMP-9 concentration between non-stimulated supernatants, and after 10 and 100μM fMLP stimulation, where there was a 3-fold increase compared with non-stimulated values (Figure 3.4 (B)). This pattern was similar for smokers and COPD patients, where there was a 4-12 fold increase, compared with non-stimulated values. There was significantly less active

MMP-9 in non-stimulated supernatants from COPD patients, compared with non-smokers ($p<0.01$ non-smokers *vs.* COPD patients). There were, however, no other significant differences in active MMP-9 concentration between subject groups.

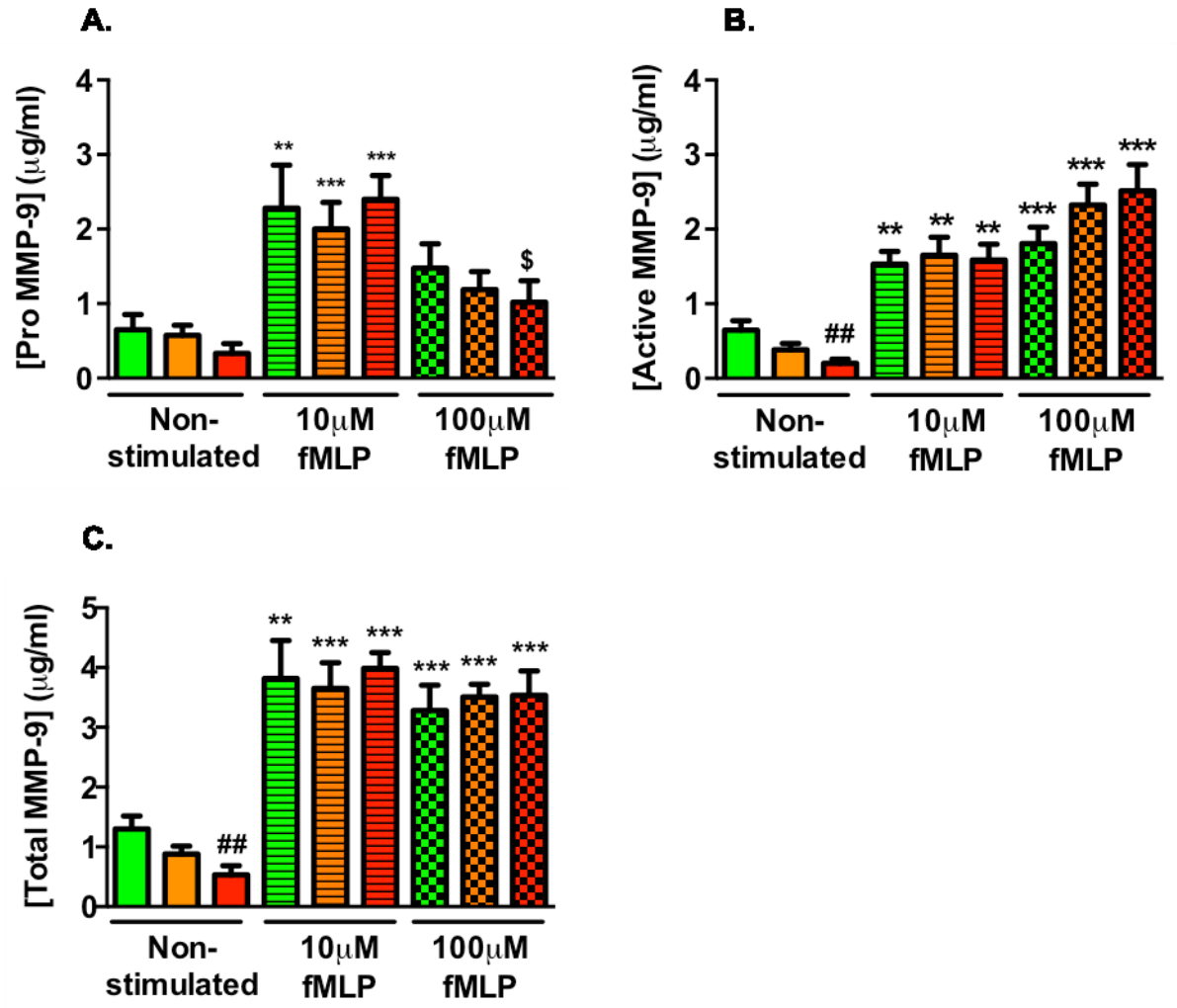


Figure 3.4. Effect of fMLP on MMP-9 isoforms in neutrophil supernatant. Non-smoker (●), smoker (●), COPD (●). (A) Pro MMP-9, non-smoker: n=11; smoker: n=11; COPD n=11-12. (B) Active MMP-9, non-smoker: n=11; smoker: n=11; COPD n=11-12. (C) Total MMP-9, non-smoker: n=11; smoker: n=11; COPD: n=11-12. Data are mean ± SEM, **p<0.01/***p<0.001 *vs.* non-stimulated, $p<0.05 10µM *vs.* 100µM, ##p<0.01 non-smoker *vs.* COPD, as determined by Kruskal-Wallis and Dunn's post-hoc tests.

3.3.4.4. Total MMP-9

In non-smokers, there was a significant three-fold increase in total MMP-9 concentration between non-stimulated supernatants, and after 10 and 100μM fMLP stimulation (Figure 3.4 (C)). This pattern was similar for smokers, which showed a four-fold increase, and COPD patients, which showed a seven-fold increase, compared with non-stimulated values. There was significantly less total MMP-9 in non-stimulated supernatants from COPD patients, compared with non-smokers ($p<0.01$ non-smokers *vs.* COPD patients). There were, however, no other significant differences in active MMP-9 concentration between subject groups.

3.3.5. Correlations with lung function

There were no significant correlations between NE, MMP-8, and pro-MMP-9 concentrations of neutrophil supernatants and any lung function parameters in subjects, at any level of stimulation (Table 3.4). There were significant positive correlations between active MMP-9 concentration in non-stimulated neutrophil supernatants and FEV_1, FEV_1 % predicted and FEV_1/FVC ratio, and between active MMP-9 concentration in 10μM fMLP stimulated neutrophil supernatants and FVC (Figure 3.5, A-C); however no correlations between active MMP-9 concentration in 100μM fMLP stimulated neutrophil supernatants and subject lung function. When non-smokers and smokers were excluded from the analysis, all of these correlations became non-significant, albeit this is more than likely due to low numbers (Table 3.6). However, there was a significant positive correlation between MPO concentration in 10μM fMLP stimulated neutrophil supernatants and FVC ($r=0.73$, $p<0.05$) in COPD subjects (Figure 3.5 (D), Table 3.6). There was a weak negative correlation between active MMP-9 concentration of supernatants after 100μM fMLP stimulation and FEV_1 % predicted ($r=-0.34$), which continued to be a trend in COPD subjects alone, despite remaining insignificant ($r=-0.4$). There were no significant correlations between protease concentrations and either age or smoking history.

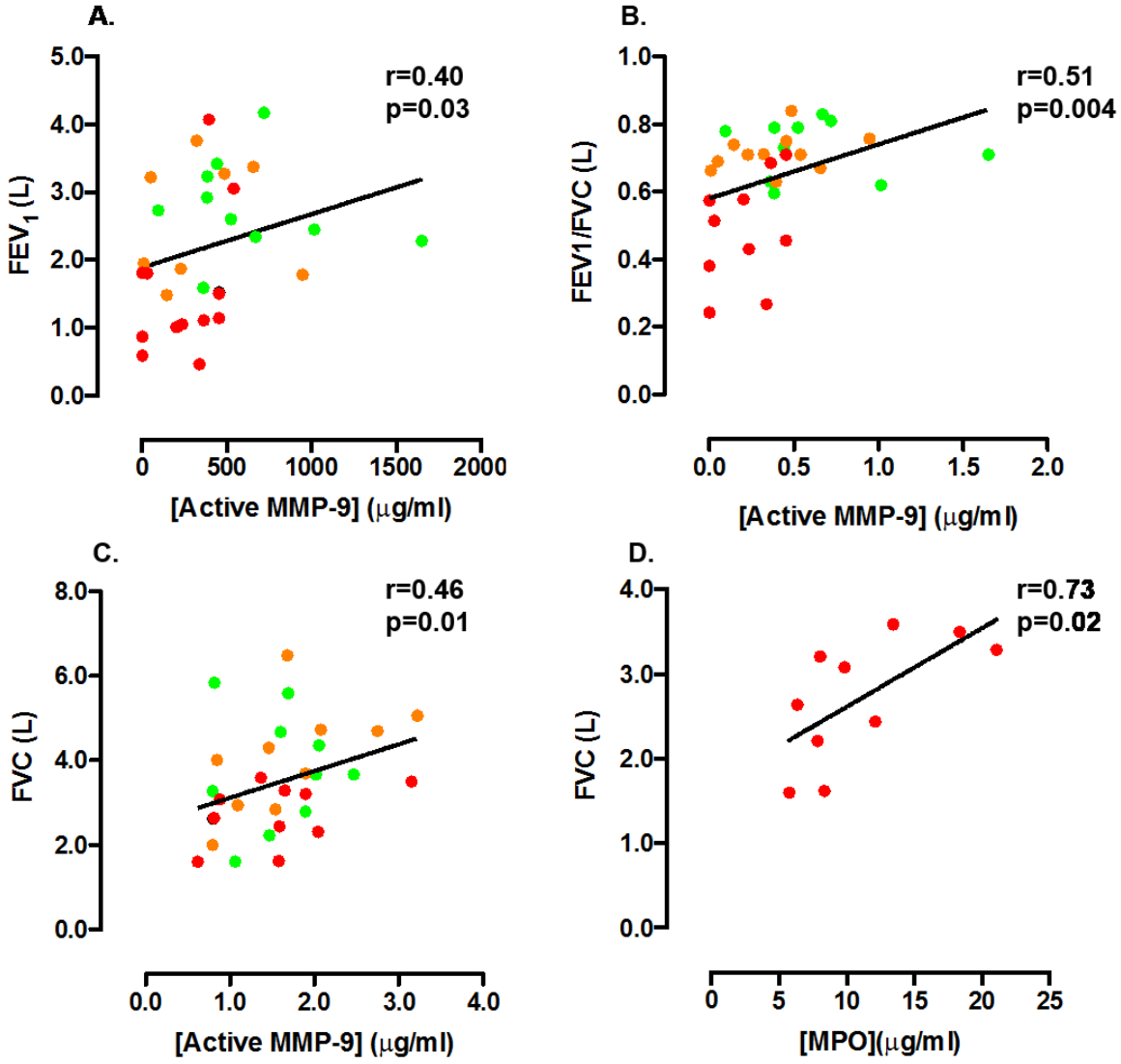


Figure 3.5. Correlation between protease concentration and lung function. Non-smoker (●), smoker (●), COPD (●). (A) [Active MMP-9] in non-stimulated neutrophil supernatants *vs*, FEV_1 (n=31), (B) [Active MMP-9] in non-stimulated neutrophil supernatants *vs.* FEV_1/FVC (n=31), (C) [Active MMP-9] in 10µM fMLP-stimulated neutrophil supernatants *vs.* FVC (n=31), (D) [MPO] in 10µM fMLP-stimulated neutrophil supernatants from COPD patients only *vs.* FVC (n=10). r= Spearman rank correlation coefficient.

Table 3.4. Correlations between supernatant protease concentration, after different fMLP stimulations, and subject lung function: all subjects

Protease	[fMLP] (μM)	FEV_1	FEV_1 %predicted	FVC	FEV_1/FVC
		R-value (n)			
[NE]	NS	-0.03 (43)	-0.06 (43)	-0.02 (43)	-0.02 (43)
	10	0.17 (44)	-0.02 (44)	0.14 (44)	0.08 (44)
	100	0.15 (37)	0.12 (37)	0.18 (37)	0.05 (37)
[MMP-8]	NS	-0.15 (34)	-0.03 (34)	-0.33 (34)	0.26 (34)
	10	-0.23 (33)	-0.20 (33)	-0.28 (33)	-0.09 (33)
	100	-0.17 (33)	-0.18 (33)	-0.06 (33)	-0.14 (33)
[Pro MMP-9]	NS	0.19 (32)	0.19 (32)	0.11 (32)	0.14 (32)
	10	0.11 (31)	-0.11 (31)	-0.04 (31)	-0.29 (31)
	100	0.19 (33)	0.24 (33)	0.24 (33)	0.03 (33)
[Active MMP-9]	NS	**0.40 (31)***	**0.42 (31)***	0.23 (31)	**0.51 (31)****
	10	0.28 (31)	0.10 (31)	**0.46 (31)****	-0.11 (31)
	100	-0.24 (33)	-0.34 (33)	-0.20 (33)	-0.16 (33)
[MPO]	NS	-0.11 (33)	-0.00 (33)	-0.27 (33)	0.28 (33)
	10	0.09 (32)	0.07 (32)	0.09 (32)	-0.02 (32)
	100	-0.15 (30)	-0.09 (30)	-0.14 (30)	-0.05 (30)

R= Spearman's Rank Correlation Co-efficient. *p<0.05, **p<0.01.

NE= Neutrophil elastase; MMP= Matrix metalloprotease; MPO= Myeloperoxidase; FEV= Forced expiratory volume; FVC= Forced vital capacity.

Table 3.5. Correlations between supernatant protease concentration, after different fMLP stimulations, and subject lung function: COPD patients

Protease	[fMLP] (μM)	FEV_1	FEV_1 %predicted	FVC	FEV_1/FVC
		R-value (n)			
[NE]	NS	-0.02 (13)	0.32 (13)	-0.14 (13)	0.24 (13)
	10	0.18 (17)	0.18 (17)	0.01 (17)	0.19(17)
	100	0.08 (10)	0.19 (10)	0.44 (10)	-0.10 (10)
[MMP-8]	NS	0.05 (11)	0.37 (11)	-0.40 (11)	0.32 (11)
	10	0.01 (11)	0.08 (11)	-0.29 (11)	011 (11)
	100	-0.10 (11)	-0.20 (11)	0.34 (11)	-0.34 (11)
[Pro MMP-9]	NS	0.43 (10)	0.26 (10)	-0.14 (10)	0.13 (10)
	10	0.05 (10)	0.26 (10)	-0.25 (10)	0.44 (10)
	100	0.36 (11)	0.09 (11)	0.13 (11)	0.30 (11)
[Active MMP-9]	NS	0.14 (10)	0.28 (10)	-0.24 (10)	0.41 (10)
	10	-0.24 (10)	-0.43 (10)	0.36 (10)	-0.58 (10)
	100	-0.11 (11)	-0.41 (11)	0.03 (11)	-0.10 (11)
[MPO]	NS	-0.03 (11)	0.36 (11)	-0.19 (11)	0.30 (11)
	10	0.02 (10)	-0.24 (10)	**0.73 (10)***	-0.43 (10)
	100	-0.24 (10)	0.01 (10)	-0.14 (10)	-0.04 (10)

R= Spearman's Rank Correlation Co-efficient. *p<0.05.

NE= Neutrophil elastase; MMP= Matrix metalloprotease; MPO= Myeloperoxidase; FEV= Forced expiratory volume; FVC= Forced vital capacity.

3.3.6. Summary of results

The results of the present chapter were:

1. There were significant increases in protease concentration/activity in peripheral blood neutrophil supernatants from non-smokers, smokers, and COPD patients, following fMLP stimulation, compared with non-stimulated samples.
2. There were no significant differences in the protease concentrations/activities between the three subject groups.
3. The effects of fMLP were not concentration-responsive, except in the case of pro-MMP-9 and active MMP-9.
4. There were conflicting correlations between active MMP-9 concentration and patient lung function, depending on the lung function parameter and level of fMLP stimulation, when considering all subject groups and COPD patients alone.

3.4. Discussion

The results of the present chapter showed that protease concentration/activity in peripheral blood neutrophil supernatants increased with fMLP stimulation; however there were no differences between subject groups. These effects were, with the exception of MMP-9, not concentration-responsive, and showed some correlation with patient lung function.

3.4.1. Differences in protease concentrations between subject groups

One of the objectives of this chapter was to try and identify whether there are inherent differences in neutrophil activity, resulting in excessive or “hypersensitive” degranulation, between non-smokers, smokers and COPD patients; to try and reconcile whether this may be associate with the lung damage, and the corresponding airway dysfunction and airflow limitation, observed in COPD. The concentration of a number of neutrophil proteases, namely NE, MPO, MMP-8 and -9, were measured in neutrophil supernatants from non-smokers, healthy smokers, and COPD patients; either non-stimulated, or stimulated with fMLP. This allows investigation of basal levels of protease release in non-stimulated neutrophil supernatants, and then how these levels increase upon neutrophil activation with fMLP, which stimulates degranulation.

The proteases investigated represented a cross-section across granule subtypes: MMP-9 is stored in gelatinase granules, MMP-8 in secretory granules, and MPO and NE in azurophil granules; so any differences associated with granule mobilisation might be identified. In all cases, the supernatants were obtained from neutrophils at a concentration of ten million cells/ml, which allowed investigation of inherent differences in neutrophil activity, without the confounding issue of different cell numbers between subject groups.

There were trends for higher MMP-8 and active MMP-9 concentrations in COPD patients, compared with non-smokers and smokers; however these were not significant. There was very little differences observed between the subject groups, with regards to NE and MPO. Previous studies have observed increased NE from fMLP-stimulated COPD neutrophils,

compared with non-smokers (Milara *et al.*, 2012). The latter study used 30nM fMLP, whereas the present study used much higher concentrations; however an fMLP concentration-response was performed herein for NE activity, and there were no differences between subject groups at any concentration of fMLP. The latter study also measured NE activity in "real-time", i.e. adding the fMLP to neutrophils resuspended with NE substrate, and immediately measuring activity. In the present study, NE activity was measured in supernatants from neutrophils already stimulated with fMLP. Therefore, differences between subject groups in NE activity, or, indeed, any protease release, may have temporal constraints that were overlooked by the methodology of the present study. Burnett *et al.*, (1987) observed increased proteolytic digestion of fibronectin by neutrophils from emphysema patients compared with controls, which was further augmented by fMLP stimulation. Interestingly, the latter study did not observe any differences in total elastase activity between controls and emphysema patients. Therefore, there are likely other proteases, not measured in the present study, which may differ in activity in COPD patients.

3.4.2. Differences in protease concentration between 10 and 100μM fMLP stimulation

There was little difference in protease release between 10 and 100μM fMLP stimulation for NE and MPO. There were, however, some differences observed with the other proteases measured. When considering MMP-8, there was a trend towards higher MMP-8 concentration following 100μM, compared with 10μM fMLP; however this was only observed in smokers. With MMP-9, there was a reduction in pro MMP-9 concentration between 10 and 100μM fMLP, observed across all subject groups, which corresponds with a higher active MMP-9 concentration following 100μM fMLP stimulation. MMP-9 is synthesized and stored as an inactive pro- isoform, which is, upon stimulation, cleaved to its active counterpart by proteases, in particular other members of the MMP superfamily. Therefore, between 10 and 100μM fMLP stimulation, more pro-MMP-9 appears to be cleaved to form active MMP-9, which explains the decrease in pro-MMP-9 between 10 and 100μM fMLP, and the corresponding increase in active MMP-9. There were almost the same concentrations of

total MMP-9 after 10 and 100μM fMLP, therefore it appears to be the cleavage of the isoforms that differs between these two stimulations.

For NE, MPO and MMP-8, 10μM elicited maximal degranulation, whereas it was submaximal for MMP-9. As there are similar concentrations of total MMP-9 after both fMLP stimulations, and because gelatinase granules have a greater propensity for exocytosis compared with the other subtypes, it is unlikely that mobilisation of granules can adequately explain this result. Pro MMP-9 is cleaved and activated by a number of other MMPs and serine proteases (Van den Steen *et al.*, 2002), therefore it may be the release of a particular protease after 100μM stimulation that further cleaves pro MMP-9 to form more of the active isoform. As there were similar concentrations of all proteases measured in this study after both 10 and 100μM stimulation, it is unlikely to be any of these. Alternatively, the results obtained may be a temporal issue. Neutrophils were stimulated with fMLP for 10min. However, at lower concentrations of fMLP, degranulation may be slower and, hence, less pro MMP-9 would be cleaved to active MMP-9. After higher concentrations of fMLP, however, degranulation may occur more quickly and, therefore, more pro MMP-9 could be cleaved. This is unlikely, however, as both 10 and 100μM fMLP are very high stimulations, and would most likely induce rapid degranulation in both cases. It is likely that there are other proteases, not measured in this chapter, that do have differing activities/concentrations between 10 and 100μM fMLP, which may change the relative composition of MMP-9 isoforms (and also induce some functional differences, see Chapter 4).

In the present thesis, fMLP was the only stimulus used to activate neutrophils. This is physiologically relevant, as fMLP is a bacterial product and could, therefore, mimic bacterial infection. However, there are a surplus of additional stimuli that could have been investigated that may yield differences in protease release. These include opsonized bacteria or LPS (Matthews *et al.*, 2012); phorbol myristate acetate (PMA) (Noguera *et al.*, 2001), or CXCL8 (Dalboni *et al.*, 2013). CSE has also been shown to prime neutrophil responses prior to fMLP stimulation (Koethe *et al.*, 2000). Therefore it may be more

physiologically relevant to investigate different stimuli, and the priming effects of different mediators on neutrophil degranulation; in order to determine whether these yield any differences in protease activity, both between subject groups and level of stimulation.

3.4.3. Correlations between protease concentration and subject demographics

There were some significant correlations between protease concentration and subjects' lung function. Active MMP-9 from non-stimulated neutrophils was positively correlated with FEV_1, FEV_1 % predicted, and FEV_1/FVC ratio; and when non-smokers and smokers were excluded from the analysis, the correlation with FEV_1/FVC, although now insignificant, remained a positive trend. However, the range of concentrations of active MMP-9 released by non-stimulated neutrophils was very low, and would, in all likelihood, not be physiologically significant.

Active MMP-9 release from neutrophils stimulated with 10μM fMLP was positively correlated with FVC; however, when non-smokers and smokers were excluded from the analysis, there were also relatively strong, but insignificant, negative correlations with FEV_1 % predicted and FEV1/FVC ratio. Although the correlations are insignificant, there were relatively strong negative associations between active MMP-9 concentration in 10μM fMLP-stimulated neutrophil supernatants and FEV_1 % predicted and FEV_1/FVC ratio, and similarly between 100μM fMLP-stimulated supernatants and FEV_1 % predicted. Due to the relatively low n, the discordant results with different lung function parameters, and the fact that most proteases measured showed no correlation with lung function: on the impact of neutrophil proteases on lung function, no firm conclusions can be drawn from these results.

The COPD subjects in this chapter were significantly older than the non-smokers and smokers, by ~8years. Age is an important issue to address, as abnormal neutrophil activity has been associated with increasing age (Lord *et al.*, 2001), with regards to reduced chemotaxis (Sapey *et al.*, 2014), phagocytosis and superoxide production (Wenisch *et al.*, 2000), and degranulation (Suzuki *et al.*, 1983, Fülöp *et al.*, 1986). However, despite there

being a statistically significant difference in age, it is unlikely to have had much of an effect on the results herein. In the aforementioned studies, the difference in age between young and old subjects where differences were observed in their neutrophil activity was substantially larger (~50years) than that of the present chapter. In this chapter, COPD patients also had significantly greater smoking history than smokers, by ~10-20 pack years. Cigarette smoke induces CXCL8 release, NF-κB activity and intracellular ROS and nitric oxide (NO) production through activation of TLR-9 in human neutrophils (Mortaz *et al.*, 2010). However, it is unclear as to whether this effect is concentration-dependent, and this warrants further investigation. Nevertheless, there were no significant correlations between concentrations of any of the proteases measured and either age or smoking history; therefore it is unlikely that either of these parameters had a considerable effect on the results herein.

Another potential reason for the inconsistent correlations between lung function and protease concentration may be that the lung function parameters used are not the most physiologically relevant with regards to the lung pathology being considered. The protease-antiprotease imbalance has most often been implicated in the development of emphysema, whereas FEV_1 and FVC, with regards to COPD diagnosis, highlight generalised airflow obstruction. As such, it may be more relevant to correlate protease concentration/activity with a measures of emphysema; such as lung volumes, including residual capacity, total lung capacity and diffusion capacity; or by using quantitative CT scans, as in Wang *et al.*, (2014).

3.4.4. COPD neutrophils: inherent differences?

Overall, it was inconclusive as to whether neutrophils from COPD patients had inherent differences in their ability to degranulate, compared with non-smokers and smokers. COPD neutrophils have been shown to have aberrant functions, including chemotaxis (Sapey *et al.*, 2011, Milara *et al.*, 2012), ROS production (Noguera *et al.*, 2001), and protease release

(Burnett *et al.*, 1987, Milara *et al.*, 2012), although there is little evidence for the latter from the present study to support this. The obvious limitation of the present study is that, due to both financial and temporal constraints, only four different neutrophil proteases were investigated, although, as described in the Introduction (section 1.4.2), these four have previously been implicated in COPD pathophysiology. A high-throughput, multiplex system, such as Luminex or Meso Scale Discovery (MSD) technology, may be a more effective way to investigate a large panel of neutrophil products. It may also be of interest to further investigate the temporal aspect of degranulation, and how this may differ between subject groups.

There is also little firm evidence in the present study to support an association between protease release and lung function. Sputum MMP-8 and -9 (Vernooy *et al.*, 2004), and MMP-12 (Demedts *et al.*, 2006) have previously been negatively associated with lung function; however, in such studies, these differences in protease concentrations and subsequent correlation with airflow obstruction are probably a function of the increased number of neutrophils observed in COPD, rather than intrinsic differences in protease release between subject groups. A larger cohort of subjects may allow more conclusive correlations to be evaluated.

3.4.5. Hypothesis: accept/reject

The hypothesis for this chapter was:

"Stimulation of neutrophils with fMLP will induce release of proteases, and there will be differences in protease composition between neutrophil supernatants from non-smokers, smokers and COPD patients."

This can only be partially accepted, as fMLP did release selected proteases from neutrophils, but there were no differences in the amount or composition of released proteases between non-smokers, smokers and COPD patients. Relatively large numbers of

subjects were used, and the lack of differences between groups was striking. Consequently, rejection of the second part of the hypothesis seems justified.

This chapter has broadly characterised the protease composition of neutrophil supernatants. The next chapter will begin to investigate the effects of these neutrophil supernatants on small airway function, and determine whether those from COPD patients induce an augmented response, compared with their non-smoker and smoker counterparts.

Chapter 4.

Effect of Neutrophil Supernatants on Small Airway Structure & Function

4. Effect of Neutrophil Supernatants on Small Airway Structure & Function

4.1. Introduction

The previous chapter (Chapter 3) showed that a number of proteases were released into supernatants by stimulated neutrophils. However, of the proteases measured, there were no significant differences between non-smokers, smokers and COPD patients. Nevertheless, neutrophils are considered to be important mediators of the lung damage associated with COPD; therefore this chapter will now investigate the consequence of neutrophil degranulation on small airway function.

As described in Chapter 1 (section 1.4.1.4), there is increased neutrophil infiltration in the subepithelial and smooth muscle layer of COPD airways (Di Stefano *et al.*, 1998, Baraldo *et al.*, 2004), and also increased neutrophil numbers in sputum (Keatings *et al.*, 1996) and BALF (Martin *et al.*, 1985) from COPD patients. These increases in neutrophil numbers have been significantly correlated with lung function decline (Hogg *et al.*, 2004, Hogg, 2004, O'Donnell *et al.*, 2004). COPD neutrophils also exhibit intrinsic differences, compared with non-smokers and smokers; demonstrating increased cytokine release and respiratory burst, and abnormal chemotaxis (Noguera *et al.*, 2001, Sapey *et al.*, 2011, Milara *et al.*, 2012). Therefore, both increased numbers and aberrant activity of neutrophils may mediate the lung damage that is characteristic, and symptomatic, of COPD.

The aim of this chapter was to directly investigate evidence for the latter: to examine whether or not COPD neutrophils cause excessive small airway dysfunction, without the confounding issue of increased numbers. In order to do this, PCLS, an *ex vivo* preparation of lung tissue (section 1.5.1), were used to investigate small airway physiology and “pathophysiology” in response to neutrophil supernatants. As the parenchymal attachments are still intact, small airways in PCLS mimic *in vivo* airway dynamics (Cooper *et al.*, 2009), making them an attractive system to examine small airway dysfunction following experimental stresses. Small airways are of particular interest in COPD as they are thought to be the main site of airflow obstruction (Hogg *et al.*, 1968); whilst remodelling and, in some cases, hyperresponsiveness

of the small airways, is central to the aetiology of the disease. In addition, a cross-sectional analysis of patients enrolled in the GLUCOLD (Groningen Leiden Universities Corticosteroids in Obstructive Lung Disease) study revealed that severity of airway hyperresponsiveness was significantly correlated with worsening lung function, and increased numbers of sputum neutrophils (van den Berge *et al.*, 2012). Therefore, there is accumulating evidence that abnormal neutrophil activity may contribute to the small airway dysfunction that is indicative of COPD pathophysiology. However, it is currently unknown exactly how neutrophils may impact upon the small airway obstruction observed in COPD; and so the aim of this chapter was to investigate this further.

4.1.2. Hypothesis

From the above introductory observations, the hypothesis for this chapter was, therefore, that:

"The products of neutrophil activation and degranulation will affect PCLS structure and viability, with associated effects on small airway function, including airway closure and capacity for reopening".

4.1.3. Aims

In order to investigate this hypothesis, the aims for this chapter were to:

1. Incubate rat PCLS overnight in neutrophil supernatants from non-smokers, smokers or COPD patients, either non-stimulated or stimulated with fMLP (10 or 100μM).
2. Following overnight incubation with neutrophil supernatants, assess the histological structure and viability of PCLS, along with the function of small airways, including baseline patency, airway closure to CCh, and passive airway reopening.

4.2. Methods

4.2.1. Generation of rat PCLS and assessment of airway function

Rat PCLS were generated and airway function assessed as described in section 2.2.1.

4.2.2. Initial assessment of airway function and grouping of PCLS

Airways were selected for use and grouped based on the criteria outlined in section 2.2.1.4. To summarise briefly, after an initial assessment of airway function, airway closure must be >50% at 10μM CCh. With regards to grouping PCLS for treatments: groups must contain at least 4 PCLS, there must be a similar number of PCLS in each group, and the average maximal closure of airways within each group must be approximately the same across all groups. Due to the large number of variables in these experiments (variation in composition of neutrophil supernatants, and inherent variability of lung tissue between rats) an additional stipulation was included for PCLS experiments in this chapter: PCLS from lungs from two rats were used for each neutrophil supernatant. This was to try to account for and minimize the effect of variation due to the lung tissue itself that may interfere with these experiments.

As described in section 2.2.1.4, airways in PCLS were subject to an initial assessment of maximal airway closure by administration of 10μM CCh. Following a series of washes in rat IB, and a 1h recovery period to allow airways to reopen, PCLS were then grouped based on the criteria outlined above.

4.2.3. Incubation of PCLS in control compounds or neutrophil supernatants

Neutrophil supernatants, that had been stored at -80°C, were thawed and warmed to 37°C in a water bath. Once PCLS groups had been established (see above), 1ml neutrophil supernatant was added to selected groups. For control compounds, 2% DMSO ($^{v}/_{v}$), 10μM Cytochalasin B, or 10 or 100μM fMLP was added to PCLS in 1ml rat IB. For untreated controls, PCLS were incubated in 1ml rat IB. All PCLS were then incubated overnight at 37°C.

4.2.4. CCh-induced airway closure and passive airway reopening

Airway closure, in response to increasing concentrations of CCh and passive airway reopening were investigated as described in 'Materials & Methods'- Sections 2.2.1.4 and 2.2.1.5.

4.2.5. Measurement of PCLS viability

PCLS viability was measured by WST-1 assay, as described in section 2.2.2.1. For the purposes of these experiments, untreated PCLS were considered to represent 100% viability, and so the absorbance of all treated PCLS was presented as a percentage of the untreated absorbance.

4.2.6. Data Analysis

In this chapter, normal distributions were not assumed for any of the analyses, therefore patient demographics were compared using Kruskal-Wallis tests, with Dunn's post-hoc comparisons; with the exception of smoking history (pack years), which were compared using a Mann-Whitney U-test. CCh concentration-response curves were constructed by plotting % airway closure (calculated with respect to Day 1 baseline airway patency), against the log CCh concentration, and constructing a 4-parameter logistic non-linear regression curve. Mean (±SEM) EC_{50} values to CCh were then calculated from PCLS used to generate each curve. Airway functional parameters and viability of PCLS treated with neutrophil supernatants from the three subject groups were compared using Kruskal-Wallis tests, with Dunn's post-hoc comparisons. For familiarity of presentation, data are displayed as mean ± SEM. The null hypothesis of no significant differences between untreated airways and those treated with control compounds or neutrophil supernatants was rejected at $p<0.05$.

