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The role of the SERPINs α_1-antitrypsin and α_1-antichymotrypsin in lung homeostasis

By

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Abstract

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The lung is under daily attack by a variety of external stimuli. To combat this, a number of proteinases have evolved to degrade this foreign material and aid in lung host defence. These proteinases can also degrade host tissue, and are therefore under strict regulation by a number of antiproteinases. Possibly the most important of these antiproteinases are the liver derived serine proteinase inhibitors (SERPINs), of which α_1-antitrysin (α_1-AT) and α_1-antichymotrypsin (α_1-ACT) are key members. The importance of these antiproteinases has been highlighted in a number of disease states which results in an insufficiency of inhibitor. In these cases, lung tissue damage is prevalent, and it has been suggested that the lung damage associated with diseases such as emphysema and asthma can be linked to a decrease in lung serpin activity. Both α_1-AT and α_1-ACT have been shown to be produced locally by lung epithelial cells, and there is still some discussion as to the importance that epithelial derived serpins have in lung host defence. Broncho-alveolarlavage (BAL) has been used to examine serpin levels from asthmatics and controls that have been subjected to ozone inhalation. The effect of allergen challenge on asthmatics has also been examined. A lung fragments model and cell culture techniques have been used to examine the possible role of locally produced serpins in the lung.

It has been shown that there are differences in serpin levels in BAL between asthmatics and non-asthmatics at rest. Asthmatics have significantly higher levels of α_1-AT but have reduced levels of α_1-ACT. However, this imbalance in serpin levels did not affect total inhibitory activity and there was no difference between total inhibitory activity in asthmatics and normal BAL. Ozone inhalation results in a significant decrease in total inhibitory activity in the asthmatic lung which is mirrored by a decrease in total and active α_1-AT. Ozone had no effect on serpin levels or total inhibitory activity in control subjects.

Allergen challenge results in significant increases in total inhibitory activity as well as increases in α_1-AT and α_1-ACT levels in BAL. It has been shown by examining the α_1-AT/albumin ratio in BAL that the increase in α_1-AT following allergen challenge may be partially derived from local synthesis. It has also been shown that the steroid fluticasone propionate inhibits this local production following allergen challenge.

We have used a lung fragment and epithelial cell model to explore local serpin production further. Fluticasone propionate had no effect on serpin production in either model, but the serine proteinase Porcine Pancreatic Elastase significantly increased levels of both α_1-AT and α_1-ACT in both models. It has been shown that the effect of this proteinase is dependent on protein activity rather than protein structure by using PMSF to inhibit proteolytic activity. The inflammatory mediator Oncostatin M (OSM) also increased α_1-AT and α_1-ACT levels in the epithelial cell model.

In summary, it has been shown indirectly that asthmatics may have a decreased anti-oxidant shield in their lung, evident by decreased levels of active inhibitor following ozone inhalation. This may explain the lung damage associated with asthmatics living in highly polluted areas. Allergen challenge leads to a series of inflammatory changes that results in elevated levels of antiproteinases in the lung. A significant proportion of this may be produced locally in the lung. Finally, lung fragments and lung epithelial cells have the ability to produce significant amounts of both α_1-AT and α_1-AT and these inhibitors can be regulated by the proteinase elastase. This may provide a feedback loop, which is used in order to control proteolytic activity in the lung.

List of Abbreviations

α_1-AT	α_1-antitrypsin
α_1-ACT	α_1-antichtmotrypsin
α_2-M	α_2-macroglobulin
α-T	α-tocopherol
AH2	Ascorbate
ARDS	Adult Respiratory Distress Syndrome
AU	Arbitrary Unit
BAL	Bronchoalveolar-Lavage
BCA	Bicinchoninic acid
CF	Cystic Fibrosis
Der p1	Dermatophagoides pteronyssinus
EGF	Epithelial Growth Factor
ELISA	Enzyme Linked Immuno Sorbent Assay
ELF	Epithelial Lining Fluid
FP	Fluticasone Propionate
GSH	Glutathione
HCl	Hydrochloric acid
HNE	Human Neutrophil Elastase
HRP	Horse Radish Peroxide
HSA	Human Serum Albumin
IgE	Immunoglobulin E
mRNA	messenger ribonucleic acid

MMP	Matrix Metalloproteinase
NFκB	Nucleotide Factor κB
NSa-NIT	N-Succinyl-ala-ala-pro-phe p-Nitroanilide
OSM	Oncostatin M
PAF	Platelet Activating Factor
PAR	Proteinase Activated Receptor
PBS	Phosphate Buffered Saline
PPB	Parts per Billion
PPE	Porcine Pancreatic Elastase
PMSF	Phenyl Methyl Sulfonyl Fluoride
SERPIN	Serine Proteinase Inhibitor
SDS	Sodium Dodecyl Sulfate
SLPI	Secretory Leukocyte Proteinase Inhibitor
TIMP	Tissue Inhibitor of Metalloproteinase
TGFβ	Transforming Growth Factor β
TMB	Tetra-methyl-benzene
UA	Urate
USG	Ultraserose G

Chapter One

Introduction

1.1 Proteinase / antiproteinase balance in the lung

The respiratory surface is constantly exposed to a variety of environmental insults. For example pollen from a variety of flora as well as fungal spores, animal hair and even dust mites (Barnes, 1987) have been shown to induce an allergic response in the lung. Similarly, inflammatory episodes can be triggered by a number of non-antigenic stimuli, including air pollution and cigarette smoke (Anto et al, 1989). Inflammation leads to the influx of migratory cells, which can secrete a number of proteinases that are critical in combating these environmental attacks.

Once these proteinases have successfully degraded and cleared the foreign material from the airways, this natural defensive system must be switched off. Failure to do so results in the progressive digestion of the lung by the body's' own enzymes. A wide variety of 'anti-proteinases' have evolved that act as a switch to turn off this proteolytic activity. The importance of these anti-proteinases is highlighted in a number of disease states, some of which actually involve a genetic deficiency in these anti-proteinases. However, the lung damage associated with diseases such as asthma and emphysema is believed to be as a result of a general imbalance in the proteinase to antiproteinase ratio. This imbalance can occur in a number ways

a) The total anti-proteinase activity in the lung may be significantly impaired, as in the case of a genetic deficiency.
b) The total proteinase activity may be significantly increased, such is the case after an inflammatory response.
c) Total anti-proteinase levels may not be significantly reduced, but the majority of these inhibitors may be present in an inactive form.

Before I go into more detail concerning the lung disease associated with a distortion of the delicate proteinase/anti-proteinase equilibrium, it is important to introduce some of the proteinases and anti-proteinases that are the subject of this thesis.

1.2 Lung Proteinases

There are a number of proteinases that are active in the lung, and these include human neutrophil elastase (HNE), proteinase-3, collagenase and the matrix metalloproteases (MMPs). Possibly the most potentially damaging of these is human neutrophil elastase (HNE).

1.2.1 Human Neutrophil Elastase (HNE)

The serine proteinase HNE exists as at least four distinct isoenzymes, which range in molecular weight from 24 to 30 kDa. It is a strongly basic glycoprotein, produced by polymorphonuclear leukocytes and released from azurophilic granules. The primary role of intracellular HNE is the degradation of foreign proteins ingested by leukocytes during phagocytosis. Extracellular HNE will degrade elastin, an integral component of lung tissue, blood vessels and other organs (Bernstein et al 1994).

HNE is a member of the extended chymotrypsin family of serine proteinases, which includes α-chymotrypsin and trypsin, of which each enzyme is capable of degrading a wide variety of proteins (Tetley, 1993). HNE is potentially one of the most destructive enzymes, as its target elastin cannot be re-synthesised in large quantities (Bernstein et al, 1994; Vignola et al, 1998). HNE is, therefore, under strict regulation by a number of anti-proteinases, both in plasma and the tissues. The levels of elastase in the lung are usually minimal and only elevated at times of lung aggravation. Neutrophils, not normally present in the lung can migrate to a site of damage and degranulate releasing elastase. Elastase may be found on the cell surface of neutrophils, and this cell surface expression can be drastically up-regulated by pro-inflammatory mediators (Owen et al, 1995). Regulatory mechanisms ensure that this activity tends to be very localized. Other homeostatic activities of HNE include stimulation of mucus secretion and degradation of fibronectin, laminin, collagen and proteoglycans. However, one of the more relevant activities includes the stimulation of platelet activating factor (PAF) from both neutrophils and endothelial cells. This inflammatory mediator has a direct role in the recruitment and activation of inflammatory cells, such as the eosinophil, but also causes bronchoconstriction (Gaillard et al, 1992).

1.2.2 Matrix Metalloproteinases

More than twenty members of the matrix metalloproteinase (MMP) family have been identified so far. All are produced and secreted in a latent, proenzyme form and require Zn^{2+} and Ca^{2+} ions for activity. This group of proteinases plays an important role in cell migration, inflammation, angiogenesis, wound healing, and, of course, tissue remodelling. Collectively, the MMPs can cleave most, if not all, the protein constituents of the extracellular matrix, including proteoglycans, laminin, fibronectin and elastin (Tetley, 1993). The purpose of these enzymes is to facilitate cell migration and dispersal. Again, MMP activity is usually under strict control by a number of antiproteinases, including the tissue inhibitors of metalloproteinases (TIMPs) and, perhaps more importantly during inflammation, the plasma protein α_2-macroglobulin which enters the lung by capillary leakage during oedema (Connor and Fitzgerald, 1994).

MMPs are produced by a wide variety of cells in the lung, including fibroblasts, neutrophils, macrophages, endothelial cells, and extrapulmonary sources include osteoblasts, keratinocytes and hepatocytes (Springman et al 1990).

1.2.3 Proteinase-3 and Cathepsin G

Like HNE, these enzymes are produced in the neutrophil, stored in the azurophilic granules, and released at the same time as HNE. Both enzymes are known to have a broad specificity and Cathepsin G is present at minimal levels on the surface of neutrophils. In comparison to HNE and the MMPs, little is known about the substrate specificities of proteinase-3 and cathepsin G.

1.3 Antiproteinases

Over 100 natural proteinase inhibitors have been identified, and antiproteinases may be grouped on the basis of their origin, reaction mechanism or structural similarity (Travis and Salvesen, 1983, Laskowski and Kato, 1980). Probably the most important group of lung antiproteinases are the Serine Proteinase Inhibitors (SERPINs).

1.3.1 – The Serpins

The term 'serpin' (SERine Proteinase INhibitor) was first used by Carrell and Travis in 1985. They are now one of the most well-known and well-understood family of proteinase inhibitors being functionally related by forming stable non-disassociating inactive complexes with their target proteinases (Travis and Salvesen, 1983). Interaction with the active site of their target proteinase is accomplished by the specific recognition of the P_1-P_1' residues at the reactive site of the inhibitor (Schechter and Berger, 1967). All serpins have a high sequence homology in a region of 350 amino acids that are involved in shaping the serpin structure (Gettins et al, 1993) and the structural homologies between two of the most important inhibitors in the lung, α_1-antitrypsin (Carrell et al, 1982) and α_1-antichymotrypsin (Chandra et al, 1983) have been known for over 15 years. As with all serpins they share a highly ordered tertiary structure, which is folded into three β sheets and nine α helixes (Huber and Carrell, 1989, Wright, 1996). These serpins interact with their target proteinase at a reactive site located within a loop structure 30-40 amino acids from the carboxyl terminus (Potempa et al, 1994). This is known as the reactive center loop, and is exposed on the surface of the inhibitor making it very susceptible to proteolysis by non-target proteinases or to oxidation at susceptible residues, either of which renders the molecule non-functional as an inhibitor (Johnson and Travis, 1977). The importance of the serpins as a family of inhibitors has been made clear by a variety of deficiency states caused by mutations in serpin genes. Consequences of dysfunctional serpins include thrombosis, emphysema, liver disease and angiodema (Stein and Carell, 1995). I shall now look more closely at one of the most important lung antiproteineses - the serpin, α_1-antitrypsin.

1.3.1.1 α_1-antitrypsin

This 52 kDa glycoprotein was originally found to inhibit trypsin and chymotrypsin, and so was given the name α_1-antitrypsin (α_1-AT), but its main physiological target is actually neutrophil elastase. It is primarily a plasma protein and is present in high concentrations (±2mg/ml) in the blood from where it readily traverses the extracellular matrix and enters the lung (Carrell et al 1982).

In fact, the main source of α_1-AT in the lung is thought to be diffusion from the plasma, but there are potentially other sources (see figure 1.2). Recent studies suggest that lung epithelial cells can synthesize and secrete α_1-AT (Cichy et al 1997). This is potentially very interesting as the exact contribution of locally synthesized α_1-AT to the total lung pool is, as yet, unknown. α_1-AT expression in these epithelial cells appears to be under the control of specific inflammatory mediators, of which Oncostatin M (OSM) is a prime example (Sallenave et al, 1997). This topic will be discussed in further detail later.

The dominant structural feature of α_1-AT is the 5 stranded A sheet from which the reactive centre loop arises (Fig 1.2). The P1 residue acts as bait for the target substrate and once bound the molecule undergoes a conformational change that locks the target molecule in place. This change is caused by the cleavage of the scissile bond between methionine 358 (P_1) and serine 359(P_1') (Potempa et al, 1994; Elliott et al, 1996; Wilczynska et al, 1997). Unfortunately, the methionine at position 358 is very sensitive to oxidative inactivation by free radicals (Gaillard et al, 1992). A number of proteinases also have the ability to inactivate α_1-AT by cleaving the molecule at non-specific sites in the reactive center loop and there is evidence that a cleaved form of α_1-AT is a potent neutrophil chemoattractant (Banda and Senior, 1988). This may amplify the inflammatory response by stimulating neutrophil migration.

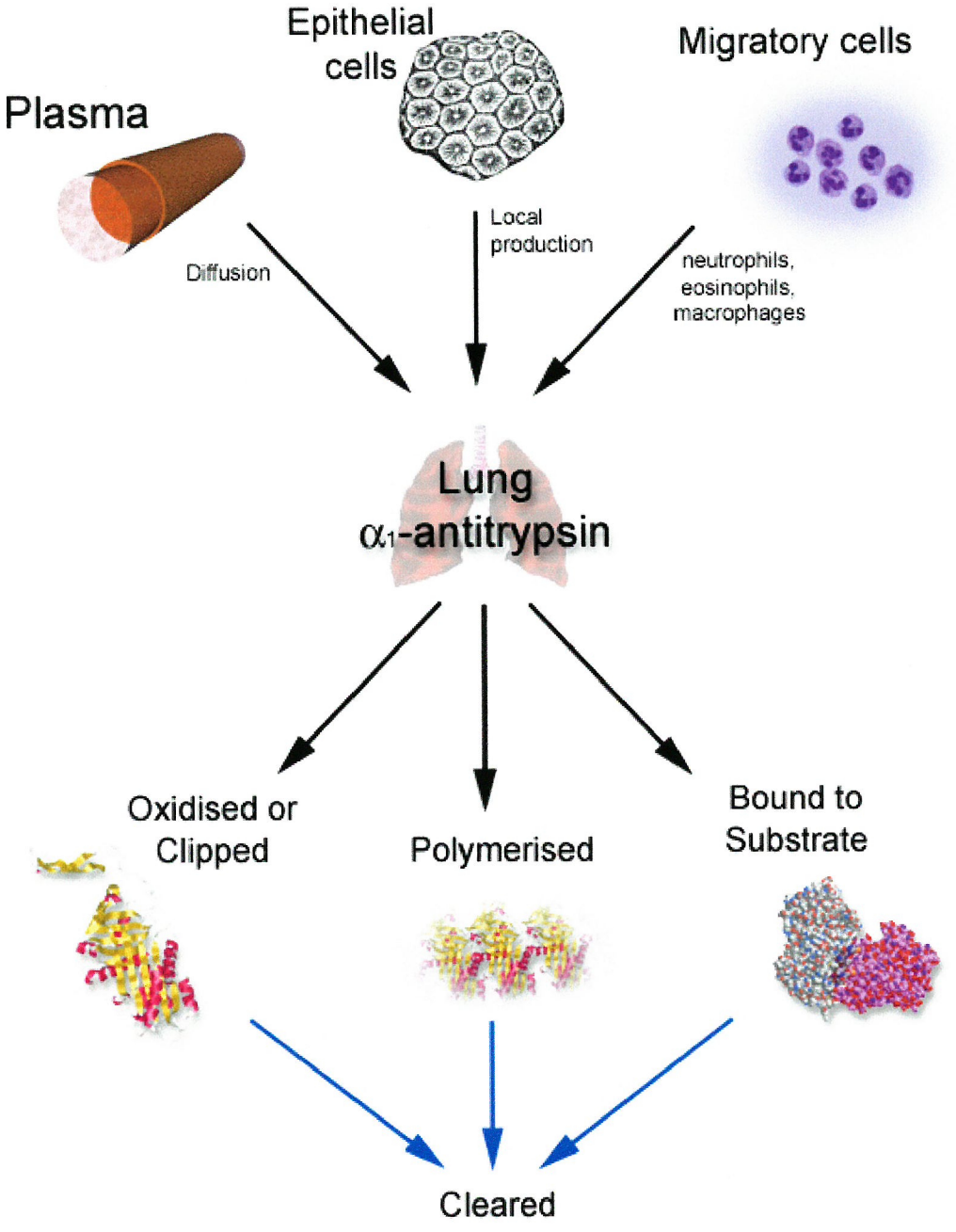

Figure 1.1 - Sources and degradation of α_1-AT. The main source of α_1-AT is believed to be the plasma, from where it can freely diffuse into the lung. Recently other sources have been identified, and these include infiltrating migratory cells and also the local production in the lung by epithelial cells. α_1-AT can be inactivated in a variety of means. This includes oxidation of the active site methionine, 'clipping' of the molecule by proteinases or upon binding to its target substrate.