4.3. Results

4.3.1. Temporal effect on small airway closure

Small airway closure on PCLS was assessed at 24h (day 1) and 48h (day 2), in order to investigate any potential changes in airway closure with time of incubation, and to establish conditions for future experiments (Figure 4.1). CCh induced a concentration-dependent increase in airway closure in both day 1 and day 2 PCLS, with mean maximal increases of 83.5 ± 3.4% for day 1 and 85.7 ± 5.0% for day 2 at 10μM CCh, and EC_{50} values of 150 ± 52nM and 230 ± 94nM (n=3), for day 1 and day 2, respectively (Figure 4.1). There was a non-significant ~10% reduction in baseline airway patency on day 2 (8.2 ± 2.5%, n=3), compared with day 1, and no significant differences in either maximal airway closure or EC_{50} to CCh between day 1 and day 2.

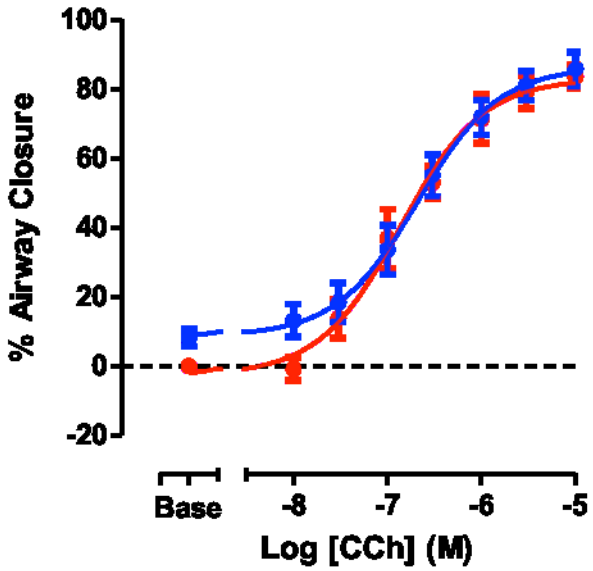


Figure 4.1. Temporal effect of small airway closure. Small airway closure in PCLS, in response to CCh, at 24h (Day 1) (●) and 48h (Day 2) (●) post generation of PCLS. N=3 rats, 10-12 PCLS/rat. Data shown are mean ± SEM. *p<0.05 10nM *vs.* 10μM CCh (Day 1), baseline *vs.* 10μM CCh (Day 2). Dashed line at 0% represents Day 1 baseline patency.

4.3.2. Effect of control compounds on airway contractility

PCLS were incubated overnight with diluent/control compounds that were used during neutrophil stimulation, in order determine whether or not these had any effects on airway closure that may interfere with interpretation of future experiments.

CCh induced a concentration-dependent increase in airway closure in PCLS that had been pre-incubated in 2% ($^{v}/_{v}$) DMSO, 10μM Cyt B or 10 and 100μM fMLP, with maximal increases above baseline of 70-84% at 3-10μM CCh, and EC_{50} values of 150-470nM CCh (Figure 4.2 (A) and Table 4.1). There was a significant 15% reduction in maximal airway

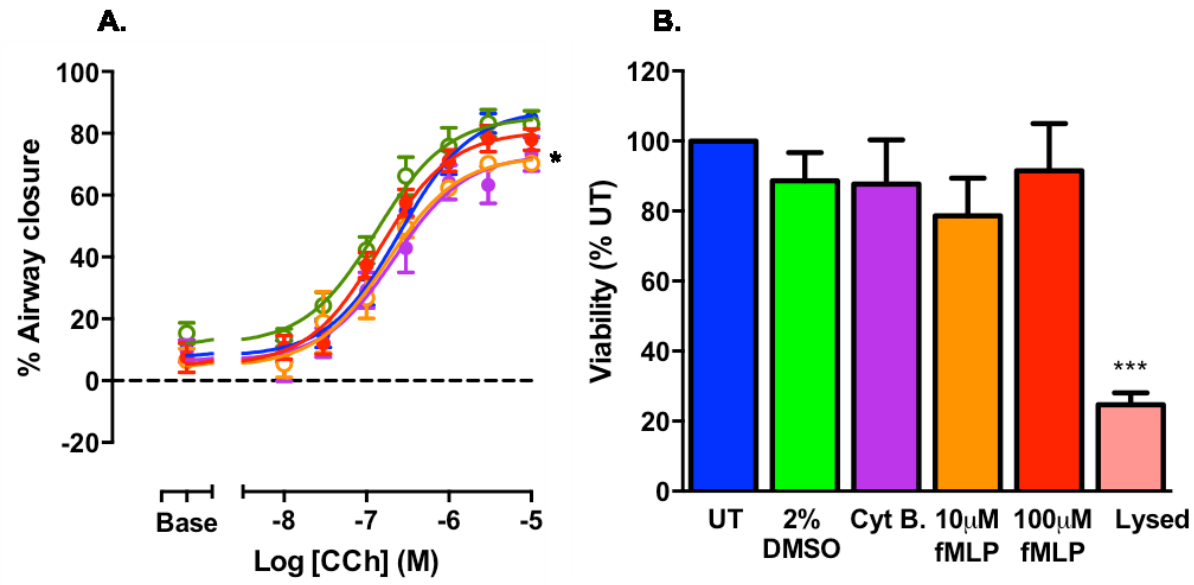


Figure 4.2. Effect of control compounds on CCh-induced small airway closure and PCLS viability. Untreated (UT,●); 2% DMSO (open circles,●); 10μM cytochalasin B (●); 10μM fMLP (open circles, ●); 100μM fMLP (●), lysed (●). (A) CCh Concentration-response curve of small airway closure following overnight incubation of PCLS in control compounds. UT n=21 rats, all compounds n=7 rats, *p<0.05 UT *vs.* 10μM fMLP maximal closure. All data sets above 30nM (UT), 100nM (all other compounds) CCh were significantly higher than baseline, p<0.05-0.001. (B) Viability of PCLS, assessed by WST-1 assay, following incubation in control compounds. Untreated were considered 100% viable, therefore viability is expressed as % UT. N=7-9 rats, ***p<0.001 lysed *vs.* UT. Data are mean ± SEM. Dashed line at 0% represents day 1 baseline patency.

Table 4.1. Effect of CCh on small airway closure in control compound pre-treated PCLS.

Compound	Baseline Airway Patency (%)	Maximal Closure (%)	[CCh] for maximal closure (μM)	EC_{50} (nM)
Untreated (n=21)	10.4 ± 2.2	84.9 ± 2.1	10	471 ± 87
2% DMSO (n=7)	15.3 ± 3.4	83.0 ± 4.4	3	262 ± 125
10μM Cyt. B (n=7)	9.6 ± 3.6	73.4 ± 5.6	10	304 ± 121
10μM fMLP (n=7)	6.5 ± 3.8	70.3 ± 2.1*	3	188 ± 42
100μM fMLP (n=7)	7.5 ± 4.8	78.0 ± 3.5	3	148 ± 31

Data are mean ±SEM. *p<0.05 *vs.* Untreated. DMSO= Dimethyl sulfoxide. CCh= Carbachol

closure of airways on PCLS incubated in 10μM fMLP; however there were no other significant differences in baseline patency, maximal airway closure or EC_{50} to CCh when PCLS were incubated in any other the compounds.

4.3.3. Effect of control compounds on PCLS viability

There were no significant differences in viability when PCLS were incubated in either 2% ($^{v}/_{v}$) DMSO, Cyt B, or either concentration of fMLP, compared with untreated PCLS (Figure 4.2 (B)). In contrast, lysed (control) PCLS showed an ~80% reduction in viability, compared with untreated PCLS.

4.3.4. Effect of neutrophil supernatants on airway contractility

4.3.4.1. Non-stimulated neutrophil supernatants

4.3.4.1.1. Subject demographics

Subject demographics for non-stimulated neutrophil supernatants are shown in Table 4.2. There were no significant differences in age or smoking history between subject groups; however, COPD patients were ~12 years older and smoked ~40% more cigarettes than the smoker group. COPD patients also had, as expected, significantly worse lung function than non-smoker and smoker volunteers, with 25-40% reductions in measured parameters.

Table 4.2. Subject demographics for non-stimulated neutrophil supernatants, in which PCLS were incubated.

	Non-smokers	Smokers	COPD
N	4	4	4
Age	61 ± 6	60 ± 3	72 ± 1
Gender (m/f)	1/3	1/3	2/2
Smoking history (Pack years)	N/A	32.6 ± 11.7	46.0 ± 3.1
FEV_1 (L)	3.0 ± 0.2	2.0 ± 0.3	1.5 ± 0.4*
FEV_1 predicted (%)	100.0 ± 4.2	83.4 ± 6.8	59.9 ± 10.6*
FVC (L)	4.1 ± 0.4	2.9 ± 0.4	2.6 ± 0.7
FEV_1/FVC (L)	0.75 ± 0.04	0.71 ± 0.02	0.57 ± 0.07

Data are mean ± SEM. *p<0.05 non-smokers *vs.* COPD, as determined by Kruskal-Wallis and Dunn's post-hoc tests. FEV_1 = Forced expiratory volume over 1sec. FVC= Forced vital capacity. One pack year is defined as smoking 20 cigarettes per day for 1 year.

4.3.4.1.2. Effects on small airway closure

CCh induced a concentration-dependent increase in airway closure in all four subject groups (untreated and those pre-incubated in non-stimulated neutrophil supernatants from non-smokers, smokers and COPD patients), with significant increases above baseline at CCh

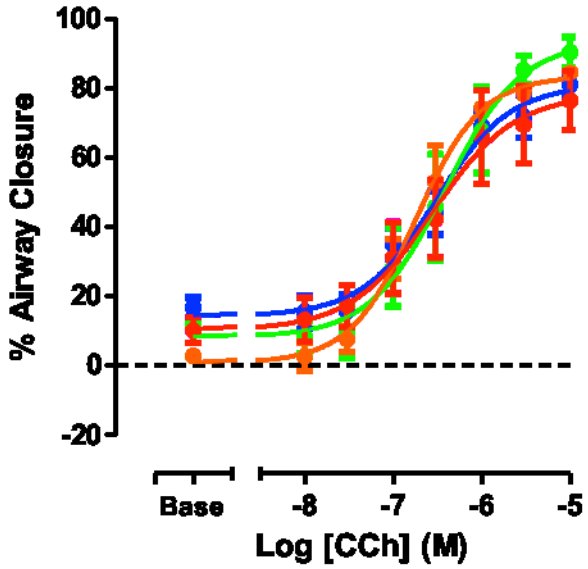


Figure 4.3. Effect on CCh-induced small airway closure of overnight incubation of PCLS in supernatants from non-stimulated neutrophils: concentration-response relationships. Untreated (●), n=12 rats; non-smoker (●), n=4 supernatants, n=8 rats; smoker (●), n=4 supernatants, n=8 rats; COPD (●), n=4 supernatants, n=8 rats. Data shown are mean ± SEM. All data sets above 1μM (UT), 3μM (smoker) and 10μM (COPD) CCh were significantly different to baseline, $p<0.05$-0.01. Dashed line at 0% represents day 1 baseline patency.

concentrations of 1-10μM CCh; maximal increases above baseline of 76-90% at 10μM CCh, and EC_{50} values of 230-750nM CCh (Figure 4.3 and Table 4.3). There were no significant differences in the maximal airway closure, EC_{50} to CCh, or baseline patency of airways of airways on PCLS incubated in non-stimulated neutrophil supernatants from any of the three subject groups (Figure 4.3).

4.3.4.2. Supernatants of neutrophils stimulated with 10μM fMLP

4.3.4.2.1. Subject demographics

Subject demographics for supernatants of neutrophils stimulated with 10μM fMLP are shown in Table 4.4. Subject groups were matched for age, but COPD patients had significantly

Table 4.3. Effect of CCh on small airway closure in PCLS pre-treated with non-stimulated neutrophil supernatants from different subject groups.

Subject Group	Baseline Airway Patency (%)	Maximal Closure (%)	[CCh] for maximal closure (μM)	EC_{50} (nM)
Untreated (n=12 rats)	16.7 ± 2.8	81.0 ± 3.5	10	422 ± 107
Non-smokers (n=4)	12.1 ± 2.0	90.1 ± 4.4	10	754 ± 497
Smokers (n=4)	2.7 ± 1.9	84.5 ± 1.9	10	229 ± 91
COPD (n=4)	10.2 ± 3.7	76.5 ± 8.6	10	505 ± 248

Data are mean ± SEM. No significant differences between groups.

greater smoking history, smoking ~128% more than smokers. COPD patients also had, as expected, significantly worse lung function than non-smoker and smoker volunteers, with ~17-47% decreases in measured parameters.

4.3.4.2.2. Effects on small airway closure

CCh induced a concentration-dependent increase in airway closure in PCLS that had been incubated with neutrophil supernatants from all subjects, as well as those that were untreated. There were significant increases above baseline at CCh concentrations of 1-3μM CCh: maximal increases above baseline of 76-85% at 10μM CCh, and EC_{50} values of 160-640nM CCh (Figure 4.4 and Table 4.5).

There were no significant differences in maximal airway closure of airways in PCLS incubated in supernatants from any of the three subject groups (Table 4.5). There were also no significant differences in EC_{50} to CCh of airways incubated in non-smoker and smoker neutrophil supernatant, compared with untreated airways; however there was a four-fold

Table 4.4. Subject demographics for neutrophil supernatants stimulated with 10μM fMLP, in which PCLS were incubated.

	Non-smokers	Smokers	COPD
N	5	6	6
Age	64 ± 3	60 ± 3	65 ± 5
Gender (m/f)	3/2	3/3	3/3
Smoking history (Pack years)	N/A	25.0 ± 4.8	57.0 ± 5.1$^{\#}$
FEV_1 (L)	3.2 ± 0.5	2.6 ± 0.4	1.4 ± 0.2*
FEV_1 predicted (%)	102.1 ± 8.4	87.9 ± 6.0	54.5 ± 6.8*
FVC (L)	4.4 ± 0.7	3.9 ± 0.7	2.8 ± 0.4
FEV_1/FVC (L)	0.75 ± 0.01	0.73 ± 0.04	0.62 ± 0.1

Data expressed mean ± SEM. $^{*/\#}p<0.05$ non-smokers/smokers *vs*. COPD, as determined by Kruskal-Wallis and Dunn's post-hoc tests. FEV_1 = Forced expiratory volume over 1 sec. FVC= Forced vital capacity. One pack year is defined as smoking 20 cigarettes per day for 1 year.

decrease in EC_{50} to CCh in those airways incubated with COPD supernatants, but this did not reach significance. There was no significant reduction in the baseline patency of airways incubated in supernatants from any of the subject groups (Table 4.5); however baseline airway patency of airways was three-fold higher in PCLS incubated with non-smoker or COPD neutrophil supernatants, compared with untreated airways.

4.3.4.3. Supernatants from neutrophils stimulated with 100μM fMLP

4.3.4.3.1. Subject demographics

Demographics for the subjects who provided neutrophils for this study are presented in Table 4.6. COPD patients were significantly older than control subjects, by ~12 years. COPD patients also had significantly greater smoking history, smoking ~38% more than healthy smokers; and, as expected, significantly worse lung function than non-smoker and smoker volunteers, with 27-53% decreases in measured parameters.

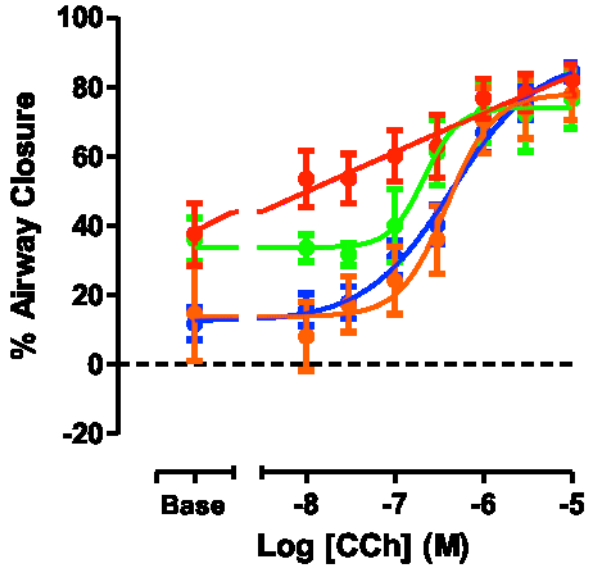


Figure 4.4. Effect on CCh-induced small airway closure of overnight incubation of PCLS in supernatants from neutrophils stimulated with 10μM fMLP: concentration-response relationships. Untreated (●), n=20 rats; non-smoker (●), n=5 supernatants, n=10 rats; smoker (●), n=6 supernatants, n=12 rats; COPD (●), n=6 supernatants, n=12 rats. Data are mean ± SEM. All data sets above 1μM (UT) and 3μM (COPD) CCh were significantly higher than baseline $p<0.05$-0.001. No significant differences compared with UT airways. Dashed line at 0% represents day 1 baseline patency.

4.3.4.3.2. Effect on airway closure

CCh induced a concentration-dependent increase in airway closure in PCLS that had been incubated with neutrophil supernatants from all subjects groups, and with untreated PCLS, with significant increases above baseline at CCh concentrations of 1-10μM CCh; maximal increases above baseline of 57-85% at 10μM CCh, and EC_{50} values of 4-382nM CCh (Fig 4.5 and Table 4.7).

Airways in PCLS incubated with non-smoker neutrophil supernatants had an ~13% reduction in maximal airway closure, compared with untreated airways, and a five-fold decrease in EC_{50} to CCh, compared with untreated airway; however none of these shifts reached

Table 4.5. Effect of CCh on small airway closure in PCLS pre-treated with supernatants from neutrophils stimulated with 10μM fMLP.

Subject Group	Baseline Airway Patency (%)	Maximal Closure (%)	[CCh] for maximal closure (μM)	EC_{50} (nM)
Untreated (n=20 rats)	11.8 ± 4.6	84.5 ± 2.8	10	641 ± 160
Non-smokers (n=5)	31.5 ± 6.6	76.5 ± 8.3	10	283 ± 62
Smokers (n=6)	16.3 ± 11.4	78.0 ± 7.5	10	520 ± 102
COPD (n=6)	37.5 ± 9.0	82.2 ± 4.5	10	162 ± 97

Data are mean ± SEM. No significant differences between groups.

Table 4.6. Subject demographics for neutrophil supernatants stimulated with 100μM fMLP, in which PCLS were incubated.

	Non-smokers	Smokers	COPD
N	7	7	7
Age	64 ± 3	58 ± 2	70 ± 2$^{\#\#}$
Gender (m/f)	2/5	5/4	3/5
Smoking history (Pack years)	N/A	28.7 ± 4.9	39.5 ± 4.8$^{\#}$
FEV_1 (L)	2.8 ± 0.3	2.7 ± 0.3	1.2 ± 0.2$^{\$\$}$
FEV_1 predicted (%)	106.1 ± 9.3	99.0 ± 4.3	49.8 ± 4.9$^{\$\$}$
FVC (L)	3.7 ± 0.4	3.8 ± 0.4	2.3 ± 0.3$^{\#}$
FEV_1/FVC (L)	0.72 ± 0.03	0.73 ± 0.02	0.53 ± 0.06$^{\$}$

Data are mean ± SEM. $^{\#}p<0.05$ smokers *vs.* COPD, $^{\#\#}p<0.01$ smokers *vs.* COPD, $^{\$\$}p<0.01$ non-smokers and smokers *vs.* COPD, as determined by Kruskal-Wallis and Dunn's post-hoc tests. FEV_1 = Forced expiratory volume over 1sec. FVC= Forced vital capacity. One pack year is defined as smoking 20 cigarettes per day for 1 year.

significance (Figure 4.5 (●) and Table 4.7). There was also no significant difference in baseline patency, compared with untreated airways.

Airways in PCLS incubated with smoker neutrophil supernatants had a 30% reduction in maximal airway closure, and an ~10-fold reduction in EC_{50} to CCh, compared with untreated airways; however neither reached significance (Figure 4.5 (●) and Table 4.7). There was also a 2-fold decrease in baseline airway patency, compared with untreated airways, but this was also not significant. Maximal airway closure of airways in PCLS incubated with COPD neutrophil supernatants was significantly reduced by -30%, with an ~100-fold significant decrease in EC_{50} to CCh, compared with untreated airways (Figure 4.5 (●) and Table 4.7). In addition, there was a significant, 4-fold reduction in baseline airway patency, compared with untreated airways.

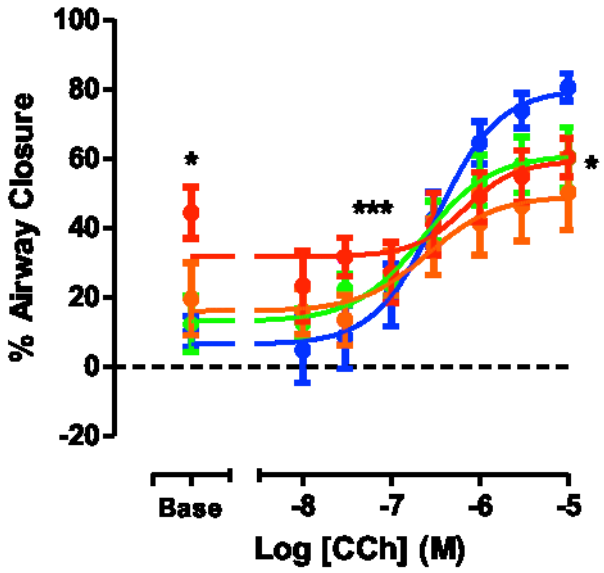


Figure 4.5. Effect on CCh-induced small airway closure of overnight incubation of PCLS in supernatants from neutrophils stimulated with 10μM fMLP: concentration-response relationships. Untreated (●), n=22 rats; non-smoker (●), n=7 supernatants, n=14 rats; smoker (●), n=7 supernatants, n=14 rats; COPD (●), n=7 supernatants, n=14 rats. Data are mean ± SEM. *p<0.05 (maximal airway closure and baseline airway patency), ***p<0.001 (EC_{50}) untreated *vs.* COPD, as determined by Kruskal Wallis and Dunn's post-hoc tests. All data sets above 1μM (UT), 3μM (non-smoker) and 10μM (COPD) CCh were significantly higher than baseline, p<0.05-0.001. Dashed line at 0% represents day 1 baseline patency.

Table 4.7. Effect of CCh on small airway closure in PCLS pre-treated with supernatants from neutrophils stimulated with 100μM fMLP.

Subject Group	Baseline Airway Patency (%)	Maximal Closure (%)	[CCh] for maximal closure (μM)	EC_{50} (nM)
Untreated (n=22 rats)	11.5 ± 4.6	82.5 ± 3.4	10	382 ± 172
Non-smokers (n=7)	12.4 ± 8.1	60.3 ± 8.7	10	70 ± 36
Smokers (n=7)	27.3 ± 8.1	57.5 ± 9.5	10	41 ± 19
COPD (n=7)	44.4 ± 7.4*	60.3 ± 5.6*	10	4 ± 2***

Data are mean ± SEM. *p<0.05, ***p<0.001 *vs.* untreated.

4.3.4.3.3. Effect on airway reopening

Following CCh washout, airways reopened in a time-dependent manner, with an initial reopening of ~20-50% immediately following washout, and then a further 20-35% after 60 min (Figure 4.6). After this time, airway closure was ~3-40%, with respect to their initial baseline patency.

Untreated airways took ~30min to reopen to half the maximal airway closure ($t_{1/2}$) following CCh washout. In contrast, airways in PCLS incubated in neutrophil supernatants reopened to baseline by ~90 s after washout (Figure 4.6, Table 4.8). Airways in untreated PCLS remained ~40% contracted after 60 min post-washout, whereas those within PCLS incubated with neutrophil supernatants were, on average, only 6% contracted at the same time-point. There was a significant reduction in $t_{1/2}$ in non-smokers and smokers, compared with untreated airways; and airway closure at 60 min was significantly reduced in non-

smokers, compared with untreated airways. There were, however, no significant differences in any of the parameters of airway reopening between subject groups.

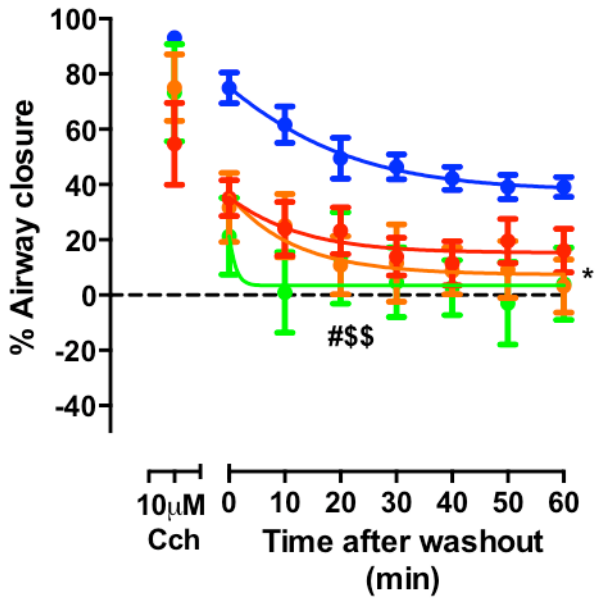


Figure 4.6. Effect of CCh washout on small airway reopening following overnight incubation of PCLS in supernatants from neutrophils stimulated with 100μM fMLP. Untreated (●), n=12 rats; non-smoker (●), n=5 supernatants, n=10 rats; smoker (●), n=6 supernatants, n=12 rats; COPD (●), n=6 supernatants, n=12 rats. Data are mean ± SEM. *p<0.05 60 min airway closure untreated *vs.* non-smoker, # p<0.05 $t_{1/2}$ untreated *vs.* non-smoker, $$ p<0.01 $t_{1/2}$ untreated *vs.* smoker, as determined by Kruskal Wallis and Dunn's post-hoc tests. All data sets are significantly different to 10μM CCh values after 40min (UT) and 60min (smoker), p<0.05-0.01. Data sets from non-smokers and COPD were not significantly different to 10μM CCh values at any time point. Dashed line at 0% represents day 1 baseline patency.

4.3.4. Effect of neutrophil supernatants on PCLS viability

4.3.4.1. Non-stimulated neutrophil supernatants

When PCLS were incubated in supernatants from non-stimulated neutrophils, there was a significant reduction in viability to ~50% of untreated PCLS (Figure 4.7 (A)). Lysed PCLS, as expected, were also significantly less viable than untreated PCLS, at ~15% of untreated.

Table 4.8. Effect of CCh on passive airway reopening in PCLS pre-treated with supernatants from neutrophils stimulated with 100μM fMLP.		
Subject Group	$T_{1/2}$ (min)	Airway closure at 60min
Untreated (n=12 rats)	27.7 ± 1.8	40.2 ± 4.2
Non-smokers (n=5)	-1.4 ± 0.2*	-8.0 ± 6.7*
Smokers (n=6)	1.2 ± 3.3**	2.1 ± 11.7
COPD (n=5)	1.3 ± 2.4	15.8 ± 9.6
Data are mean ± SEM. *p<0.05, **p<0.01 *vs.* untreated, as determined by Kruskal-Wallis and Dunn's post hoc tests.		

4.3.4.2. Supernatants of neutrophils stimulated with 10μM fMLP

When PCLS were incubated in supernatants from neutrophils stimulated with 10μM fMLP, there was a reduction in viability to ~60% of untreated PCLS; however this was not significant (Figure 4.7 (B)). Again, lysed PCLS, as expected, were significantly less viable than untreated PCLS, at ~10% of untreated.

4.3.4.3. Supernatants of neutrophils stimulated with 100μM fMLP

When PCLS were incubated in supernatants from neutrophils stimulated with 100μM fMLP, there was a significant reduction in viability to ~50-60% of untreated airways, across all three subject groups (Figure 4.7 (C)). Again, lysed PCLS were significantly less viable than untreated airways, at ~10% of untreated.

4.3.4.4. Viability of PCLS incubated with fresh or frozen media

Supernatants from both non-stimulated and stimulated neutrophils reduce PCLS viability to a similar extent. One consistent difference between the neutrophil supernatants and the

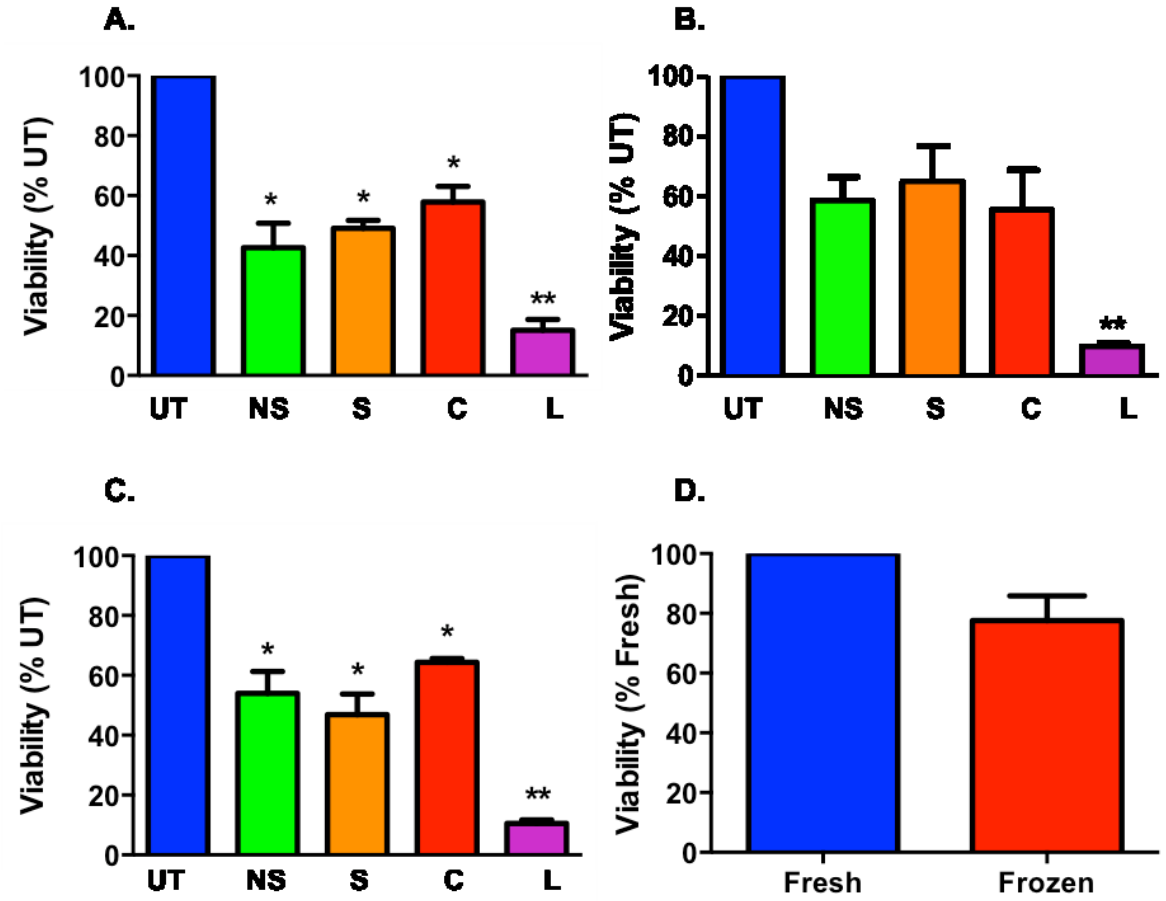


Figure 4.7. Effect on viability of PCLS, as assessed by WST-1 assay, of overnight incubation in neutrophil supernatants. Untreated (●), non-smoker (●), smoker (●), COPD (●), lysed (●). (A) non-stimulated neutrophil supernatants, n=6 supernatants, n=12 rats, (B) supernatants from neutrophils stimulated with 10μM fMLP, n=4-6 supernatants, n=8-12 rats, and (C) supernatants from neutrophils stimulated with 100μM fMLP, n=6-7 supernatants, n=12-14 rats. Data are % untreated PCLS, which were considered 100% viable. (D) Viability of PCLS following overnight incubation in either fresh or frozen media. N=4 rats, viability is expressed as % viability of PCLS in fresh media, which were considered 100% viable. Data are mean ± SEM. *p<0.05, **p<0.01 *vs.* untreated, determined by Wilcoxon signed-rank test.

untreated PCLS is that the media from the neutrophil supernatants has been stored at -80°C and thawed prior to use; whereas untreated PCLS are incubated in fresh media. In order to determine whether this had an effect on viability, untreated PCLS were incubated overnight in either fresh media, or media that had been stored at -80°C and thawed. Assuming that PCLS incubated in fresh media were 100% viable, there was a ~23% reduction in viability in

PCLS incubated in frozen media, compared with those incubated in fresh media, but this was not significant (77.6 ± 8.2% fresh, n=4, Figure 4.7 (D)).

4.3.5. Correlations between supernatant protease composition and airway function

There was a trend towards a weak negative correlation between maximal airway closure and supernatant NE concentration; however this was not significant (Figure 4.8 (A)). There was a significant negative correlation between maximal airway closure and supernatant active MMP-9 concentration (Figure 4.8 (B)). There were, however, no other correlations between supernatant protease composition and any other functional parameters measured.

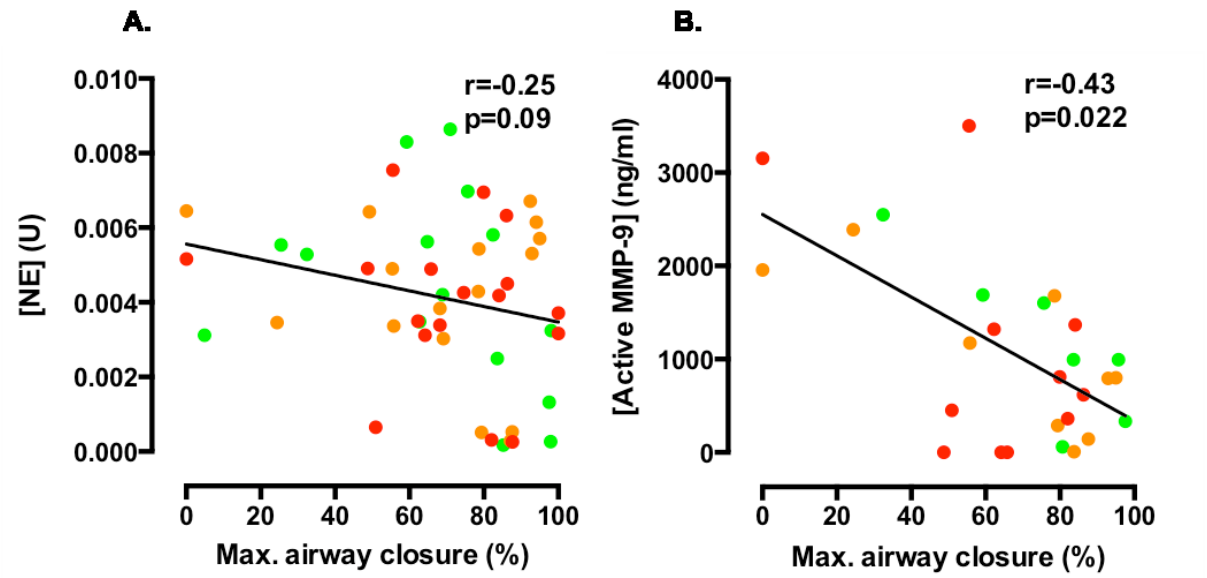


Figure 4.8. Correlation between supernatant protease composition and maximal contraction of airways. (A) Neutrophil elastase (NE), n=46; (B) Active MMP-9, n=27, *p<0.05. r= Spearman rank correlation coefficient.

4.3.6. Histology of PCLS

In order to investigate why neutrophil supernatants alter small airway function, it was important to determine whether overnight incubation in these supernatants had altered airway structure. Therefore, sections of PCLS were stained with EVG, which stains elastin fibres purple and collagen pink, in order to histologically assess small airways.

Untreated PCLS (Figure 4.9, near left panel) have a thick, pseudostratified epithelium, stained pink-brown, with cilia on their apical edge. Below the epithelium, there is clear elastin staining in purple running along the basement membrane. Collagen fibres can also be seen, stained in pink, running around the edge of the airway walls.

4.3.6.1. Non-stimulated neutrophil supernatants

PCLS incubated with non-stimulated supernatants from non-smokers, smokers and COPD patients show some shedding of the epithelium; however elastin and collagen in the airway wall appear intact (Figure 4.9, top panel).

4.3.6.2. Supernatants from neutrophils stimulated with 10μM fMLP

PCLS incubated with supernatants from neutrophils stimulated with 10μM fMLP show extensive shedding of the epithelium, particularly in those incubated with smoker or COPD neutrophil supernatants. There is virtually no elastin in the airway wall in PCLS incubated with supernatants from all subject groups; however collagen fibres remain present. There are also far fewer parenchymal attachments to the airways, compared with untreated PCLS, and those incubated with non-stimulated supernatants (Figure 4.9, middle panel).

4.3.6.3. Supernatants from neutrophils stimulated with 100μM fMLP

PCLS incubated with supernatants from neutrophils stimulated with 100μM fMLP show extensive shedding and destruction of the epithelium. This does not occur to any greater extent than in PCLS incubated with supernatants from neutrophils stimulated with 10μM fMLP, except with regards to non-smoker supernatants where there is quite a pronounced difference in the structure of the epithelium. Again, there is virtually no detectable elastin in the airway walls in PCLS incubated with supernatants from all subject groups, and parenchymal attachments to the airway walls are vastly diminished, compared with untreated PCLS and those incubated with non-stimulated supernatants. However, collagen fibres remain present (Figure 4.9, bottom panel).

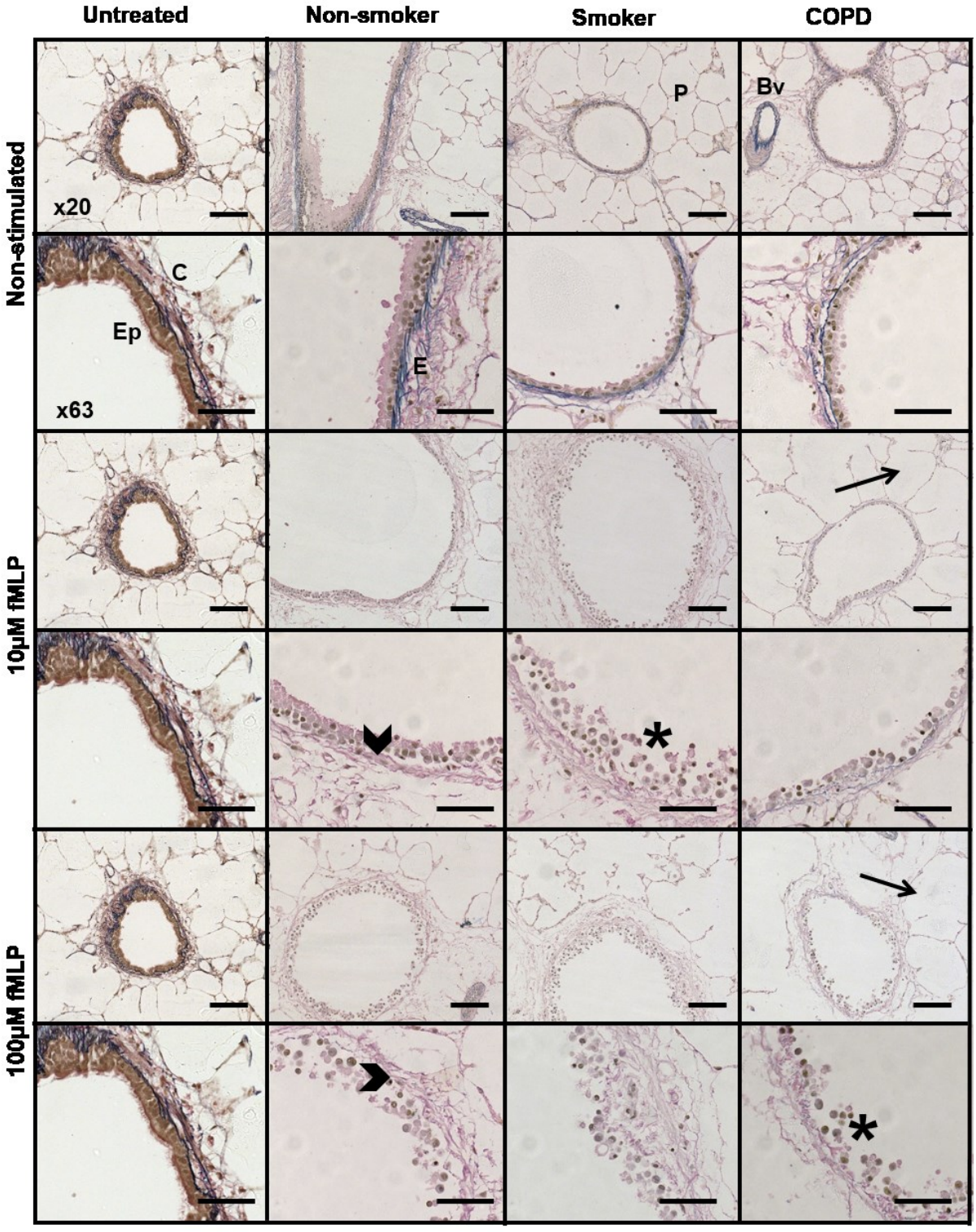


Figure 4.9. Histology of PCLS following overnight incubation in neutrophil supernatants. Sections have been stained with EVG, which stains elastin purple and collagen pink. Ep= epithelium; E= elastin, P= parenchyma, Bv= blood vessel, C= collagen. Arrows indicate destruction of parenchymal attachments; stars indicate epithelial shedding, and arrow heads indicate elastin degradation in airway wall. Scale bar = 50μm.

4.3.7. Summary of results

The main results of the present chapter were:

1. There were no significant differences in rat small airway closure in response to CCh between days 1 and 2.
2. With the exception of 10μM fMLP, which slightly reduced maximal airway closure compared with untreated airways, the compounds used to stimulated neutrophils had no significant effect on either airway function or PCLS viability.
3. Non-stimulated neutrophil supernatants had no effect on small airway function; however they significantly reduced PCLS viability.
4. Supernatants from neutrophils stimulated with 10μM fMLP had no significant effects on airway function; however they caused a ~60% reduction in PCLS viability.
5. Supernatants from neutrophils stimulated with 100μM fMLP reduced maximal closure of airways and, in the case of COPD, significantly reduced both EC_{50} to CCh and baseline airway patency. There was also a significant reduction in PCLS viability.
6. There were weak negative correlations between supernatant NE and active MMP-9 concentration and maximal airway closure; however no other correlations between airway function and neutrophil supernatant composition.
7. Histological analysis of PCLS revealed extensive shedding of the epithelium and destruction of elastin fibres in the airway walls of PCLS incubated in supernatants from neutrophils stimulated with either 10 or 100μM fMLP, but with no obvious differences between subject groups.

4.4. Discussion

In this chapter, it was demonstrated that supernatants from neutrophils stimulated with 100μM fMLP significantly affected small airway function in rat PCLS. This was significant when PCLS were incubated with neutrophil supernatants from COPD patients. In addition, both unstimulated and stimulated neutrophil supernatants significantly reduced PCLS viability following overnight incubation.

4.4.1. Concentration-responsive effects of fMLP

The effect of supernatants from neutrophils stimulated with 100μM fMLP, compared with those stimulated with 10μM, was a ~20% reduction in maximal airway closure. Although this was only statistically significant for COPD supernatants, the mean maximal closure was virtually identical in all subject groups (the difference in statistical significance may be due to a greater range in values in non-smokers and smokers). Conversely, the mean maximal closure in airways treated with neutrophil supernatants stimulated with 10μM fMLP was <10% less than the untreated. This suggests that there are additional products released from neutrophils following 100μM fMLP stimulation that are not released at lower concentrations of fMLP. From the results obtained in Chapter 3, one candidate could be active MMP-9. This is supported by the observation that there was a significant negative correlation between concentration of active MMP-9 in the supernatant, and maximal closure of the airways. MMP-9 induces transforming growth factor (TGF)-β production in human airway epithelial cells, peaking at 6h and fading by 24h at the mRNA level, and peaking at 24h for levels of TGFβ protein (Perng *et al.*, 2011). TGFβ is associated with cell proliferation (Huang *et al.*, 2005), and, hence, possible hypertrophy of the airway wall; however, it is unlikely that, even if TGFβ was produced in a similar way in rat epithelial cells, it would be able to elicit these effects in the time-frame in which the present PCLS were incubated in supernatant. As highlighted in Chapter 3, there are a plethora of additional proteases, not measured in this study, that may be differentially released at 10 and 100μM fMLP that may account for the differences observed in airway closure. Effects of individual neutrophil

products, namely ROS and elastase, on PCLS are investigated in Chapter 5, in order to try to reconcile these effects.

4.4.2. Paracrine factors mediating reduction in baseline airway patency

Supernatants from COPD neutrophils stimulated with 100μM fMLP significantly reduced the EC_{50} to CCh and baseline airway patency of airways on PCLS. In functional terms, airways became more sensitive to CCh and close at lower concentrations; however the airways are more contracted at baseline, prior to any addition of CCh. The effect on EC_{50} is therefore most likely an artefact caused by the reduction in maximal airway closure and reduced baseline patency. Airways in PCLS incubated with smoker neutrophil supernatants also demonstrated a slight (~20%) reduction in baseline airway patency; however this effect was not seen with supernatants from non-smoker neutrophils. Reduced baseline patency suggests that a product released by neutrophils is either directly causing bronchoconstriction of airways, stimulating the release of a bronchoconstrictor from the lung tissue, or inhibiting a bronchodilator. Lipid mediators, such as prostaglandins, are commonly associated with mediating airway tone. NE has been shown to stimulate release of PGE_2 from human airway epithelial cells (Perng *et al.*, 2003). PGE_2 activates specific G-protein coupled receptors (GPCR) on target cells, known as EP receptors, of which there are 4 known subtypes. Depending on the receptor subtype activated, PGE_2 can elicit different bronchomotor effects: PGE_2 is thought to predominantly provoke bronchodilatory effects via EP_2 and EP_4 receptors, but has been shown to have bronchoconstrictor effects via the activation of EP_1 and EP_3 receptors (Clarke *et al.*, 2009). In addition, PGE_2 has been shown to mediate airway constriction in rat PCLS in response to epithelial injury via EP_3 receptor activation (Zhou *et al.*, 2013). Therefore, the reduction in baseline patency may be due to release of PGE_2 from epithelial cells, in response to elastolytic stress, which then causes constriction of the small airways prior to CCh-challenge. In order to investigate this, the prostaglandin content of PCLS supernatants could be determined. PCLS could also be incubated in neutrophil supernatants in the presence of indomethacin, a non-selective cyclooxygenase (COX)-

inhibitor. Prostanoids are derived from arachidonic acid released by the phospholipid membrane, and converted to various prostaglandins via COX. COX exists in 2 isoforms: COX-1, which is constitutively expressed; and COX-2, which is induced by cytokines during inflammation (Kalinski, 2012). Indomethacin inhibits both COX-1 and -2, and therefore inhibits the production of any prostaglandins that may modulate airway tone. Selective EP receptor antagonists could also be used to identify the mechanism further.

4.4.3. Mechanical factors mediating aberrant airway function

In addition, some of the altered functional effects may be due to reduced mechanical constraints imposed on the PCLS small airways in response to degradation of the parenchymal attachments by neutrophil proteases. Airway closure is a compound process mediated by airway smooth muscle fibres contracting and shortening, and the opposing forces exerted by parenchymal attachments to the airway wall. Khan *et al.*, (2010) reported increased luminal narrowing (i.e. reduced baseline patency) in airways incubated overnight in elastase and/or collagenase. However, they also reported increased maximal airway closure to acetylcholine (Ach) and reduced reopening of airways following ACh washout, compared with untreated airways. These results are in direct opposition to those of the present study, wherein there was reduced maximal closure of airways and augmented reopening. Khan *et al* (2010) reasoned that "a loss of wall matrix proteins will both remove a load which opposes airway closure, thereby increasing the velocity of airway closure, as well as decrease the restorative force that helps the wall spring back upon relaxation (decreasing the velocity of relaxation)". It is difficult to reconcile the results in the present study with those reported by Khan *et al* (2010); however, they investigated the effects of elastase and collagenase, which are only 2 components of the variety of neutrophil products that airways would have been exposed to in the present study. The results observed herein are, more than likely, a combination of mechanical effects due to degradation of matrix proteins; and a paracrine response that may involve various lipid mediators, as described above. In order to

investigate this further, PCLS were incubated in various inhibitors, in order to try to elucidate the mechanisms underlying the observed functional differences (see Chapter 6).

4.4.4. Non-specific effects of fMLP

The functional effects and toxicity of the compounds used to stimulate the neutrophils was assessed in order to determine whether any effects observed when incubating PCLS in neutrophil supernatants could be attributed to degranulated neutrophil products, rather than vehicles or stimulants. Neither DMSO (the vehicle for these compounds), Cyt B, nor 100μM fMLP had any significant effect on either small airway function or PCLS viability; however 10μM significantly reduced the maximal closure of airways. Despite this statistical significance, it is unlikely that this result represents a true effect of 10μM fMLP on small airway closure. Firstly, the mean maximal closure of airways incubated in 10μM fMLP was only ~3% less than those incubated with 10μM Cyt. B, and this did not yield a statistically significant result. Secondly, the concentration-response curve for a higher concentration of fMLP (100μM) is almost identical to the untreated curve, with no differences in any of the functional parameters measured. Finally, the actual difference in maximal closure between untreated airways and those incubated in 10μM fMLP was only ~14%, which is unlikely to be physiologically significant, even if it is statistically. There is some evidence of fMLP causing bronchoconstriction in rabbits *in vivo* (Berend *et al.*, 1986); however the authors of this study determined that this effect was, at least in part, mediated by a neutrophil response. The same group also suggested that fMLP causes bronchoconstriction in human airways *in vitro* (Armour *et al.*, 1985, Berend *et al.*, 1988) and *in vivo (Berend et al., 1988)*. However, as the effects observed in the present study were not concentration-responsive, and also not markedly different from the effects of other compounds that did not produce a statistically significant difference in airway closure, it is possible that these results are due to inherent variation in airway function, which is one of the disadvantages of the PCLS system. In order to circumvent this when assessing future results, statistically significant (or insignificant)

results should be interpreted with caution, and a greater emphasis applied to the physiological significance of differences in airway function.

4.4.5. Relationship between airway function and PCLS viability

One notable observation of the results in the present chapter was that airway function and PCLS viability were not necessarily concordant. This was most profound in PCLS incubated in non-stimulated supernatants: there were no significant differences in airway function, compared with untreated airways; however there was a significant reduction in PCLS viability. This suggests that all neutrophil supernatants reduce PCLS viability, irrespective of fMLP stimulation and, hence, active degranulation. Although non-stimulated neutrophils have not, by definition, been treated with fMLP, they may still be activated to a certain extent. Neutrophil activation is a spectrum of processes, rather than being a discreet "activated"/ "not-activated" state. This begins with neutrophil "rolling" in response to adhesion molecules on the endothelium, followed by "firm adhesion" and transmigration to the site of injury. Once in the interstitial space, neutrophils continue travelling along chemotactic gradients until finally, when there is no longer an evident gradient of chemoattractant, oxidative burst and degranulation ensues (Amulic *et al.*, 2012). Therefore, although the neutrophils in the "non-stimulated" samples have not been stimulated with fMLP, they may still be activated, albeit to a lesser extent, possibly during the isolation process.

There was some evidence for the above activation in Chapter 3, as the concentration of MMP-9 was slightly elevated compared with other proteases measured. The reduction in viability does not appear to be augmented when PCLS were incubated in fMLP-stimulated neutrophil supernatants, which might be expected if the detrimental effects on viability were due to proteolytic or oxidative stress. The effects on viability were also not due to, nor caused by interference with, any of the compounds used to stimulate the neutrophils. It is unlikely that the supernatant is interfering with the assay because, as mentioned previously, none of the compounds used to treat the neutrophils (DMSO, Cyt B or fMLP) have any

effect; and, as the neutrophils have not been stimulated, there should not be any neutrophil products that could potentially impede the assay. Overall, it is unclear as to what may be causing the loss in viability. Oxidative stress is commonly associated with cell toxicity (Ryter *et al*. 2007), and reduces PCLS viability (see Chapter 5). As such, there may be low levels of ROS being released by neutrophils, even without fMLP stimulation, that detrimentally effect PCLS viability.

Another possibility was that media that had been freeze/thawed may be affecting the viability of the PCLS. Neutrophils are resuspended in rat IB and the subsequent products are frozen at -80°C until ready for use; however, when performing the experiments, untreated PCLS are incubated in fresh media. Nevertheless, these experiments were performed (Figure 4.7 (D)) and, despite a slight decrease in viability, there were no significant differences between the two conditions.

An additional reason for the dichotomy between airway function and PCLS viability may be due to the PCLS representing a heterogeneous cell population that is differentially affected by the stresses imposed on it by the neutrophil supernatants. PCLS are, in essence, a cross-section of the lung microenvironment, which makes them extremely useful at investigating airway function as, with all of the parenchymal attachments still intact, the airways contract as they might *in vivo*. However, this does also mean that PCLS contain a number of different types of cell, and each may respond differently to an imposed stress. Therefore, the overall "net" effect on function and viability will be observed, without any regard for individual cell responses. This may be the case with viability: airway smooth muscle cells may be far more resilient to the cytotoxicity of the supernatants and, hence, continue to contract; whereas epithelial cells may be more sensitive. Hence, the overall effect will be a net loss of viability, even though the airway smooth muscle cells remain quite viable. To circumvent this, similar experiments could be performed *in vitro* on specific cell types, namely airway epithelial and smooth muscle cells, and viability assessed to determine if there is a difference in cytotoxicity. Alternatively, sections could be stained with Terminal

deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to determine which cells are apoptotic.

4.4.6. Hypothesis: accept/reject

The hypothesis of this chapter was:

"Supernatants from fMLP-stimulated neutrophils will affect PCLS structure and viability, with associated effects on small airway function, including airway closure and capacity for reopening."

The results of this chapter demonstrated that supernatants from neutrophils stimulated with fMLP did significantly affect a number of functional parameters of small airways in rat PCLS. In contrast, supernatants from non-stimulated neutrophil supernatants had little effect on small airway function, suggesting that degranulated neutrophil products mediate these effects. In addition, COPD neutrophil supernatants had more of an effect than supernatants from non-smokers and smokers. As such, this hypothesis can be accepted.

This chapter has provided some evidence that neutrophils may be driving the aberrant small airway function observed in COPD, independently of the increased burden of neutrophil numbers. However, when activated, neutrophils release a plethora of proteolytic enzymes and ROS, any of which may be the driving force behind the aberrant effects induced by COPD neutrophils. As such, the next Chapter will investigate potential mechanisms behind these effects, specifically, the effects of elastase and oxidative stress.

Chapter 5.

Effect of H_2O_2 and Elastase on Small Airway Structure & Function

5. Effect of Hydrogen Peroxide and Elastase on Small Airway Structure and Function

5.1. Introduction

The previous two chapters showed that a number of proteases, including elastase, were released from neutrophils (Chapter 3), and these products induced aberrant small airway function in rat PCLS (Chapter 4). The present chapter takes these observations forward by investigating two potential mechanisms influencing the small airway dysfunction observed following incubation of PCLS with COPD neutrophil supernatants: elastase treatment, using PPE as a substitute for NE; and oxidative stress, in the form of H_2O_2 treatment.

Two of the leading hypotheses regarding the onset of COPD are those of an oxidant/antioxidant imbalance (Rahman *et al.*, 2006), and a protease/antiprotease imbalance (Churg A *et al.*, 2005). ROS and proteases are vital components of the neutrophil armamentarium that enables them to efficiently destroy invading pathogens. Under normal, healthy conditions, these ROS and proteases are quenched by endogenous antioxidants and antiproteases, which limits damage to healthy host tissue. However, under pathological conditions, where there is an excess of inflammatory cells and/or a chronic pro-inflammatory environment, endogenous antiproteases and antioxidants cannot sufficiently counteract these oxidative and proteolytic stresses, leading to destruction of healthy lung tissue, and pathological remodelling of airways (Owen, 2005).

Oxidative stress is a common feature of COPD. The source of oxidants can either be exogenous, as in cigarette smoke; or endogenous, derived from immune cells in response to inflammatory stimuli (MacNee, 2000). H_2O_2 is increased in the exhaled breath condensate of COPD patients compared with never smokers, which is indicative of the increased oxidative burden in the lungs (Dekhuijzen *et al.*, 1996). Neutrophils from COPD patients have increased intracellular ROS production (Milara *et al.*, 2012) and increased respiratory burst (Noguera *et al.*, 2001) in response to fMLP and PMA stimulation, respectively, compared with controls, which may also contribute to the perpetuated oxidative stress characteristic of COPD.

PPE is often used in animal models of elastase-induced lung damage (Tolnai *et al.*, 1985, van de Lest *et al.*, 1995, Khan *et al.*, 2007, Khan *et al.*, 2010). PPE and NE share 40% sequence homology, and are especially similar in the active site regions (Bode *et al.*, 1989); however the products of digestion differ between the two elastases, irrespective of the type of elastin substrate. Nevertheless, PPE is a financially viable substitute for NE when investigating elastolytic lung damage.

Following the results of the previous chapter (Chapter 4), whereby neutrophil supernatants, in particular those from COPD patients, caused aberrant small airway function, the present chapter set out to investigate the effects of elastolytic and oxidative stresses, to investigate whether either of these treatments reproduced the effects of the neutrophil supernatants. In addition, some of the aberrant effects on small airway function observed in the previous chapter may be attributed to mediator release, e.g. cytokines and/or bronchodilator/constrictors, from rat PCLS in response to neutrophil supernatants; therefore this also warranted investigation.

5.1.2. Hypothesis

From the above introductory observations, the hypothesis for this chapter was therefore that:

"H_2O_2 or PPE will affect small airway structure and function, with associated effects on PCLS viability and mediator release; and will, at least in part, reproduce the effects of neutrophil supernatants on small airway function."

5.1.3. Aims

In order to investigate this hypothesis, the aims of this chapter were to:

1. Incubate rat PCLS overnight in increasing concentrations of either H_2O_2 or PPE and, the following day, assess the function of small airways, including baseline patency and airway closure to CCh assess; and the viability and histological structure of PCLS.

2. Measure CINC-1, TNF-α and PGE_2 release from rat PCLS in response to either H_2O_2 or PPE.

5.2. Methods

5.2.1. Generation of rat PCLS and assessment of airway function

Rat PCLS were generated and airway function assessed as described in section 2.2.1.

5.2.2. Initial assessment of airway function and grouping of PCLS

As described in section 2.2.1.4, airways on PCLS were subject to an initial assessment of maximal airway closure by administration of 10μM CCh. Following a series of washes in rat IB, and a 1 h recovery period to allow airways to reopen, PCLS were then grouped based on the criteria outlined in section 2.2.1.4.

5.2.3. Incubation of PCLS in H_2O_2

PCLS were placed in 1ml rat IB in individual wells of a 24-well plate. H_2O_2 was prepared at the following stock concentrations in rat IB: 300mM, 100mM, 30mM, and 10mM. These were then added to 1ml rat IB (1% $^{v}/_{v}$), to give final concentrations of 3mM, 1mM, 0.3mM and 0.1mM. For untreated controls, PCLS were incubated in 1ml rat IB. All PCLS were then incubated overnight at 37°C. Small airway CCh concentration-responses were then performed the following day, as described in section 2.2.1.4.

5.2.4. Incubation of PCLS in PPE

PCLS were placed in 1ml rat IB in individual wells of a 24-well plate. PPE was reconstituted at 1U/μl in rat IB. PPE was added to 1ml rat IB to give the following final concentrations: 3U, 1U, 0.3U and 0.1U. For untreated controls, PCLS were incubated in 1ml rat IB. All PCLS were then incubated overnight at 37°C. CCh concentration-responses were then performed the following day, as described in section 2.2.1.4.

5.2.5. Measurement of PCLS viability

PCLS viability was measured by WST-1 assay, as described in section 2.2.2.1. For the purposes of these experiments, untreated PCLS were considered to be 100% viable, and so

the absorbance of all treated PCLS was presented as a percentage of the untreated absorbance.

5.2.6. Measurement of TNF-α and CINC-1 in PCLS supernatant

To assess release of TNF-α and CINC-1 from PCLS, the rat IB in which the PCLS were incubated overnight (± H_2O_2 or PPE) was isolated and stored at -80°C.

TNF-α and CINC-1 were both detected using a sandwich enzyme-linked immunosorbant assay (ELISA). The basis of the assay was that microplates were first coated with an analyte-specific “capture” antibody, which forms the first layer of the “sandwich”. Samples or standards are then added to this microplate, and any analyte present binds to this immobilised capture antibody. A biotin-labelled detection antibody, which also binds to the analyte, is then added and forms the final layer of the “sandwich”. Streptavidin- horseradish peroxidase (HRP) binds to the biotin on the detection antibody, and substrate solution, a mix of 3,3', 5,5' tetramethylbenzidine (TMB) and H_2O_2, reacts with the HRP bound to the streptavidin, resulting in a colour change that is proportional to the amount of analyte present. Sulphuric acid is added to stop this reaction.

Clear, 96-well Nunc™ microplates were coated with capture antibody at final concentrations of 0.4 and 4µg/ml, for CINC-1 and TNF-α respectively, and incubated overnight at room temperature. Manufacturers data sheets indicated that the CINC-1 antibody was specific for natural and recombinant rat CINC-1 and did not cross-react with other rat CINC proteins (CINC-2α, -2β and -3), nor related proteins in human and mouse (GRO-α, -β, or –γ in human, and MIP-2 in mouse); although there was 10.5% cross-reactivity with mouse KC. Similarly, TNF-α antibody was indicated as being specific for natural and recombinant rat TNF-α and did not cross-react with human and porcine TNF-α and –β homologues; however there was 2.4% cross-reactivity with mouse TNF-α.

The following day, the plates were washed multiple times in wash buffer (0.05% Tween®20 in PBS), and blocked with 1% ($^{w}/_{v}$) bovine serum albumin (BSA) in PBS for 2h, to prevent

non-specific binding. After several more washes, 100µL samples and standards were added to the microplate: standards were serially diluted from 1000pg/ml for CINC-1 and 4000pg/ml for TNF-α; and samples were diluted 1/50 for CINC-1, and left undiluted for TNF-α. These were incubated at room temperature for 2h. Plates were then washed several times in wash buffer, and 100µL detection antibody added to each well at 50ng/ml for CINC-1 and 225ng/ml for TNF-α. Plates were, again, incubated at room temperature for 2h. After several more washes in wash buffer, 100µL Streptavidin-HRP was added at a final concentration of 100µg/ml, and incubated in the dark for 20min. Plates were washed several times, and 100µL TMB substrate solution added to each well, and incubated in the dark for 20min. Fifty µL 1M H_2SO_4 is added to each well to stop the reaction, and microplates read on a microplate reader at λ450nm, with a reference λ of 570nm. Lower limit of detection for CINC-1 and TNF-α were 15.6 and 62.5pg/ml, respectively. To construct the standard curve, absorbance was plotted against concentration of CINC-1/TNF-α standards and a non-linear regression curve fit to the data (Figure 5.1 (A-B)), from which test samples were interpolated.

5.2.7. Measurement of PGE_2 in PCLS supernatant

To assess release of PGE_2 from PCLS, the rat IB in which the PCLS were incubated overnight (± H_2O_2 or PPE) was isolated and stored at -80°C.

PGE_2 was measured using PGE_2 Express EIA kit. This assay is based on competition between PGE_2 in samples and a PGE_2-acetylcholinesterase (AChE) conjugate (called PGE_2 tracer) for binding to a limited amount of monoclonal PGE_2 antibody. The amount of PGE_2 tracer and PGE_2 monoclonal antibody are kept constant; however the amount of PGE_2 in samples will change. Therefore, the amount of PGE_2 tracer that is able to bind to the PGE_2 monoclonal antibody will be inversely proportional to the concentration of PGE_2 in the sample. A 96-well plate is pre-coated with a goat polyclonal anti-mouse IgG antibody, which binds to the PGE_2-antibody complex. The amount of PGE_2 tracer that was able to bind to the PGE_2 antibody, which then binds to the polyclonal antibody on the plate, was detected by

adding Ellman's Reagent, which contains a substrate of AChE and reacts to form a coloured product that can be measured spectrophotometrically.