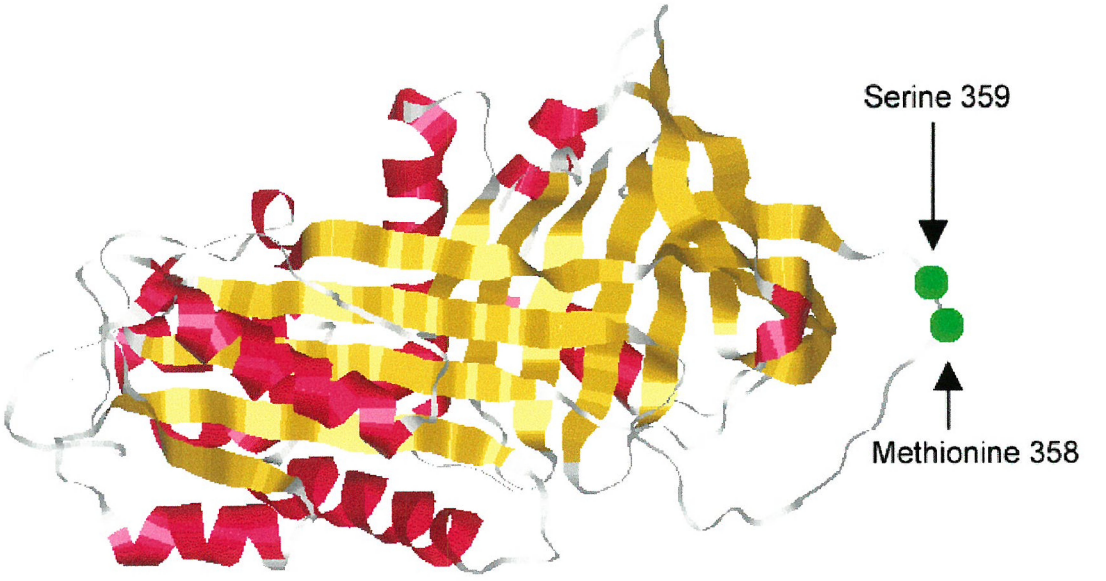

Figure 1.2 Structure of α_1-AT. α helices are shown in red, β sheets in yellow. The reactive centre loop comprises of the MET 358 and the SER 359 shown in green. Taken from the software RASMOL.

Finally, α_1-AT can polymerise where the reactive center loop of one molecule inserts into the β pleated sheet of another (Elliot et al, 1998). This is known as loop sheet polymerisation or loop sheet linkage, and is the basis of α_1-AT deficiency (Stein and Carrell, 1995). In this disease the synthesis of α_1-AT by hepatocytes is not impaired, but the secretion into the blood is severely compromised (Hall et al, 1976). The decrease in circulating α_1-AT levels caused by blockage of the terminal secretory pathway from the hepatocyte has two major consequences. Firstly, the α_1-AT polymers in the liver may accumulate as large intracellular inclusions that can cause severe liver damage. Secondly, the decreased levels of plasma α_1-AT lead to a disruption of normal homeostasis of the proteinase/ antiproteinase balance in the lung. Unchecked, the loss of protective α_1-AT activity leads to escalating elastase activity that increases the likelihood of tissue breakdown, and hence lung disease (Carrell et al, 1996).

1.3.1.2 α_1-antichymotrypsin

This member of the serpin family was first isolated and characterized without knowledge of its function but was subsequently shown to inhibit chymotrypsin along with α_1-AT and α_2-

macroglobulin. However the main physiological target of α_1-antichymotrypsin (α_1-ACT) is the neutrophil proteinase, Cathepsin G (Heimburger and Haupt, 1965). In competition experiments the association rate of α_1-ACT with cathepsin G was 100 times greater than that of α_1-AT (Beatty et al 1980). The main source of α_1-ACT is believed to be the liver, from where it is secreted into the plasma. In fact, plasma concentrations of α_1-ACT are approximately 0.5mg/ml, which makes it the second most predominant serine antiproteinase in human plasma (Heimburger et al, 1971). However, whether α_1-ACT has an important physiological role has yet to be established. It has been shown in bronchitics that up to 85% of α_1-ACT from lung is inactive (Berman et al 1986). This large percentage of inactive α_1-ACT may actually be beneficial to the lung as α_1-ACT that is complexed with chymotrypsin inhibits the secretion of neutrophil superoxide. This powerful oxidative agent can cause significant lung damage and host tissue destruction (Schuster et al, 1992) and the inactive α_1-ACT may therefore be playing an important role in lung tissue defence against free radicals.

α_1-ACT is an *acute phase* protein and levels are high in most inflammatory and malignant conditions (Kelly et al 1978, Matsuzaki et al 1981). α_1-ACT actually shows the most immediate response of all acute phase proteins, doubling in eight hours after insult (Travis and Salvesen, 1983). Initially there was some confusion as to the exact molecular weight of α_1-ACT and this was believed to be due to different methods of purification. Sizes ranging from 55-68kDa were reported, but the accepted molecular weight is now 68kDa (Travis et al, 1978; Katsuna 1980; Laine 1981).

Evidence for the local production of α_1-ACT came into focus in the early 1980s, where it was observed that levels of lung α_1-ACT couldn't be attributed to plasma influx alone (Stockley and Burnet, 1980). Recent studies have shown that lung epithelial cells have the ability to secrete substantial amounts of α_1-ACT after stimulation with OSM (Cichy et al, 1995; Baumann and Gauldie, 1994). There is now evidence that human cortical astrocytes have a specific receptor for OSM that once bound to OSM triggers α_1-ACT transcription (Kordula et al 1998).

1.3.2 Other proteinase inhibitors

1.3.2.1 α_2-macroglobulin

α_2-macroglobulin (α_2-M) is a non-specific endopeptidase inhibitor and so is active against serine, aspartate, cysteine and metalloproteinases. α_2-macroglobulin is a very effective and fast acting plasma inhibitor of HNE (Tetley, 1993; Bernstein et al, 1994). Like α_1-AT, it is produced by hepatocytes, but its method of proteinase inhibition is via a unique entrapment system (Barrett and Starkley, 1973). Low molecular weight enzymes that bind to α_2-M still retain proteolytic activity, compared to the almost total inhibition of large sized proteins such as elastase. α_2-M (725 kDa) is a glycoprotein, significantly larger than both α_1-AT and α_1-ACT, which makes diffusion from the plasma to the lung less efficient. α_2-M reacts with such a variety of proteinases and its rapid clearance from the bloodstream once complexed suggests that α_2-M should be considered part of the general immune defence system (Debanne et al, 1975; Imber and Pizzo, 1981).

1.3.2.2 Secretory leukocyte protease inhibitor

Secretory leukocyte protease inhibitor (SLPI) is a 12 kDa nonglycosylated protein produced by lung epithelial cells (Kramps et al, 1988) and there are increased numbers of SLPI-containing cells in human bronchioles in small airway diseases (Bernstein et al, 1994).

HNE has the ability to increase SLPI transcript levels in airway epithelial cells. Other neutrophil products such as cathepsin G, myeloperoxidase and lysozyme have no effect on SLPI transcription suggesting that SLPI is specifically regulated by HNE, its target proteinase (Marchand et al, 1997). SLPI also has the ability to bind heparin, a highly sulphated glycosaminoglycan found in the ECM. It has been shown that upon binding, SLPI becomes a more efficient inhibitor of both HNE and mast cell chymase, a chymotrypsin-like proteinase stored in mast cell granules. This suggests an important role for SLPI as a proteinase inhibitor in the ECM (Walter et al, 1996).

1.3.3.3 Tissue Inhibitors of Metalloproteinases.

The first Tissue Inhibitor of Metalloproteinase (TIMP) was cloned in 1985 (Docherty et al, 1985), and since then a further three have been identified. Unlike other proteinase inhibitors such as α_2-M, which is a fluid phase inhibitor, the TIMPs are believed to be the main inhibitors in tissue. Their main targets are the MMPs, but these proteinase inhibitors have many other biological activities including mitogenic like activity (Hayakawa et al, 1994), cell growth promoting activity (Hayakawa et al, 1992) and erythroid potentiating activity (Stetler-Stevenson et al, 1992). These physiological actions are independent of MMP activity, and because they are multifunctional inhibitors the mechanism of action of these cellular effects are still poorly understood (Hayakawa et al, 1994; Chesler et al 1995, Baker et al, 1999).

1.3.3.4 Elafin

Elafin is a 57 amino acid peptide (6 kDa) that was first isolated from the skin of patients with psoriasis and subsequently from bronchial secretions. Elafin was shown to be a specific inhibitor of HNE and proteinase-3, both of which digest elastin. It has no effect on any of the other serine proteinases such as trypsin, chymotrypsin or cathespin G (Bernstein et al, 1994). The structure of elafin is particularly interesting as it has high homology to SLPI. The structure consists of a central β-hairpin, accompanied by two external segments linked by the proteinase binding loop. Like many proteinase inhibitors, elafin has a high content of cysteine residues. A cluster of 3 disulphide bridges connects the external segments to the central β-sheet and a single fourth disulphide bridge links the binding sheet to the central β-turn. It is this spatial distribution of disulphide bridges that is similar to that of SLPI. It has been found that segments 22 to 27 are the proteinase binding loop of elafin, with a scissile peptide between Ala24 and Met25 (Francart et al, 1997).

All of these antiproteinases are crucial for the maintenance of a healthy lung. It has been suggested that in many disease states it is an imbalance or a decrease in antiproteinase levels that gives rise to, or is a major contributing factor to the disease.

1.4 SERPIN sources – the argument for local production.

As mentioned earlier, there are three potential sources for serpin antiproteinases found in the lung. The most important of these is believed to be the plasma where α_1-AT and α_1-ACT are present in relatively high concentrations (2mg/ml and 0.5mg/ml, respectively). However, recent studies have demonstrated that both α_1-AT and α_1-ACT can be synthesised and secreted by lung epithelial cells and that this secretion can be upregulated by a cytokine known as oncostatin M (OSM). OSM is produced by activated T cells, macrophages and neutrophils (Grenier et al, 1999). It has several interesting properties that influence the course of the inflammatory response (Zarling et al, 1986) and although identified as a member of the IL-6 superfamily, its *in vivo* properties and physiological functions are ill defined (Wallace et al, 1999). However, it has numerous *in vitro* properties including the regulation of cholesterol metabolism, differentiation of megakaryotes and more interestingly, an inhibitor of tumor growth (Lorenz et al, 1996; Bruce et al 1993). OSM acts on a variety of lung epithelial cells to significantly up regulate production of the serpins α_1-AT and α_1-ACT. This includes the stimulation of α_1-AT and α_1-ACT expression in the HTB55 lung cell line (Cichy et al 1997, Cichy et al 1995), the increased expression of α_1-AT in the A549 lung cell line (Sallenave et al, 1997) and an increased expression of α_1-AT and α_1-ACT in the HTB58 lung cell line (Cichy et al 1998). It is not too surprising that epithelial cells could be the main target of OSM, as the expression of OSM receptors on these cells are up to 20 times the number compared to other cell types (Linsley et al, 1989). There is some evidence to suggest that OSM is a pro-inflammatory mediator (Modur et al, 1997), but the fact that there is such a significant response in proteinase inhibitor expression after exposure to OSM suggests that this may be misleading. In fact I believe that OSM is a potent stimulator of the lung defensive system, and these recent studies have clearly shown that OSM is one of the most important stimulators of α_1-AT and α_1-ACT production by lung epithelial cells.

1.5 Lung diseases

The lung is a very delicate environment, and as mentioned earlier, small changes in the homeostasis of proteinases and antiproteinases can result in large pathological changes to the

lung. Highlighted below are a few examples of lung disease in which lung damage results from an imbalance between proteinases and inhibitors.

1.5.1 Asthma

Asthma is one of the most well known lung diseases and the symptoms; shortness of breath, difficulty of breathing, cough and a sense of constriction of the chest are well known to the 10% of the population that suffer from asthma (Pride, 1992). Despite major improvements in medication, asthma is increasing both in prevalence and severity (Holgate and Busse, 1998).

Asthma is characterised by airway obstruction (that may be fully reversible), airway inflammation (due to infiltration of inflammatory cells) and increased airway responsiveness to a variety of stimuli (Costello 1991). It can be broadly classified into two types, intrinsic and extrinsic. Intrinsic asthma, also known as non-atopic asthma, does not involve immunoglobulin-E (IgE) mediated mechanisms. Extrinsic asthma is more common and an attack is triggered by an external agent or allergen, to which the individual has become sensitised. In short, it can be described as an abnormal response to normal environmental allergens (Sheffer, 1991). A good marker for an *atopic* individual are elevated levels of IgE, especially in young adults (Burrows et al, 1989). Both atopic and non-atopic asthma have well defined morphological and pathological characteristics; thickening of the basement membrane, mucosal disruption and infiltration of a particular white blood cell, the eosinophil (Djukanovic et al, 1990). However, the rest of this report will focus on atopic asthma.

Asthma ranges in severity from mild episodic attacks to frequent life threatening exacerbations that require periods in hospital. Asthma also tends to run in families suggesting the existence of an underlying genetic predisposition to the disease (Townley et al 1986, Levitt et al, 1990). However, at the heart of an asthmatic attack is the environmental allergen that once inhaled by atopic individuals goes on to trigger an attack.

1.5.1.1 Current asthma therapy

Asthmatics now have a wide variety of medication to choose from, most of which can be used in parallel which each other. Broadly, the medication fits into two categories, the corticosteroids

(anti-inflammatory) and the β-agonists (bronchodilators). The inhaled β-agonists, of which salbutamol is a classic example, act through β-adrenoreceptors which belong to a family of G-protein linked receptors (Tattersfield, 1987). The exact mechanism of action of the β-agonists is unimportant, but the main consequence of this treatment is the bronchodilation of the airway smooth muscle allowing ease of breathing (O'Shaughnessy et al, 1991). They are fast acting and are simply a means to ease an asthmatic attack. However, this class of drugs will not be discussed further in this thesis. The inhaled corticosteroids, of which fluticasone propionate is a classic example, act in an entirely different way. Their mechanism of action is different as they are thought to diffuse across the cell membrane and bind to specific corticosteroid receptors within the cell cytoplasm. They have the potential to modulate many of the components of the inflammatory response that occurs within the airways of asthma. It is therefore, primarily, an anti-inflammatory agent, and must be taken daily. Corticosteroids have a number of positive effects on the lung including the inhibition of circulating neutrophils (Lomas et al, 1991) and an increase in local SLPI production (Stockley et al, 1986). The most beneficial effect of steroid though is probably a decrease in the general inflammatory response, which includes an inhibition of histamine bronchial reactivity and a decrease in the permeability of the blood-airway lumen barrier (Nocker et al, 1999).

It is clear then, that the corticosteroids are anti-inflammatory, but little is known about the effect of steroids on serpin levels in the airways following steroid treatment.

1.5.2 Emphysema

Emphysema is a slowly progressive disease characterised by enlargement of the respiratory airspaces of the lungs and the destruction of their walls. Important risk factors for the development of this disease are smoking and increased age (Tetley, 1993). There are a number of different forms of emphysema in humans all with a slightly different pathophysiology. However, in all types of emphysema it appears the main cause is an increase in free elastase levels in the lung. There are several hypotheses concerning the development of emphysema, and a number of animal models have been used to investigate the relationship between this increased elastase activity and the onset of the disease. It has also been suggested that oxidative stress, hypoxia

and even starvation may contribute to the development of emphysema (Snider et al 1994). What is clear, however, is that emphysema is caused primarily by the degradation of elastin by uninhibited elastase activity.

1.5.3 Cystic Fibrosis

Cystic Fibrosis (CF) is a lethal, autosomal, recessive hereditary disorder caused by mutations of the cystic fibrosis transmembrane conductance gene. It is a disease affecting chloride channels and is characterised clinically by chronic purulent bronchitis culminating in pulmonary insufficiency, exocrine pancreatic insufficiency, sterility in males and abnormally elevated concentrations of sodium chloride in sweat (Hunt and Geddes, 1985). However, the most common cause of death in patients with CF are the respiratory symptoms, which include a build up of thick mucus in the lung, making the clearance of bacteria and foreign bodies difficult. It is known that people with CF have an intense chronic neutrophil-dominated epithelial inflammation, and consequently increased lung neutrophil elastase levels. The increase in neutrophil numbers is due to the constant irritation of the lung surface by allergens that have not been cleared effectively and consequently induce an inflammatory response (Birrer et al, 1994). It is this upset of the proteinase/antiproteinase balance that occurs at a very early age that is thought to be responsible for the subsequent lung damage and pulmonary insufficiency associated with CF.