The assay was performed as per the manufacturer's instructions. Briefly, PCLS supernatants were diluted 1:100 in EIA buffer provided in the kit, in order to fit on the linear part of the

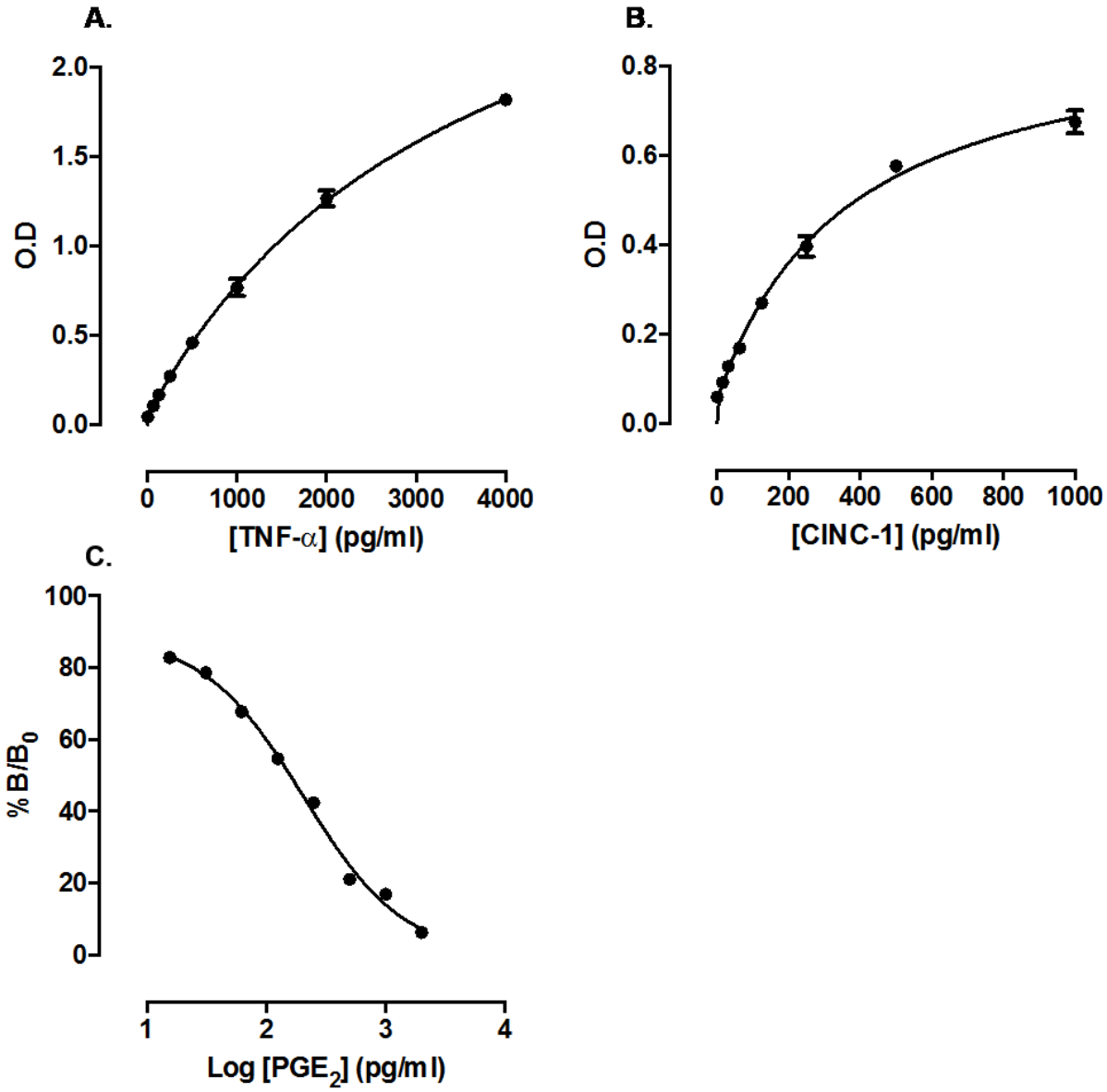


Figure 5.1. Standard curves of ELISAs and EIA assays. (A) TNF-α concentration against optical density (O.D) at λ450-570nm, lower limit of detection 62.5pg/ml. (B) CINC-1 concentration against O.D at λ450-570nm, lower limit of detection 15.6pg/ml. (C) Log PGE_2 concentration against $\%B/B_0$ (% sample binding/maximum binding) , lower limit of detection 36pg/ml. Data are mean ± SEM of duplicate readings.

standard curve. PGE_2 standards were serially-diluted from 2000-15.6pg/ml in EIA buffer. A number of controls wells were run alongside standards and samples: a maximal binding (B_0) control, which contains 50µl each of PGE_2 tracer and PGE_2 monoclonal antibody alone and is a measure of the maximal amount of tracer able to non-competitively bind to the antibody; a non-specific binding (NSB) control, which contains 50µl tracer alone and measures non-specific binding of the tracer to the antibody coated on the plate; a blank (Blk) control, which contains only 200µl Ellman's Reagent at the development stage, and hence measures background absorbance caused by Ellman's Reagent; and a total activity (TA) control, where 5µl PGE_2 tracer was added to 200µl Ellman's Reagent at the development stage, which measures the total enzymatic activity of the tracer in an excess of substrate. Fifty µl of standards and samples were incubated, in duplicate, with 50µl each of PGE_2 tracer and monoclonal antibody for 1h at room temperature on an orbital shaker. Following this, the plate was washed several times to remove all unbound sample/PGE_2 tracer, and incubated with 200µl Ellman's Reagent for 60-90min at room temperature, protected from light. The microplate was then read on a microplate reader at λ420nm. To prepare the data for constructing a standard curve, the average absorbance from the NSB wells was subtracted from all wells. The corrected samples and standards (B) were then divided by the average corrected B_0, and multiplied by 100, to get a $\%B/B_0$ (% standard or sample bound/ maximum bound). To construct the standard curve, the $\%B/B_0$ was plotted against log concentration of PGE_2 standards, and four-parameter logistic non-linear regression curve fit to the data (Figure 5.1 (C)). Sample PGE_2 concentrations were then interpolated from this curve. Limit of detection was 36pg/ml. Monoclonal PGE_2 antibody has ≥1% cross-reactivity with the following compounds: PGE_2-ethanolamide (100%), PGE_2-1-glyceryl ester (100%), PGE_3 (43%), PGE_1 (18.7%), 8-*iso*-PGE_2 (2.5%), sulprostone (1.25%), and 6-keto-$PGF_{1\alpha}$ (1%).

5.2.8. Data analysis

In this chapter, normal distributions were not assumed for any of the analyses, therefore non-parametric Kruskal-Wallis tests, with Dunn's post-hoc comparisons were performed

when comparing airway parameters and mediator release. CCh dose-response curves were constructed by plotting % airway closure (calculated with respect to Day 1 baseline airway patency), against the log CCh concentration, and constructing a four-parameter logistic non-linear regression curve. Mean (±SEM) EC_{50} values to CCh were then calculated from the PCLS used to generate each curve. Airway functional parameters and viability of PCLS treated with H_2O_2 or PPE were compared using Kruskal-Wallis tests, with Dunn's post-hoc comparisons. For familiarity of presentation, data are displayed as mean ± SEM. The null hypothesis of no significant differences between untreated airways and those treated with control compounds or neutrophil supernatants was rejected at $p<0.05$.

5.3. Results

5.3.1. Effect of H_2O_2 on small airway closure

CCh induced a concentration-dependent increase in small airway closure in PCLS that had been pre-incubated in H_2O_2, with maximal increases above baseline of 20-90% at 10μM CCh, and EC_{50} values of 300-850nM CCh (Fig 5.2 (A) and Table 5.1).

At the lower concentrations of H_2O_2 (0.1-0.3mM), there were minimal effects on maximal airway closure, with decreases of <10% compared with untreated airways (Table 5.1). However, at higher concentrations of H_2O_2 (1 and 3mM), the maximal airway closure was

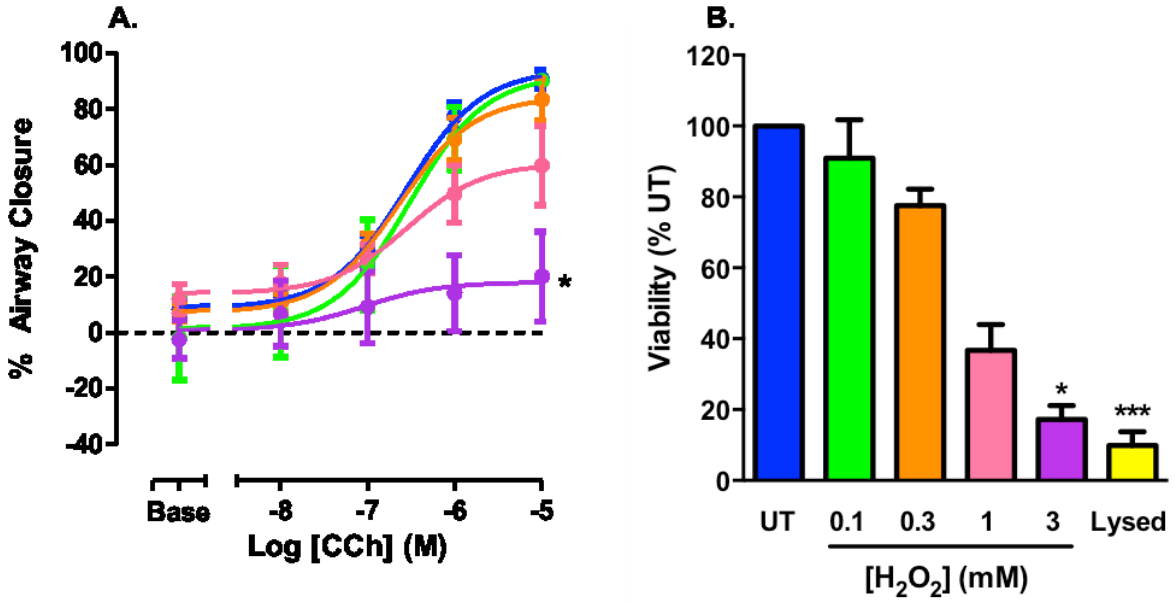


Figure 5.2. Effect of H_2O_2 on small airway function and PCLS viability. (A) Concentration-response curve of small airway closure in response to overnight incubation of PCLS in H_2O_2. Untreated (●), n=6 rats; 0.1mM (●), n=4 rats; 0.3mM (●), n=5 rats; 1mM (●), n=6 rats; 3mM (●), n=6 rats. Data are mean ± SEM. *p<0.05 untreated *vs.* 3mM, as determined by Kruskal Wallis and Dunn's post-hoc tests. All data sets, except for 3mM H_2O_2, above 1μM CCh were significantly higher than baseline (p<0.05-0.001). (B) Viability of airways, as assessed by WST-1 assay, following overnight incubation in H_2O_2. Untreated, n=6 rats; 0.1mM, n=6 rats; 0.3mM, n=4 rats; 1mM, n=6 rats; 3mM, n=6 rats; lysed, n=6 rats. Data are expressed as % untreated PCLS, which were considered 100% viable. Data are mean ± SEM. *p<0.05, ***p<0.001 *vs.* untreated, as determined by Wilcoxon signed-rank test.

reduced to ~60% and 20% respectively, compared with untreated airways that were ~90% closed. There was a two-fold increase in baseline airway patency of airways incubated in 3mM H_2O_2; however this was not significant, and there were no notable effects on baseline patency at any other concentration of H_2O_2. The CCh response of small airways on PCLS incubated with 3mM H_2O_2 showed a shift in EC_{50}, with a ~three-fold increase compared with untreated airways; however this did not reach significance. There were no notable differences in EC_{50} at any other concentrations of H_2O_2.

Table 5.1. Effect of H_2O_2 on small airway function

[H_2O_2] (mM)	Maximal Closure (%)	[CCh] for maximal closure (μM)	Baseline Airway Patency (%)	EC_{50} (nM)
Untreated (n=6)	90.7 ± 3.2	10	8.3 ± 3.6	307 ± 48
0.1mM H_2O_2 (n=4)	90.3 ± 2.1	10	-2.1 ± 15.0	379 ± 137
0.3mM H_2O_2 (n=5)	83.4 ± 7.5	10	7.1 ± 3.1	320 ± 102
1mM H_2O_2 (n=6)	59.8 ± 14.2	10	11.9 ± 5.3	378 ± 116
3mM H_2O_2 (n=6)	20.1 ± 16.1*	10	20.1 ± 16.1	852 ± 619

Data are mean ± SEM. *p<0.05 *vs*. untreated, as determined by Kruskal-Wallis test and Dunn's post hoc tests. H_2O_2= hydrogen peroxide

5.3.2. Effect of H_2O_2 on PCLS viability

Having shown that high concentrations of H_2O_2 reduced maximal airway closure in response to CCh, it was important to determine if this was due to alterations in tissue viability.

Incubation in increasing concentrations of H_2O_2 resulted in a concentration-dependent decrease in PCLS viability (Fig 5.2 (B)). At the lower concentrations of H_2O_2, 0.1 and 0.3mM, there was ~10-20% decrease in PCLS viability, compared with untreated PCLS. However, at higher concentrations of H_2O_2, 1 and 3mM, viability of PCLS was ~40-20% of untreated PCLS, and this was significant at 3mM H_2O_2. At 3mM H_2O_2, PCLS viability was similar to that of lysed PCLS, which were, as expected, ~10% of untreated viability.

5.3.3. Effect of PPE on small airway closure

After determining the effects of H_2O_2 on small airway function and PCLS viability, it was of interest to investigate the role of elastolytic stress on these parameters, as this was another mediator potentially implicated in neutrophil supernatant-induced small airway dysfunction.

CCh induced a concentration-dependent increase in small airway closure in PCLS that had been pre-incubated in PPE, with maximal increases above baseline of 40-80% at 10μM CCh, and EC_{50} values of 730-3000nM CCh (Fig 5.3 (A) and Table 5.2).

Incubation in increasing concentrations of PPE resulted in a concentration-dependent decrease in maximal airway closure (Fig 5.3 (A)). At the lower concentrations of PPE (0.1 to 0.3U) this was only ~4-10% reduction, compared with untreated airways; however, at higher concentrations of PPE (1 and 3U), there was an ~20-40% reduction, compared with untreated airways (Table 5.2). There were no significant differences in EC_{50} values between untreated airways and airways treated with PPE; however there was 1.5-4.5-fold increase in EC_{50} to CCh with PPE treatments, compared with untreated airways. There was a ~2-fold increase in baseline airway patency at 3U PPE, compared with untreated airways; however this was not significant.

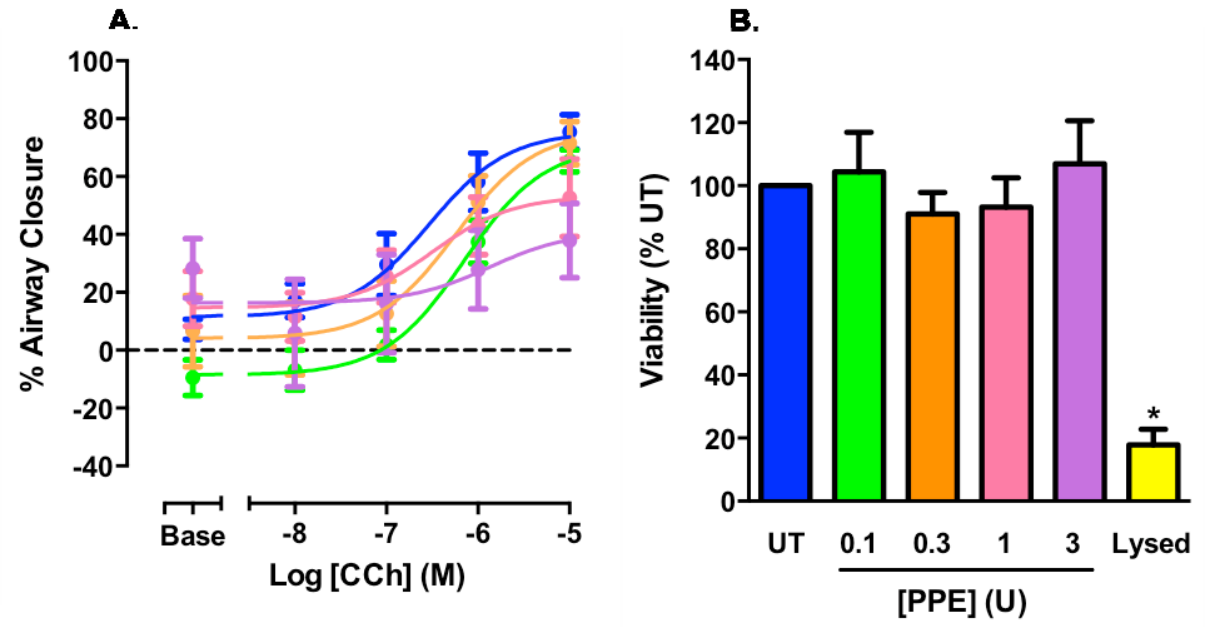


Figure 5.3. Effect of PPE on small airway function and PCLS viability. (A) Concentration-response curve of small airway closure in response to overnight incubation of PCLS in PPE. Untreated (●), n=5 rats; 0.1U (●), n=6 rats; 0.3U (●), n=6 rats; 1U (●), n=6 rats; 3U (●), n=6 rats. Data are mean ± SEM. The only data set significantly higher than baseline ($p<0.05$) was with 0.1U PPE at 10μM CCh. (B) Viability of airways, as assessed by WST-1 assay, following overnight incubation in PPE. Untreated, n=8 rats; 0.1U, n=8 rats; 0.3U, n=8 rats; 1U, n=8 rats; 3U, n=8 rats; lysed, n=8 rats. Data are expressed as % untreated PCLS, which were considered 100% viable. Data are mean ± SEM. *$p<0.05$ *vs.* untreated, as determined by Wilcoxon signed-rank test.

5.3.4. Effect of PPE on PCLS viability

PPE had no significant effect on PCLS viability (Fig. 5.3 (B)), as PCLS incubated with PPE were 91-107% of untreated viability. Lysed PCLS, as expected, were ~18% untreated viability.

5.3.5. Effect of H_2O_2 and PPE on PGE_2 release from PCLS

Maximal airway closure of small airways in PCLS incubated with both H_2O_2 and PPE was reduced, compared with untreated airways. One possible mechanism behind this could be the release of bronchodilators from PCLS, in response to the added stresses. As such,

Table 5.2. Effect of PPE on small airway function				
[PPE] (U)	Maximal Closure (%)	[CCh] for maximal closure (μM)	Baseline Airway Patency (%)	EC_{50} (nM)
Untreated (n=5)	75.4 ± 6.0	10	7.2 ± 3.4	738 ± 309
0.1U (n=6)	65.4 ± 3.8	10	-9.5 ± 6.1	1386 ± 757
0.3U (n=6)	71.6 ± 7.5	10	6.6 ± 12.4	3376 ± 2804
1U (n=6)	52.6 ± 13.3	10	17.8 ± 9.5	2198 ± 1520
3U (n=6)	37.9 ± 12.8	10	28.3 ± 10.3	1185 ± 518
Data are mean ± SEM. PPE = Porcine pancreatic elastase				

PGE_2 was measured in the supernatant of PCLS incubated with either H_2O_2 or PPE. There was a ~1.5fold decrease in PGE_2 concentration in PCLS supernatant following treatment with H_2O_2 or PPE, compared with untreated PCLS; however this effect did not appear to be concentration-dependent. Untreated PCLS released ~12ng/ml, and all concentrations of H_2O_2 induced release of ~7-9ng/ml from PCLS, and all concentrations of PPE induced release of ~8.7-11.5ng/ml (Figure 5.4).

5.3.6. Effect of H_2O_2 and PPE on CINC-1 release from PCLS

Following investigation of PGE_2 in PCLS supernatants, it was of interest to also investigate the release of other mediators, namely CINC-1 and TNF-α, to assess the nature of the microenvironment induced by H_2O_2 and PPE. Treatment of PCLS with increasing

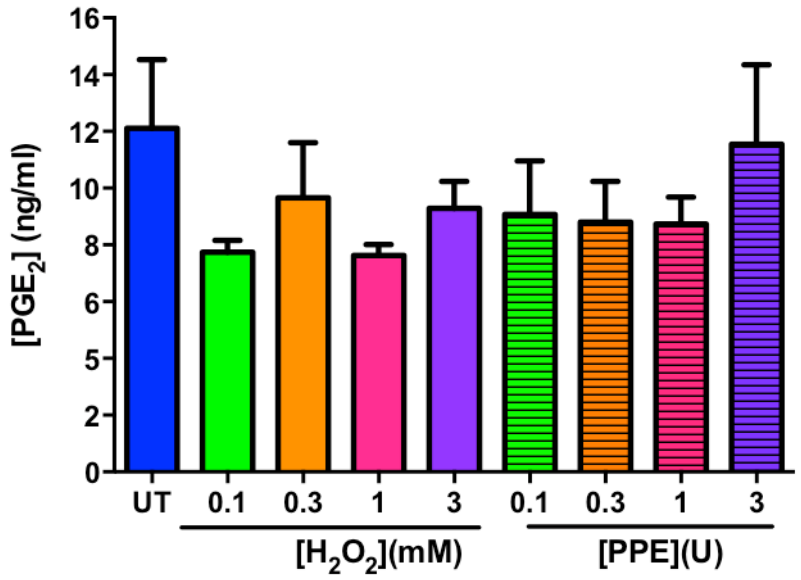


Figure 5.4. Effect of H_2O_2 and PPE on PGE_2 release in PCLS supernatant, following overnight incubation. All groups are n=4 rats. Data are mean ± SEM.

concentrations of H_2O_2 resulted in a concentration-dependent decrease in CINC-1 release from PCLS (Figure 5.5). Untreated PCLS released ~4.5ng/ml CINC-1, and 0.1mM H_2O_2, released similar amounts at ~4.2ng/ml. However, this was reduced ~1.5-8 fold at 0.3, 1 and 3mM H_2O_2. PPE also resulted in a concentration-dependent decrease in CINC-1 (Figure 5.5); however, unlike with H_2O_2, there was a 2-fold reduction of CINC-1 at the lowest concentration of PPE, and ~4-11-fold decreases at 0.3-3U PPE. This reduction was significant at 3U PPE.

5.3.7. Effect of H_2O_2 and PPE on TNF-α release from PCLS

Levels of TNF-α were below the limit of detection (i.e. 62.5pg/ml) in supernatants from PCLS treated with either H_2O_2 or PPE. This may reflect a true lack of response, or be due merely to a problem with the assays. As such, PCLS TNF-α release was investigated following addition of LPS, a known inducer of the TNF-α pathway, either alone or in conjunction with H_2O_2 or PPE.

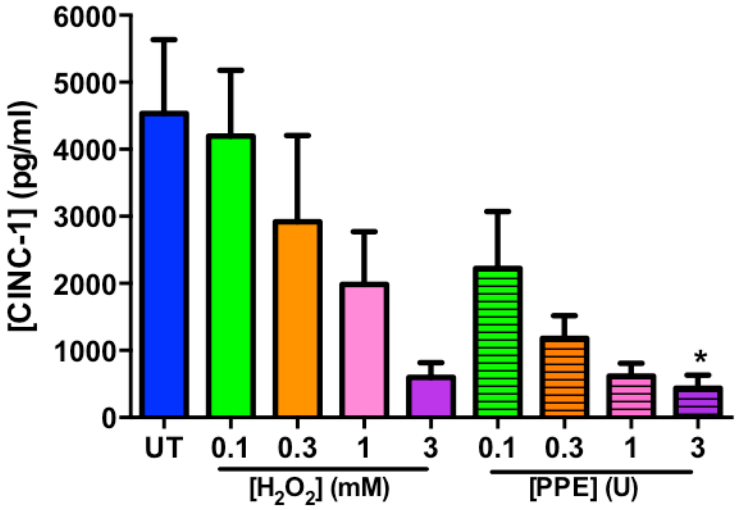


Figure 5.5. Effect of H_2O_2 and PPE on CINC-1 in PCLS supernatant, following overnight incubation. All groups are n=4 rats. Data are mean ± SEM. *p<0.05 *vs.* untreated, as determined by Kruskall-Wallis test and Dunn's post hoc comparisons

5.3.7.1. Effect of LPS on PCLS TNF-α release and viability

An LPS concentration-response was performed on PCLS, and TNF-α release measured, in order to determine the optimal concentration of LPS to use in conjunction with H_2O_2 or PPE. As expected, increasing concentrations of LPS induced TNF-α release in a concentration-dependent manner, with 10-15-fold increases in TNF-α release at 100 and 1000ng/ml LPS (Fig. 5.6 (A)). As such, 100ng/ml LPS was chosen for use in subsequent experiments. LPS had no significant effect on PCLS viability (data not shown).

5.3.7.2. Effect of LPS, in conjunction with H_2O_2 and PPE, on TNF-α release from PCLS

In response to 100ng/ml LPS, there was a 6.5-fold increase in TNF-α release from PCLS, compared with untreated PCLS (Fig. 5.6 (B)). However, in conjunction with increasing concentrations of H_2O_2, there was a concentration-dependent decrease in TNF-α release, with a 9-fold decrease at 1mM H_2O_2, and a 100-fold decrease at 3mM H_2O_2. In conjunction with PPE, there were ~100-fold decreases in TNF-α release at 0.1U PPE, and levels were virtually undetectable at higher concentrations of PPE (Fig. 5.6 (B)).

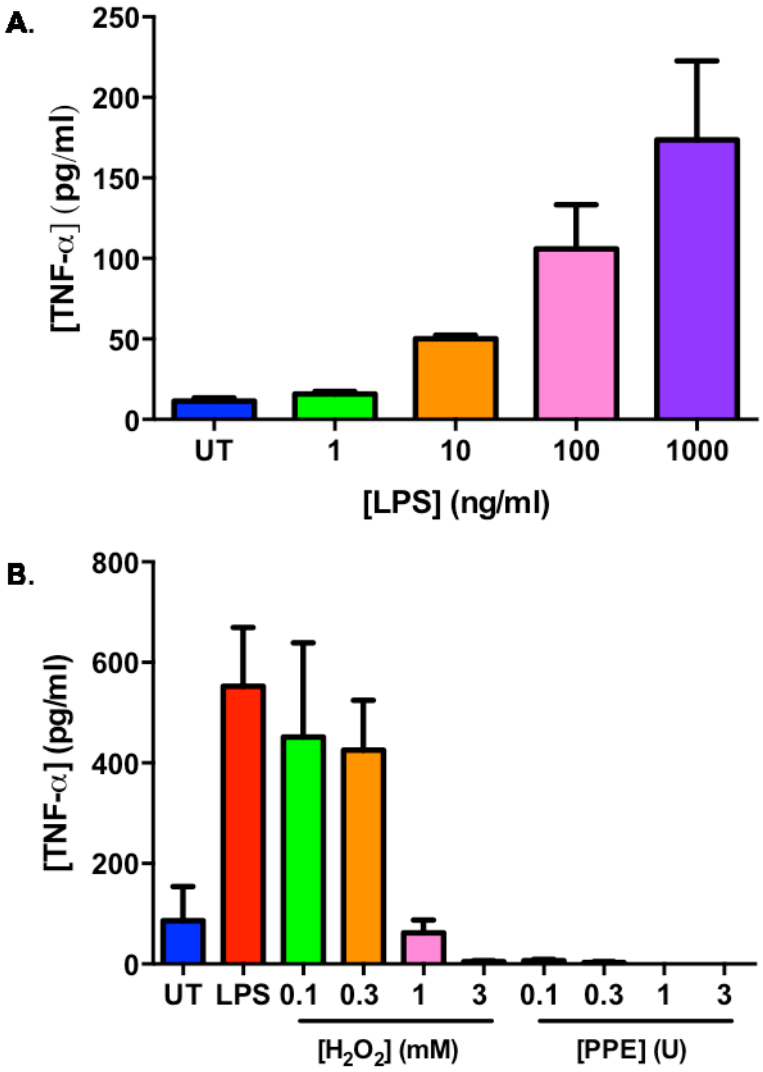


Figure 5.6. Effect of H_2O_2 or PPE on LPS-induced TNF-α release. (A) LPS concentration-response. All groups n=4 rats. (B) TNF-α release in response to 100ng/ml LPS and increasing concentrations of either H_2O_2 or PPE. All groups n=3 rats. Data are mean ± SEM.

5.3.8. Effect of H_2O_2 and PPE on mediator levels in rat IB 'spiked' with either CINC-1 or TNF-α

The previous section (Section 5.3.7.2) showed that H_2O_2 and PPE reduced LPS-induced TNF-α concentrations in rat PCLS supernatants. Consequently, in order to assess whether H_2O_2 or PPE alone had any direct effect on CINC-1 or TNF-α detection, rat IB was "spiked"

with either 500pg/ml CINC-1 or 1000pg/ml TNF-α and incubated overnight with either 0.1-3mM H_2O_2 or 0.1-3U PPE.

There was a ~1.2-2-fold decrease in TNF-α and CINC-1 levels in untreated (UT) rat IB, and those incubated with increasing concentrations of H_2O_2, with respect to the 500pg/ml CINC-1 "spike" (Figure 5.7 (A)) or 1000pg/ml TNF-α 'spike' (Figure 5.7 (B)). However, H_2O_2 did not appear to have any effect on CINC-1 or TNF-α levels (Fig. 5.7). In contrast, in rat IB treated with PPE, there were virtually undetectable levels of CINC-1 and TNF-α at all concentrations of PPE (Figure 5.7).

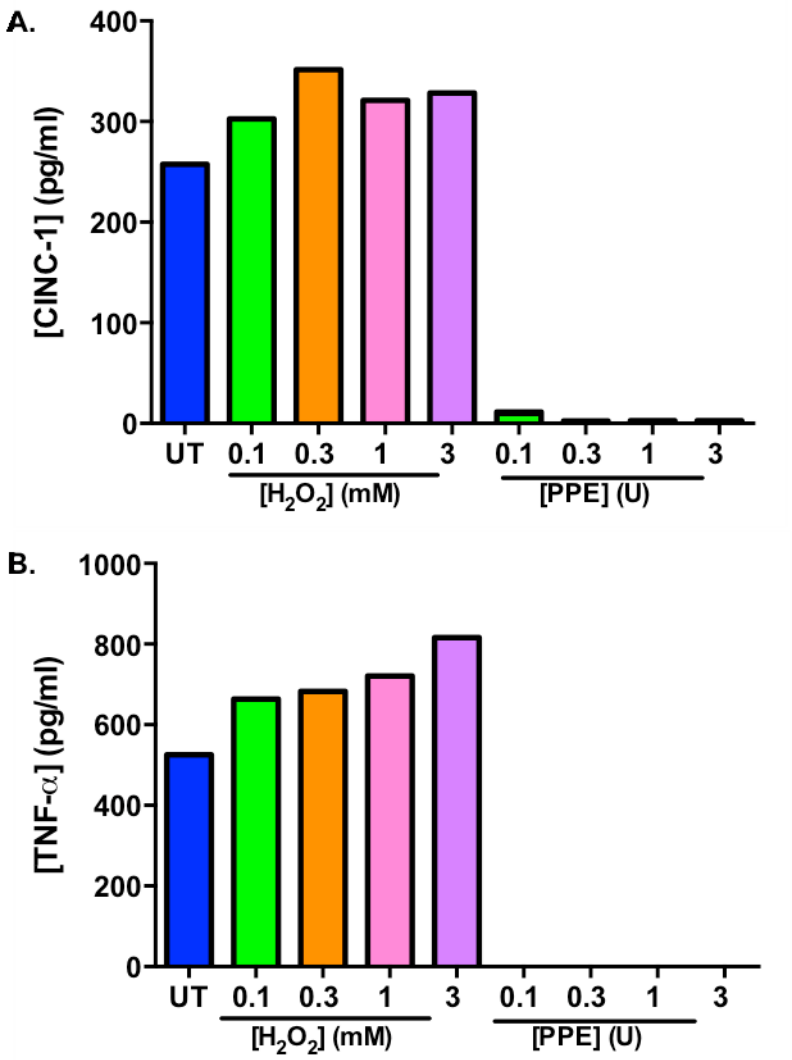


Figure 5.7. Effect of overnight incubation in H_2O_2 and PPE on measurement of "spiked" concentrations of (A) CINC-1 and (B) TNF-α levels in rat IB. Data were a single measurement.

5.3.9. Histology of PCLS

As small airway function and mediator release from PCLS in response to H_2O_2 and PPE treatment had already been assessed, it was important to also assess the architecture of the small airways in response to these stresses, and to elucidate any abnormalities that may be associated with the previous results in this chapter. As such, histological analysis of small airways in PCLS treated with either H_2O_2 or PPE was performed.

Untreated PCLS (Figure 5.8 and 5.9, near left panel) have a thick, pseudostratified epithelium, stained pink-brown, with cilia on their apical edge. Below the epithelium, there is clear elastin staining in purple running along the basement membrane. Collagen fibres can also be seen, stained in pink, running around the edge of the airway walls.

5.3.9.1. H_2O_2

PCLS that were incubated with 0.1-1mM H_2O_2 show little destruction to the epithelium; however, at the highest concentration of H_2O_2 (3mM), there is clear destruction of the epithelium as it appears to be only ~1 cell thick, compared with the epithelium of untreated airways, which appears more pseudo-stratified. At 0.1 and 0.3mM, there are clear, intact parenchymal attachments, which are reduced at 1 and 3mM H_2O_2. Elastin and collagen in the airway wall appears to be intact at all concentrations of H_2O_2 (Figure 5.8).

5.3.9.2. PPE

PCLS incubated with all concentrations of PPE have no detectable elastin in the airway walls. At all concentrations, there is some evidence of epithelial detachment, with portions of the epithelium missing around the airway wall. Parenchymal attachments are only visible in 3U PPE; however they appear to be intact. Collagen staining is evident at all concentrations of PPE. Unlike with other treatments, damage to the airway is not particularly consistent with increasing concentration of PPE; i.e. is not concentration-responsive (Figure 5.9).

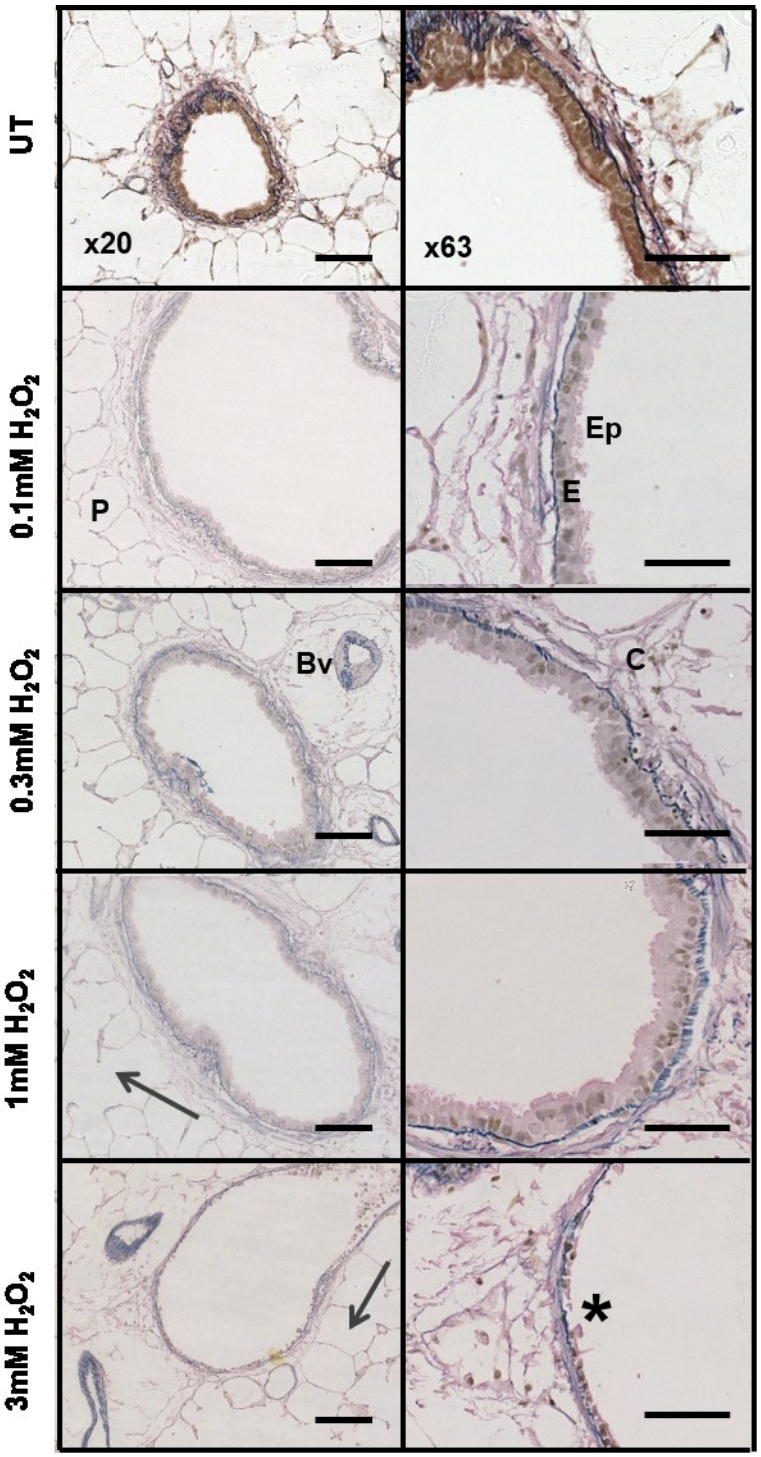


Figure 5.8. Histology of small airways in PCLS incubated overnight with increasing concentrations of H_2O_2. Sections have been stained with Elastin Van Giesons (EVG), which stains elastin purple and collagen pink. UT= untreated; Ep= epithelium; E= elastin; P= parenchyma; Bv= blood vessel; C= collagen. Stars indicate epithelial shedding, arrows indicate parenchymal destruction. Scale bar = 50μm.

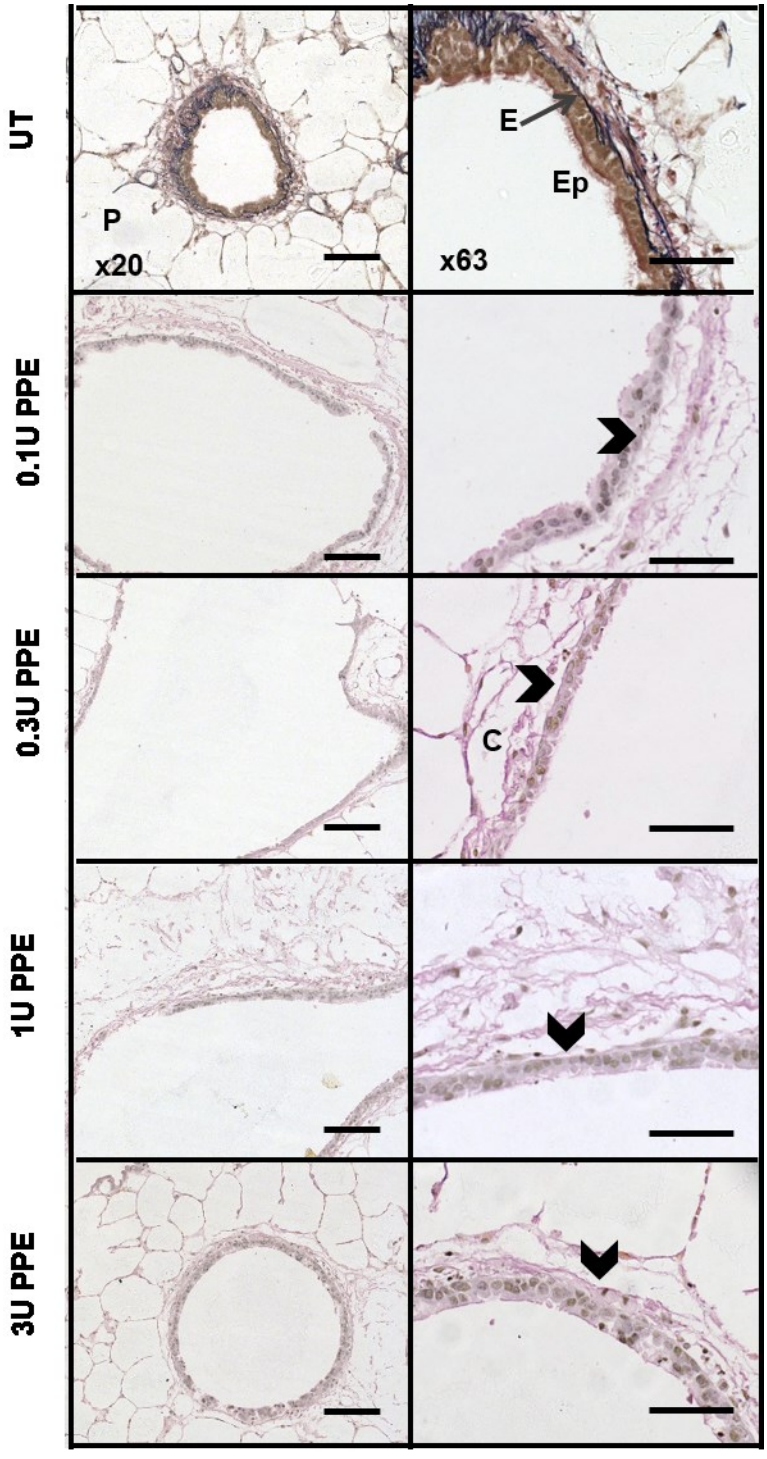


Figure 5.9. Histology of PCLS incubated overnight with increasing concentrations of PPE. Sections have been stained with Elastin Van Giesons (EVG). Ep= epithelium; E= elastin; P= parenchyma; C= collagen. Arrowheads indicate loss of elastin in airway wall. Scale bar = 50μm.

5.3.10. Summary of results

The results of the present chapter were:

1. Increasing concentrations of H_2O_2 caused concentration-dependent decreases in maximal closure of small airways in rat PCLS; however had no notable effects on either EC_{50} to CCh or baseline airway patency. Increasing concentrations of H_2O_2 also caused concentration-dependent decreases in PCLS viability.
2. Increasing concentrations of PPE caused concentration-dependent decreases in maximal closure of small airways in rat PCLS; however had no notable effects on either EC_{50} to CCh or baseline airway patency. Increasing concentrations of PPE had no effect on PCLS viability.
3. Both H_2O_2 and PPE had no significant effect on PGE_2 release, but caused concentration-dependent decreases in CINC-1 release from PCLS.
4. There were no detectable levels of TNF-α from PCLS following overnight incubation in either H_2O_2 or PPE; however LPS could concentration-dependently induce TNF-α release from PCLS. H_2O_2 caused concentration-dependent decreases in LPS-induced TNF-α release from PCLS; however all concentrations of PPE abolished LPS-induced TNF-α release from PCLS.
5. H_2O_2 had no effect on recovery of either CINC-1 or TNF-α from “spiked” media; however all concentrations of PPE virtually abolished both proteins.
6. Histological analysis of PCLS revealed that with increasing concentrations of H_2O_2 there is increasing epithelial damage; however elastin and collagen appear to remain intact. All concentrations of PPE resulted in complete obliteration of elastin staining and epithelial damage; however collagen remained intact.

5.4. Discussion

The results of the previous chapter (Chapter 4) demonstrated that incubation of rat PCLS in supernatants from stimulated neutrophils resulted in aberrant airway function and a reduction in PCLS viability. Exactly why neutrophil products should elicit these responses is unclear. In order to investigate some putative mechanisms, this chapter examined the effect of oxidative and elastolytic stresses, as they are important components of the neutrophil arsenal against invading pathogens. Therefore, the present chapter investigated the effects of H_2O_2 and PPE on small airway function, viability and mediator release.

5.4.1. Effects of H_2O_2 on small airway closure

Both H_2O_2 and PPE caused concentration-dependent decreases in maximal small airway closure. In the case of H_2O_2, this reduction in maximal airway closure was concomitant with reduced PCLS viability, suggesting a link between these two parameters. There are a number of different ways in which oxidants can damage cells: they can directly damage structural components, for example collagen (Curran *et al.*, 1984); they can initiate lipid peroxidation of membrane phospholipids (Gutteridge, 1995); and can inhibit ATP synthesis and cause direct DNA damage (Cochrane, 1991). H_2O_2 can initiate apoptosis pathways (Ryter *et al.*, 2007), and has previously been shown to cause cell death in rat lung explants (Martin *et al.*, 1981); therefore the result on PCLS viability is concordant with the literature.

H_2O_2 has also been shown to mediate contractile responses of airways. Treatment with 1mM H_2O_2 alone has been shown to initiate transient contractile responses in rat tracheal explants (Szarek *et al.*, 1990) and in lung parenchymal strips (Kramer *et al.*, 1987). This response was attenuated by indomethacin, a non-selective COX inhibitor, and abolished by mianserin, a 5-HT antagonist, suggesting a role for both prostaglandins and 5-HT in H_2O_2-induced contraction of rat trachea (Szarek *et al.*, 1990). Pre-treatment with H_2O_2 has also been shown to significantly reduce cholinergic contractions in tracheal explants (Kramer *et al.*, 1987, Szarek *et al.*, 1990) and lung parenchymal strips, and, in addition, attenuate relaxation

via isoprenaline (Kramer *et al.*, 1987); suggesting both muscarinic and/or contractile pathways, as well as adrenergic and/or relaxant pathways, are affected by H_2O_2. Similar observations have been found in other species: in strips from rabbit intrapulmonary bronchioles, high concentrations of H_2O_2 (>30μM) attenuated ACh-induced contractions, and this effect was further attenuated in strips with their epithelium removed (Asano *et al.*, 2001), indicating H_2O_2-induced "removal" of an epithelial airway dilatory factor (e.g. PGE_2); and H_2O_2 induced relaxation of ACh-contracted canine bronchial strips, and indomethacin attenuated these relaxations (Gao *et al.*, 1992), thereby further implicating prostaglandins in the response. In the latter study, ACh significantly increased release of PGE_2 and PGI_2, and this was further augmented in the presence of H_2O_2. These results suggest that, at high concentrations, H_2O_2 can attenuate cholinergic contraction of airways, and this effect is mediated by bronchodilatory prostaglandins. With respect to the results of the present chapter, the effects observed may be a combination of both loss of viability, and direct effects of H_2O_2 on modulating airway tone. One important aspect here may be the time-frame: the experiments herein were all performed following overnight incubation in H_2O_2, whereas those discussed from the literature were all following much shorter exposures to H_2O_2. Prostaglandins are potentially important mediators of H_2O_2-induced small airway responses. PGE_2 release was measured in this chapter; however the results were inconclusive (see section 5.4.4). Therefore, in order to reconcile these results, the present contractile experiments could be repeated in the presence of indomethacin, to see if the reduction in maximal airway closure is attenuated and, thereby, implicate any involvement of prostaglandins. A time-course of H_2O_2 exposure could also be performed investigating both airway function and PCLS viability, to determine the effect of H_2O_2 at shorter time-points, and whether there is a temporal factor that mediates the effects of H_2O_2 on PCLS viability and/or paracrine responses.

5.4.2. Effects of PPE on small airway closure

With regards to PCLS incubated overnight with PPE, this had no effect on PCLS viability; therefore the reduction in maximal airway closure, unlike that of H_2O_2, cannot be attributed to a loss of viability. Similar experiments have been performed previously with mice PCLS and showed that *in vivo* treatment with a single exposure to PPE followed, 14 days later, by sacrifice and subsequent PCLS generation, caused a significant and concentration-dependent reduction in the magnitude of response to ACh; however overnight treatment of PCLS from naïve mice with PPE *in vitro* caused larger and faster contractile responses to ACh (Khan *et al.*, 2010). *Ex vivo* preparations of tracheal and parenchymal strips from guinea pigs also demonstrated hyperresponsiveness to cholinergic stimulation following *in vivo* elastase instillation for 24 or 48h (Willet *et al.*, 1988). However, *ex vivo* preparations of trachea and bronchi from hamsters that were given a single instillation of elastase *in vivo* and sacrificed 4-weeks later, demonstrated reduced contraction to ACh; although parenchymal strips from the same animals demonstrated increased contraction (Qian *et al.*, 1989), indicating a possible differential response between proximal and distal airways.

The results demonstrated in the present chapter are concordant with the *in vivo* data from the (Khan *et al.*, 2007) study, whereby overnight incubation of PPE caused concentration-dependent decreases in maximal airway closure; however they are in conflict with the *in vitro* results. Similarly, the results with respect to the trachea and bronchi from the Qian & Mitzner (1989) study were also concordant with those in the present chapter. Some of the differences observed here might be due to species differences, as the experiments in the present chapter were performed in rats, whereas those in the literature were performed in mice, guinea pigs and hamsters. It is also difficult to reconcile the concentrations of PPE used between the experiments herein and those in the literature; therefore this may also be a confounding factor. The effects of PPE seem to be largely attributed to mechanical alterations in the contractile apparatus of the airway; however, NE has been shown to induce the release of PGE_2 from human airway epithelial cells, therefore there may also be a paracrine response involved that warrants investigation. In addition, according to histological

analysis of PCLS treated with PPE, there appeared to be complete loss of elastin fibres at all concentrations of PPE, thereby being not concentration-dependent, as the functional results may suggest. This indicates that the mechanical alterations caused by elastin destruction would be uniform across all PPE treatments, therefore further implicates paracrine factors released in response to PPE treatment that may help explain the concentration-dependent loss of airway function.

As discussed in section 5.1, PPE is widely used in the literature in animal models of emphysema or COPD-like symptoms. However, there are quite substantial differences between PPE and NE, particularly with regards to their substrate specificity. This made it difficult to establish a concentration, or activity, of PPE that would accurately mimic the levels of NE found within the neutrophil supernatants; and therefore maintain consistency across the experiments. Despite the financial constraints, in the experiments herein it may have been more physiologically relevant, and consistent with the experiments in Chapter 4, to use NE rather than PPE; particularly as the PCLS system accommodates relatively small volumes of drug/treatment.

5.4.3. Comparative effects of H_2O_2 and PPE with those of neutrophil supernatants

One of the objectives of the present chapter was to elucidate whether H_2O_2 or PPE could reproduce the effects of neutrophil supernatants on small airway function that were observed in Chapter 4. To recap, neutrophil supernatants from COPD patients caused significant reductions in maximal closure, EC_{50} to CCh and baseline patency of airways in rat PCLS, and also significantly reduced PCLS viability. The highest concentrations of both H_2O_2 and PPE caused reductions in maximal airway closure; therefore oxidative and/or elastolytic stresses may contribute to the decreased maximal airway closured caused by neutrophil supernatants. However, neither H_2O_2 nor PPE had any notable effects on either EC_{50} or baseline airway patency, suggesting other mechanisms are at play with regards to these supernatant-induced effects. The highest concentrations of H_2O_2, but not PPE, significantly

Table 5.3. Summary of the effects of neutrophil supernatants, H_2O_2 and PPE on small airway function and PCLS viability			
	Stimulated (100μM fMLP) neutrophil supernatants	**H_2O_2**	**PPE**
Maximal airway closure	↓	↓	↓
EC_{50} to CCh	↓	No effect	↑ (NS)
Baseline airway patency	↓	No effect	No effect
PCLS viability	↓	↓	No effect
↑ and ↓ denote increased and reduced, respectively. NS= non-significant			

reduced PCLS viability, suggesting that oxidative stress may be involved in neutrophil supernatant-induced reduction in PCLS viability. Therefore, H_2O_2 (or oxidative stress) may be implicated in neutrophil supernatant-induced reduced maximal airway closure and PCLS viability (however, it is worth noting here that the former may be a consequence of the latter, as described above in Section 5.4.1). PPE, or neutrophil elastase, may also be implicated in neutrophil supernatant-induced reduced maximal airway closure, but had no effect on any other functional parameters or PCLS viability. Neither H_2O_2 nor PPE had any significant effect on baseline airway patency or EC_{50} to CCh, although this was non-significantly increased at both the highest concentration of H_2O_2 and with all PPE treatments, suggesting other mechanisms are involved. With regards to baseline airway patency, this may include the release of bronchoconstrictors, either from neutrophils themselves, or induced from the lung epithelium in response to neutrophil products. Conversely, this may be due to inhibition of bronchodilators, again, either directly by neutrophil products, or via an epithelial response

to neutrophil products. The effect on EC_{50} may be mediated by the destruction of parenchymal attachments on the airways, which thus facilitates small airway closure by minimising the load opposing smooth muscle contraction. This may be more pronounced in PCLS incubated with neutrophil supernatants, as these airways are exposed to more proteases than just PPE alone. This is summarised in Table 5.3.

5.4.4. Mediator release in response to H_2O_2 and PPE treatment

In order to investigate potential mechanisms mediating the effects observed in the present chapter, mediator release from PCLS following treatment with H_2O_2 and PPE was investigated. PGE_2 release was investigated, due to its role as a bronchodilator, which has been shown to be released from airway smooth muscle in response to both H_2O_2 (Szarek *et al.*, 1990, Gao *et al.*, 1992, Asano *et al.*, 2001) and elastase treatment (Perng *et al.*, 2003). The results of this were inconclusive. There appeared to be no significant effect of either H_2O_2 or PPE on PGE_2 release; however this is more than likely due to problems with the assay. The assay measures PGE_2 directly; however this is rapidly metabolized, with a very a short half-life (Samuelsson *et al.*, 1975). Therefore, it is possible that any PGE_2 released by the PCLS had already been metabolized. In addition, PGE_2 in the PCLS supernatants was measured at two different dilutions, and there was little agreement between the final PGE_2 concentrations at these two dilutions (data not shown). This suggests some interference/contamination between the samples and the assay, as the assay is sensitive to contamination with organic solvents and rat antibodies that may be found in the PCLS supernatant. To remedy this in future, samples could be purified prior to running them on this assay. Alternatively, measuring a more stable PGE_2 metabolite may yield more conclusive results.

CINC-1 and TNF-α were chosen due to their inductive roles in inflammation and in response to a variety of stresses, in order to evaluate the inflammatory environment induced by H_2O_2 or PPE. CINC-1 mRNA expression and protein secretion is induced *in vitro* in rat C6 glioma

cells by a number of different stresses, including LPS, H_2O_2 and TNF-α (Uehara *et al.*, 1998); and CINC-1 protein levels are elevated in BALF, lung tissue homogenates and serum from rats following *in vivo* exposure to nickel oxide nanoparticles (Nishi *et al.*, 2009), diesel exhaust particles (DEP) (Yokota *et al.*, 2005), and LPS (Mitsuhashi *et al.*, 1999). TNF-α is an important pro-inflammatory cytokine heavily implicated in a plethora of inflammatory diseases, including COPD (Barnes, 2009) , and causes bronchial hyperresponsiveness in rats (Kips *et al.*, 1992) and guinea pigs (Makwana *et al.*, 2012). In addition, rat alveolar epithelial cells release TNF-α *in vitro* in response to LPS stimulation (McRitchie *et al.*, 2000).

The results of the present chapter showed that neither H_2O_2 nor PPE treatment had any inductive properties for these cytokines, either with or without LPS treatment, but caused concentration-dependent decreases in both CINC-1 and TNF-α. Neither H_2O_2 nor PPE alone induced TNF-α secretion, therefore LPS was used, firstly to confirm that PCLS had the propensity to release TNF-α; and, secondly, to investigate how this was affected by H_2O_2 or PPE treatment. LPS induced TNF-α secretion from PCLS in a concentration-dependent manner; however both H_2O_2 and PPE caused concentration-dependent decreases in LPS-induced TNF-α secretion. With respect to PPE, this was most likely due to the observation that PPE most probably degrades both CINC-1 and TNF-α protein, as demonstrated by the spiking experiments in Figure 5.5. The same experiments demonstrated that H_2O_2 did not affect either CINC-1 or TNF-α proteins; therefore the reduction in their expression in PCLS treated with H_2O_2 may be due to other mechanisms, as, at low concentrations, H_2O_2 has been shown to mediate signalling pathways (reviewed by (Gough *et al.*, 2011)). Therefore, an explanation of the results of the present chapter may be due to the concurrent reduction in PCLS viability with increasing concentrations of H_2O_2. In order to investigate this further, lower, non-lethal concentrations of H_2O_2 could be used to treat PCLS, and cytokine output measured.

One profound pitfall in the aforementioned experiments was the observation that PPE most probably degraded both CINC-1 and TNF-α proteins, therefore measuring their release from

PCLS using ELISA was not possible. It may, therefore, be better to measure mRNA expression of these cytokines, as this may not be as susceptible to degradation compared with protein secretion. However, this result may allude to a potential anti-inflammatory mechanism, whereby the release of proteases by inflammatory cells, such as neutrophils, regulates the concentration of pro-inflammatory cytokines, such as TNF-α. *In vivo*, this could represent a self-limiting mechanism of inflammatory cell recruitment and maintenance of a pro-inflammatory environment, which may be dysregulated in chronic inflammatory conditions.

5.4.5. Hypothesis: accept/reject

The hypothesis at the beginning of this chapter was:

"Addition of H_2O_2 or PPE will affect small airway structure and function, with associated effects on PCLS viability and mediator release; and will, at least in part, reproduce the effects of neutrophil supernatants on small airway physiology."

The results of the chapter showed that H_2O_2 and PPE did indeed affect small airway function in that they both reduced maximal airway closure to CCh. Consequently, this part of the hypothesis can be accepted. However, as neither stress was able to completely reproduce the effects of neutrophil supernatants in Chapter 4, this part of the hypothesis can be rejected.

This aim of this chapter was to investigate potential mechanisms that may mediate neutrophil supernatant-induced small airway dysfunction and loss of PCLS viability. The results allude to potential roles of both H_2O_2 and PPE in certain aspects of this, namely reduced maximal airway closure and PCLS viability; however much remains unclear. The next chapter will attempt to reconcile this using an alternative approach: inhibiting particular components of the neutrophil supernatants, to determine whether or not small airway dysfunction and PCLS viability can be restored.

Chapter 6.

Effect of Pre-Incubation with Antioxidants and Protease Inhibitors on Neutrophil Supernatant-Mediated Small Airway Dysfunction

6. Effect of Pre-Incubation with Antioxidants and Protease Inhibitors on Neutrophil Supernatant-Mediated Small Airway Dysfunction

6.1. Introduction

In the previous two chapters (Chapters 4 and 5), neutrophil supernatants, H_2O_2 and PPE have all been shown to induce small airway dysfunction and, with the exception of PPE, reduction in PCLS viability. It was, therefore, of interest to ascertain whether or not the aberrant effects induced by these stresses can be inhibited, using antioxidants or inhibitors of various proteases.

There have been numerous clinical attempts at utilizing protease inhibitors and antioxidants in the treatment of COPD, but with very limited success. Patients enrolled in clinical trials investigating the efficacy of AZD9668, a neutrophil elastase inhibitor, over a three-month period demonstrated no significant improvements in lung function or quality of life, either in conjunction with treatment with the LAMA tiotropium (Vogelmeier *et al.*, 2012), or without (Kuna *et al.*, 2012). Clinical trials investigating the efficacy of a selective inhibitor for MMP-9 and MMP-12 demonstrated that the drug did not have any significant effects on lung function; however there was a trend for a reduction in levels of plasma and urinary desmosine, an indicator of elastolytic degradation in the lung (Dahl *et al.*, 2012).

N-acetyl-cysteine (NAC) elicits both direct and indirect antioxidant properties: it has a free thiol group that directly interacts with ROS, and can also be utilized as a precursor for GSH, which is an important endogenous antioxidant (Dekhuijzen, 2004). Treatment with NAC protects against cigarette smoke-induced lung damage in rats *in vivo* (Balansky *et al.*, 1992, Cai *et al.*, 2009) and *in vitro (Fu et al., 2010)*; and daily NAC treatment significantly reduced concentration of H_2O_2 in exhaled breath condensate of COPD patients after one year (Kasielski *et al.*, 2001).

Chapter 3 of this thesis showed that supernatants of neutrophils from COPD patients stimulated with 100μM fMLP contain a number of different proteases, including MMP-8, MMP-9, MPO and NE (Section 3.3). These supernatants had adverse effects on small

airway function and viability when incubated overnight with rat PCLS (Section 4.3). In order to investigate what may be causing these adverse effects, the present chapter pre-incubated neutrophil supernatants with NAC or inhibitors of NE or MMP-9, in order to determine whether the effects on small airway function and/or PCLS viability could be attenuated, and thereby assess the contribution of oxidants and proteases to the supernatant-induced effects on small airways and PCLS.

6.1.2. Hypothesis

From the above introductory observations, the hypothesis for this chapter was therefore:

"The antioxidant NAC, and/or inhibitors of NE and/or MMP-9 will inhibit small airway dysfunction and reduced PCLS viability in PCLS incubated in stimulated neutrophil supernatants from COPD patients."

6.1.3. Aims

In order to investigate this hypothesis, the aims of this chapter were to:

1. Investigate the conditions for NAC pre-incubation of PCLS pre-treatment with 1mM H_2O_2, with respect to small airway function and PCLS viability, in order to optimise conditions for pre-incubation with neutrophil supernatants.
2. Pre-incubate NAC in neutrophil supernatants, as per conditions identified in (1), and investigate small airway function and PCLS viability after neutrophil supernatant treatment.
3. Optimize conditions (i.e. pre-incubation time and concentration) for the pre-incubation of NE inhibitor in neutrophil supernatants, using neutrophil supernatants and a NE activity assay.
4. Pre-incubate NE inhibitors in neutrophil supernatants, as per conditions identified in (3), and then incubate rat PCLS overnight with neutrophil supernatants, and measure small airway function and PCLS viability.

5. Optimize conditions for pre-incubation with MMP-9 inhibitor (i.e. pre-incubation time and concentration) using neutrophil supernatants and MMP-9 activity assay.
6. Pre-incubate MMP-9 inhibitor in neutrophil supernatants, as per conditions identified in (5), and then incubate rat PCLS overnight with neutrophil supernatants, and measure small airway function and PCLS viability.

6.2. Methods

6.2.1. Generation of rat PCLS and assessment of airway function

Rat PCLS were generated and airway function assessed as described in section 2.2.1.

6.2.2. Initial assessment of airway function and grouping of PCLS

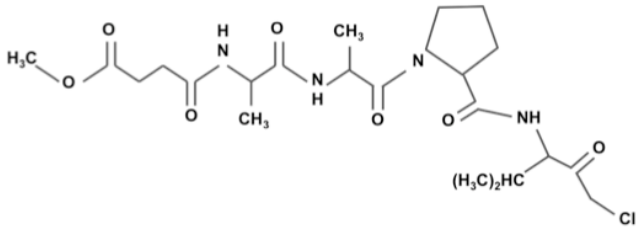
As described in section 2.2.1.4, airways on PCLS were subject to an initial assessment of maximal airway closure by administration of 10μM CCh. Following a series of washes in rat IB, and a 1h recovery period to allow airways to reopen, PCLS were then grouped based on the criteria outlined in section 2.2.1.4. As in Chapter 4, in order to minimize the impact of lung tissue variability on the results, a mix of PCLS from 2 different rats were used when investigating the effect of neutrophil supernatants in conjunction with protease inhibitors or NAC.

6.2.3. Optimization of NE inhibitor

A selective, non-reversible inhibitor of NE (324745; Table 6.1) was reconstituted at 10mM in DMSO. Aliquots were stored at -20°C. Neutrophil supernatants were thawed to 37°C in a water bath prior to use in the following experiments.

To determine the concentration of inhibitor to use, a selection of COPD patient neutrophil supernatants were pre-incubated with varying concentrations of inhibitor, ranging from 100nM to 1mM, for 30min at 37°C. NE activity assay was then performed as described in chapter 2, section 2.2.5.1.

To determine pre-incubation time with inhibitor, a selection of COPD patient neutrophil supernatants were pre-incubated with 10μM inhibitor for varying amounts of time, ranging from 10min to 4h. NE activity assay was then performed as described in section 3.2.2.

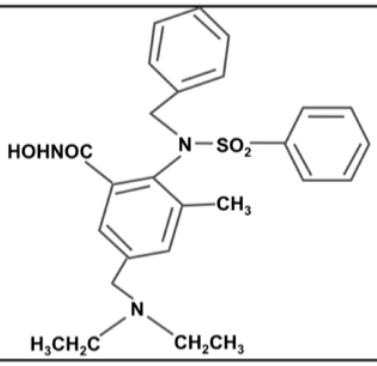
Table 6.1. Data sheet for NE Inhibitor	
Structure	
Cat. Number (Company)	324745 (Calbiochem)
Empirical formula	$C_{22}H_{35}ClN_4O_7$
Peptide sequence	MeOSuc-Ala-Ala-Pro-Val-CMK
Molecular mass	503.0
Primary target	Neutrophil elastase
K_i	10μM
Reversible (Y/N)	N

To determine if inhibition was maintained overnight, a selection of COPD patient neutrophil supernatants were pre-incubated overnight at 37°C in varying concentrations of inhibitor, ranging from 1nM to 10μM. The following day, NE activity assay was performed as described in section 3.2.2.

6.2.4. Optimisation of MMP-9 inhibitor

A selective inhibitor of MMP-9 (444278; Table 6.2) was reconstituted at 10mM in DMSO. Aliquots were stored at -20°C.

To determine the concentration of inhibitor to use, a selection of neutrophil supernatants were pre-incubated in varying concentrations of inhibitor, ranging from 10nM to 100μM ($^{v}/_{v}$), for 30min or overnight, at 37°C. MMP-9 concentration in supernatants was then determined, as described in section 2.2.5.2.

Table 6.2. Data sheet for MMP-9 Inhibitor	
Structure	
Cat. Number (Company)	444278 (Calbiochem)
CAS Number	1177749-58-4
Empirical formula	$C_{27}H_{33}N_3O_5S$
Molar mass	511.6
Primary target	MMP-9 (MMP-1 (IC_{50} 1.1μM) and MMP-13 (IC_{50}113nM) at higher concentrations)
IC_{50}	5nM
Reversible (Y/N)	Y

6.2.5. Incubation of PCLS in H_2O_2 and NAC

To assess the concentration of NAC that would likely be appropriate to inhibit oxidant activity in neutrophil supernatants, PCLS were pre-incubated for 30min at 37°C with varying concentrations of NAC, ranging from 1μM to 1mM. Following this, 1mM H_2O_2 was added and PCLS were then incubated overnight at 37°C. CCh concentration-responses were then performed the following day, as described in section 2.2.1.4. This protocol is summarized in Figure 6.1.