1.5.4 Adult respiratory distress syndrome (ARDS)

ARDS is an acute condition caused by a massive influx of neutrophils in the lung. This condition develops after a 'latent' period that can be hours or days subsequent to trauma which may be caused by a variety of events including sepsis and pancreatitis. The inflammatory mediators that are generated as a result of the injury act on inflammatory cells. These cells accumulate abnormally in the pulmonary microvasculature where they secrete a number of destructive proteinases including human neutrophil elastase (Seamas et al, 1995). This may lead to an increase in total elastase in the lung, which causes extensive tissue damage due to its broad proteolytic specificity. There does not appear to be any clear differences between a person who develops ARDS after trauma, and those who do not. However, a common theme of both ARDS

and CF appears to be increased numbers of neutrophils in the lung, and emphysema is primarily caused by increased elastase activity. The neutophil, therefore, appears to be one of the main contributors to the lung damage associated with these diseases.

1.6 Proteinase and Proteinase inhibitors in lung disease

In order to fully understand the complex interactions between proteinases and antiproteinases, it is first important to know something about the *levels* of various proteinases and their respective inhibitors in the lung.

1.6.1 Methods of examination

Arguably, the most useful tool that a clinician has to examine the levels of various mediators in the lung is for the patient to undergo a bronchoscopy (Reynolds and Newball, 1974). This involves placing a fibreoptic bronchoscope into the lungs of the patient before distilling a volume of saline solution through the bronchoscope and into the lungs. This solution is then aspirated, spun down, and a cell count can be obtained. The liquid that remains is known as bronchoalveolar lavage or BAL, and is a very useful way for examining levels of mediators in the lung. Another technique is to examine sputum mediator levels for changes in proteinase / antiproteinase activity. Finally, levels of SERPINs in particular, may be examined in plasma, as this is the body's largest pool of these inhibitors.

1.6.2 Levels of mediators in asthma and lung disease

One of the most interesting observations made in asthmatic patients, is that there is a significant increase in elastase from sputum, which also correlates with the number of neutrophils found (Vignola et al, 1998). This is perhaps the biggest clue as to why there is so much lung degradation in asthma. If total levels of elastase are increased due to excess inflammation then levels of proteinase inhibitors are insufficient to counter the effects of this potent neutrophil enzyme. It appears then, that the neutrophil is one of the contributing factors to the lung damage associated with asthma. At face value, this evidence from sputum would suggest that it is the increase in proteinase activity that leads to the resulting lung damage. However, in asthmatic

BAL, there is also a significant increase in the anti-elastase inhibitor, α_1-AT (Gaillard et al, 1992; Vignola et al, 1998). This should complement the increased elastase levels and result in proteinase/inhibitor homeostasis. However, asthmatics not only have an increase in total elastase content, but also in the total active free elastase (Gaillard et al, 1992). Perhaps the increase in α_1-AT may not be sufficient to counteract the increase in elastase levels. In fact the majority of this α_1-AT may be inactive (Sibille et al, 1988). In fact, 50% of the α_1-AT in BAL from bronchitic patients is in an inactive form (Morrison et al, 1987).

Neutrophils, the main candidate cells for inflicting lung damage, are elevated in BAL from α_1-AT deficient patients, suggesting a greater potential elastase burden (Elliot et al, 1998). An increase in neutrophil count, combined with a decrease in total α_1-AT levels due to a decrease in secretion by the liver could lead to unrestrained elastase activity in the lung (Morrison at al, 1987).

1.6.3 Plasma protein influx and capillary permeability in asthma.

Along with increased elastase activity, there is evidence that plasma influx and increased capillary permeability are other characteristic features of asthma. This is relevant in view of the fact that three important proteinase inhibitors originate in plasma, α_1-AT, α_1-ACT and α_2-M. Firstly though, we must look at the mediators that cause the increase in capillary leakage. Are the levels of these mediators significantly higher in asthmatic BAL? Histamine is probably the most potent inducer of plasma influx and levels of this mediator are found in increased levels in asthmatics (Barnes et al, 1982; Casale et al, 1987). PAF, another inflammatory mediator that increases capillary permeability is found in elevated levels in the asthmatic lung (Nakamura et al, 1987) and both PAF and histamine probably act via receptors on the endothelial cell surfaces to induce their effects (Evans et al, 1987).

One of the best indicators of plasma influx is the appearance of albumin in BAL. Albumin is a plasma protein and is not secreted locally by epithelial cells or by any migrating cell types found in the lung (Ryley and Brogan, 1968). If albumin is present in plasma, the only explanation

is plasma protein influx into the lung. Not surprisingly, in asthmatics, this plasma protein is found in elevated levels in BAL (Brogan at al, 1975; Van Vyve et al 1995). What about other protein levels though?

α_2–macroglobulin is also found in the plasma and is not secreted locally by any of the cell types found in the lung. Levels of this inhibitor are significantly increased in asthmatic BAL, another good indicator of plasma influx (Van Vyve et al, 1995, Gaillard et al 1993). However, although it is evident that there is a plasma protein influx into the lung that would account for the increased levels of α_1-AT, α_1-ACT and α_2-MG, this rise in total proteinase inhibitors is still not sufficient to counteract the increased levels of elastase.

1.7 Effect of oxidative stress on lung pathophysiology

Oxidative stress in the lung can come from a variety of forms. Degranulating neutrophils release harmful peroxidases and free radicals directly on the lung epithelia, but other sources of oxidative stress includes external pollution, such as ozone, nitric oxide and sulphur dioxide.

Ozone is one of the most potentially harmful substances to the lung environment. However, it is important to realise that ozone is not actually a free radical itself, and that most of the damage surrounding ozone inhalation are as a result of its powerful oxidising action (Ryrfeldt et al, 1993). This can cause three main effects in the lung.

a) The formation of free radicals.
b) The inactivation of lung anti-oxidants
c) The promotion of airway inflammation

1.7.1 Free radical formation with ozone.

Ozone can react with all hydrocarbon molecules in the lung, to cause the release of cytotoxic products, including reactive free radicals (Kelly et al, 1995). Oxygen free radicals have been known for some time to be mediators of tissue destruction and they achieve this in a number of ways. Firstly, free radicals can inactivate proteinase inhibitors in the lung, including α_1-AT (Smith

et al, 1987), and any decrease in total antiproteinase activity will result in a damaging tissue environment in the lung (Winrow et al, 1993). Secondly, radicals readily react with hyaluronic acid, which forms the central axis of proteoglycan. Following exposure proteoglycans will therefore destabilise, causing a general degradation of lung connective tissue (Greenwald and Moy 1980). Finally, free radicals can activate NFκB, which is an important transcriptional factor for many inflammatory systems (Schreck et al, 1991). However, the effects of oxygen free radical species on the production of inhibitors are still largely unknown.

1.7.2 The inactivation of lung anti-oxidants.

Ozone can inactivate the lungs natural defence against free radical attack, the anti-oxidants. These include glutathione, ascorbic acid (Vitamin C), uric acid and α-tocopherol (Vitamin E). Following ozone exposure, levels of ascorbic acid (Mudway et al, 1996, Mudway et al, 1999) and uric acid (Mudway et al, 1996) have been shown to be significantly depleted. Even more concerning is the fact that patients with asthma have low levels of ascorbic acid and α-tocopherol (Kelly et al, 1999). This may leave asthmatic subjects vulnerable to oxidative stress and the effect on their lungs could be potentially devastating. The proteinase inhibitor shield could become inactivated and total proteinase activity could escalate due to neutrophil recruitment and airway inflammation.

1.7.3 Ozone as a promoter of inflammation

It is well documented that ozone inhalation results in airway inflammation in mice (Kleeberger and Hubak, 1992), ferrets, monkeys, rats (Sterner-Kock et al, 2000) and humans (Scannell at al, 1996). Inflammation brings about an infiltration of leukocytes such as the neutrophil and these provide another source of free radicals that will enhance lung damage and increase airway inflammation (Mudway et al, 1999). Ozone also increases vascular permeability, evident from increased plasma proteins in the lung following exposure (Bassett et al, 2000). Ozone is also associated with a decrease in lung function (Frischer et al, 1999), again highlighting its damaging effects. In fact severe asthmatics appear to be far more prone to airway inflammation following ozone exposure than mild asthmatics (Vagaggini et al, 1999).

1.8 Conclusion

The lung is an incredibly complex and rapidly changing environment. Proteinase levels are constantly fluctuating as they are released by activated neutrophils and other inflammatory cell types. Antiproteinase levels must therefore mirror the levels of active proteinases, but there are a variety of sources from which the inhibitors may be derived. An increase in plasma influx will result in increased levels of both α_1-AT and α_1-ACT, which may achieve the correct homeostasis. Neutrophils and other migrating cell types may also secrete α_1-AT, although levels produced by these cells must surely not be sufficient, or there would not be the lung damage observed in diseases associated with chronic neutrophilia. Finally, the lung epithelia has been vastly overlooked, and only relatively recently has it been identified as a significant source of proteinase inhibitors.

The importance of the serpins in lung physiology is not in dispute. Diseases such as α_1-AT deficiency highlight the importance of these inhibitors and the role they play in lung homeostasis. What is less obvious is what contribution the plasma derived inhibitors play in day to day maintenance of the lung compared to the production of these inhibitors by lung epithelial cells. Even less clear is what variety of stimuli are responsible for the upregulation of these locally derived inhibitors. Oncostatin has been shown to cause a significant increase in the secretion of both α_1-AT and α_1-ACT in lung derived epithelial cells, but what of other mediators such as neutrophil elastase. Levels of SLPI have been shown to be upregulated by elastase exposure, but to date there has been no research investigating the effects of proteinases or oxidative stress on the levels of locally derived inhibitors either in vitro or in vivo.

1.9 Aims

The aims of this project are to examine the levels of various proteinase inhibitors in asthmatic patients compared to control subjects. I also aim to investigate changes in these inhibitors under varying environmental conditions. To achieve this I will examine the effect of ozone on mediator levels in BAL from patients with asthma. I will also examine what effect

allergen challenge has on levels of α_1-AT, α_1-ACT and other mediators in BAL from atopic individuals with asthma. I also aim to examine the local pulmonary production of α_1-AT and α_1-ACT and assess the actions of some of the inflammatory mediators and therapeutic agents, which may modify their synthesis. Two complementary experimental models will be used to achieve these aims - human lung fragments, consisting of a mixed cell population, and a more well defined primary bronchoepithelial cell culture system.

Chapter 2

Materials and Methods

2.1. Materials

HSA, chymotrypsin, N-Succinyl-ala-ala-pro-phe p-Nitroanilide (NSa-NIT, chymotrypsin substrate), RPMI 1640 culture medium, penicillin-streptomycin, gentamycin, human neutrophil elastase, hydrocortisone, insulin, porcine pancreatic elastase and N-succinyl-ala-ala-ala p-nitroanilide (elastase substrate), Protein A agarose, goat anti-human α_1-antitrypsin and general laboratory chemicals were all obtained from Sigma Chemicals Co (Poole, UK). Tris was bought from ICN. $NaHCO_3$, Na_2HPO_4, Na_2CO_3, PEG 4000 were purchased from BDH Ltd (Poole, UK). Collagen and epithelial growth factor (EGF) were purchased from Roche Diagnostics (Basel Switzerland). Trypsin EDTA, HAMS F12:DMEM media, Glutamine, pen-strep antibiotics and Ultraserose G (USG) was purchased from Gibco Life Technologies (Paisley, UK). High molecular weight rainbow standards, streptavidin horseradish peroxidase conjugate, biotinylated standards, anti-rabbit Immunglobulin conjugated to horseradish peroxidase, L-^{35}S Methionine (Specific activity > 1000Ci.mmol^{-1}) and supersignal ECL Western blotting kits were bought from Amersham (Buckinghamshire, UK). Bicinchoninic acid (BCA) assay kits were purchased from Pierce (Illinois, USA). Rabbit anti-human α_1-antitrypsin, rabbit anti-human α_1-antichymotrypsin, rabbit anti-human serum albumin (conjugated and non-conjugated to HSA) were purchased from DAKO chemicals (Buckinghamshire). Nitrocellulose membranes were obtained from Sartorius (Surrey, UK). Scintillant was obtained from LKB. Purified dust mite allergen, Der p1, from Dermatophagoides pteronyssinus was a generous gift from Professor N.Kalsheker at the University of Nottingham. Finally, α_1-antitrypsin was a kind donation from Prof David Lomas, University of Cambridge, UK.

2.2. Buffers

PBS-Tween contained 0.14M NaCl, 2.7mM KCl, 1.8mM KH_2PO_4 and 10mM Na_2HPO_4 and 0.1% Tween, pH 7.4 with HCl. Towbins transfer buffer for Western blotting contained 25mM Tris, 0.95M glycine and 20%v/v methanol and was adjusted to pH 7.0 with HCl. Separation gel buffer contained 0.375M Tris and was adjusted to pH 8.9 with HCl and was used for native (non-

reducing gels). Stacking gel buffer for native gels contained 0.47M Tris and adjusted to pH 6.9 with H_3PO_4. Anode running buffer contained 100mM Tris and was adjusted to pH 7.8 with HCl. Cathode running buffer contained 52.9mM Tris and 64.8mM Glycine and adjusted to pH 8.2 with HCl. Running buffer for SDS PAGE contained 0.325M Tris and 0.2% SDS and the pH was adjusted to 8.8 with HCl. Stacking buffer contained 0.125M Tris and 0.2% SDS and the pH was adjusted to 6.8 with HCl. Electrode buffer contained 2mM Tris, 192mM glycine and 0.1% SDS. Low Salt buffer for immunoprecipitation contained 10mM Tris/HCl pH 7.5, 1%w/v Tritaxol, 0.15M NaCl and 2mM EDTA. High salt buffer was the same as above but with 0.5M NaCl .

Coating buffer for coating the 96 well plates with antibody to α_1-antitrypsin, α_1-antichymotrypsin and albumin contained 1.5mM Na_2CO_3 and 3.5mM $NaHCO_3$, pH 9.6. Sodium phosphate buffer contained 30mM $NaHCO_3$ and 30mM Na_2HPO_4, 0.16M NaCl and 0.1%w/v PEG 4000 and adjusted to pH 7.4 with HCl. Tyrodes' buffer which was used in the preparation of human lung tissue contained 137mM NaCl, 2.68mM KCl, 0.42mM NaH_2PO_4, 1.36mM $CaCl_2.2H_2O$, 2.63mM $MgCl_2.6H_2O$ and 1%w/v glucose and the pH adjusted to 7.4 by the addition of 1g $NaHCO_3$. Lysis buffer contained 1M NaOH and 1% SDS.

2.3 Clinical studies

2.3.1 Ozone exposure chamber study

We obtained samples of BAL from a study conducted on asthmatics and controls using an ozone exposure chamber. The work was performed at the Medical Division, National Institute of Occupational Health, Umea, Sweden.

The chamber was built out of aluminum and the volume measured 14.1m^3. During the exposures ambient air was continuously drawn through the exposure chamber at a ventilation rate of 15-20m^3/hr. This represents the flow of fresh air to produce a constant circulation of 400m^3/hr through the chamber. Humidification was achieved using a silica gel humidifier, while the temperature could be precisely regulated ($\pm 0.5^{\circ}C$) by using a heat exchanger operated by warm

or cold circulating water. This was made possible by the recirculation of the air inside the chamber.

Ozone was generated using a Fischer's ozone generator (500MM; Fischer Labor and Wefahrens-Tecknik-Gmbh, Tyksland, Germany). When oxygen passes into the generator, it is converted into ozone using a high voltage field, the concentration of which can be regulated by changing the voltage applied. Concentrations of ozone in the exposure chamber were measured continuously using a Dasibi model 1109 UV photometric ozone analyser (Dasibi Environmental Corp, Glendale, CA, USE). This equipment has the ability to measure ozone concentrations between 0-2000 parts per billion (ppb) ±0.1ppb. For the duration of the two studies, ozone concentrations remained stable at 198 ±8ppb. Temperature in the chamber was maintained at 20°C and relative humidity was 50%. 15 control subjects and 11 mild asthmatics were exposed on two separate occasions, one to 200 ppb ozone and one to filtered air. Exposure lasted 2 hours with subjects performing a standard alternate 15 minute exercise and rest protocol which resulted in a mean minute ventilation of 20 litres/min/m^2 body surface area. The study was conducted in a single blind, crossover control fashion. Individuals underwent bronchoalveolar lavage (BAL) of 3 x 60mls 6 hours after the 2-hour exposure.