6.2.6. Incubation of PCLS in neutrophil supernatants with inhibitors or NAC

Neutrophil supernatants, that had been stored in 1ml aliquots, were thawed and warmed to 37°C in a water bath. Once PCLS groups had been established, 1ml neutrophil supernatant

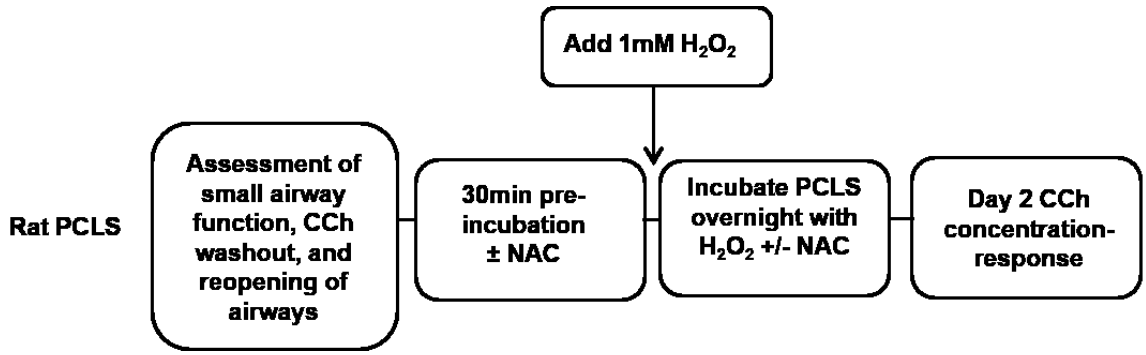


Figure 6.1. Summary of protocol for pre-incubation of PCLS with NAC, prior to addition of H_2O_2.

was added to selected groups. For those groups for investigation of the effects of protease inhibitors or NAC, neutrophil supernatants were pre-incubated for 30min at 37°C in 10μM NE inhibitor (324745) or MMP-9 inhibitor (444278), or 1mM NAC, prior to their addition to PCLS. For untreated controls, PCLS were incubated in 1ml rat IB. All PCLS were then incubated overnight at 37°C. CCh concentration-responses were then performed the following day, as described in section 2.2.1.4. This protocol is summarized in Figure 6.2.

6.2.7. Measurement of PCLS viability

PCLS viability was measured by WST-1 assay, as described in section 2.2.2.1. For the purposes of these experiments, untreated PCLS were considered to be 100% viable, and so the absorbance of all treated PCLS was presented as a percentage of the untreated absorbance.

6.2.8. Data analysis

In this chapter, normal distributions were not assumed for any of the analyses, therefore patient demographics were compared using Kruskal-Wallis tests, with Dunn's post-hoc comparisons. CCh dose-response curves were constructed by plotting % airway closure (calculated with respect to Day 1 baseline airway patency), against the log CCh concentration, and constructing a 4-parameter logistic non-linear regression curve. Mean (±SEM) EC_{50} values to CCh were then calculated from the PCLS used to generate each

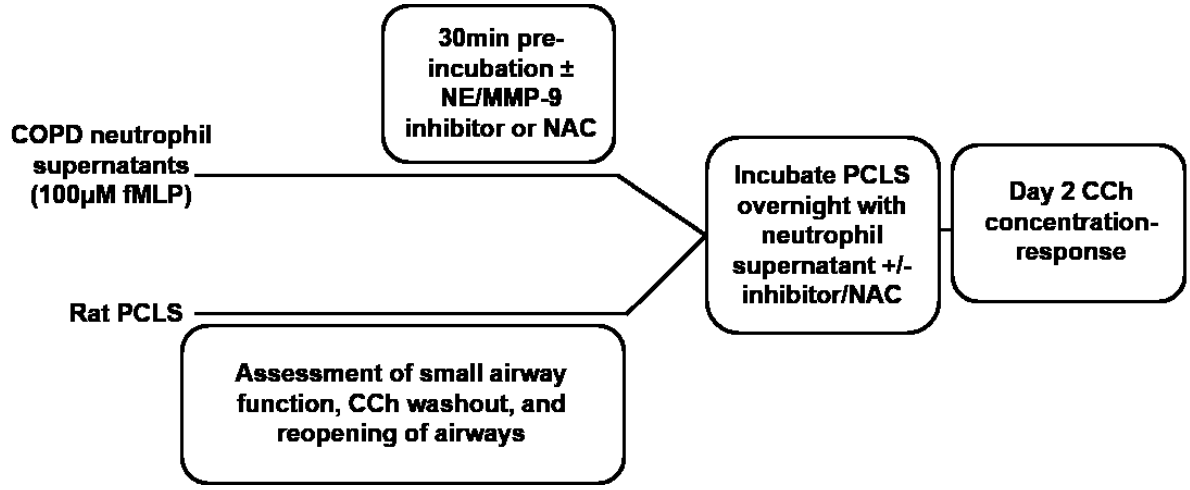


Figure 6.2. Summary of the protocol for pre-incubation of inhibitors/NAC with neutrophil supernatants, prior to overnight incubation with PCLS.

curve. Airway functional parameters and viability of PCLS treated with neutrophil supernatants from the three subject groups were compared using Kruskal-Wallis tests, with Dunn's post-hoc comparisons. For familiarity of presentation, data are displayed as mean ± SEM. The null hypothesis of no significant differences between untreated airways and those treated with control compounds or neutrophil supernatants was rejected at $p<0.05$

6.3. Results

6.3.1. Effect of pre-incubation with NAC on H_2O_2-induced small airway dysfunction

In order to determine an appropriate concentration of NAC to use with neutrophil supernatants, its activity against 1mM H_2O_2-induced airway/PCLS effects were investigated. This concentration of H_2O_2 was selected as its effects on small airway function and PCLS viability (see Section 5.3.1 & 5.3.2) were comparable with those induced by supernatants from neutrophils stimulated with 100μM fMLP (see Section 4.3.4.3).

PCLS were pre-incubated with NAC for 30 min prior to overnight incubation with 1mM H_2O_2 treatment. The following day, small airway closure in response to CCh and PCLS viability was assessed, to determine whether NAC could attenuate the effects of H_2O_2. CCh induced a concentration-dependent increase in small airway closure in PCLS that had been treated with H_2O_2, with or without NAC pre-incubation, with maximal increases above baseline of ~65-90% at 10μM CCh, and EC_{50} values of ~240-490nM CCh (Figure 6.3 (A), Table 6.3).

Treatment with 1mM H_2O_2 alone resulted in ~20% reduction in the maximal airway closure, but had little effect on either EC_{50} or baseline airway patency, compared with untreated airways (Table 6.3). Pre-incubation with NAC at 1, 10 and 100μM prior to H_2O_2 treatment had little effect on maximal airway closure, as there were significant ~25-30% decreases in maximal airway closure, compared with untreated airways. However, pre-incubated with 1mM NAC prior to H_2O_2 treatment significantly restored maximal airway closure to 95% of that of untreated airways. There were no effects on EC_{50} to CCh or baseline airway patency at any concentration of NAC. In response to the results of these experiments, 1mM NAC was chosen for use in subsequent experiments.

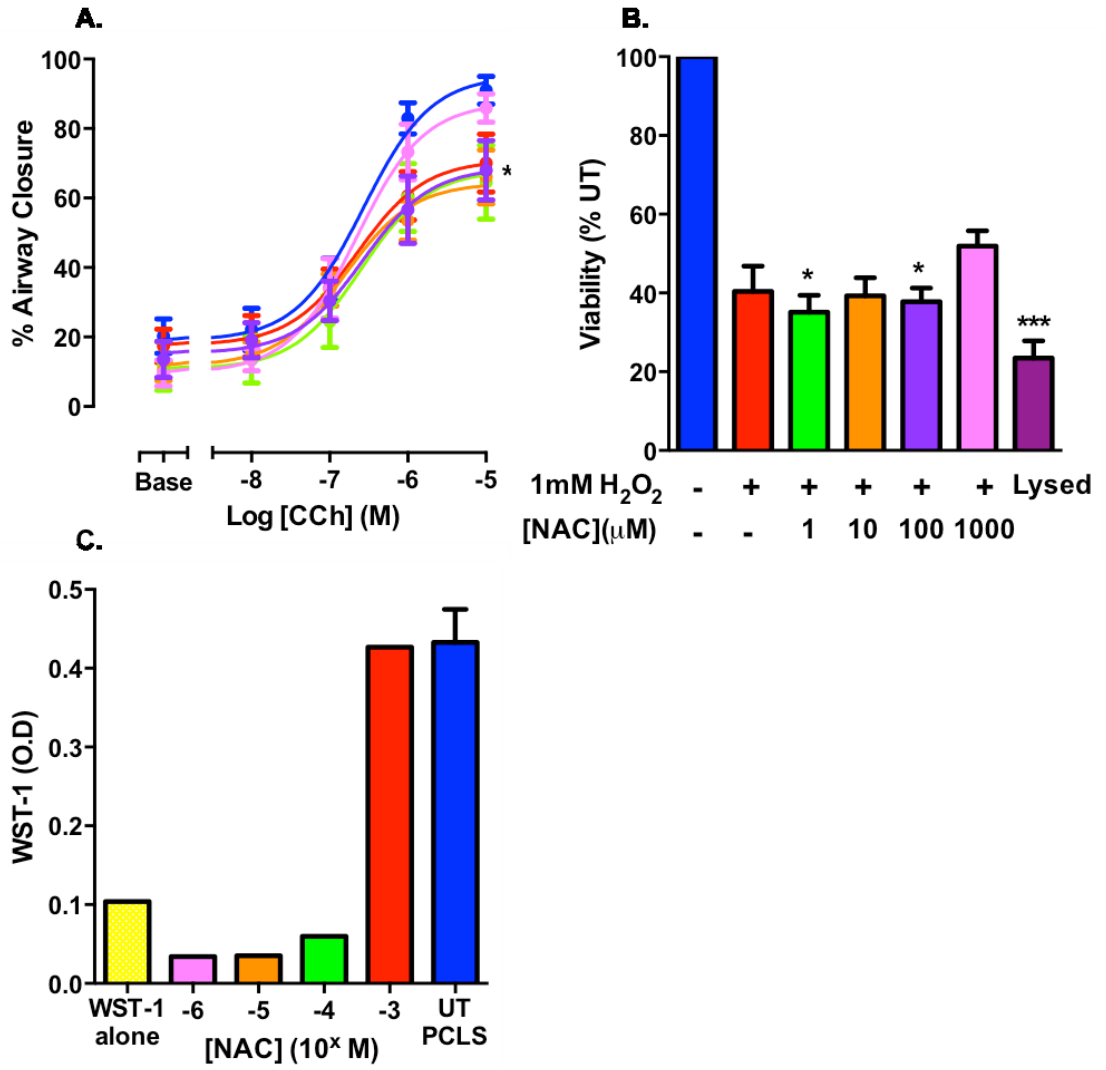


Figure 6.3. **Effect of NAC on H_2O_2-induced small airway closure and PCLS viability**. (A) Effect on small airway closure of pre-treatment with NAC on overnight incubation with 1mM H_2O_2. Untreated (●), n=8 rats; 1mM H_2O_2 (●), n=8 rats; 1μM NAC+1mM H_2O_2 (●), n=6 rats; 10μM NAC+1mM H_2O_2 (●), n=7 rats; 100μM NAC+1mM H_2O_2 (●), n=5 rats; 1mM NAC+1mM H_2O_2 (●), n=6 rats. *p<0.05 1, 10 and 100μM NAC *vs.* untreated. Data are mean ± SEM. All data sets above 1μM CCh were significantly higher than baseline (p<0.05-0.0001). (B) Effect on PCLS viability, as determined by WST-1 assay, of pre-incubation with NAC and overnight incubation with 1mM H_2O_2. Lysed (●), n=6-7 rats per group. Data are mean ± SEM. *p<0.05, ***p<0.001 *vs*, untreated. (C) Effect of NAC on reduction of WST-1 solution. Optical density of an example untreated PCLS shown as a reference point.

Table 6.3. Effect of pre-incubation with NAC on treatment with 1mM H_2O_2 on small airway function

Treatment	Maximal Closure (%)	[CCh] for maximal closure (μM)	Baseline Airway Patency (%)	EC_{50} (nM)
Untreated (n=8)	91.0 ± 4.1	10	20.3 ± 5.0	277 ± 45
1mM H_2O_2 alone (n=8)	70.1 ± 8.3	10	17.4 ± 4.8	373 ± 130
1μM NAC (n=6)	64.5 ± 10.6*	10	11.6 ± 7.0	240 ± 62
10μM NAC (n=7)	66.1 ± 7.8*	10	10.3 ± 2.9	491 ± 235
100μM NAC (n=5)	68.0 ± 8.6*	10	13.6 ± 5.2	314 ± 60
1mM NAC (n=6)	85.9 ± 4.1	10	9.6 ± 3.8	331 ± 167

Data are mean ± SEM. *p<0.05 *vs.* untreated, as determined by Kruskal-Wallis test and Dunn's post hoc comparisons. n= number of rats. NAC= *N*-acetyl cysteine; CCh= carbachol.

6.3.2. Effect of pre-incubation with NAC prior to H_2O_2 treatment on PCLS viability

Treatment with 1mM H_2O_2 resulted in a loss of PCLS viability to ~40% that of untreated airways (Figure 6.3 (B)). Pre-treatment with 1, 10 or 100μM NAC did not inhibit this H_2O_2-induced loss of viability, with significant reductions in PCLS viability of ~35-40% that of untreated airways. Pre-incubation with 1mM NAC slightly attenuated this loss of viability to ~50% that of untreated airways. As, expected, viability of lysed PCLS was ~25% that of untreated airways.

The lack of any marked effect of NAC on viability was inconsistent, at least for 1mM NAC, with its reversal of airway contractility. It was possible that the apparent lack of effect was due to NAC interfering with the viability assay. The basis of the viability assay is that WST-1 is reduced by mitochondrial enzymes to form a coloured tetrazolium salt. As NAC is an antioxidant, and therefore is a reducing agent, its effect on reduction of WST-1 was investigated to determine any potential assay interference (Figure 6.3. (C)). Low concentrations of NAC (1-100μM) did not appear to additionally reduce WST-1 solution, as there were decreases in optical density comparable with WST-1 solution alone. However, 1mM NAC caused a four-fold increase in optical density, compared with WST-1 solution alone. This optical density was comparable to that caused by incubation of WST-1 solution with untreated (i.e. viable) PCLS. This suggests that, at a concentration of 1mM, NAC reduces WST-1 solution, and therefore may interfere with assessment of PCLS viability. However, concentrations <1mM do not appear to interfere with the WST-1 assay; therefore it can be assumed that the loss of viability at these concentrations represents an accurate reflection of experimental outcomes.

6.3.3. Pre-incubation of neutrophil supernatants with NAC, an NE inhibitor or an MMP-9 inhibitor

6.3.3.1. Subject demographics

The COPD patients from whom neutrophils were isolated for the following experiments are shown in Table 6.4. Subjects were all, on average, 68 years-old, and had very similar lung function, with FEV_1/FVC ratios of ~0.6L. However, smoking history varied between the groups, with those patients used in the NE inhibitor experiments smoking significantly over double those used in the NAC experiments. There were no other significant differences in demographics between any of the experimental groups.

Table 6.4. Subject demographics: Neutrophil supernatants pre-incubated with NAC, NE inhibitor, or MMP-9 inhibitor

	NAC	NE Inhibitor (324745)	MMP-9 inhibitor (444278)
N	4	7	5
Age	68 ± 3	68 ± 2	68 ± 3
Gender (m/f)	2/2	2/5	3/2
Pack years	24.8 ± 4.2*	53.9 ± 11.6	32.4 ± 6.5
FEV_1 (L)	1.6 ± 0.2	1.5 ± 0.1	1.5 ± 0.2
FEV_1 predicted (%)	60.3 ± 4.7	68.4 ± 4.4	62.0 ± 6.4
FVC (L)	2.8 ± 0.1	2.7 ± 0.2	2.5 ± 0.2
FEV_1/FVC (L)	0.62 ± 0.1	0.59 ± 0.1	0.58 ± 0.1

Data are mean ± SEM. *p<0.05 *vs*. NE inhibitor.

NAC= *N*-acetylcysteine, NE= neutrophil elastase, MMP= matrix metalloprotease, FEV= forced expiratory volume, FVC= forced vital capacity.

6.3.3.2. Effect of pre-incubation with 1mM NAC on supernatant-induced small airway dysfunction

Supernatants from neutrophils from COPD patients that were stimulated with 100μM fMLP were pre-incubated with 1mM NAC for 30min, prior to overnight incubation in the continued presence of NAC with PCLS. The following day, small airway function was assessed. CCh induced a concentration-dependent increase in small airway closure in untreated PCLS and in those that had been pre-incubated in 1mM NAC alone, with maximal increases above baseline of ~75% at 10μM CCh, and EC_{50} values of ~200-1000nM CCh (Figure 6.4, Table 6.5). However, in PCLS that were incubated with COPD neutrophil supernatants, either with or without pre-incubation with NAC, the maximal increase above baseline was ~7-27%, which was a significant ~65-90% reduction in maximal airway closure compared with

untreated airways. There was a five-fold increase in EC_{50} to CCh between untreated airways and those incubated in NAC alone; however this was not significant and there were no other significant effects on EC_{50} to CCh between untreated airways and those incubated in COPD neutrophil supernatants alone. The EC_{50} to CCh for airways incubated in neutrophil supernatants and NAC could not be calculated due to the shallowness of the curve. There was a ~50% reduction in the baseline airway patency of airways incubated with the neutrophil supernatants, with or without NAC pre-incubation; however this was not significant. Therefore, NAC did not inhibit the effect of neutrophil supernatants on small airway closure.

Due to the aforementioned interference between 1mM NAC and WST-1 solution (Section 6.3.2), PCLS viability was not assessed in these experiments.

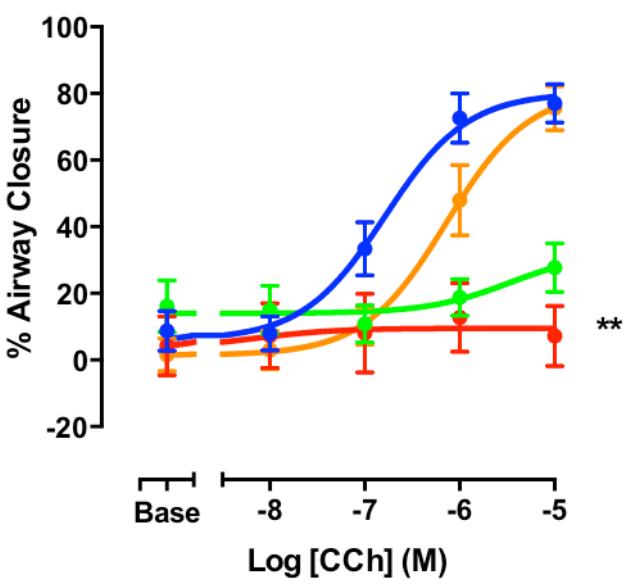


Figure 6.4. Effect of NAC on small airway contractility induced by neutrophil supernatants. Rat PCLS were pretreated with NAC prior to overnight incubation in supernatants from COPD neutrophils stimulated with 100μM fMLP. Untreated (●), n=6 rats; 1mM NAC alone (●), n=4 rats; COPD supernatants alone (●), n=5 supernatants, n=10 rats; COPD supernatant + 1mM NAC (●), n=5 supernatants, n=10 rats. Untreated data sets above 1μM CCh are significantly higher than baseline ($p<0.01$). Data are mean ± SEM, **$p<0.01$ *vs.* untreated.

Table 6.5. Effect of pre-incubation of COPD neutrophil supernatants with NAC on small airway function

Treatment	Maximal Closure (%)	[CCh] for maximal closure (μM)	Baseline Airway Patency (%)	EC_{50} (nM)
Untreated (n=6)	77.1 ± 5.8	10	8.8 ± 6.0	200 ± 48.8
NAC alone (n=4)	75.6 ± 6.6	10	1.5 ± 4.9	1075 ± 652
COPD (n=5 supernatants)	27.7 ± 7.3	10	16.1 ± 7.8	521 ± 309
COPD + NAC (n=5 supernatants)	7.2 ± 9.0**	1	4.2 ± 8.8	-

Data are mean ± SEM. NAC= *N*-acetyl cysteine. **p<0.01 *vs.* untreated, as determined by Kruskall-Wallis test and Dunn's post-hoc comparisons

6.3.4. Efficacy of NE inhibitor in neutrophil supernatants: time-course and concentration-response

In order to determine the appropriate concentration and pre-incubation time for the NE inhibitor (324745), a time-course and concentration-response was performed in supernatants from neutrophils from COPD patients that had been stimulated with 100μM fMLP (Figure 6.5). After 30 min pre-incubation, there was a step-wise decrease in NE activity in neutrophil supernatants with increasing concentrations of inhibitor. There was ~50% decrease in NE activity at 1μM inhibitor, and, at concentrations above 10μM, the inhibitor almost completely abolished NE activity, with levels below the limit of detection at 100μM and 1mM (Figure 6.5 (A)). After overnight incubation, there was also a step-wise reduction in NE activity with increasing concentrations of inhibitor, with an ~50% decrease at 100nM of inhibitor, and complete abolishment of NE activity at concentrations above 1μM of inhibitor, with levels being below the limit of detection (Figure 6.5 (B)). There was a 75-fold decrease

in IC_{50} to inhibitor following overnight incubation with inhibitor (11.4 ± 0.2nM), compared with 30 min pre-incubation (919 ± 107nM); however this was not significant (Figure 6.6).

NE activity was markedly reduced following 10 min pre-incubation with 10μM inhibitor, and completely abolished after 30 min (Figure 6.5 (C)). Following these results, the conditions chosen for subsequent experiments were 10μM inhibitor, and a 30min pre-incubation time.

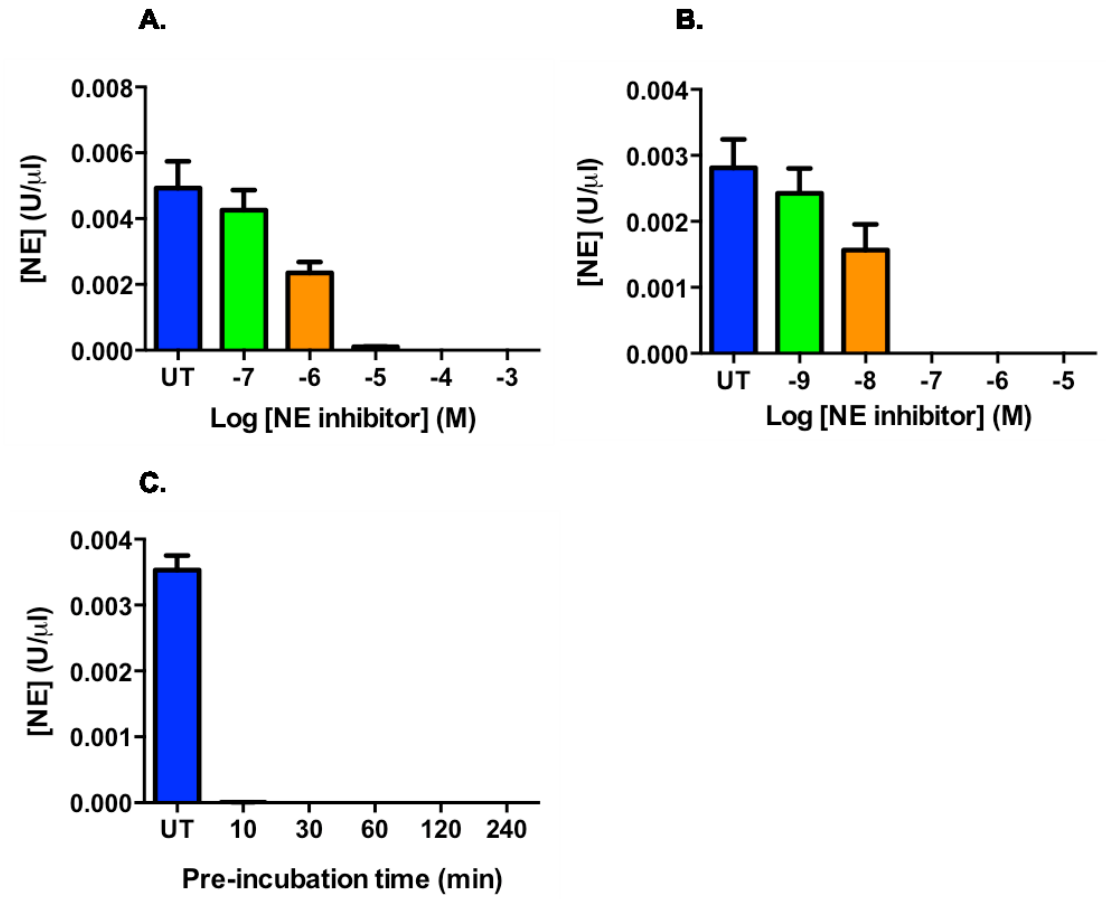


Figure 6.5. Optimization of concentration and pre-incubation time of NE inhibitor (324745) in neutrophil supernatants. (A-B) Concentration-response of inhibition of NE in neutrophil supernatants following (A) 30 min and (B) overnight incubation with NE inhibitor (324745), n=3 supernatants. (C) Time-course of pre-incubation with 10μM NE inhibitor, n=3 supernatants. Data are mean ± SEM.

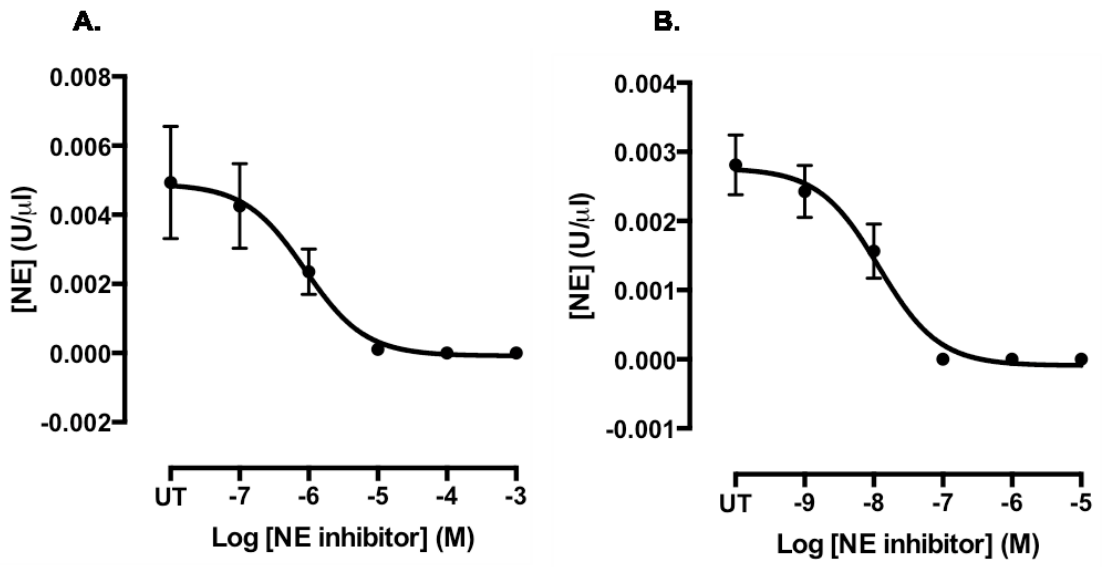


Figure 6.6. Measurement of NE activity in COPD neutrophil supernatants following pre-incubation with increasing concentrations of NE inhibitor. Supernatants pre-incubated for A) 30 min and B) overnight. Note different axes scales. Data are mean ± SEM, n=3-4 supernatants.

6.3.5. Effect of pre-incubation of neutrophil supernatants with NE inhibitor on small airway function and PCLS viability

Supernatants from neutrophils from COPD patients that were stimulated with 100μM fMLP were pre-incubated for 30min with 10μM NE inhibitor (324745), prior to overnight incubation with PCLS. The following day, small airway function was assessed. CCh induced a concentration-dependent increase in small airway closure in PCLS that had been treated with neutrophil supernatants, with or without NE inhibitor, with maximal increases above baseline of ~50-85% at 10μM CCh, and EC_{50} values of ~160-370nM CCh (Figure 6.7 (A), Table 6.6). Treatment with NE inhibitor alone had no significant effect on small airway function. Incubation with neutrophil supernatants without NE inhibitor resulted in an ~40% decrease in maximal airway closure and ~30% decrease in EC_{50} to CCh, compared with untreated airways. Pre-incubation of neutrophil supernatants with NE inhibitor restored the maximal airway closure to ~90% that of untreated airways; although there was still a >two-fold decrease in EC_{50} to CCh, compared with untreated airways. There was an ~15%, non-

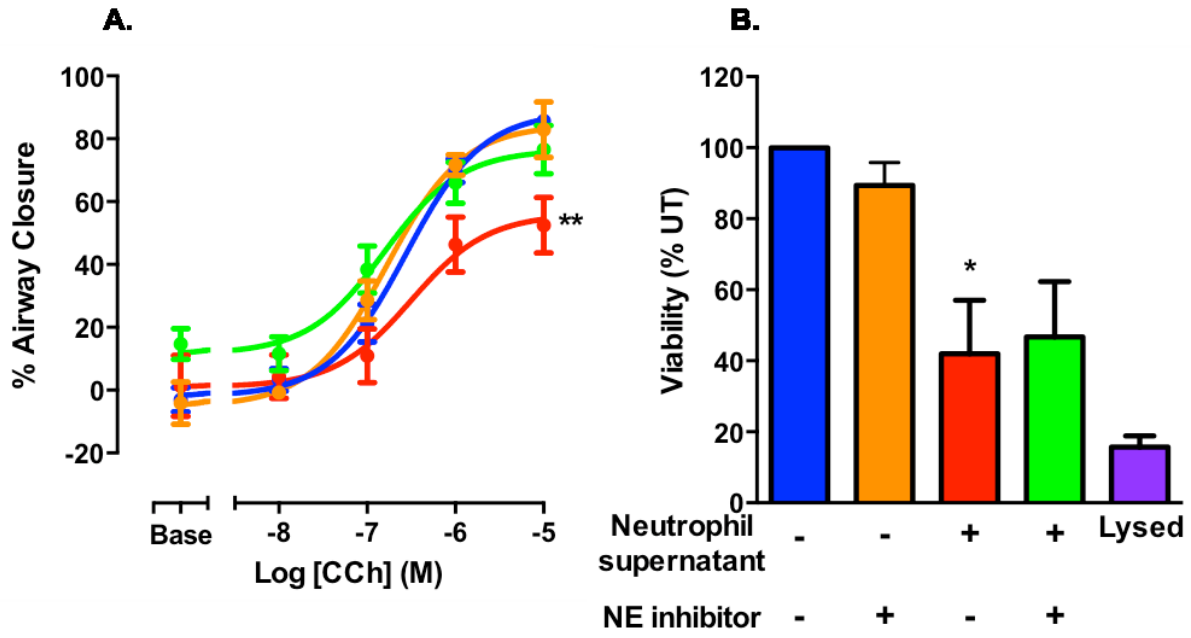


Figure 6.7. Effect of pre-incubation with NE inhibitor (324745) on neutrophil supernatant-induced small airway dysfunction and PCLS viability. (A) Effect on small airway closure. Untreated (●), n=10 rats; NE inhibitor alone (●), n=4 rats; COPD neutrophil supernatant alone (●), n=7 supernatants, n=14 rats; COPD neutrophil supernatants + pre-incubation with 10μM NE inhibitor (●), n=7 supernatants, n=14 rats. All data sets above 1μM CCh were significantly higher than baseline ($p<0.05$-0.0001) (B) Effect on PCLS viability. Untreated (●), n=4 rats; NE inhibitor alone (●), n=4 rats; COPD neutrophil supernatant alone (●), n=7 supernatants, n=14 rats; COPD neutrophil supernatants + pre-incubation with 10μM NE inhibitor (●), n=7 supernatants, n=14 rats; lysed (●), n=4 rats. Data are mean ± SEM. **$p<0.01$ *vs.* untreated, as determined by Kruskal-Wallis and Dunn's post-hoc tests; *$p<0.05$ *vs.* untreated, as determined by Wilcoxon Signed Rank test.

significant reduction in baseline airway patency in PCLS treated with neutrophil supernatants pre-incubated with NE inhibitor; however no other significant effects were observed.

NE inhibitor alone had no significant effects on PCLS viability (Figure 6.7 (B)). However, PCLS incubated with neutrophil supernatants, both with and without inhibitor, were ~41-46% viable, compared with untreated airways. Therefore, pre-incubation with NE inhibitor did not appear to restore PCLS viability. As expected, lysed PCLS were ~15% viable, compared with untreated airways.

Table 6.6. Effect of pre-incubation of COPD neutrophil supernatants with NE inhibitor on small airway function

Treatment	Maximal Closure (%)	[CCh] for maximal closure (μM)	Baseline Airway Patency (%)	EC_{50} (nM)
Untreated (n=10 rats)	85.7 ± 2.1	10	-3.1 ± 3.8	367 ± 78.6
NE inhibitor alone (n=4 rats)	82.9 ± 8.9	10	-4.1 ± 6.8	234 ± 115
COPD (n=7 supernatants)	52.5 ± 8.8**	10	1.3 ± 9.7	262 ± 93
COPD + NE inhibitor (n=7 supernatants)	76.6 ± 7.7	10	14.7 ± 4.9	161 ± 47

Data are mean ± SEM. **p<0.01 *vs.* untreated, as determined by Kruskal-Wallis test and Dunn's post hoc comparisons. NE= Neutrophil Elastase.

6.3.6. Efficacy of MMP-9 inhibitor on neutrophil supernatants

In order to determine the efficacy of the MMP-9 inhibitor (444278), concentration-responses were performed in supernatants from neutrophils from COPD patients stimulated with 100μM fMLP (Figure 6.8). After 30 min pre-incubation, active MMP-9 was not inhibited at any concentration tested (Figure 6.8. (A)); however, following overnight incubation, there was a step-wise decrease in active MMP-9 concentration with increasing concentrations of MMP-9 inhibitor, with ~92% inhibition at 10μM and an IC_{50} of ~11nM (Figure 6.8 (B) and (C)). The protocol for the MMP-9 assay made it difficult to perform a time-course, as "active" samples (i.e. supernatants + inhibitor) incubate on the plate for 2h, with no step to stop the inhibition before adding the samples. Therefore, due to the temporal constraints of the MMP-9 activity assays, it was not possible to investigate multiple time-points for this inhibitor. It would be unfeasible to pre-incubate the inhibitor with the neutrophil supernatants overnight, prior to then commencing experiment with PCLS, as it is unclear how this might affect the

composition of the neutrophil supernatants, and there would likely be some degradation of products after this length of time. Therefore, 10μM inhibitor with a 30 min pre-treatment with neutrophil supernatants before overnight incubation with PCLS was chosen for subsequent experiments.

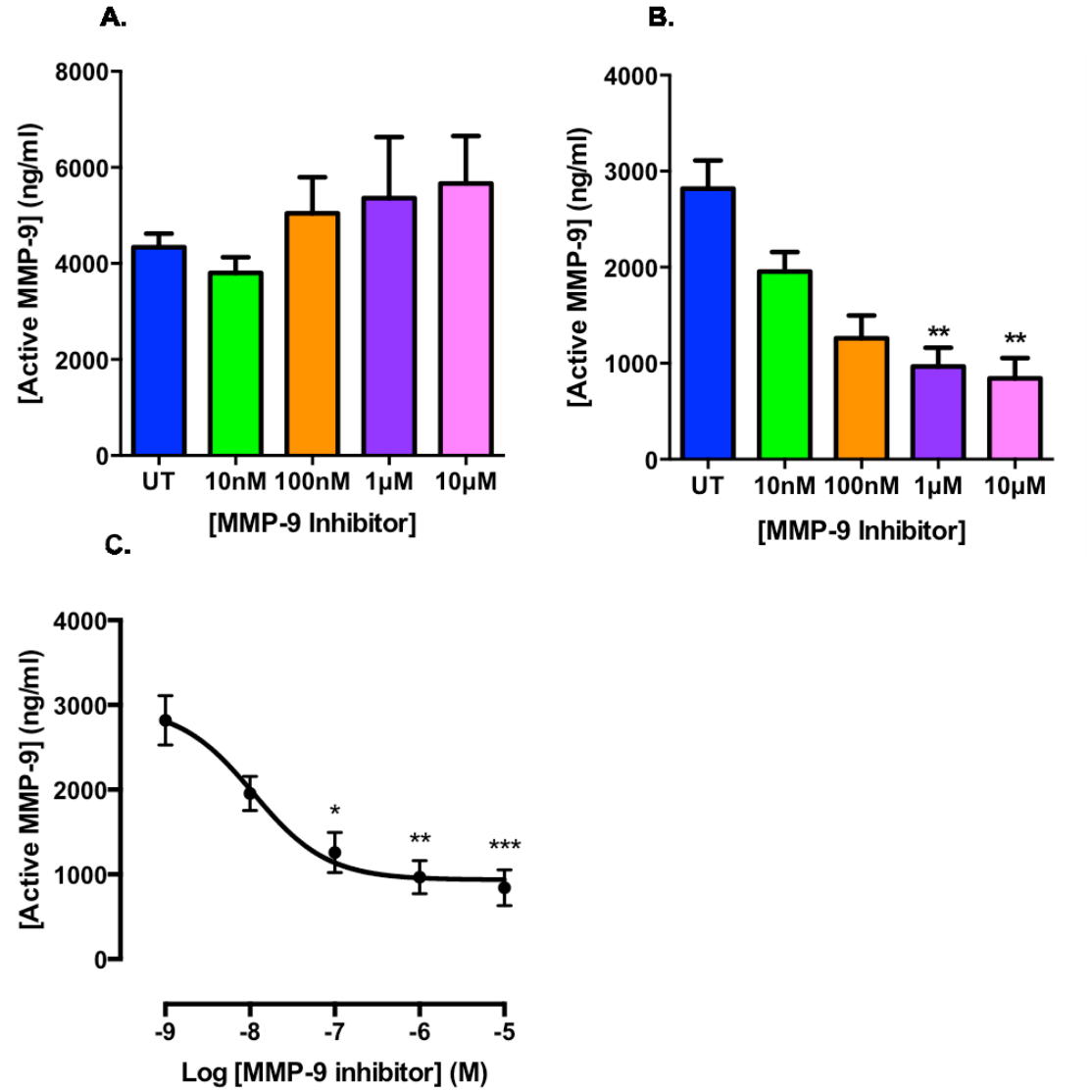


Figure 6.8. Effect of pre-incubation with an MMP-9 inhibitor (444278) on active MMP-9 concentration in neutrophil supernatants. (A) 2.5h (30min pre-incubation + 2h on plate) and (B) overnight incubation with supernatants from neutrophils stimulated with 100μM fMLP. (C) Concentration-response of active MMP-9 concentration in neutrophil supernatants following overnight incubation with increasing concentrations of MMP-9 inhibitor. N=7 supernatants per group, data are mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 *vs*. untreated, as determined by Kruskal-Wallis and Dunn's post-correctional tests.

6.3.7. Effect of pre-incubation of neutrophil supernatants with MMP-9 inhibitor on small airway function and PCLS viability

Supernatants from neutrophils from COPD patients that were stimulated with 100μM fMLP were pre-incubated for 30 min with MMP-9 inhibitor, prior to overnight incubation with PCLS. The following day, small airway function and PCLS viability was assessed. CCh induced a concentration-dependent increase in small airway closure in untreated PCLS and those incubated in MMP-9 inhibitor alone, with maximal increases above baseline of ~78% at 10μM CCh, and EC_{50} values between 200-500nM (Figure 6.9 (A), Table 6.7). However, maximal airway closure of small airways in PCLS incubated with neutrophil supernatants, either with or without inhibitor, was ~17-24%, which was an ~70-80% decrease compared

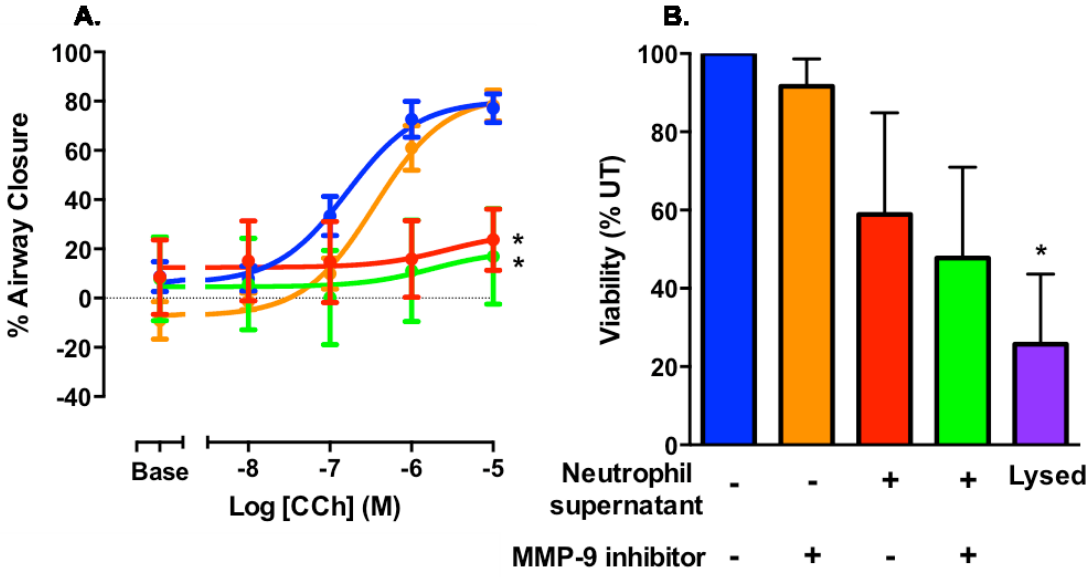


Figure 6.9. Effect of an MMP-9 inhibitor (444278) on neutrophil supernatant-induced small airway dysfunction and PCLS viability. (A) Effect on small airway closure. Untreated (●), n=6 rats; MMP-9 inhibitor alone (●), n=6 rats; COPD neutrophil supernatant alone (●), n=5 supernatants, n=10 rats; COPD neutrophil supernatants + pre-incubation with 10μM MMP-9 inhibitor (●), n=5 supernatants, n=10 rats. *p<0.05 *vs.* untreated, as determined by Kruskal-Wallis and Dunn's post-hoc tests. All untreated and MMP-9 inhibitor alone data sets above 1μM CCh were significantly higher than baseline (p<0.05-0.01). (B) Effect on PCLS viability, expressed as % untreated, which were considered 100% viable. Lysed, all groups n=4-5 supernatants; n=8-10 rats. Data are mean ± SEM. *p<0.05 *vs.* Untreated, as determined by Wilcoxon Signed Rank tests.

with untreated airways. There was a ~two-fold increase in EC_{50} to CCh in airways incubated with neutrophil supernatants and inhibitor, but this was not significant. There were no significant differences in the baseline airway patency between any groups. Therefore, MMP-9 inhibitor (444278) did not inhibit small airway dysfunction.

MMP-9 inhibitor alone had no significant effect on PCLS viability (Figure 6.9 (B)); however PCLS incubated with neutrophil supernatants, either with or without MMP-9 inhibitor, had ~48-58% the viability of untreated PCLS. Pre-incubation with MMP-9 inhibitor did not restore PCLS viability. As expected, lysed PCLS were significantly less viable than untreated PCLS, at ~ 25% untreated viability.

Table 6.7. Effect of pre-incubation of COPD neutrophil supernatants with MMP-9 inhibitor (444278) on small airway function

Treatment	Maximal Closure (%)	[CCh] for maximal closure (μM)	Baseline Airway Patency (%)	EC_{50} (nM)
Untreated (n=6)	77.1 ± 5.8	10	8.7 ± 3.5	200 ± 48.8
MMP-9 inhibitor alone (n=5)	78.2 ± 6.4	10	-9.1 ± 7.7	488 ± 171
COPD (n=5)	16.9 ± 9.4	10	7.8 ± 17.1	541 ± 299
COPD + MMP-9 inhibitor (n=5)	23.7 ± 12.5	10	8.5 ± 15.1	1068 ± 400

Data are mean ± SEM. MMP= Matrix metalloprotease.

6.3.8. Summary of results

The results of the present chapter were:

1. NAC (1mM) was able to restore maximal airway closure following overnight treatment with 1mM H_2O_2. Lower concentrations of NAC had no notable effects. At 1mM, NAC appeared to interfere with WST-1 viability assay, therefore it was inconclusive as to whether PCLS viability was also restored.
2. At 1mM, NAC did not inhibit neutrophil-supernatant induced small airway dysfunction. PCLS viability was not assessed due to aforementioned interference between NAC and WST-1 assay.
3. At 10μM, NE inhibitor (324745) was able to abolish NE activity after 30min pre-incubation with neutrophil supernatants.
4. Pre-incubation of NE inhibitor in neutrophil supernatants, in the conditions outlined above, prior to overnight incubation with PCLS resulted in restoration of the maximal closure of small airways in PCLS; however there was no significant restoration of PCLS viability.
5. Pre-incubation of MMP-9 inhibitor (444278) in neutrophil supernatants prior to overnight incubation with PCLS did not inhibit supernatant-induced small airway dysfunction or loss of PCLS viability.

6.4. Discussion

In order to assess the contribution of oxidants and proteases present in neutrophil supernatants on rat small airway function and PCLS viability, the present chapter investigated the effect of the antioxidant, NAC, and inhibitors of NE and MMP-9, to determine whether or not these could attenuate the effects of supernatants from COPD neutrophils stimulated with 100μM fMLP on small airway dysfunction and loss of PCLS viability.

6.4.1. NAC could attenuate H_2O_2-induced small airway dysfunction, but not that induced by neutrophil supernatants.

Prior to pre-treating neutrophil supernatants with NAC, a series of experiments were performed with H_2O_2 in order to optimise the concentration of NAC that may be appropriate to inhibit ROS in neutrophil supernatants. Treatment with 1mM H_2O_2 was chosen, as the maximal airway closure in response to this treatment was ~60%; similar to that observed when PCLS were incubated in supernatants from COPD neutrophils stimulated with 100μM fMLP. Therefore, it was postulated that if the effects of the neutrophil supernatants were due to oxidative stress, then this concentration of H_2O_2 would most closely mimic that.

Pre-incubation with 1mM NAC appeared to attenuate H_2O_2-induced reduction in maximal airway closure. Despite this result being non-significant, there was an ~18% increase in maximal airway closure with 1mM NAC pre-incubation, compared with 1mM H_2O_2 treatment alone. However, the effects of NAC on H_2O_2-induced reductions in maximal airway closure were not concentration-dependent: at all other concentrations of NAC tested, the maximal airway closure was lower than that of H_2O_2 alone. When incubated alone, 1mM NAC did not augment maximal airway closure, which suggests that there are not any off-target effects of NAC that increase maximal airway closure independent of its effects on H_2O_2. Therefore, these results suggest that there is a threshold level of NAC that can attenuate small airway dysfunction induced by H_2O_2; and that below this level, NAC is ineffective. This may be

explained by the observation that the reaction between NAC and H_2O_2 is slow, with a small reaction rate constant (Aruoma *et al.*, 1989); which suggests that high concentrations of both reactants may be required to react. In addition, NAC must be in considerable excess of H_2O_2 for the reaction between them to occur at any significant rate (Moldéus *et al.*, 1986). NAC attenuates H_2O_2-induced apoptosis in rat alveolar type II cells *in vitro* at similarly high concentrations: cells were pre-treated for 30min with 5mM NAC prior to treatment with 500μM H_2O_2 (Fu *et al.*, 2010). This supports the notion that high concentrations of NAC are required to elicit any effects when using H_2O_2 as an oxidative stress.

One of the issues encountered with using high concentrations of NAC was the interference of this antioxidant with the viability assay used herein. The basis of the viability assay is the reduction of the tetrazolium salt, WST-1, by cellular dehydrogenases, to the coloured compound formazan. However, as NAC is a reducing agent, this resulted in interference at high NAC concentrations. A similar effect has also been reported by Bruggisser *et al.*, (2002). In order to circumvent this, a viability assay that does not rely on reduction of tetrazolium salts would need to be utilized in future experiments. One way may be to homogenize PCLS into a single cell suspension and use flow cytometry to detect early and late apoptotic/necrotic cells by staining for Annexin-V and 7- Amino-Actinomycin (AAD). A foreseeable problem with this approach may be that the homogenizing procedure could further damage cells in the PCLS, resulting in an underestimate of PCLS viability. Other methods utilized in the literature have been to use confocal microscopy to examine PCLS stained with propidum idodide (PI) and/or calcein acetoxymethyl/ethidium homodimer-1 (calcein AM/EthD-1), which stain the nuclei of dead cells (Henjakovic *et al.*, 2008, Nassimi *et al.*, 2010); although it is worth noting that the PCLS used in those experiments were approximately half the thickness of those used in the present study, which may be an important factor to consider.

Pre-incubating neutrophil supernatants with 1mM NAC did not attenuate the effects of maximal airway closure, which suggests that oxidative stress is not involved to any great

extent in neutrophil supernatant-induced small airway dysfunction. This is not altogether unsurprising, as reactive oxygen species are highly unstable, so it is unlikely that they will remain present in neutrophil supernatants throughout their storage. However, it is possible that NAC was required in greater excess in order to be effective, therefore neutrophil supernatants could be pre-incubated with even higher concentrations of NAC in order to establish whether or not oxidative stress is involved. To the author's knowledge, there is not a validated way to measure the concentration of ROS in supernatant; therefore it is difficult to establish whether or not oxidative stress is a feasible target. Due to the interference with the assays, it was not possible to establish whether or not NAC could attenuate neutrophil supernatant-induced loss of PCLS viability; therefore alternative viability assays, as described above, could be utilized in order to investigate this. However, the current data suggest that oxidative stress does not contribute significantly to small airway dysfunction in rat PCLS associated with neutrophil supernatants.

6.4.2. NE inhibition could attenuate reduction in maximal airway closure caused by neutrophil supernatants

The results of the current chapter suggest that inhibition of NE may attenuate the reduction in maximal airway closure caused by incubation with neutrophil supernatants. Although not significant, there was a ~45% increase in maximal airway closure when supernatants were pre-incubated with NE inhibitor (324745), compared with COPD supernatant alone. The effects of elastolytic activity on small airway dynamics were discussed in detail in Section 5.4; however, as the results from Chapter 5 are somewhat discordant with the literature, it is difficult to reconcile the effect that elastase inhibition may have on small airway function. One possible explanation may be that NE has been shown to induce PGE_2 release from bronchial epithelial cells, and PGE_2 is a known bronchodilator (Perng *et al.*, 2003). As such, inhibition of NE may reduce this release of PGE_2, and thus increase maximal airway closure. In order to validate this hypothesis, levels of PGE_2 in the PCLS supernatant could be measured. Alternatively, neutrophil supernatants could be pre-incubated with indomethacin,

a non-selective prostaglandin inhibitor, to investigate whether this also attenuates small airway dysfunction.

NE inhibition did not have any significant protective effects against loss of PCLS viability. This result is concordant with the results of Chapter 5, where elastase was shown to have no effect on PCLS viability (section 5.3.4), and, therefore, does not appear to be involved in neutrophil supernatant-induced loss of PCLS viability.

6.4.3. MMP-9 inhibition had no effect on neutrophil supernatant-induced small airway dysfunction and loss of PCLS viability

Inhibition of MMP-9 had no protective effects against the reduction in maximal airway closure, nor the loss of PCLS viability caused by incubation with neutrophil supernatants. However, this may be because the inhibitor used was not adequately inhibiting MMP-9. The results of the present chapter suggest that after 30 min there was no inhibition of MMP-9 in the sample; however there is an ~three-fold reduction in MMP-9 activity following overnight incubation. Neutrophil supernatants were pre-incubated with the inhibitor for 30 min, therefore it is possible that MMP-9 activity in the sample was still high and, thus, continued to damage PCLS.

One of the reasons why the inhibitor may have been so ineffective is that it was a reversible inhibitor, therefore may not be permanently inhibiting MMP-9 throughout the incubation period. In addition, the assay used to determine MMP-9 activity may not have been appropriate to optimise the inhibitor. The assay used to measure active MMP-9 in supernatants is a fluorometric assay that measures the enzyme activity of active MMP-9, and is described in detail in Section 3.2.3. The assay utilizes a plate coated with a monoclonal antibody that binds to MMP-9 in the sample; therefore binding of the inhibitor may alter the conformation of MMP-9 so it no longer effectively binds to the antibody coated on the plate. However, as there was observable inhibition following overnight incubation with the inhibitor, this is unlikely. It is difficult to perform a time-course using this assay, as

samples need to be incubated on the plate for two hours; therefore this is the minimum time-point for measurement. An activity assay, similar to that used to measure NE activity (section 3.2.2) might be more appropriate, as it enables immediate determination of enzyme activity. However, to the author's knowledge, there are currently no specific fluorogenic or colormetric substrates for MMP-9. Zymography is a semi-quantitative method to measure MMP-9 activity through the digestion of gelatin that is incorporated into a Tris-Glycine gel. Samples are temporarily denatured, separated on these gelatin gels, and then renatured and stained to reveal white bands on a dark background where MMP-9 has digested the gelatin. Pro- and active-MMP-9 concentration can then be semi-quantitatively determined by comparing them against an MMP-9 standard. This could be used to optimise conditions for the inhibitor: the inhibitor could be added to the renaturing buffer at different concentrations or for different lengths of time, and then the gel developed as normal. The intensity of the band can then be compared with samples renatured in buffer without inhibitor (from the same gel) to determine whether the inhibitor was working. Although this will only provide semi-quantitative data, it may be a more appropriate way to optimize the conditions for the inhibitor.

Given the variable results with the chosen inhibitors, it may have been a better approach to use more broad-spectrum inhibitors of MMP's or serine proteases initially, prior to narrowing down to more specific inhibitors. This may have given better focus as to which specific proteases would best be targeted for inhibition, and provide a stronger basis for assessing the effectiveness of more specific inhibitors.

6.4.4. Discrepancy in the effect of neutrophil supernatants

One notable observation in the present chapter is that the effects of COPD neutrophil supernatants were dissimilar to those previously reported in Chapter 4. In Chapter 4, there were significant reductions in baseline airway patency, EC_{50} to CCh and maximal airway closure when PCLS were incubated with COPD neutrophil supernatants. In the present

chapter, not all of these effects were replicated. The supernatants used to investigate NE inhibition again caused reductions in maximal airway closure, but had no effects on either EC_{50} or baseline patency; and the supernatants used to investigate the effects of NAC and MMP-9 inhibition resulted in virtually no small airway function following overnight incubation with PCLS. This difference is difficult to reconcile, as neutrophils were treated in exactly the same way, and there were no significant differences in subject demographics of patients used between the Chapters 4 and 6. Therefore, this result most likely reflects the variability in the PCLS. In order to try and minimise the effects of variability between different animals, PCLS from two different rats were used when investigating the effects of supernatants from each patient, and at least four PCLS were used for each condition investigated. However, this method is clearly not sufficiently robust to adequately minimise airway/PCLS variability. Another caveat is that PCLS are grouped on Day 1 by airway closure at 10μM CCh, and not by EC_{50}; therefore there may be differences that exist between the airways prior to any treatment. However, it would not be feasible to attempt to equally group PCLS based on both of these parameters. As such, increasing the number of rats per supernatant, and the number of PCLS per condition, may further minimise the variability between PCLS and thus provide more consistent results.

6.4.5. Hypothesis: accept/reject

The hypothesis at the beginning of this chapter was:

"The antioxidant NAC, and/or inhibitors of NE and/or MMP-9 will inhibit small airway dysfunction and reduced PCLS viability in PCLS incubated in stimulated neutrophil supernatants from COPD patients."

With the exception of pre-incubation with NE inhibitor and its effects on restoring small airway function, the results of the present chapter mean that this hypothesis has to be rejected.

Chapters 4 investigated the effects of neutrophil products on rat PCLS, and reported that neutrophil supernatants from COPD patients induce aberrant small airway function and loss of PCLS viability. Chapter 5 examined two potential mechanisms (oxidative and elastolytic stress) that may contribute to neutrophil-induced dysfunction; and the present chapter (Chapter 6) attempted to attenuate the neutrophil supernatant-induced damage using protease inhibitors and antioxidants. The final chapter (Chapter 7) will therefore investigate small airway function in human PCLS from COPD patients, and determine whether or not this is concordant with the results obtained from Chapters 4 and 5.

Chapter 7.

Small Airway Function in Human PCLS

7. Small Airway Function in Human PCLS

7.1. Introduction

Small airways have been implicated as the main site of airflow obstruction in COPD (Hogg *et al.*, 1968), and dysfunction of these airways has been significantly associated with decline in lung function (O'Donnell *et al.*, 2004). Airflow resistance is inversely proportional to the radii of the airways raised to the fourth power; therefore even a small decrease in the airway lumen will result in a comparatively large increase in airflow resistance. Airway wall remodelling, as a result of fibrosis and hypertrophy of the various layers of the airway wall; occlusion of the airway lumen with inflammatory cells and exudates; and/or destruction of alveolar attachments, can all contribute to narrowing of the airway lumen and, thus, increased resistance to airflow.

Despite their importance in the pathophysiology of COPD, small airways are notoriously difficult to investigate *in vivo*. Standard pulmonary function tests are not particularly reliable at specifically assessing small airway dysfunction; however single and, more recently, multiple breath nitrogen washouts; exhaled nitric oxide; and impulse oscillometry are used as non-invasive measures of small airway dysfunction in humans (Stewart *et al.*, 2013). Imaging techniques, such as high resolution computed tomography (HRCT) and magnetic resonance imaging (MRI) are also used to assess and diagnose small airways disease. As described in previous chapters (Chapters 1, and 4-6), PCLS is an *ex vivo* preparation of lung tissue that includes functional small airways in their native microenvironment, which enables direct investigation of small airway dynamics, independent of *in situ* inflammatory responses that may confound their investigation.

In Chapters 4-6, rat PCLS were used as an *ex vivo* test system to investigate small airway function in a “pathological” microenvironment, that may be extrapolated to indicate certain mechanisms of COPD pathophysiology; for example the effect of neutrophil activation. In order to investigate the relevance of this acute rat model to COPD, small airway function was assessed in the present chapter in PCLS from resected human lung tissue samples.

7.1.2. Hypothesis

From the above introductory observations, the hypothesis of the present chapter was therefore that:

"*Ex vivo* small airway function in human PCLS will differ between tissue from non-smokers, smokers and COPD patients, and this will correlate with lung function parameters."

7.1.3. Aims

In order to investigate this hypothesis, the aims for this chapter were to:

1. Generate PCLS from resected human lung tissue, and investigate small airway function.
2. Assess any correlations between *ex vivo* small airway function and subject demographics.

7.2. Methods

7.2.1. Subject selection

Lung tissue samples were obtained from patients undergoing surgical resection at the Royal Brompton Hospital, London, which were determined macroscopically normal by a resident pathologist. Through accessing patient notes and lung function data, patients with COPD were categorised as those with FEV_1: FVC ratio <0.7, and smoking history of >10pack years, as defined by GOLD, 2014. Smokers had >10 pack-year smoking history (50 pack-years, Table 7.1), but otherwise normal lung function; and non-smokers had variable, but negligible smoking history (two were never smokers, one patient smoked occasional cigars) and normal lung function. All subjects gave written, informed consent as approved by the Royal Brompton, Harefield and NHLI Research Ethics Committee (ethics number 10/H0504/9).

7.2.2. Human tissue inflation and PCLS generation

Human incubation buffer (human IB) comprised Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 100µg/ml (1% $^{v}/_{v}$) penicillin/streptomycin, 4mM (2% $^{v}/_{v}$) L-glutamine, and 50µg/ml (0.5% $^{v}/_{v}$) gentamycin. Tissue inflation was undertaken on ice, in a small water bath filled with ice-cold slicing buffer. Evenly-spaced injections of ~100µl 3% ($^{w}/_{v}$) agarose were administered throughout the tissue until adequately inflated (approximately 1-2ml/g tissue). Inflated tissue samples were then left to solidify. The outer membranous layers of the tissue were sliced off and the orientation of airways and blood vessels determined. Eight mm cores were cut through the tissue, and placed in slicing buffer prior to slicing. PCLS were generated as described previously (see Section 2.2.1.2), and were sliced at 400-450µm thickness. PCLS were placed in individual wells of a 24-well plate in 1ml human IB and then washed as described previously. Contraction and relaxation analysis was performed as described previously (Sections 2.2.1.4 and 2.2.1.5).

7.2.3. Data Analysis

As data sets were not assumed to be normally distributed, non-parametric statistical tests were used in all cases. However, for familiarity of presentation, data are presented as mean ± SEM. Comparisons between subject demographics, and between airway function parameters were conducted using Mann-Whitney t-tests, or Kruskall-Wallis (ANOVA) tests followed by Dunn's post-correctional tests. Correlations between small airway function and subject demographics were performed using Spearman's rank correlation co-efficient. Concentration-response curves for contraction analyses were constructed by log-transforming CCh concentration, and plotting against airway closure (Section 2.2.1.4). Non-linear regression curves were then constructed, from which EC_{50} values were determined. The null hypothesis of no significant differences between groups was rejected at $p<0.05$. All statistical analysis was performed using GraphPad Prism v5.0 software.

7.3. Results

7.3.1. Subject demographics

Subject demographics are shown in Table 7.1. Non-smoker subjects were approximately 25 years younger than both smokers and COPD patients ($p<0.05$ *vs*. COPD patients); however smokers and COPD patients were matched for age and smoking history. Smokers and COPD patients had similar FEV_1 % predicted, and both were higher than non-smokers. COPD patients did, however, have significantly lower FEV_1/FVC ratio, compared with non-smokers and smokers. Detailed patient history (including medication, diagnosis and reason for surgery) was unavailable for most subjects; however one non-smoking subject was diagnosed with pulmonary hypertension, and another with α1-AT deficiency. It is of note that with the COPD patients, despite FEV_1/FVC values falling within the criteria of a COPD diagnosis, the FEV_1% predicted values are very high. This may allude to a misdiagnosis; however, as mentioned previously, detailed patient history is not available for most patients, therefore this is difficult to check. It is, nevertheless, an important point to bear in mind when interpreting any future results.

7.3.2. Airway closure of airways in human PCLS

In response to CCh, airways in PCLS from all subject groups closed in a concentration-dependent manner, with maximal increases above baseline of approximately 38-56% at 3-10μM CCh. Despite there being no significant differences, airways in PCLS from COPD patients had ~1.5-fold higher maximal airway closure at 10μM CCh, compared with non-smokers. All three subject groups had similar EC_{50} values within 50nM of each other (Table 7.2, Figure 7.1).

7.3.3. Passive airway reopening of airways in human PCLS

Following CCh washout, airways in PCLS passively reopened to >95% of their original pre-CCh patency (0%) by 60 min, and all had reopened to <50% maximal airway closure in <0 min, as reflected by $t_{1/2}$ values (Figure 7.2, Table 7.2)). Airways in PCLS from the single non-

smoker available for use in the experiment reopened to ~10% larger than their pre-CCh patency almost immediately after washout, and then closed back to 0% over a further 40 min. This was much quicker than airways in PCLS from smokers, which took ~20 min to reach their pre-CCh patency, and then continued to enlarge beyond their baseline patency over 60min. Airways in PCLS from two COPD patients took around 40 min to reach their pre-CCh patency; however, unlike the other two subject groups, these airways did not enlarge beyond their pre-CCh patency, and, despite being very close, did not even reopen back to 0% after 60 min.

Table 7.1. Subject demographics for human PCLS experiments

	Non-smokers	**Smokers (Current/Ex)**	**COPD**
N	3	8 (2/6)	5
Age	42 ± 6	74 ± 2	74 ± 3*
Gender (m/f)	0/3	4/3[$]	3/2
Smoking history (Pack years)	N/A[#]	50.8 ± 21.8	43.7 ± 9.3
FEV_1 (L)	2.5 ± 0.3	2.2 ± 0.3	1.9 ± 0.2
FEV_1 predicted (%)	75.0 ± 11.4	85.2 ± 5.4	90.8 ± 11.5
FVC (L)	3.3 ± 0.4	2.8 ± 0.4	3.4 ± 0.3
FEV_1/FVC (L)	0.78 ± 0.07	0.77 ± 0.02	0.57 ± 0.04**

Data are mean ± SEM. *p<0.05 non-smokers *vs.* COPD; **p<0.01 smokers *vs.* COPD. FEV_1= Forced expiratory volume in one second. FVC= Forced vital capacity.

[#]One patient smoked occasional cigars. [$]Some patient data unavailable.

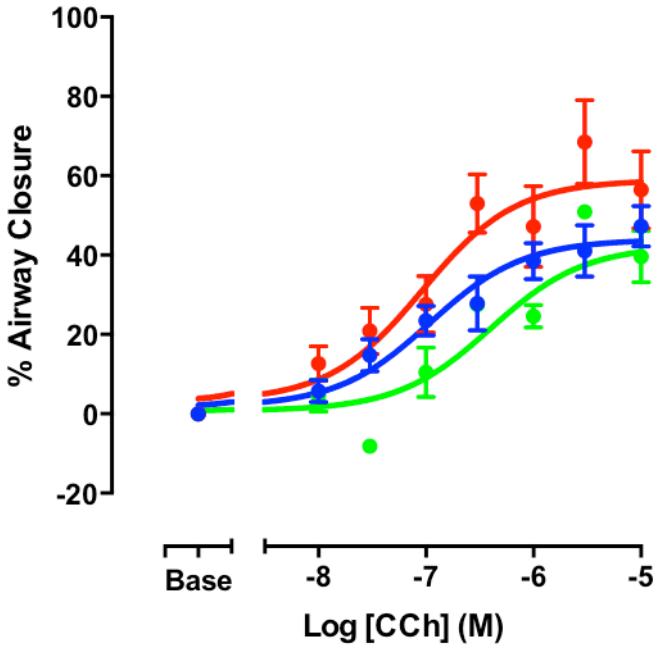


Figure 7.1. **CCh concentration-response curve of small airway closure in PCLS from human lung samples.** Non-smokers (●), n=3; smokers (●), n=8; COPD (●), n=4. Data are mean ± SEM. There are no significant differences between any data points.