2.3.2 - Allergen challenge study

This study protocol was approved by the institutional review board of the University of Maryland. All patients (n=20) who participated in this study were non-smokers, met the American Thoracic Society criteria for the clinical diagnosis of asthma and had a positive skin test to ragweed. Patients underwent a control bronchoscopy before having purified ragweed extract (480 PNU in 4ml saline, ALK laboratories, Milford, CT, USA) instilled into the anterior segment of the right upper lobe. 24 hours later they underwent a second bronchoscopy. Patients were then split into two groups, one receiving a 6 week course of placebo, the other receiving a 6 week course of the steroid fluticasone propionate (FP; 250µg/ml twice daily). Following the course, patients underwent a third BAL followed by another allergen challenge. 24 hours later patients underwent a final bronchoscopy. All BAL was conducted using 2x 40ml of saline making a total of 80ml.

2.4. Experimental procedures.

2.4.1 Human lung tissue

Human lung tissue from Guys Hospital, London, was obtained from the normal margin of tissue resections from patients with lung carcinoma. The tissue was finely chopped and washed with Tyrodes' buffer and lung fragments were incubated overnight at 37°C and 5% CO_2 in a 24 well plate with RPMI-1640 containing 1mg/ml penicillin, streptomycin and gentamycin. The overnight incubation was important to allow the tissue to fully recover. The following day, fragments were incubated with appropriate stimulus over a time course, before removing the medium for anaylsis and the lung fragments weighed.

2.4.2 Primary Bronchoepithelial cells

Epithelial cells were a generous gift from Dr Jane Collins and Kate Hayes at the University Pathology group at Southampton General Hospital. Briefly, a section of bronchus from lung sample was taken and slit along its length. The sample was washed in HAMS F12: DMEM five times. The sample was then placed into 25mls of sterile protease solution and left at 4°C overnight. 6 well plates were coated with collagen (17μl collagen in 1ml media) in F12: DMEM media (500ml of media was supplemented with 3.6mg hydrocortisone, 57.3mg insulin, 100 mg EGF, 1ml USG, 5ml glutamine and 5mls penicillin-streptomycin antibiotics) and left to dry for one hour. The sample is then opened up by the previously cut slit, and epithelial cells washed from the inner surface using protease solution. Cells are resuspended with F12: DMEM and 1 ml foetal bovine serum before being centrifuged at 1100 rpm at 4°C for 10 minutes. After decanting the supernatant, cells were washed again before adding 3mls of cell suspension to each collagen-coated plate. Cells were received in 6 well plates at ~90% confluency and media was removed before being replaced with 2ml fresh media containing appropriate stimulus. Cells were incubated for 24 hours at 37°C, 5% CO_2 before the supernatant was removed and stored. Cells were washed briefly in PBS before adding 1ml of 0.4M NaOH lysis buffer. Cell lysates were then assayed for total protein content and cell supernatants assayed for α_1-antitrypsin and α_1-antichymotrypsin.

2.4.3 Acrylamide Gels

A 10% non-denaturing gel was prepared as described by Goldberg, 1989. However, for increased separation of proteins of a similar molecular weight, a gradient gel was made. This technique involves making 2 different concentrations of gel which are then combined prior to polymerization, so that it forms a density gradient from the top of the gel (7.5%) to a (15%) higher density at the bottom. Gradient gels were prepared as described by McPeek 1986 and placed in a gradient maker which then makes the gradient gel. Once polymerization has occurred, a 1.7% stacking gel was added. A 10% SDS acrylamide gel was prepared as described by Laemmli 1970.

2.4.4 Western Blotting

Gels were run at 27mA, 250V and 15W with electrode buffer. Once completed, the gel was removed from the glass plates and placed in Towbins transfer buffer (see section 2.2 for composition) for 30 minutes to allow for gel shrinkage before being placed into a 'sandwich' for the blotting process (Figure 2.1)

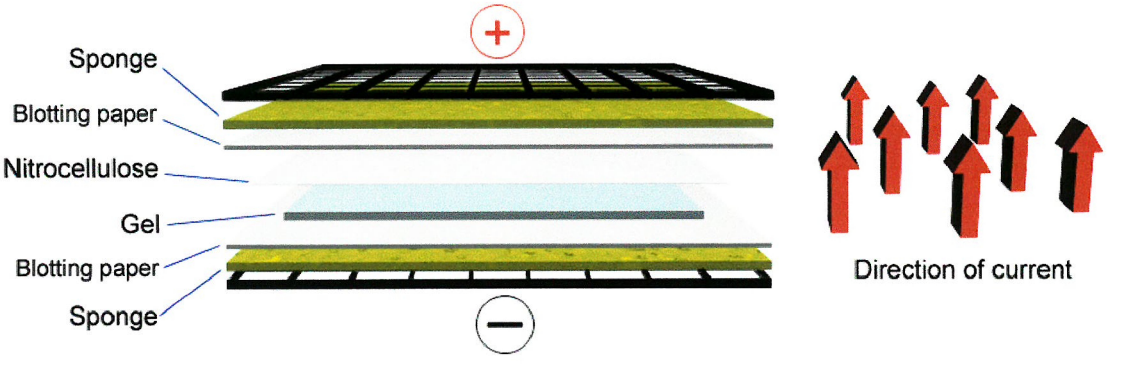

Figure 2.1 – Diagram showing the correct layering of the blot 'sandwich'. The direction of current ensures transfer of proteins from the gel to the nitrocellulose paper.

The proteins in the gel were transferred to nitrocellulose in Towbins for 3 hours at 200mA, 250V and 15W as described by Towbins *et al* in 1979. The nitrocellulose was then washed 4 times in PBS-Tween and non-specific binding sites were blocked overnight in PBS-Tween containing 5% powdered milk. The blot was then washed 4 times in PBS-Tween again and incubated with the appropriate antibody in 2mls PBS-Tween. This was incubated for 2 hours at room temperature with constant mixing, and then washed again with PBS-Tween. The blot was then sealed in a bag

containing 2ml of PBS-Tween and secondary antibody (usually goat anti-rabbit) conjugated to horseradish peroxidase at 250ng/ml. The nitrocellulose was left mixing with the secondary antibody at room temperature for 2 hours before 4 more washes in PBS-Tween. α_1-antitrypsin band visualization was conducted using the ECL kit and blue-sensitive x-ray film.

2.4.5 Pulse chase radiolabelling.

Lung fragments were incubated with 75μCi radiolabelled $^{S}35$ L-methionine for 45 minutes. Following incubation fragments were washed in fresh medium to remove unincorporated radioactivity. Following a 24 hour incubation period, lung fragments were removed and stored. Supernatants were then washed by firstly adding an equal volume of low salt precipitation buffer and then by mixing for 2 minutes. 3μl of a 0.5mg/ml solution of rabbit immunoglobulins were added and mixed for 1 hour at room temperature. 20μl of a 1:1 slurry of swollen protein A agarose in low salt buffer was then added to the sample. Mixing was continued for a further 90 minutes before the sample was spun at 12,000 rpm for 4 minutes and the supernatant retained (removing any non specific protein from the sample). Immunoprecipitation of α_1-AT continues as follows. 3μl of 1.2mg/ml antibody to human α_1-AT was added to the supernatant and mixed for an hour at room temperature. Following incubation, 20μl of protein A agarose suspension was added and mixed over 90 minutes before spinning down and aspirating off the supernatant. The pellet was washed three times in low salt precipitation buffer followed by one wash in high salt precipitation buffer, one wash in distilled water and finally re-suspended in 30μl sample buffer. This was heated for 5 minutes at 96^{0}C before centrifugation at 12,000 rpm. The supernatant was then kept for scintillation counting. Briefly, 5 μl of sample was placed onto filter paper and dried. Papers were then inserted into scintillation tubes and scintillant was added before being counted on a scintillation counter.

2.4.6 Ninety-six well plate assays

2.4.6.1. Bicinchoninic acid (BCA) protein assay

The BCA assay was developed using human serum albumin (HSA) as the standard. Standards ranged from 1mg/ml to 4μg/ml. Briefly, 100 μl of standard or unknowns were added to a 96 well plate before adding 100μl of BCA reagent. After 30 minutes samples were read at 550nm and a standard curve was plotted. Figure 2.2 shows a typical standard curve produced by this assay. Interplate coefficient for this assay was 9%, intraplate coefficient was 8% and the limit of detection was 8μg/ml.

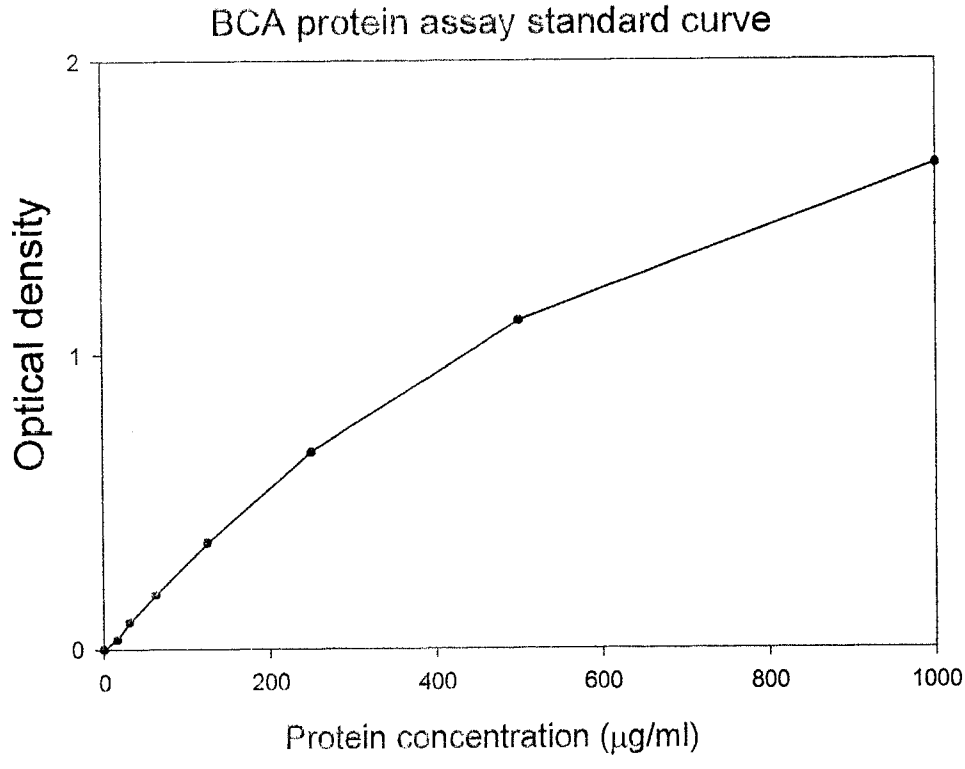

Figure 2.2 Typical standard curve produced by the BCA protein assay. Coefficient of variation for this assay was 9%, intraplate coefficient of variation was 8% and limit of detection was 8μg/ml

2.4.6.2 α_1-antitrypsin ELISA

Initially we had no way of accurately measuring α_1-antitrypsin, active α_1-antitrypsin, α_1-antichymotrypsin, human serum albumin or total inhibitory activity in our BAL samples. I therefore developed and characterized a number of assays for this purpose. Each assay had to be validated to ensure that they were accurate enough for our needs. This involved rigorously testing each assay for inactive and/or complexed protein to ensure that it was detecting 100% of total protein content and not just a small percentage. It was also important to measure the limit of detection and intraplate and interplate variation for each assay to ensure that these values fell within acceptable limits.

To measure total immunoreactive α_1-antitrypsin we used a 'sandwich' ELISA. Briefly, 96 well plates were coated with goat anti-human α_1-antitrypsin at a concentration of 10μg/ml in coating buffer (see section 2.2 for composition). The plates were incubated at 4°C for 6 hours. The plates were washed extensively and non-specific binding sites blocked by overnight incubation at 4°C with PBS-Tween containing 5% powdered milk. The following day the plate was washed again and 100μl of standard or sample added. The plate was incubated at 4°C for 2 hours, washed and incubated with rabbit anti-human α_1-antitrypsin at a concentration of 50ng/ml. The plates were then washed 4 times with PBS-Tween and then incubated for a further 2 hours at 4°C with 50ng/ml anti-rabbit antibody conjugated to horseradish peroxidase. After a final wash in PBS-Tween, bound α_1-antitrypsin was visualized using the HRP substrate (TMB substrate system). This substrate reacts with horseradish peroxidase forming a blue product. This reaction was stopped by the addition of 100μl 3M sulphuric acid, which results in a deep yellow colour. Optical density was measured at a wavelength of 450 nm. This assay detected >98% inactive protein (see Chapter 2.6 for more information) and the interplate coefficient of variation for this assay was 7%, intraplate coefficient of variation was 5% and limit of detection was 10ng/ml (Figure 2.3).

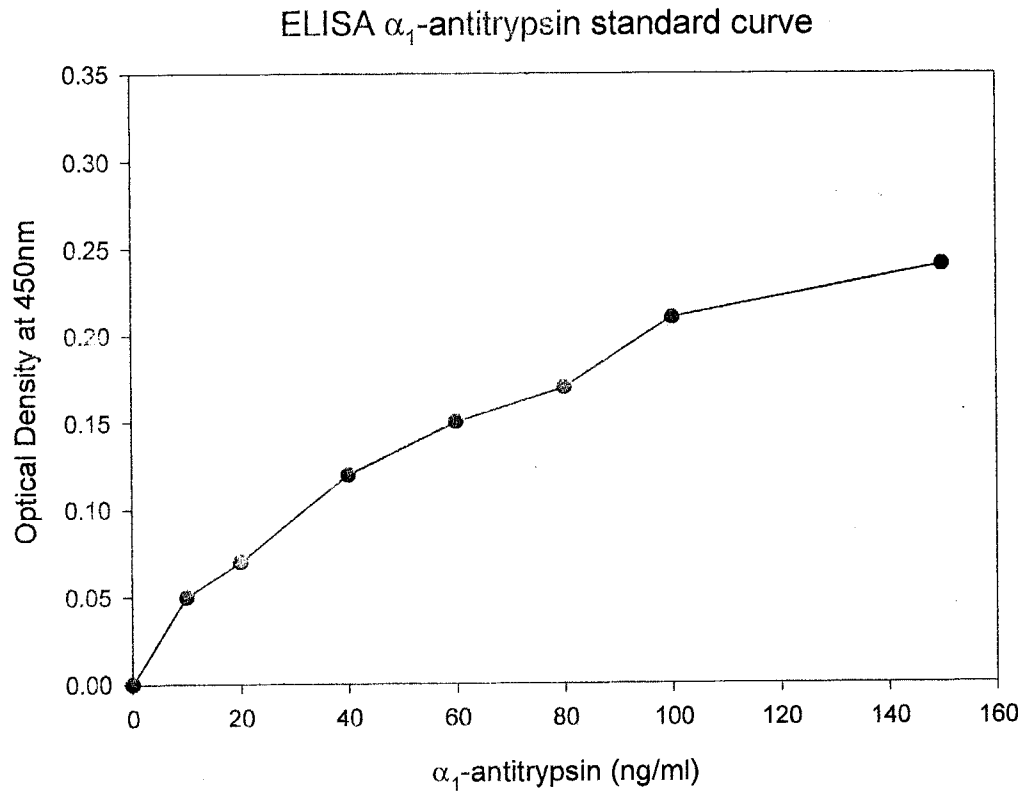

Figure 2.3 – Typical standard curve produced from the α_1-antitrypsin ELISA. Coefficient of variation for this assay was 7%, intraplate coefficient of variation was 5% and limit of detection was 10ng/ml

2.4.6.3 α_1-antichymotrypsin Assay

An α_1-antichymotrypsin ELISA assay was developed as follows. Briefly, 96 well plates were coated with goat anti-human α_1-antichymotrypsin at a concentration of 10μg/ml in coating buffer (see section 2.2 for composition). The plates were incubated at 4°C for 6 hours. The plates were washed extensively and non-specific binding sites blocked by overnight incubation at 4°C with PBS-Tween containing 5% milk. The following day the plate was washed again and 100μl of standard or sample added. The plate was incubated at 4°C for 2 hours, washed and incubated with rabbit anti-human α_1-antichymotrypsin at a concentration of 0.5μg/ml. The plates were then washed 4 times with PBS-Tween and then incubated for a further 2 hours at 4°C with 50ng/ml

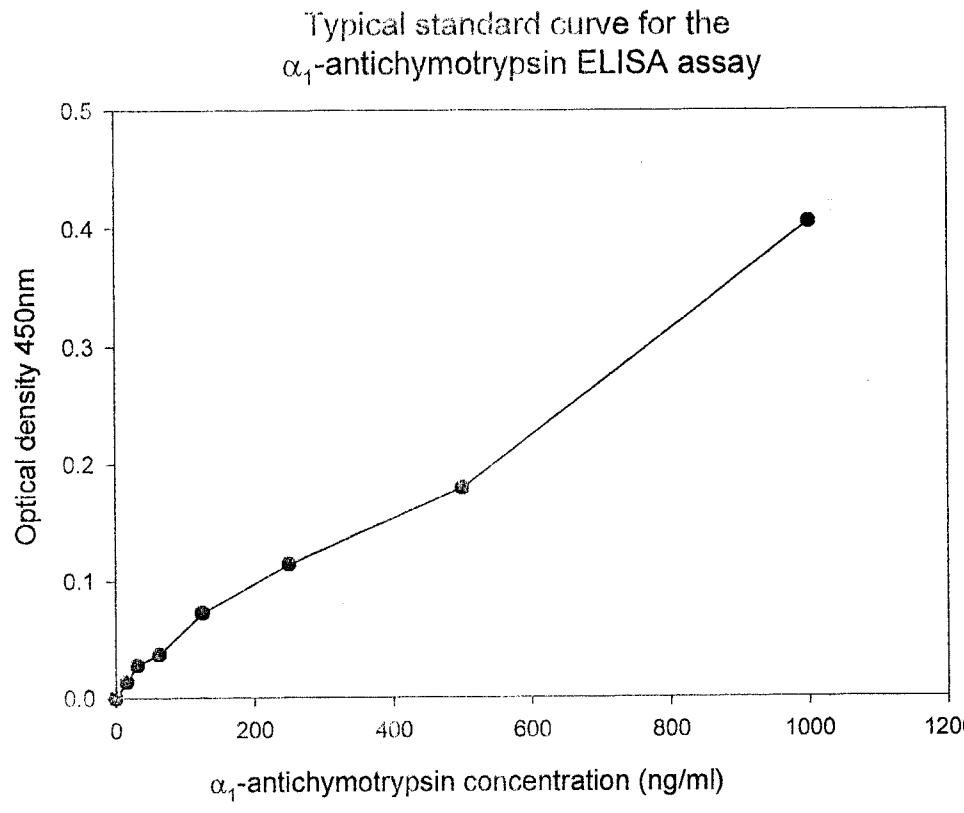

Figure 2.4 – Typical standard curve for the α_1-antichymotrypsin ELISA assay. Coefficient of variation for this assay was 8%, intraplate coefficient of variation was 5% and limit of detection was 15ng/ml

anti-rabbit antibody conjugated to horseradish peroxidase. Washing and development of samples continued as explained previously. A typical standard curve produced by this assay is shown in Figure 2.4. This assay detected >98% of inactive protein (data not shown), interplate coefficient of variation for this assay was 8%, intraplate coefficient of variation was 5% and limit of detection was 15ng/ml.

2.4.6.4 Chymotrypsin Immobilization Assay

To assess how much α_1-antitrypsin was active and able to bind to a substrate, we used a chymotrypsin immobilization assay. Chymotrypsin was added at a concentration of 25μg/ml in sodium phosphate buffer (pH 7.4) to a 96 well plate. Plates were incubated at 4°C for 6 hours. The plate was washed and non-specific binding blocked using PBS-Tween containing 5% milk overnight. The plate was then washed again and an α_1-antitrypsin standard curve (10ng/ml – 150ng/ml) or samples were added in sodium phosphate buffer (Figure 2.5). This was incubated at 4°C for 2 hours allowing α_1-antitrypsin to bind to the chymotrypsin. An antibody to α_1-antitrypsin

was then used to detect bound α_1-antitrypsin, as explained previously. The plate was developed with the HRP substrate system (TMB), the reaction stopped with 3M H_2SO_4 and optical density read at 450nm. As expected, this assay did not detected inactive protein (See Chapter 2.6 for more information), interplate coefficient of variation for this assay was 16%, the intraplate coefficient of variation was 8% and limit of detection was 7ng/ml

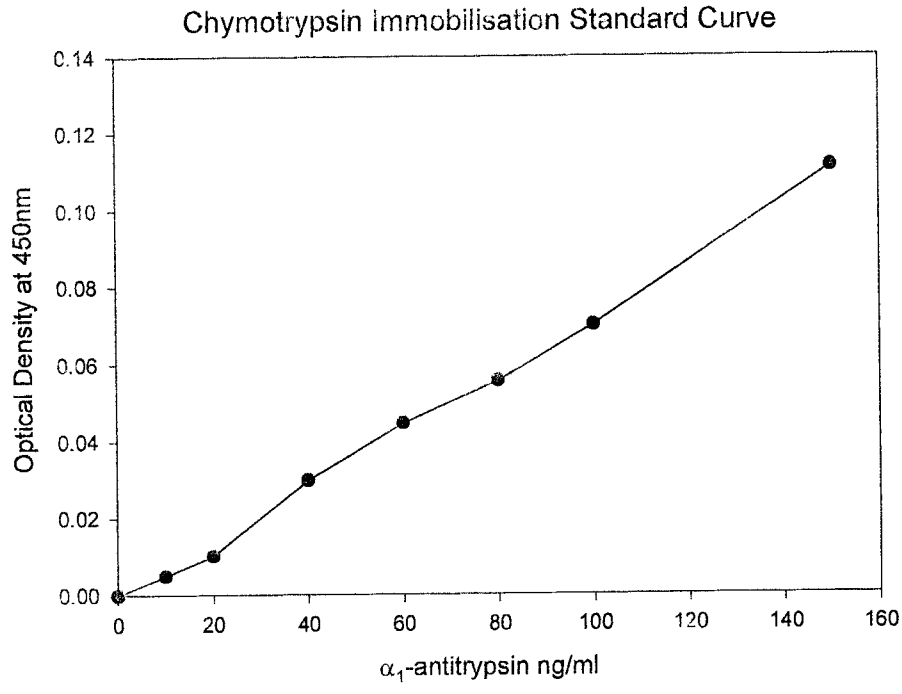

Figure 2.5 – Typical standard curve produced from the chymotrypsin immobilisation assay. Coefficient of variation for this assay was 16%, intrapiate coefficient of variation was 8% and limit of detection was 7ng/ml

2.4.6.5 Chymotrypsin Inhibition Kinetic Assay

To measure the total amount of active proteinase inhibitors in a sample, we used a chymotrypsin inhibition kinetic assay. An α_1-antitrypsin standard curve (50µl) was made up in sodium phosphate buffer (pH 7.4) with a range from 0 to 1500ng/ml. Samples were also added into the wells (50µl) and then 50µl of an 18µM chymotrypsin standard was added. A brief incubation period was necessary to allow the α_1-antitrypsin and the chymotrypsin to complex.

100µl of a 6.2mg/ml chymotrypsin substrate solution (NSa-NIT substrate) was added in 30mM Na_2PO_4 buffer (pH 7.4). The optical density was then read at 405nm for 5 minutes, taking a reading every 30 seconds. The gradient of the slopes was then taken and plotted to form a standard curve (figure 2.6), from which the relative amounts of chymotryptic inhibition were calculated. As expected, this assay did not detect inactive inhibitors (See chapter 2.6), the interplate coefficient of variation for this assay was 9%, the intraplate coefficient of variation was 4% and limit of detection was 15ng/ml

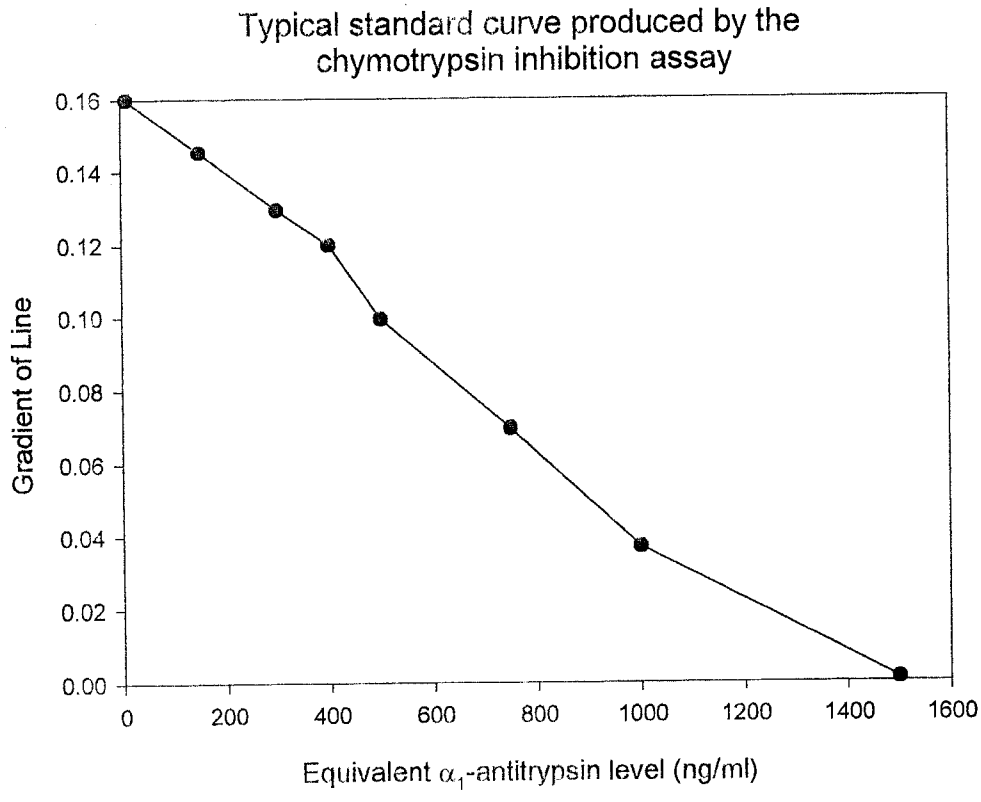

Figure 2.6 – Typical standard curve produced from the chymotrypsin inhibition assay. The value obtained from the curve is typically in arbitrary units (AU) as the assay measures *total* inhibitory activity. Coefficient of variation for this assay was 9%, intraplate coefficient of variation was 4% and limit of detection was 15ng/ml

2.4.6.6 Human Serum Albumin ELISA Assay

For measuring total levels of human serum albumin (HSA) in samples we used an HSA ELISA assay. Briefly, a 96 well plate was incubated with 14mg/ml of antibody to HSA in coating buffer at 4^0C for 6 hours. Following incubation, the plate was washed as described previously and incubated overnight with PBS-Tween containing 5% milk. The following day the plate was washed again and an HSA standard curve (0-1mg/ml) or samples were added and incubated at 4^0C for 2 hours. Following incubation, the plate was washed and an antibody to HSA conjugated to HRP was added at a concentration of 130ng/ml for 2 hours before a final wash. Development of the plate continued as described previously. Figure 2.7 shows a typical standard for this assay. It was not possible to detect inactive or complexed HSA for this assay but interplate coefficient of variation was 11%, intraplate coefficient of variation was 8% and limit of detection was 16μg/ml. There appears to be possible saturation after 200 μg/ml although further investigations came up with no explanation, as the curve did not plateau out, even at high HAS concentrations. However, using known concentrations of HSA in this assay determined that it was highly accurate.

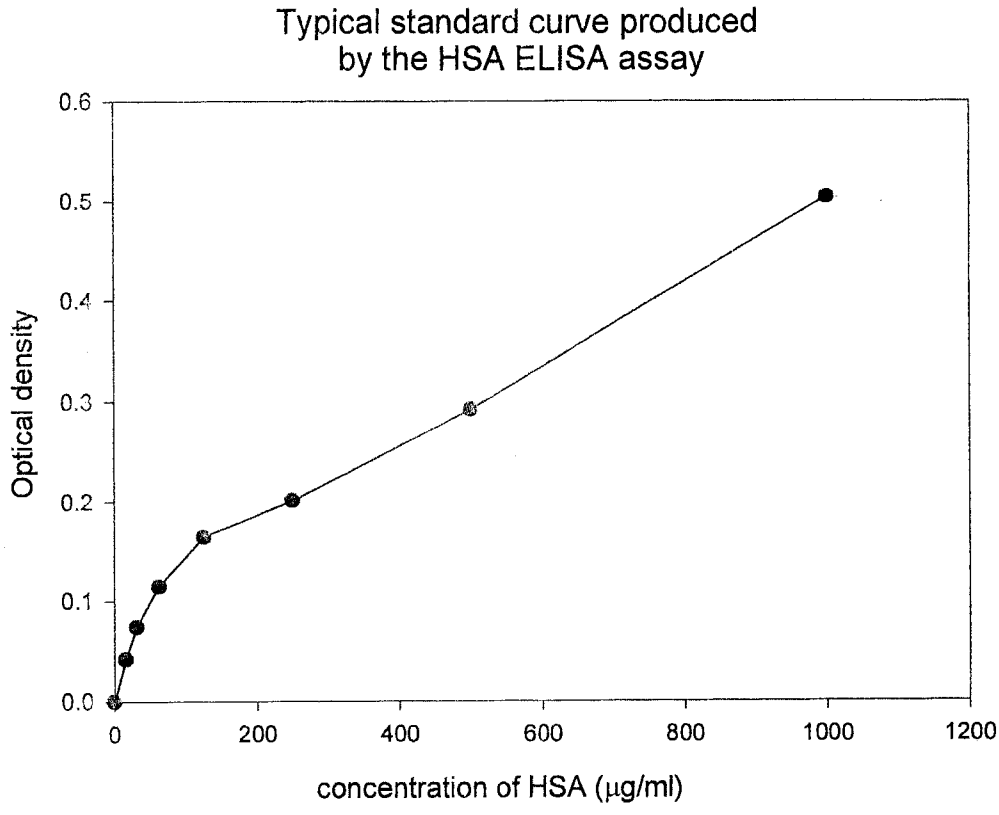

Figure 2.7 – Typical standard curve produced by the HSA ELISA assay Coefficient of variation for this assay was 11%, intraplate coefficient of variation was 8% and limit of detection was 16µg/ml

2.5 Generation of polymerized, oxidised and clipped α_1-antitrypsin

2.5.1. Polymerization

α_1-antitrypsin was polymerised by heating a 2mg/ml solution at 65°C for 1 hour. The presence of polymers was checked with the use of a non-denaturing gradient gel (Figure 2.8).

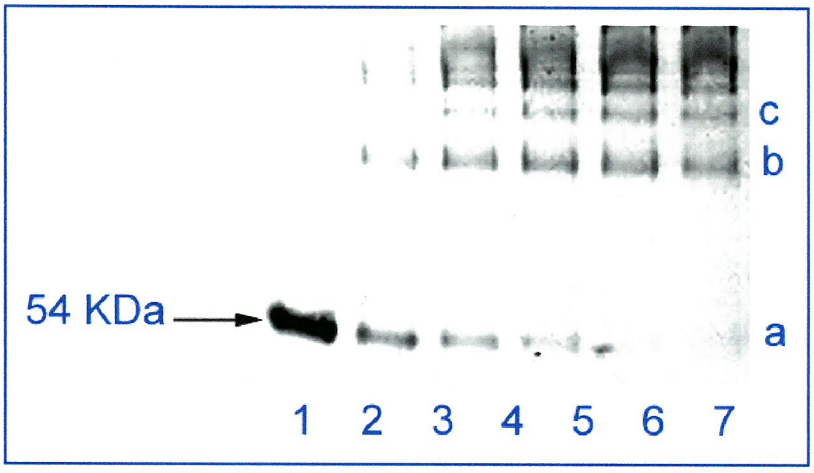

Figure 2.8– Non denaturing gradient gel showing the effect heating α_1-antitrypsin over 75 minutes. 8 samples containing 6.2µg of α_1-antitrypsin were heated at 65°C and the polymerization reaction stopped at each timepoint (lane 1 = 0min, lane 2 = 4min, lane 3 = 10min, lane 4 = 15min, lane 5 = 30min, lane 6 = 45min, lane 7 = 60min, lane 7 = 75min). (a) Monomers, (b) dimers and (c) trimers are all visible. Dimers will have a molecular weight of around 104 KDa, and trimers around 156 KDa. (Gel used with the kind permission of Diana Oakley.)

2.5.2. Oxidation

α_1-antitrypsin was oxidised by adding different concentrations of hydrogen peroxide (10^{-2}M – 10^{-8}M) to the 2mg/ml stock. The α_1-antitrypsin was incubated for 30 minutes at room temperature to allow for oxidation. α_1-antitrypsin activity was assessed using the chymotrypsin immobilization assay as described in 2.4.6.4.

2.5.3. Clipped

α_1-antitrypsin was clipped by adding various concentrations of chymotrypsin 2.5mg/ml – 0.1mg/ml) to the 2mg/ml stock. The α_1-antitrypsin was incubated for 30 minutes at room temperature to allow complexes to form. The presence of clipped α_1-antitrypsin was checked using a non-denaturing gel (Figure 2.9.