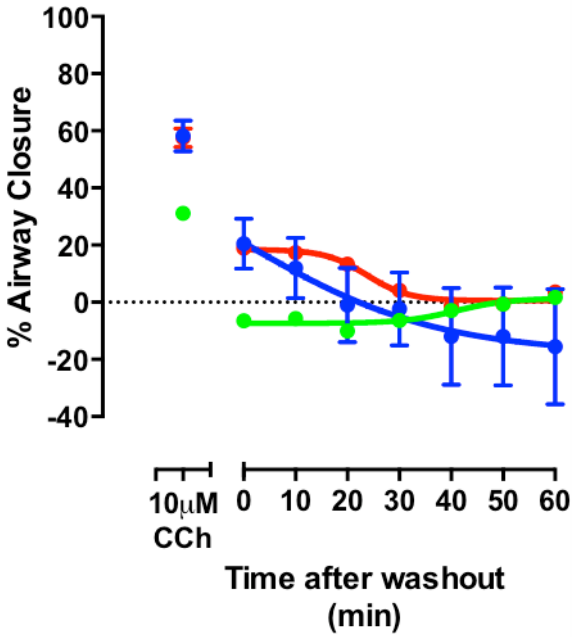


Figure 7.2. **Passive reopening of small airways in PCLS from human lung samples, following CCh-washout.** Non-smokers (●), n=1; smokers (●), n=4; COPD (●), n=2. Data shown are mean ± SEM. There are no significant differences between any data points.

Table 7.2. Function of small airways in human PCLS, in response to CCh					
	Airway Closure			**Airway Reopening**	
Subject Group	**Maximal Closure (%)**	**[CCh] for maximal closure (μM)**	**EC_{50} (nM)**	**$T_{1/2}$ (min)**	**Time to (0%) (min)**
Non-smokers	39.6 ± 6.5	3	233 ± 155	<0	<0
Smokers (Current + Ex)	47.2 ± 5.1	3	180 ± 55	<0	22.2
COPD	56.5 ± 9.7	10	206 ± 82	<0	-
Data are mean ± SEM. $T_{1/2}$= Time taken to reach 50% maximal airway closure.					

7.3.4. Correlations with lung function

There were no significant correlations or trends between any lung function parameters and maximal airway closure (Table 7.3). An example for the FEV_1/FVC ratio is shown in Figure 7.3.

7.3.5. Summary of results

The results of the present chapter show:

1. Small airways in PCLS from resected lung tissue from COPD patients have greater maximal airway closure than either smokers or non-smokers.
2. Airways from all subject groups reopened >50% to their pre-CCh patency almost immediately after washout; however those from COPD patients took longer to reopen completely, compared with either non-smokers or smokers.
3. There were no significant correlations between small airways function and subject demographics.

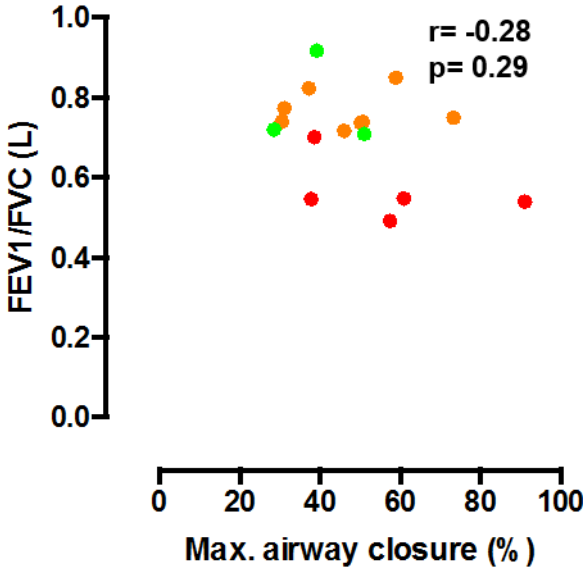


Figure 7.3. Relationship between lung function (FEV_1/FVC) and small airway closure in a group of non-smokers (●), smokers (●) and COPD patients (●). No significant correlation (Spearman rank correlation coefficient).

Table 7.3 Correlations between *ex vivo* airway function and subject demographics

Maximal airway closure *vs.*	Spearman Rank Correlation Coefficient (r)	P-value	n
Age	0.34	0.22	15
Pack years	0.11	0.70	15
FEV_1 (L)	-0.22	0.41	16
FEV_1 % predicted	0.29	0.34	13
FVC (L)	-0.07	0.80	16
FEV_1/FVC (L)	-0.28	0.29	16

FEV_1= Forced expiratory volume in one second. FVC= Forced vital capacity.

7.4. Discussion

The present chapter investigated the function of small airways in PCLS from resected tissue samples from non-smokers, smokers and COPD patients. Despite being non-significant, the results indicate a trend for the sensitivity and maximal closure of small airways to cholinergic stimulation being greater in COPD patients, compared with either smokers or non-smokers. These results also suggest that this is an effect of disease state, rather than smoking history, as COPD patients' airways were more hyperresponsive than smokers without airflow obstruction.

7.4.1. Airway hyperresponsiveness in COPD

Airway hyperresponsiveness has historically been associated with asthma; however, there is increasing evidence that it is also a prominent feature of COPD. In the GLUCOLD study (van den Berge *et al.*, 2011, 2012), 94% of COPD patients were found to be hyperresponsive. In this same study, severity of AHR was significantly correlated with high sputum neutrophilia and worse airflow obstruction, as determined by lower FEV_1 and FEV_1/IVC ratios. (Lancas *et al.*, 2011) also demonstrated that parenchymal strips from COPD patients were hyperresponsive to cholinergic stimulation, compared with non-smoking controls; and also reported a significant reduction in elastic fibres in COPD parenchymal strips, compared with controls.

7.4.2. Potential mechanisms

7.4.2.1. Increase in smooth muscle mass

There are a number of potential mechanisms that may contribute to airway hyperresponsiveness in COPD. Firstly, a number of studies have demonstrated increases in airway smooth muscle in COPD patients. Saetta *et al.*, (1999), Bosken *et al.*, (1990) and Cosio *et al.*, (1980) have all histologically investigated small airways in lung sections from COPD patients and asymptomatic smokers, and found there was a significant increase in smooth muscle area in the former group, compared with the latter. A computational model of

small airway dynamics demonstrated that increased smooth muscle mass could cause airway hyperresponsiveness (Lambert *et al.*, 1993); therefore, this is a feasible mechanism to explain the results of the present chapter. In order to explore this further, sections of PCLS could be stained for airway smooth muscle and the thickness quantified, to investigate whether or not this correlates with *ex vivo* small airway function.

7.4.2.2. Reduced elastic recoil

Another potential mechanism is reduced elastic recoil of the airways, caused by proteolytic destruction of the extracellular matrix. As described in more detail in previous chapters, a protease-antiprotease imbalance is one of the leading hypotheses regarding COPD onset and progression. Proteases, including elastases, collagenases and MMPs, degrade components of the extracellular matrix, which may alter airway dynamics. Airway closure is an active process that is dependent on a balance between the force of contraction elicited by shortening of the smooth muscle fibres, and the opposing forces generated by parenchymal attachments to the airways. Damage to these parenchymal attachments may reduce the load opposing smooth muscle contraction, leading to exaggerated narrowing of the airways. This was demonstrated by Khan *et al.*, (2010): overnight incubation of mouse PCLS with elastase and/or collagenase resulted in increased velocity and magnitude of airway contraction. There is a reduction in the elastin fibres in the alveoli and small airway walls in COPD patients, compared with control subjects, and this reduction correlates with worsening lung function (Black *et al.*, 2008). This loss of elastin is thought to contribute to reduced elastic recoil of the airways, which, alongside diminished alveolar attachments, leads to increased airway narrowing. In order to investigate this further in the present study, sections of PCLS could be stained for structural components of the airways, to assess whether or not there are any correlations between airway function and structural damage.

The results of the present chapter also suggest that airways from smokers and COPD patients take longer to reopen than those from non-smokers. Although the numbers are very

low in these experiments, so no firm conclusions can be drawn, this would also be concordant with the results of the Khan *et al* study (2010), which also demonstrated reduced relaxation velocity in mouse PCLS treated with elastase and/or collagenase. Only passive relaxation was measured in the present study, so it would be interesting to also investigate the dynamics of drug-induced relaxation, or the effects of pre-treatment with drugs such as β_2-adrenergic agonists, which induce smooth muscle relaxation.

7.4.2.3. Increase in muscarinic receptors

One other potential mechanism that may mediate airway hyperresponsiveness could be an increase in, or dysfunction of, muscarinic receptors. Cholinergic stimulation of airways is mediated by muscarinic receptors; in particular, in humans, M_2 and M_3 subtypes. Airway contraction is thought to be mediated predominantly by M_3 receptors; however M_2 receptors are thought to have indirect effects on airway contraction by inhibiting relaxation pathways (Fryer *et al.*, 1998). Therefore, increased numbers of these receptors could result in enhanced, or hypersensitive, contraction of the airways in response to cholinergic stimulation. To the author's knowledge, there is currently no evidence to suggest that there is an increase in muscarinic receptor expression in COPD; however this is something that could be investigated by immunohistochemically staining sections of PCLS for the different receptor subtypes, and assessing whether or not there are differences between subject groups.

7.4.3. Correlations with lung function

The present study did not find any correlations with any lung function parameters. This may be due to low numbers of subjects, although the r-values were also very low. Increasing the number of experiments may allude to some correlations between *ex vivo* airway dynamics and subject demographics, although these may be more statistically significant than biologically/pathophysiologically significant. One possible issue that may be "masking" significant correlations is the inclusion of non-smokers and smokers who, by definition, have

normal lung function. To circumvent this, COPD patients could be considered independently of the other two subject groups; however, at this stage, the numbers of COPD patients are too low. Another potential reason may be that airways from COPD patients do indeed have small airway disease, and are, therefore, already partially closed and/or less able to close in response to CCh stimulation, thus resulting in a lack of meaningful correlations. This was investigated herein, and there were no significant differences in the baseline airway lumen size between subject groups (data not shown), making this possibility unlikely, but not to be ruled out completely.

7.4.4. Hypothesis: Accept/Reject

The hypothesis of the present chapter was:

"Ex vivo small airway function in PCLS will differ between tissue from non-smokers, smokers and COPD patients, and this will correlate with lung function parameters."

Although there were no significant results, there were some interesting trends with regards to disease state and small airway function. However, there were no correlations with patient demographics, so, at this stage, I can neither accept nor reject this hypothesis.

Chapter 8.

General Discussion

8. General Discussion

8.1. Summary of results

The present thesis investigated the role of neutrophil products primarily on rat small airway structure and function. Stimulated neutrophil supernatants from COPD patients reduced PCLS viability and caused reductions in maximal airway closure, EC_{50} to CCh, and baseline airway patency, compared with non-COPD neutrophil supernatants; however, there were no significant differences in protease composition of neutrophil supernatants between non-smokers, smokers and COPD patients. In order to assess the contribution of different components of neutrophil supernatants to this aberrant function, PCLS were then incubated overnight with either H_2O_2 or PPE. Both H_2O_2 and PPE significantly reduced the maximal airway closure of small airways; however neither had any effect on either EC_{50} to CCh or baseline airway patency. H_2O_2 nevertheless significantly reduced viability of PCLS, whereas PPE had little or no effect. Aside from an NE inhibitor, which significantly attenuated the reduction in maximal airway closure, pre-incubation with protease inhibitors or antioxidants had no effect on the destructive effects of neutrophil supernatants. Finally, small airways in resected lung tissue from COPD patients displayed a trend towards having increased maximal airway closure and, thus, being more sensitive to CCh stimulation, compared with small airways from smokers and non-smokers. These combined observations merit some discussion of general principles, as outlined below.

8.2. Use of PCLS as a model of small airways disease

One of the objectives of this thesis was to establish whether or not PCLS could be used as a model of small airways disease (SAD), or at least the neutrophil-involved component of SAD. The pathophysiology of small airways disease in humans is complex and multifaceted, and ultimately results in increased narrowing of the airway lumen and, thus, increased airflow resistance. This can be due to proteolytic degradation of the attachments between the airway wall and the surrounding parenchyma, which can result in hypercontractility of the

airways due to the lack of parenchymal load opposing smooth muscle contraction; to mucus and inflammatory cell infiltrate occluding the airway lumen; and to remodelling and thickening of the airway wall through goblet and squamous cell metaplasia, smooth muscle hypertrophy and hyperplasia, and fibrosis (Baraldo *et al.*, 2012).

Of these mechanisms, the PCLS model in this thesis only addressed the destruction of the parenchymal attachments, and not the chronic remodelling aspect, which is in part due to the short exposure time of the PCLS to their relative insults, and the restricted range, and combination, of insults. The model in the present thesis was acute exposure of PCLS to either neutrophil supernatants, oxidative stress or elastolytic stress; therefore, there is, most likely, not enough time for these remodelling and repair processes to get underway. However, this may not be a limitation of the PCLS system in general, just the methodology used in the present thesis. In other studies, PCLS have been maintained in culture for up to 5 days (Bergner *et al.*, 2002); therefore, it may be possible to design a system to model more sub-acute exposure, involving low-level stresses over a longer time-frame, to reproduce some of the remodelling aspects of SAD pathophysiology.

In addition, the present thesis only addressed airway function in response to cholinergic stimulation. This is physiologically relevant, as anticholinergics, such as tiotropium bromide, are a popular therapeutic strategy in the treatment of COPD (Montuschi *et al.*, 2015). However, there are a number of other agonists that can mediate airway tone, including 5-HT, histamine, bradykinin, cysteinyl leukotrines (e.g. LTD_4), and prostanoids (e.g. PGD_2); and sensitivity to many of these factors is additionally enhanced by a pro-inflammatory environment. (Pelaia *et al.*, 2008). Therefore, there are, more than likely, a number of additional agonists that modulate the aberrant airway tone observed in COPD that were not investigated in the present study. As such, it would be of interest to investigate the effect of neutrophil supernatants, H_2O_2 or PPE on airway function mediated by these other agonists, to identify whether or not particular agonist/receptor pathways are differentially affected in addition to the cholinergic pathway.

With regards to human PCLS, one of the advantages of the PCLS system is that it allows isolated investigation of small airway dynamics, without the potentially confounding impact of an inflammatory environment that would be present in *in vivo* studies. Therefore, the results obtained using PCLS, as a model, can be interpreted as being more due to long-term structural/physiological changes to the airways and/or the surrounding parenchyma, and more independent of the effects of inflammatory cells and/or an inflammatory environment. As such, PCLS may bridge a significant gap between current diagnostic and investigative tools with respect to small airways disease, as both airway function and histological structure can be assessed, whilst also being able to reconcile these results with actual patient data.

One of the main disadvantages of the system is that the technique is invasive, as it requires lung tissue samples. Most patients also have significant co-morbidities, such as lung cancer, pulmonary hypertension or α1-AT deficiency; the effects of which are invariably difficult to predict accurately. In addition, a complete medical history is rarely provided, which makes it difficult to account for co-morbidities or drug intervention. In addition, lung tissue from patients with very severe disease and significant emphysema is very difficult to inflate. This potentially eliminates a whole cohort of patients that can be studied and, as these will be the subjects with the most severe disease, they would, in some ways, be the most interesting for our purposes.

All of these factors should be taken into account when interpreting results using human PCLS. In order to circumvent this in the future, a larger cohort of patients should be used, so that variability due to co-morbidities is diluted. More extensive patient histories would also be advantageous with respect to possible sub-group analysis of the results.

8.3. The use of neutrophils to model SAD pathophysiology

One of the limitations of the present study with regards to modelling SAD is that only the effects of neutrophils were investigated. As described in Section 1.4.1, a number of additional inflammatory cells are thought to be involved in COPD pathophysiology, including

macrophages, T-cells, epithelial cells and fibroblasts; therefore there will be significant interplay between these cell types and, hence, their involvement in the development and progression of COPD. Nevertheless, it would be challenging methodologically to concurrently model the effects of different inflammatory cells, due to the number of variables it would entail. As such, any conclusions drawn from the present thesis should be considered in a wider context, taking into account the potential contribution of other inflammatory cells and mediators. However, one of the advantages of the PCLS system is that it does take in to account the potential involvement and interaction of lung structural cells, which are an important source of inflammatory mediators. This was demonstrated in the present thesis in Chapter 5, wherein the release of TNF-α and CINC-1 from PCLS following H_2O_2 or PPE exposure was investigated. As discussed in Chapter 5, this was not without its difficulties (namely, the presumed degradation of TNF-α and CINC-1 proteins by PPE); however, the PCLS system does provide a platform for investigating the role of structural cells in response to different stresses and their interaction with other inflammatory cells, and relating this with functional information.

An additional limitation of the present thesis is that supernatants from the same number of neutrophils ($10x10^6$cells/ml) from non-smokers, smokers and COPD patients were investigated. There is evidence pertaining to a step-wise increase in the number of neutrophils in smokers, compared with non-smokers; and then COPD patients, compared with asymptomatic smokers, in sputum (Ilumets *et al.*, 2008), BALF (Lofdahl *et al.*, 2006), and bronchial epithelium (Di Stefano *et al.*, 2009). As such, the characteristics of the PCLS system in the present thesis are not indicative of the *in vivo* environment. However, this system does enable investigation of inherent neutrophil defects or aberrant activity between the subject groups, without the confounding issue of different cell numbers; and the present thesis did display evidence for this in Chapter 4, whereby COPD neutrophil supernatants caused significant small airway dysfunction that neither smoker nor non-smoker supernatants could reproduce. In addition, when attempting to address this limitation, a

number of issues arose. Firstly, it was difficult to establish from the literature an appropriate ratio of neutrophil numbers between subject groups, as numbers varied considerably throughout the literature and from work in our own laboratory, in particular depending on where the count was obtained (i.e. sputum (Ilumets *et al*, 2008), BALF (Lofdahl *et al*, 2006), or airways (Di Stefano *et al*, 2009)). Secondly, the volume of supernatant would ideally need to stay constant; therefore neutrophils would have to be resuspended either more or less concentrated. It has been observed in our laboratory that when attempting to resuspend >10x10^{6}cells/ml, neutrophils tend to "clump" together and cannot be used; and, based on the minimal effects on airway function of supernatants from neutrophils stimulated with lower concentrations of fMLP, particularly in smokers and non-smokers (see Section 4.3.4.2), it was posited herein that diluting the neutrophils would result in very few observable effects. Therefore, it was difficult to develop a system whereby the relative numbers of neutrophils in non-smokers, smokers and COPD patients could be established.

Another potential limitation in the present thesis is that the neutrophils were isolated from peripheral blood; therefore they may behave differently to neutrophils that have migrated into the lungs. Additionally, they were only exposed to one stimulus, fMLP, which, again, is probably not necessarily indicative of the environment *in situ*. However, neutrophils isolated from sputum or BALF will be in various states of activation and cell death; therefore, it would be difficult to ensure consistency between samples. As these neutrophils will be at least partly activated, it would be problematic to further stimulate them, and might also cause cell death. It may also be quite difficult to obtain adequate samples, particularly induced sputum from non-smokers, which would further complicate the experimental design. Overall, peripheral blood neutrophils are quiescent at the time of isolation; therefore, stimulation is relatively uniform and, importantly, can be controlled, as cells should be in similar activation states. The procedure is also relatively non-invasive, compared with induced sputum or bronchoscopy. Therefore, despite their limitations, peripheral blood neutrophils were a reasonable choice for the work in the present thesis.

8.4. The effect of other parenchymal structures on airway contraction

One of the main advantages of the PCLS system is that airways are maintained in their native microenvironment. This environment will include intact parenchymal attachments to the airways, in addition to blood vessels also within the parenchyma. Particularly within the airway generations that are of interest in the present study, airways are often situated alongside blood vessels. Blood vessels also contain smooth muscle, and so may also be subject to any constrictor/relaxant mediators that are either released by neutrophils and thereby contained within the supernatants (see Table 1.1), or released from the lung tissue in response to the stresses added, such as prostaglandins and leukotrines.

Cholinergic stimulation has vasodilatory effects via the release of NO from vascular endothelial cells that act on vascular smooth muscle (Furchgott *et al.*, 1980). Blood vessels in rat PCLS do dilate in response to acetylcholine, but only following contraction with a thromboxane mimetic, and this response is dependent on NO and endothelial-derived hyperpolarizing factor (EDHF) (Moreno *et al.*, 2006). As such, it is a possibility that the experimental stresses imposed in the present thesis (e.g. neutrophil supernatants, PPE and oxidative stress), in addition to carbachol, may also affect vascular function. Therefore, altered blood vessel function may impact on the dynamics of small airway closure within the PCLS, and blood vessel contractility may alter airway calibre. To date, few studies have concurrently investigated both small airway and vascular function within the same PCLS (Perez *et al.*, 2005, Perez *et al.*, 2005), possibly as it requires a modified inflation technique using gelatin to perfuse the pulmonary vasculature. However, this may be of interest to investigate in any future work, and would be more representative of the *in vivo* environment, whereby the dynamics of these structures work in concert.

8.5. The use of WST-1 as suitable viability assay

Throughout this thesis, WST-1 has been used as a measure of PCLS viability. This assay measures mitochondrial function via the reduction of WST-1, a tetrazolium salt, into

formazan; which elicits a measureable colour change. This assay is simple, low cost, and takes very little time; thus was utilized throughout this study. However, the drawbacks of the use of this particular assay are that, as the PCLS are made up of multiple cell types, it was impossible to establish any heterogeneity in cell viability across these populations. There was often a disconcert between airway function and PCLS viability; suggesting that the airway smooth muscle cells were still quite viable in contrast to other cell types, which then decrease the "net" viability of the PCLS.

One way to circumvent this in future experiments may be to use a universal contractile agonist, for example potassium (K^+) that will indiscriminately depolarise smooth muscle. This will determine whether or not the smooth muscle is able to contract and is, as such, viable. K^+ depolarises the smooth muscle cell membrane, opening voltage-gated Ca^{2+} channels that facilitate the influx of extracellular Ca^{2+}, which, in turn, initiate smooth muscle contraction (see Section 1.5.1). Therefore, a high K^+ solution will initiate smooth muscle contraction independent of agonist binding. This would allow distinction between aberrant smooth muscle contraction due to defective cholinergic stimulation (i.e. downregulation of muscarinic receptors), and that due to loss of viability in smooth muscle cells. In addition, TUNEL staining could be utilized on histological sections of PCLS in order to determine any heterogeneity between viability of cell types.

8.6. The use of the rat to model human respiratory disease

There may also be contention with regards to the use of rat tissue to model human disease. There are a number of anatomical differences between rat and human lungs, including different numbers of lobes: in the rat, there are one and four lobes comprising the left and right lungs, respectively (Kling, 2011); whereas in the human there are two lobes comprising the left, and three lobes comprising the right lung. Additionally, the airway branching pattern in human lungs is fairly symmetrical, dichotomous branching; whereas in rat there is a monopodial branching pattern (Yeh *et al.*, 1979). There are also inevitable differences in

their functional responses to cytokines: rodent airways have also been shown to respond poorly to leukotrines (Held *et al.*, 1999), which cause significant contraction human airways (Dahlén *et al.*, 1980). With regards to more closely mimicking human airway function, guinea pigs would have been a more physiologically relevant model organism to use. Guinea pigs are not rodents (D'Erchia *et al.*, 1996) and, like human airways, bronchoconstrict in response to leukotrines (Drazen *et al.*, 1980). Guinea pig and human airways also respond similarly to electric field stimulation and capsaicin, compared with airways in rat and mice; again highlighting species differences with respect to neuronal innervation (Schleputz *et al.*, 2012).

However, guinea pig PCLS have not been as widely used, presumably due to the issue that the airways contract whilst generating the PCLS- a problem that has been encountered in our own laboratory, There has been some attempt to reconcile this issue by adding isoprenaline to the media, in order to prevent spontaneous airway closure (Ressmeyer *et al.*, 2006); however, in the present thesis, the addition of a bronchodilator may have affected the interpretation of results.

However, there are advantages to using rats over other model organisms: they are inexpensive, easy to handle, quick to breed and mature, and large enough to be able to competently perform lung dissections and obtain PCLS. As all rats were the same strain, housed in the same environment, and treated similarly throughout their lifetime, this limits the effects of environmental and genetic factors potentially confounding any results. This may even be an advantage over the use of human tissue samples, which, even if it were ethically viable to obtain samples from healthy, non-smoking individuals, would still be subject to genetic and environmental variability. As mentioned previously, all human lung samples used in the present thesis were obtained following lung resection for surgery or transplantation, which, by definition, indicates the presence of serious lung disease in the patient, not to mention any other co-morbidities. Therefore, human PCLS would be impractical for studying the effects of neutrophil supernatants, as the considerable amount of variability in both tissue and supernatant would confuse data interpretation without very large

sample sizes, which would also have been unfeasible in the time-frame of the present study. As such, the use of rats provides a reasonable alternative for study of small airway structure and function in the present thesis.

8.7. Reproducibility of results

Another limitation in the present thesis was the lack of reproducibility between experiments, most notably in the effects of COPD neutrophil supernatants in Chapter 4 compared with Chapter 6, whereby there was a reduction in EC_{50} and baseline airway patency in the former that was not observed in the latter. This is difficult to reconcile, as neither the rats nor the neutrophils were treated any differently between these experiments. One possibility is that the sample sizes are too low to account for the inherent variability between airways on lung slices, and between patients and their neutrophil supernatants. This may be in the number of PCLS used per condition, the number of rats used per supernatant, and/or the number of supernatants tested overall. When testing drugs or invariable compounds (e.g. H_2O_2 and PPE), the conditions set out appeared to be adequate, as the number of PCLS/rats are only controlling for the variability in lung tissue. The experiments with the neutrophil supernatants are clearly far more complex, as the lung tissue, small airways, and neutrophil supernatants will have considerable variability. Consulting a statistician to determine the appropriate sample size for the number of PCLS per conditions, rats, and supernatants could help remedy this in future experiments.

8.8. Clinical implications

As mentioned previously, the work carried out in the present thesis represents one aspect of a complex, multifaceted disease; therefore, the clinical implications are limited. Neutrophil supernatants from COPD subjects had increased effects on small airway function, compared with neutrophil supernatants from non-smokers and smokers, which suggests innate differences in supernatant content. This may be important in COPD pathophysiology. H_2O_2 and PPE mimicked some, but not all, of the effects of neutrophil supernatants. This suggests

that these mediators are involved in neutrophil supernatant-induced small airway dysfunction, but that there are other factors involved. Further analysis showed that, with the exception of NE, protease inhibitors and antioxidants had little or no effect on neutrophil supernatant-induced damage. Inhibition of NE did restore maximal airway closure, which indicates a restricted, but potential therapeutic effect; however, NE inhibitors have been used in a number of clinical trials and have shown little clinical efficacy (Kuna *et al.*, 2012, Vogelmeier *et al.*, 2012). Nevertheless, the work in the present thesis indicates that there are inherent differences in the degranulation products of neutrophils between COPD patients and smokers and non-smokers, owing to their differential effects on small airway function; however, none of the proteases measured in Chapter 3 seem to account for these differences. In addition, neither H_2O_2 nor PPE could completely reproduce the effects of the neutrophil supernatants. Therefore, this work suggests that there are additional neutrophil products responsible for the aberrant small airway function that may prove to be viable therapeutic targets in the future.

8.9. Accept or reject overall thesis hypothesis

The overall hypothesis for this thesis was:

"Supernatants from fMLP-stimulated human neutrophils will significantly affect PCLS structure and small airway function in rat PCLS, and the effects of neutrophils from COPD patients will be more pronounced.

In addition, these effects will be mediated by neutrophil proteases and/or oxidative stress, and addition of exogenous inhibitors or antioxidants will attenuate these effects.

Finally, small airways in human PCLS will behave differently, compared with those from asymptomatic smokers and non-smokers"

With regards to the first part of this hypothesis, fMLP-stimulated neutrophil supernatants did affect PCLS structure and viability and small airway function, and the effects of COPD

neutrophil supernatants were more pronounced, particularly with respect to EC_{50} to CCh and baseline airway patency. As such, this part of the hypothesis can be accepted. This suggests that the composition of COPD neutrophil supernatants are different to those of non-smokers and smokers, and this may be important in COPD pathophysiology.

With regards to the second part of this hypothesis, H_2O_2 and PPE could partially reproduce the effects of neutrophil supernatants, with respect to maximal airway closure and PCLS viability (only H_2O_2). However, neither had any effect on the other airway parameters affected by neutrophil supernatants, and, with the exception of a NE inhibitor, protease inhibitors or antioxidants did not attenuate neutrophil-supernatant induced damage. As such, this part of the hypothesis can only be partially accepted. This suggests that oxidative stress and elastase may be involved in neutrophil supernatant-induced small airway responses; however additional mediators, and complex interactions between mediators, are likely to be involved.

With regards to the final part of this hypothesis, small airways in PCLS from resected lung tissue from COPD patients displayed a trend for greater maximal airway closure; therefore this part of the hypothesis can be tentatively accepted. This suggests that small airways from COPD patients may be hyperresponsive; however increased sample sizes are required to validate this observation.

8.10. Future work

The acceptance and, more particularly, rejection of various aspects of the thesis hypothesis indicate avenues for future research, as outlined below.

- Further characterise the protease composition of neutrophil supernatants, possibly using a high-throughput system such as MSD. This should enable assessment of a number of different proteases and/or mediators from one sample, without the fiscal and temporal constraints of optimizing and undertaking different assays for each protease.

- Measure the ROS content of the neutrophil supernatants, either directly or indirectly by measuring respiratory burst from neutrophils. This would allow assessment of whether or not ROS are a possible mediator in supernatant-induced airway dysfunction.
- Further investigate mediator release from PCLS, following treatment with either neutrophil supernatants, H_2O_2 or PPE; in particular looking at lipid mediators. Measurement of PGE_2 concentrations in PCLS supernatant was attempted as part of the work of this thesis using a commercially-available kit (Cayman Chemical); however, the results were inconclusive. Fluorescence-based immunoassays have been used successfully in the literature (Kirkby *et al.*, 2013); therefore, these may be a better option. Additionally, experiments could be performed in the presence of indomethacin, a COX inhibitor that will block prostaglandin release. In order to circumvent the issue of degradation of mediators in the PCLS supernatant by proteases, PCLS could be homogenized and protein expression of mediators investigated using western blotting, or mRNA expression using PCR.
- Further investigate the effects of H_2O_2 on small airway function, to try and reconcile its bronchoconstrictor effects, as discussed in the literature, and the effects observed in the present thesis. This would involve detailed time-courses and concentration-responses, to elucidate whether the effects observed in the present thesis were a product of the decreasing viability of the PCLS, or whether H_2O_2 has a more complex involvement in mediating airway tone.
- Perform detailed histological analysis to assess, in more detail, the viability of PCLS following the stresses induced in the present thesis. As the PCLS are a heterogeneous population of cells, it would be of interest to elucidate whether loss of viability is a uniform effect, or whether certain cell types are more susceptible. This may help explain some of the observed functional results; for example, the effect of

PPE, which significantly attenuated small airway closure, but had no effect on PCLS viability.

- Further optimise and expand the inhibitors used in Chapter 6, in order to investigate further targets that may be causing small airway dysfunction. As discussed previously, NE was revealed as a relatively promising target from Chapter 6; however, NE activity was similar in supernatants from the three subject groups, suggesting it was not involved differentially in COPD supernatant-induced small airway dysfunction. This opens up the possibility of additional targets, that may be identified through, firstly, broad-spectrum inhibitors of particular protease classes (e.g. serine proteases), and then more specific inhibitors.
- Increase the n-values for human PCLS samples, in order to validate (or otherwise) the observations in Chapter 7.
- Investigate the effects of bronchodilators on human PCLS, to determine whether there are differences in responses between subject groups. In addition, use immunohistochemistry to investigate the numbers of muscarinic receptors on airway smooth muscle, and inflammatory cell infiltration into the airways, to see if either of these correlate with *ex vivo* airway function. Also, investigate the release of mediators in PCLS supernatant in response to different stimuli, e.g. LPS or H_2O_2, and determine if there are any differences between subject groups.
- Finally, directly investigate the effects of sputum or BALF from COPD patients and controls on rat small airway function. These fluids should contain a variety of inflammatory cells and mediators that are upregulated in COPD, and therefore, would provide a more accurate microenvironment in which to incubate PCLS and examine small airway dysfunction in COPD.

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