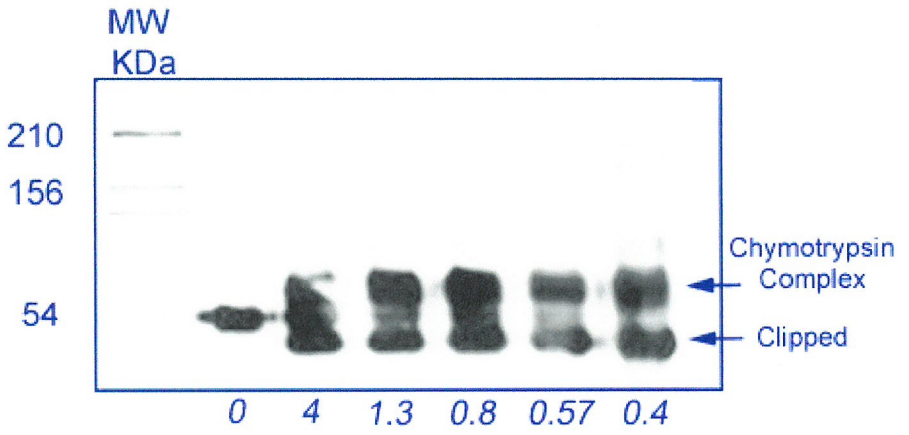

Figure 2.9- Non-denaturing 10% gel showing effect of chymotrypsin on α_1-antitrypsin. Both the lower molecular weight 'clipped' α_1-antitrypsin and the complexed α_1-antitrypsin are visible.

2.6 Validation of Assays and Stability of BAL Samples

As explained earlier, it was important to determine the binding capacity of the various forms of α_1-antitrypsin. It was hypothesized that all forms of α_1-antitrypsin would be able to bind efficiently to the α_1-antitrypsin ELISA assay. However, any inactive α_1-antitrypsin that was oxidized, clipped, or polymerized would not be able to bind efficiently to the chymotrypsin immunoassay, and would also not be able to inhibit chymotrypsin effectively in the kinetic assay. As BAL samples were being assayed over long periods of times it was important to investigate the effect of storage conditions on BAL protein. We determined that BAL stored at -70^0C retained more that 98% of its α_1-antitrypsin activity after 6 months, and that this was within acceptable limits (data not shown).

2.6.1. α_1-antitrypsin ELISA validation

The α_1-antitrypsin ELISA assay detected 100% of oxidised α_1-antitrypsin and 98% of clipped α_1-antitrypsin. However, the α_1-antitrypsin ELISA assay only detected 75% of the original α_1-antitrypsin after polymerisation. This may be because the polymers tend to aggregate making accurate dilutions difficult. (See Appendix i for further information)

It was also important to examine the percentage recovery of a known amount of α_1-antitrypsin when added to a BAL sample. This was accomplished by adding a standard sample of

BAL containing a known amount of α_1-antitrypsin to a known quantity of purified α_1-antitrypsin. The percentage of α_1-antitrypsin that was recovered from the BAL sample was then calculated. 'Spiking' our samples resulted in a recovery of >95% of α_1-antitrypsin. This verified the assays and proved that there was no external interference with the protein.

2.6.2. Chymotrypsin Immobilisation Assay Validation

It was found that oxidation heavily impaired the activity of the α_1-antitrypsin as expected. Figure 2.10 shows binding capacity of the α_1-antitrypsin to chymotrypsin after exposure to various concentrations of peroxide, the effect of 'clipping' the α_1-antitrypsin with molar ratios of chymotrypsin and the effect of polymerising the protein at 65 degrees for 1 hour. Recovery fell to 7% of control at a concentration of just 10^{-6} M H_2O_2. After adding α_1-antitrypsin to chymotrypsin at various molar ratios (4M -0.4M) a proportion of the α_1-antitrypsin became complexed, and a proportion became clipped. Recovery rapidly fell to just 30% of control. Finally, polymerised α_1-AT retained less that 25% of its original activity. The recovery of 'spiked' α_1-antitrypsin (<95%) using methods as described previously goes further to validate this assay.

2.6.3. Chymotryptic inhibition assay validation

We examined the effects of oxidising α_1-antitrypsin on total inhibitory activity. We used different concentrations of hydrogen peroxide to achieve this. Even at low levels of peroxide, inhibitory activity fell 50%. Clipping the α_1-antitrypsin with chymotrypsin also resulted in the loss of around 40% inhibitory activity. Finally, as expected, polymerisation of the α_1-antitrypsin by methods described in 2.5.1 resulted in a significant loss of inhibitory activity (>75%, See Figure 2.11). The recovery of 'spiked' α_1-antitrypsin in the kinetic assay was also >95% which again went further to validate this assay.

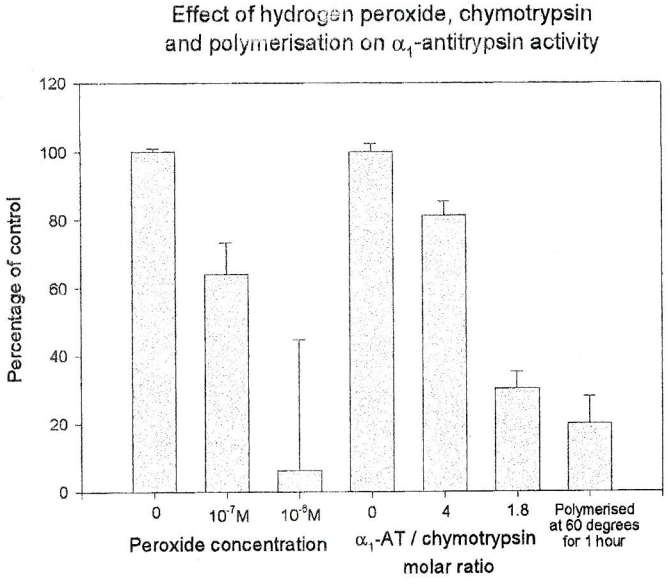

Figure 2.10 – The effect of hydrogen peroxide, chymotrypsin and polymerisation on α_1-antitrypsin activity compared to control. The binding capacity of α_1-antitrypsin after oxidation by hydrogen peroxide, after complexing with chymotrypsin for the formation of 'clipped' α_1-antitrypsin and after heating at 65 degrees for 1 hour is shown. α_1-antitrypsin activity decreases as the hydrogen peroxide and chymotrypsin concentrations increase and activity is severely impaired after the formation of polymers.

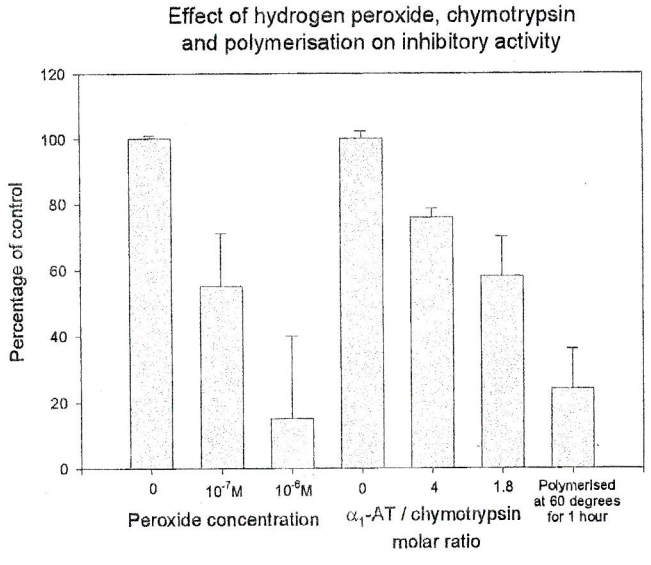

Figure 2.11 – The effect of hydrogen peroxide, chymotrypsin and polymerisation on total chymotryptic inhibitory activity compared to control. Inhibitory activity after oxidation by hydrogen peroxide, after complexing with chymotrypsin for the formation of 'clipped' α_1-antitrypsin and after heating at 65 degrees for 1 hour is shown. Total inhibitory activity decreases as the hydrogen peroxide and chymotrypsin concentrations increase and activity is severely impaired after the formation of polymers.

2.7 Statistical analysis.

Groups were compared using non-parametric statistics. Repeated measures ANOVA using the Student-Newman-Keuls method (with randomized samples sets if needed) was used to determine significant differences in the ozone and allergen study. The Wilcoxon Signed Rank and the Mann-Whitney U-Test were used to determine significant differences between other sample groups and Spearman rank was used to compare sample sets for correlations.

Chapter 3

Effect of ozone on serpin levels in BAL

3.1 Introduction

α_1-antitrypsin is one of the most important antiproteinases in the lung. It inhibits a wide range of biological proteinases including human neutrophil elastase, potentially one of the most devastating digestive proteinases in the lung environment. Unfortunately, α_1-antitrypsin is very susceptible to inactivation by a variety of means, including the oxidation of methionine 358, a key part of the reactive centre loop (Gaillard et al, 1992; Wallaert et al, 1993). A combination of increased elastase levels, and increased oxidative inactivation of α_1-antitrypsin in the lungs of asthmatics has been suggested to contribute to irreparable lung damage and airway re-modelling (Smith et al 1987).

A common oxidative agent is the man-made pollutant, ozone, and once inhaled, ozone has the ability to degenerate into free radicals. Although ozone is not a radical species itself, it is thought to mediate its harmful effects through free radical reactions (Kelly et al, 1995). This can come about either by the direct oxidation of biomolecules to give species such as –OH$^{\bullet}$ or by driving radical-dependent production of cytotoxic, *non radical* species such as aldehydes and ozonides (Kelly et al, 1995). Ozone can potentially react with all hydrocarbon molecules, and it is this broad range of reactivity that makes it such a dangerous pollutant. Ozone does not react directly with the lung epithelia, but instead is thought to exert its effect on the pulmonary epithelial lining fluid (ELF). Figure 3.1 shows some of the possible mechanisms of ozone induced injury of the lung. Other reports have indicated that asthmatics have a decreased anti-oxidant shield in their lungs (Kelly et al, 1999) and following exposure to ozone the antioxidant defence decreases further still (Mudway et al, 1999). They would therefore be less able to cope with oxidative stress and this could result in the oxidation of the active site methionine of α_1-AT, rendering it useless as a proteinase inhibitor. This would decrease the total amount of active antiproteinases in the lung and lead to a state of possibly unrestrained proteolytic activity in the lung.

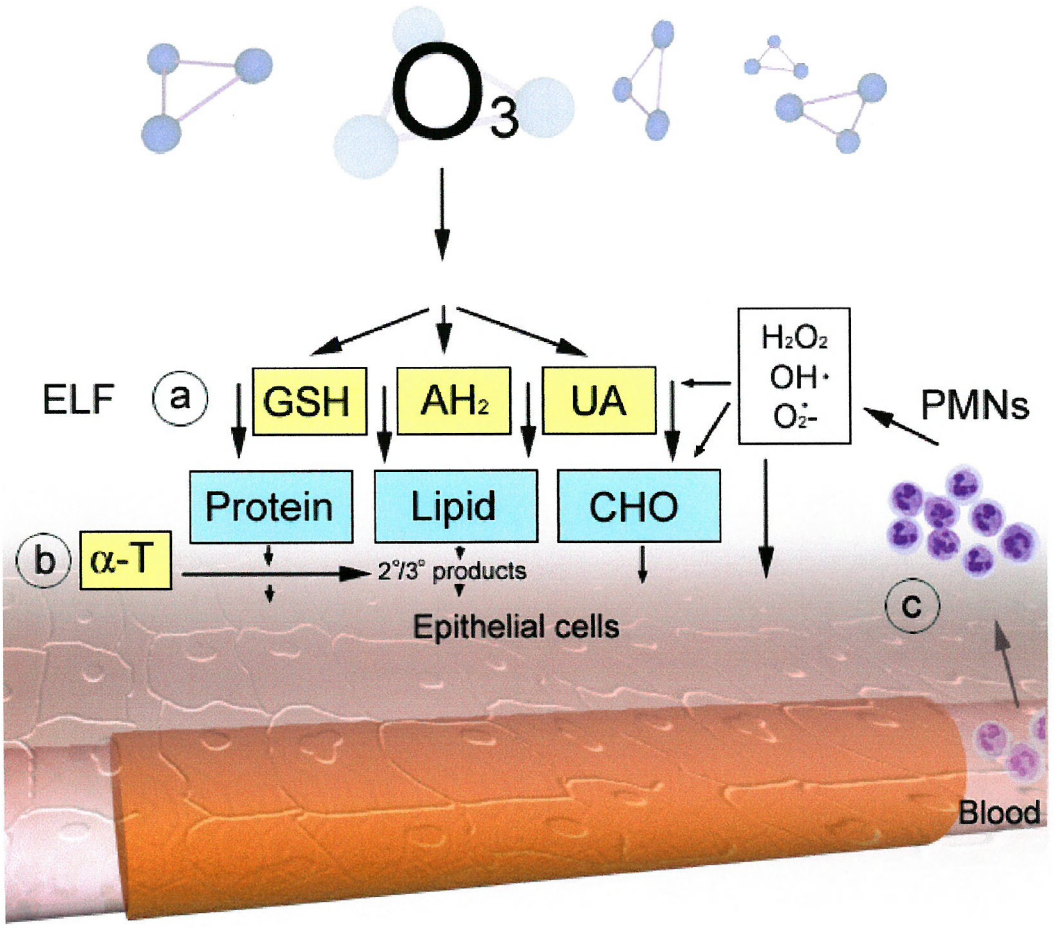

Figure 3.1 – The effects of ozone in the lung and lung anti-oxidant interaction. The first lines of defence against ozone are the antioxidants glutathione (GSH), ascorbate (AH_2) and urate (UA). (a) These act as sacrificial substrates scavenging ozone and thereby preventing the oxidation of macromolecules such as protein, lipid and carbohydrate. However, if these anti-oxidants are compromised, or if the oxidative burden is too great, ozone can then react with the macromolecules and generate harmful cytotoxic secondary and tertiary products. (b)The second line of defence is then vitamin E (α-tocopherol, α-T) Finally ozone exposure results in airway inflammation and activated neutrophils can migrate into the ELF and release further cytotoxic agents (c) *(Image modified from Kelly et al, 1995)*

3.2 Methods and Aims.

The following study was conducted on both asthmatics (n=11) and control subjects (n=15). Subjects inhaled air or ozone (200 ppb) for two hours before undergoing BAL as described in chapter 2.6. The aim of this study was initially to examine if there was a difference in the baseline levels of α_1-AT and α_1-ACT between asthmatic and control subjects. We were also aiming to examine whether asthmatics were more susceptible to oxidative stress than control subjects. We

were especially interested in the effect of ozone on antiproteinase activity in the airways, and to examine this we first looked at levels of total chymotryptic inhibitory activity, as well as total immunoreactive α_1-AT, active α_1-AT and finally α_1-ACT.

3.3 Results

3.3.1 Comparisons between control and asthmatic subjects at baseline.

Our first aim was to compare the levels of proteolytic inhibitory activity and serpin levels in BAL from the control and asthmatic subjects. Figure 3.2 shows the levels of total chymotryptic inhibitory activity in control and asthmatic subjects.

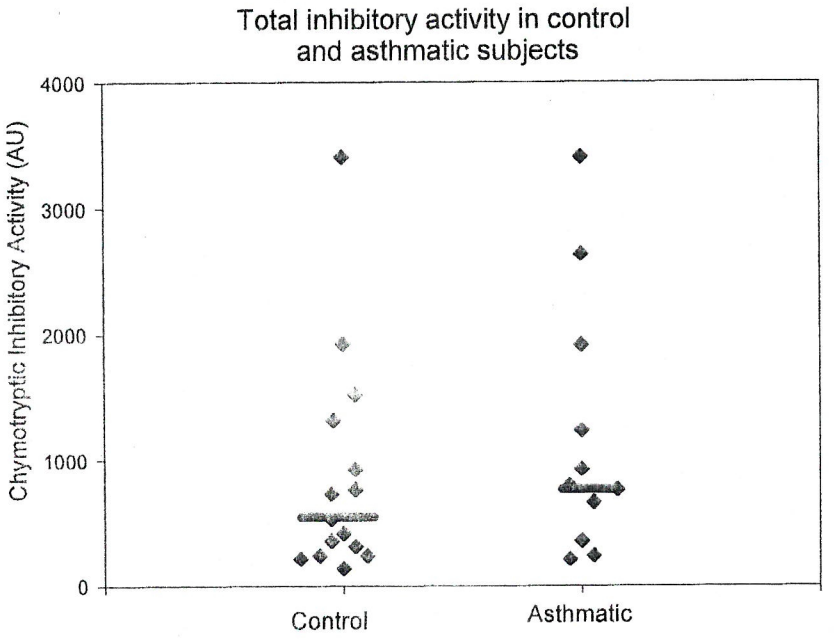

Figure 3.2 – Total inhibitory activity in control and asthmatic subjects. Total inhibitory activity was measured using a chymotrypsin inhibitory kinetic assay and values given in arbitrary units (AU). Individuals are shown by a black diamond and medians indicated by a black bar. Medians are 530AU for controls and 794AU. Repeated measures ANOVA was used to determine statistical significance.

Although median levels of total inhibitory activity in the asthmatic BAL were slightly higher that control BAL, this did not reach statistical significance. We then went on to examine the antiproteinases and serpins in BAL in more detail and looked at differences in individual serpin

levels between asthmatics and control subjects. Figure 3.3 shows the differences in α_1-AT between control and asthmatic patients, whereas figure 3.4 compares levels of *active* α_1-AT.

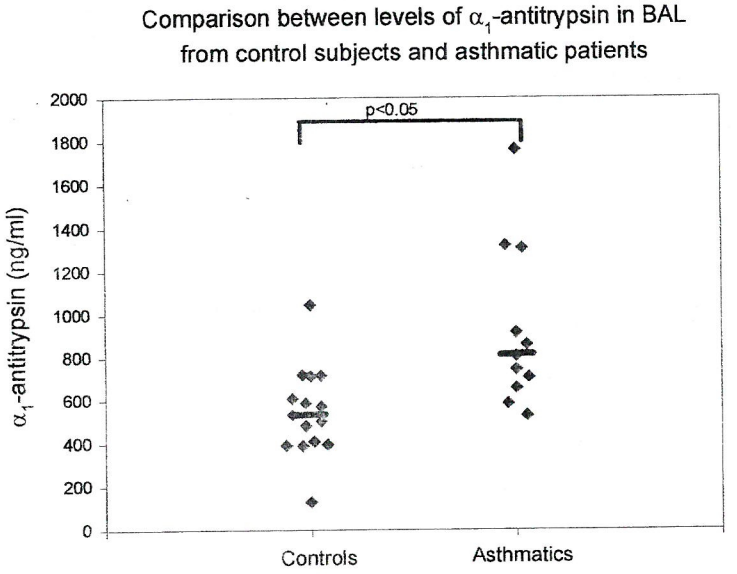

Figure 3.3– Comparison between levels of α_1-antitrypsin in BAL from control subjects and asthmatic patients. An α_1-antitrypsin ELISA was used to measure total immunoreactive α_1-antitrypsin. Individual patients are shown by a black diamond and medians indicated by a black bar. Medians were 532 ng/ml for controls compared to 804ng/ml for asthmatic subjects. ($p<0.05$) Repeated measures ANOVA was used to determine statistical significance.

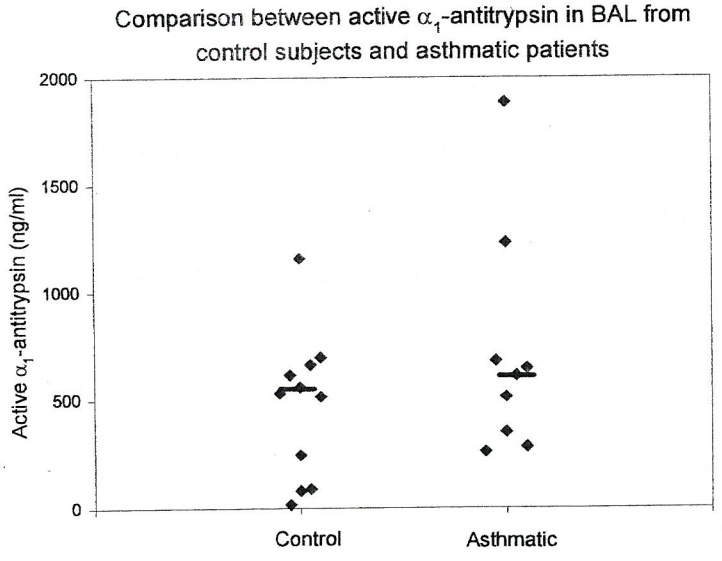

Figure 3.4– Comparison between levels of active α_1-antitrypsin in BAL from control subjects and asthmatic patients. A chymotrypsin immobilisation assay was used to measure active α_1-antitrypsin. Individual patients are shown by a black diamond and medians indicated by a black bar. Medians were 530ng/ml for controls compared to 611ng/ml for asthmatic subjects. Repeated measures ANOVA was used to determine statistical significance.

Median levels of α_1-AT in BAL from controls were 532ng/ml compared to 804ng/ml for asthmatics (Figure 3.3). This was a significant increase ($p<0.05$), suggesting asthmatics have a greater potential elastase inhibitory capacity compared to controls. Due to restricted sample volumes we were only able to measure active α_1-AT in 11 out of 15 control subjects and 9 out of the 11 asthmatic patients. However, median levels of active α_1-AT were 530ng/ml for controls compared to only 611ng/ml for asthmatic subjects (figure 3.4, p=ns). This data suggests that more of the α_1-AT in asthmatics is inactive, but in fact the percentage of active α_1-AT in these asthmatic patients is 71%. This is not significantly different from the percentage of active α_1-AT for controls, which is 84%. Therefore this data suggests that the α_1-AT shield against proteolytic attack in the resting asthmatic lung is not compromised.

We then went on to look at α_1-ACT and found no difference between in α_1-ACT between controls compared to asthmatic subjects (medians were 707ng/ml and 438 ng/ml respectively, see figure 3.5).

To summarise, the only main difference between asthmatic patients and control subjects at baseline appears to be an increase in total α_1-AT in the asthmatic BAL. Overall, there is no significant difference in the total proteolytic inhibitory shield in asthmatics and controls.

3.3.2 The effect of ozone on antiproteinase activity and serpin levels in BAL from controls and asthmatic subjects.

We examined the effect of inhaling ozone (200ppb) on proteolytic inhibitory activity and serpin levels in BAL from control and asthmatic subjects. Firstly we examined whether asthmatics are more prone to oxidative stress than controls, and secondly we examined if the ozone had caused an inflammatory response in the subject groups

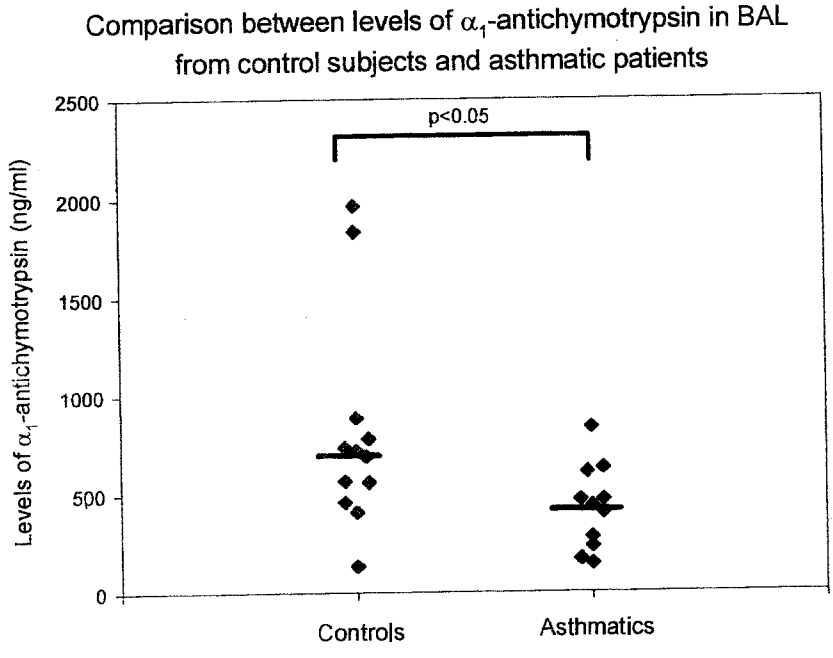

Figure 3.5 – Comparison between levels of α_1-ACT in BAL from control and asthmatic subjects at baseline. Individual patients are shown by a black diamond and medians indicated by a black bar. Medians were 707ng/ml for controls compared to 438ng/ml for asthmatic subjects. (p=ns) Repeated measures ANOVA was used to determine statistical significance.

3.3.2.1 Effect of ozone on total inhibitory activity and serpin levels in control subjects.

Levels of total inhibitory activity in control subjects did not significantly change following ozone inhalation (medians were 530AU to 661AU after ozone, see Table 3.1) We went on to examine levels of α_1-AT, active α_1-AT and α_1-ACT in these subjects following ozone inhalation. Levels of α_1-AT did not change significantly in control subjects (medians were 532ng/ml before ozone, 586ng/ml after ozone, see table 3.1). Levels of active α_1-AT fell from 530AU to 440AU following ozone, although this did not attain statistical significance. Finally, median levels of α_1-ACT in control subjects were 704ng/ml prior to ozone inhalation and 562ng/ml after ozone inhalation. This was *not* a significant decrease and in fact we observed a rise in α_1-ACT for a third of the control subjects. Overall the effect of 200ppb ozone on serpin levels and total inhibitory activity in the airways of the control subjects was minimal and is summarised in table 3.1. We then went on to examine the effect of ozone on serpins in BAL from asthmatic subjects.

	Median pre ozone	Median post ozone
Chymotryptic inhibitory activity (AU)	530 *(140-3415)*	661 *(42-1647)*
Total α_1-AT (ng/ml)	532 *(129-1044)*	586 *(141-917)*
Active α_1-AT (ng/ml)	530 *(15-1157)*	440 *(18-1083)*
Total α_1-ACT (ng/ml)	707 *(124-1966)*	562 *(215-1413)*

Table 3.1 – Effect of ozone on antiproteinase activity in control BAL. Medians and standard errors are shown. There is no significant difference between levels of either total inhibitory activity or any serpins measured following ozone inhalation. Ranges are also shown in brackets.

3.3.2.2 Effect of ozone on total inhibitory activity and serpin levels in asthmatic subjects.

Analysis of total inhibitory activity in BAL from asthmatic subjects indicates that ozone is causing a general inactivation of inhibitory proteins. Total inhibitory activity in BAL decreases from 794AU to 530AU following ozone inhalation ($p<0.05$, figure 3.6)

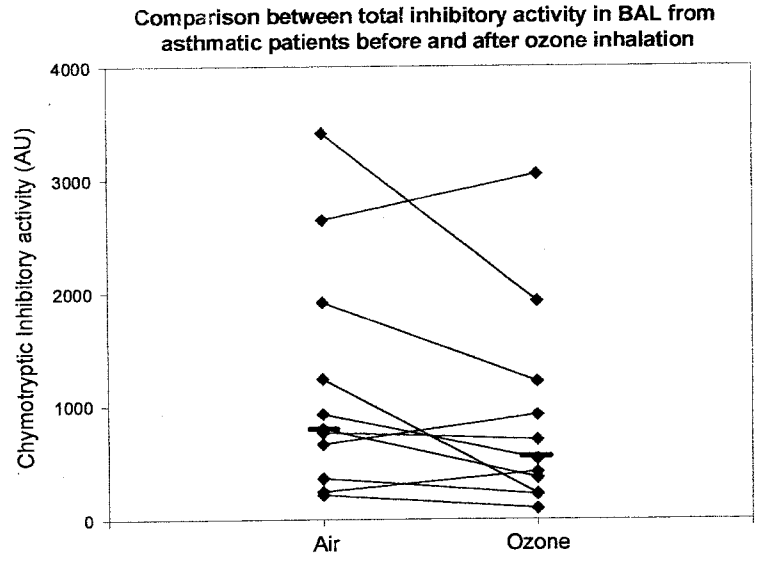

Figure 3.6- Comparison between total inhibitory activity levels in BAL from asthmatic subjects before and after ozone inhalation. Total inhibitory activity was measured using a chymotrypsin inhibitory kinetic assay. Individuals are shown by a black diamond and medians indicated by a black bar. Medians were 794AU before ozone and 530AU after ozone inhalation. ($p<0.05$). Repeated measures ANOVA was used to determine statistical significance.

Asthmatics are evidently more prone to oxidative stress than control subjects. We then went on to examine in more detail if serpin inactivation is responsible for this decrease in total proteolytic inhibitory activity? The level of total α_1-AT in asthmatic BAL does not significantly change following ozone inhalation (804ng/ml to 703ng/ml p=ns, figure 3.7). Similarly, levels of *active* α_1-AT drop slightly from 611ng/ml to 465ng/ml following ozone inhalation but this is not significant (Figure 3.8). Levels of α_1-ACT also diminish slightly, (levels were 438ng/ml before ozone and 334ng/ml after ozone inhalation) but this drop in α_1-ACT did not achieve statistical significance (data not shown).

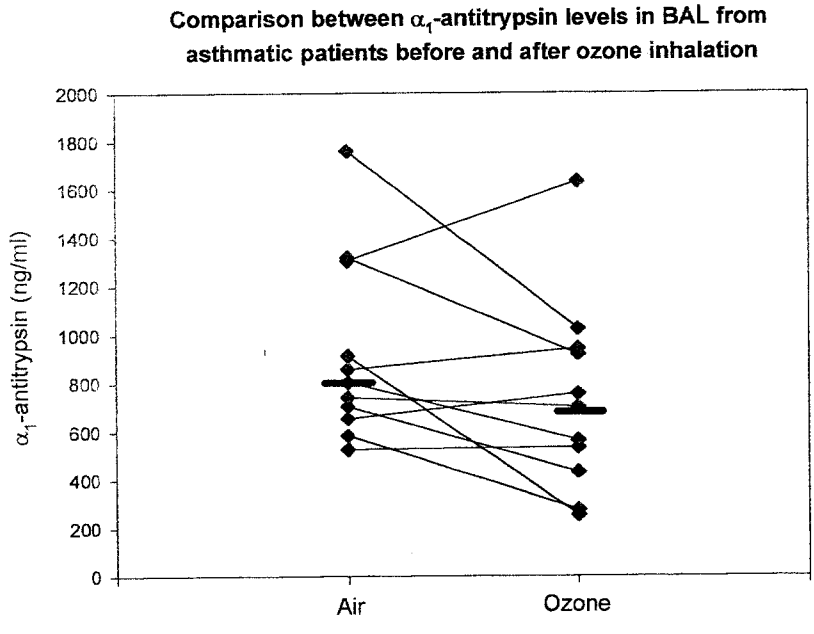

Figure 3.7 – Comparison between α_1-antitrypsin levels in BAL from asthmatic patients before and after ozone inhalation. ELISA was used to measure total immunoreactive α_1-antitrypsin. Individuals are indicated by a black diamond and medians are shown by a black bar. Medians were 804ng/ml before ozone and 703ng/ml following ozone inhalation (p=ns) Repeated measures ANOVA was used to determine statistical significance.

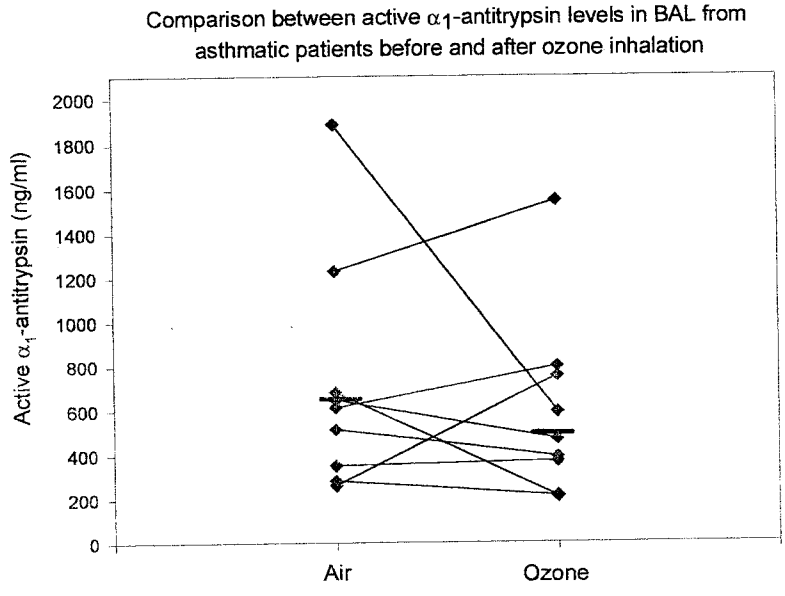

Figure 3.8 Comparison between active α_1-antitrypsin levels in BAL from asthmatic patients before and after ozone inhalation. A substrate binding assay was used to measure active α_1-antitrypsin. Individuals are indicated by a black diamond and medians are shown by a black bar. Medians were 611ng/ml before ozone and 465ng/ml following ozone inhalation (p=ns). Repeated measures ANOVA was used to determine statistical significance.

3.3.3 Effect of ozone on albumin levels and neutrophil numbers.

Albumin is the most abundant of the plasma proteins, and albumin present in BAL is a good indicator of plasma influx and increased capillary permeability. However, there was no evidence of a plasma influx after ozone in either the controls or the asthmatic patients. Albumin levels were 35μg/ml before and 44μg/ml after ozone for controls and 49μg/ml and 55μg/ml for asthmatics. In addition to albumin levels, neutrophil numbers are good indicators of airway inflammation. There was a significant change in neutrophil count in the control subjects (Figure 3.9). Levels rose from $0.112x10^4$ cells/ml before ozone to $0.232x10^4$ cells/ml following ozone inhalation ($p<0.05$). Levels of neutrophils in asthmatic BAL did not change, but remained consistently high. Neutrophil count for asthmatics was $0.297x10^4$ cells/ml before ozone and $0.256x10^4$ cells/ml following ozone inhalation. If there was an inflammatory response to be observed via white cell influx, it may be masked by the high level of baseline neutrophils in the asthmatic lung. There is no evidence from

BAL whatsoever, that this level of ozone treatment (200ppb) has invoked an inflammatory response in the asthmatic lung. However, it is quite clear that ozone *is* having a damaging effect by reducing the overall chymotryptic inhibitory activity, probably due to the oxidative inactivation of these inhibitors.

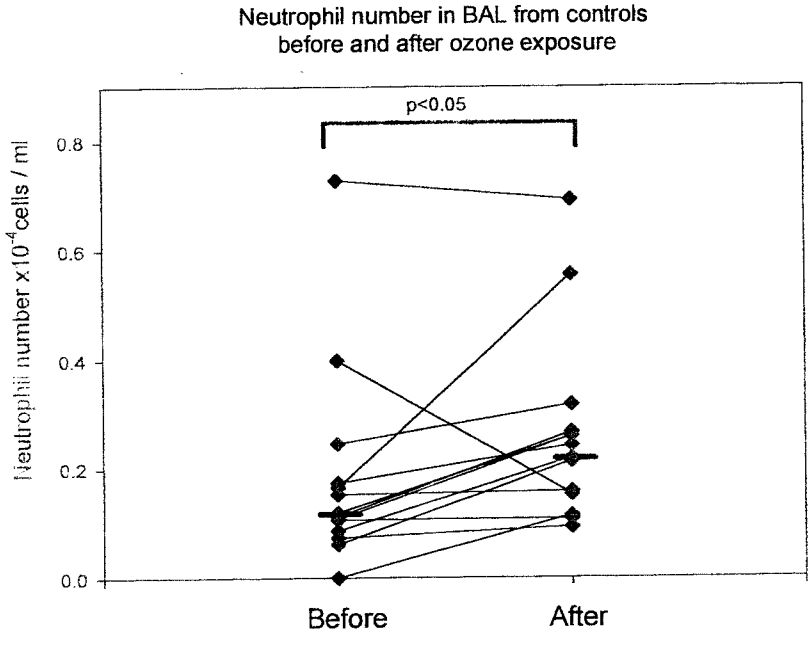

Figure 3.9 – Effect of ozone on neutrophil number in control subjects. Individuals are indicated by a black diamond and medians are shown by a black bar. Medians were 0.112 $x10^{-4}$ cells/ml before ozone and 0.232 $x10^{-4}$ cells/ml following ozone inhalation. ($p<0.05$) *(Thanks go to Nikolai Stenfors MD, Department of Respiratory Medicine and Allergy, University Hospital, Umea, for the neutrophil data)*

3.4 Discussion

We felt that existing methods for the determination of α_1-AT and α_1-ACT (rocket immunoelectrophoresis and nephelometry) were not sufficiently precise for the low levels of these proteins found in BAL. We therefore began by devising several sensitive 'in house' ELISAs for the study of inhibitory activity and serpin levels. Our initial experiments examined levels total inhibitory activity in BAL from control subjects and mild asthmatics. We decided to use chymotrypsin rather than human neutrophil elastase for this assay due to the high cost of HNE

and the fact that chymotrypsin is a good substrate for both α_1-AT and SLPI (Tremblay et al, 1996). Although there was a slight increase in total chymotryptic inhibitory activity for the asthmatic patients this did not reach statistical significance. The next step was to examine in more detail the contribution of α_1-AT and α_1-ACT to the total chymotryptic inhibitory activity. Our first observation was a significant ($p<0.05$) increase in the total immunoreactive α_1-antitrypsin in BAL from the asthmatic group (Figure 3.3). This datum does supports evidence from an earlier study conducted by Sibille et al, (1988) that showed levels of α_1-AT in BAL from asthmatic patients are actually increased compared to that of control. However, it contradicts the findings by Van Vyve et al, 1995, in which levels of α_1-AT in asthmatic BAL was significantly decreased compared to that of controls. There does not appear to be any difference between the patients asthmatic status or medication in our study compared to the study by Van Vyve, nor are there differences in the way proteins such as α_1-AT were measured. Both our study and Van Vyve used immunoassays for α_1-AT detection.

Although levels of total α_1-AT were significantly higher in asthmatic patients in our study, there was no difference in the levels of *active* α_1-AT in the lungs from asthmatics and controls. This may suggest that although asthmatics have increased levels of α_1-AT, a higher percentage is inactive. However, although the percentage of active α_1-AT in controls was higher than for asthmatics this was not significant. We went on to examine levels of immunoreactive α_1-ACT in controls and asthmatics and found that total α_1-ACT levels in asthmatics were not significantly different than controls.

In summary, my initial observations have shown that although there are fluctuations in serpin levels, mild asthmatics do not have a reduced proteolytic shield compared to control subjects. Although we do not have any data regarding levels of other inhibitors such as SLPI or elafin, the fact that total chymotryptic inhibitory activity is unimpaired in asthmatics suggests that levels of these inhibitors are also similar to that of control. We then went on to examine the effects of ozone exposure on antiproteinase defences.

Initially we looked at the effect of ozone inhalation on total inhibitory activity in control subjects and asthmatic patients. Following ozone inhalation, levels of proteolytic inhibitory activity remained unchanged in controls suggesting that these subjects have the ability to cope with this levels of oxidative stress in their airways. However levels of total inhibitory activity in asthmatic patients significantly decreased following ozone (figure 3.6). It is evident then, that asthmatics are less able to cope with this level of oxidative stress in the lung and this leads to a general inactivation of proteolytic inhibitors. There is good evidence that asthmatics have a reduced anti-oxidant shield in their lungs (Kelly et al 1999, Mudway et al 1996). A decreased anti-oxidant shield would lead to more free radical activity in the lung. Ozone has been shown to reduce both α_1-AT and α_1-ACT activity in vitro (Smith et al 1987), and so we investigated the effect of ozone on serpin levels in our subjects.

Levels of total α_1-AT and active α_1-AT did not significantly change in control subjects following ozone inhalation. This supports the previous data of an unimpaired proteolytic shield. However, levels of α_1-AT and active α_1-AT in asthmatic subjects had not significantly decreased following ozone inhalation (Figure 3.7 and 3.8, respectively). Previous studies have shown that a 2 hour exposure to 400ppb ozone leads to an *increase* in α_1-AT (Devlin et al, 1996). Devlin et al also showed that total protein significantly increases following ozone exposure, but neither the controls nor asthmatics in our study showed any increase in total protein following ozone inhalation (Data not shown). However, Devlin used ozone at a concentration of 400ppb, (twice as concentrated as this study), his subjects had an inhalation rate of 66 litres/min due to intermittent heavy exercise, (three times as much as this study which was only 20 litres/min) and only waited for 1 hour after exposure before performing BAL. These are all important factors that must be considered when comparing these data groups. If we consider that in one minute we exposed our subjects to the equivalent of 200ppb x 20 litres, which is 4000 units of ozone. Devlin used 400ppb ozone and his subjects inhaled ~60 litres which in one minute is the equivalent of 24,000 units of ozone, six time as much as this study. It is obviously very difficult to compare these two sample groups and is therefore not surprising that we have not observed the mediator changes observed

by Devlin. Not only is the concentration of ozone inhaled by his subjects more than 6 times greater, but the time of BAL after exposure is earlier, 5 hours before this study.

Finally, although levels of α_1-ACT were slightly reduced in both asthmatics and control subjects following ozone exposure, this did not reach statistical significance.

This data is indirect evidence to suggest that asthmatics have an impaired antioxidant shield in their lungs as suggested by Powell et al, 1994. Levels of total inhibitory activity are severely impaired following ozone inhalation even though levels of total α_1-AT are not significantly reduced following ozone.

According to literature, ozone induces an inflammatory response (Kelly et al 1995). Two consequences of an inflammatory response that we can measure in this study are significant quantities of albumin in BAL due to increased vascular permeability and also increased numbers of neutrophils in BAL due to leukocyte influx. However, levels of albumin do not change significantly in either control subjects or asthmatic patients following ozone inhalation. However, ozone does appear to induce some aspects of inflammation, as neutrophil number actually doubles in the control subjects following ozone, suggesting oxidative stress causes leukocyte recruitment (Figure 3.9). This supports work conducted by Devlin et al, 1996, that shows increased levels of neutrophils in BAL following ozone inhalation.

Neutrophil levels in asthmatics were significantly higher than controls even in the *resting* lung. Eosinophilia is well documented as a classic sign of an asthmatic lung (Djukanovic et al, 1990), but this data would suggest that increased neutrophil recruitment is also a significant indicator of asthma. The increase in neutrophil number *may* be partly responsible for the increase in total α_1-AT at baseline. Neutrophils have been reported to have the ability to synthesize α_1-AT (Paakko et al, 1996) although the contribution of neutrophil derived α_1-AT to the total lung pool is probably minimal. Unfortunately we do not have any data concerning eosinophil cell counts for control subjects, making it is impossible to comment on any potential differences between control and asthmatic eosinophil number.

Ozone is one of the main man made pollutants and is often found in conjunction with nitrogen dioxide (NO_2), another highly reactive and toxic oxidant pollutant (Huang et al, 1991).

Previous data had showed that inhalation of NO_2 (30ppm) resulted in the upregulation of several mRNAs for leukocyte chemoattractant proteins in mouse lungs. A similar effect was observed with ozone, and induction of these messages was associated with the duration and concentration of exposure (Johnston et al, 2000). This suggests that these pollutants exert their toxic action through a similar mechanism. The upregulation of neutrophil chemoattractants in the lung would explain our observations of the influx of neutrophils in the control lung following ozone exposure. In another study by Devlin (1999), a 4 hour inhalation of 2000 ppb NO_2 caused significant increases in neutrophil recruitment compared to control. Although this concentration of NO_2 is higher than the concentration of ozone used in this study, it still suggests that NO_2 and ozone exert their oxidant effects in similar ways. The effects of NO_2 on serpin levels are also similar to observations made in this study as following a three-hour exposure to 3ppm NO_2, active α_1-AT fell to 45% of control (Mohsenin and Gee, 1987).

In summary, in this study ozone does not initiate inflammation in the asthmatic lung but does bring about a decrease in the proteolytic shield probably by oxidative inactivation of a variety of inhibitors other than α_1-AT. Whether this is due to decreased anti-oxidant levels in asthmatics is unknown. In the control lung, ozone shows little effect apart from an increase in neutrophil recruitment.

Chapter 4

Serpin levels in the asthmatic lung after allergen challenge.

4.1 Introduction

Asthma can be broadly divided into two types; allergic asthma and non-allergic asthma. Allergic asthma is easily recognised because it is triggered by a distinct allergen, such as cat hair or pollen (Sheffer, 1991). Whether or not an allergic response is triggered will usually depend on the degree of exposure to the allergen, as well as individual sensitivity (Barnes, 1987). The effect of an allergen is usually an acute inflammatory response, which results in bronchoconstriction and oedema, then chronic inflammation that leads to an influx of inflammatory cells. Current thinking emphasises the chronic inflammatory nature of asthma (Holgate, 1996) and many of the cell types that are involved in this disease have been implicated either in the local synthesis or the functional inactivation of α_1-AT. We have looked at the effect of allergen challenge on levels of total α_1-AT, active α_1-AT, total α_1-ACT and total chymotryptic inhibitory activity.

It was hypothesised that if the allergen were causing an influx of plasma proteins, due to increased capillary permeability, then levels of total α_1-AT would increase following allergen challenge (Gauvreau et al, 1999). An increase in total α_1-AT should bring about a parallel increase in active inhibitor. We have also looked at the effect of the commonly used steroid fluticasone propionate on this system.

4.2 Patients

This study was conducted by Wendy Moore in Baltimore, US. Twenty mild asthmatic volunteers underwent bronchoalveolar lavage (BAL) followed by allergen challenge. The following day patients underwent another BAL to examine the effect of allergen on the lung. The volunteers were then randomly assigned into two groups, of which one underwent a course of the fluticasone propionate (FP; 250μg twice a day) for 6 weeks. The other group received placebo. After the 6 weeks, the patients returned for another BAL before undergoing a final allergen challenge, 24 hours after which they underwent a final BAL. BAL was stored on dry ice before being shipped to the UK where it was stored at –70°C. The asthmatic status of these volunteers were classified by their clinical history, based on the American Thoracic Society (ATS) guidelines (ATS, 1987) and were taking as needed β2 agonists as their only medication.

4.3 Results

4.3.1 – Effect of allergen challenge on mediator levels in the asthmatic lung

Our initial aim was to examine the effect of allergen on total inhibitory activity and serpin levels in BAL. Figure 4.1 shows total chymotryptic inhibitory activity both pre and post allergen challenge. Inhibitory activity increased significantly from a median of 1430 AU pre challenge to 3547 AU post challenge (Figure 4.1 $p<0.01$).

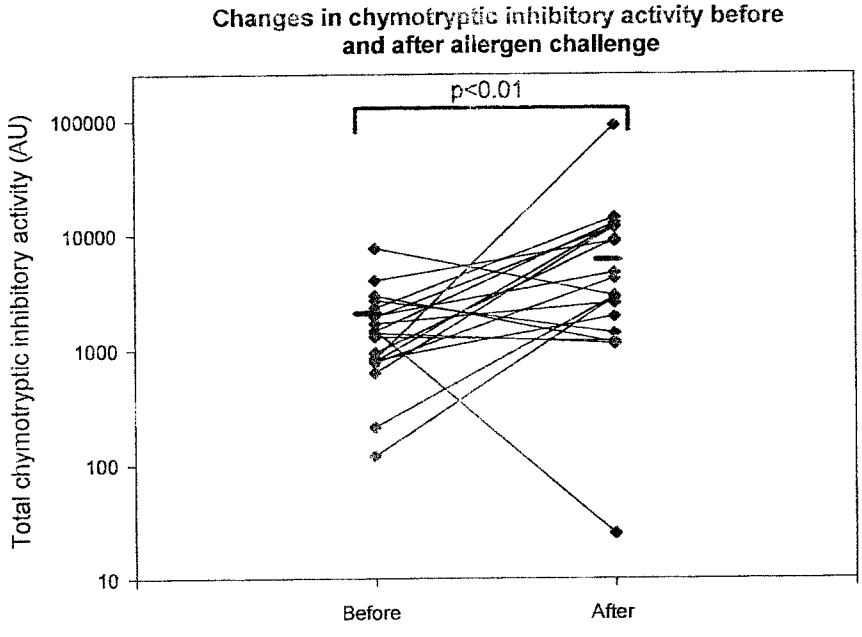

Figure 4.1 – Changes in chymotryptic inhibitory before and after allergen challenge. Mild asthmatics (n=20) underwent BAL before allergen challenge. 24 hours later they returned and underwent another BAL. BAL was then assayed for total chymotryptic inhibitory activity. Individual patients are shown by black diamond and group medians are indicated by the black bar. (Before=1430 AU, after=3547 AU, $p<0.01$) Repeated measures ANOVA was used to determine statistical significance.

Although we observed a significant increase in total chymotryptic inhibitory activity, at this point we could only speculate where this increase in inhibition was derived from. To investigate this further we examined levels of α_1-AT, active α_1-AT and α_1-ACT using specific ELISAs. Figure 4.2 shows that levels of immunoreactive α_1-AT rose from a median of 710ng/ml at baseline to 3277ng/ml 24 hours after allergen challenge. ($p<0.001$)

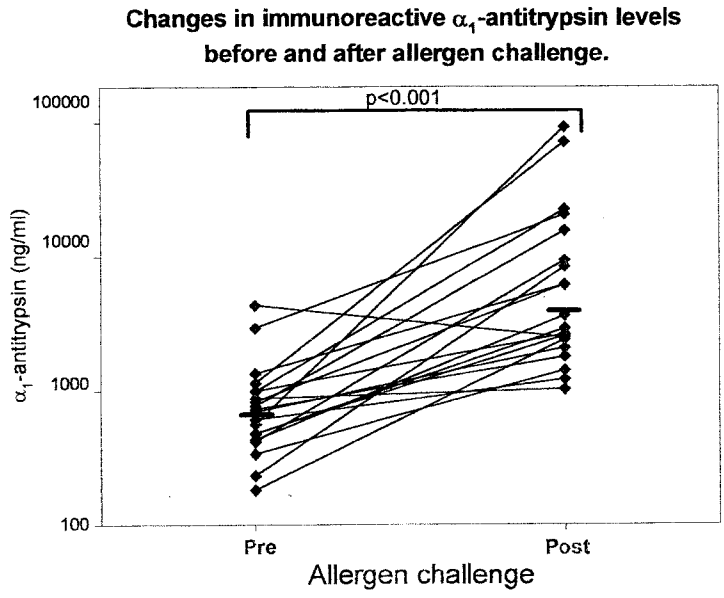

Figure 4.2 – Changes in immunoreactive α_1-antitrypsin levels before and after allergen challenge.
Mild asthmatics (n=20) underwent BAL and then underwent allergen challenge. 24 hours later they returned and underwent another BAL. BAL was assayed for immunoreactive α_1-AT by ELISA. Individual patients are shown by black diamonds, and group medians are indicated by a black bar (before=710ng/ml, after=3277ng/ml, p<0.001) Repeated measures ANOVA was used to determine statistical significance.

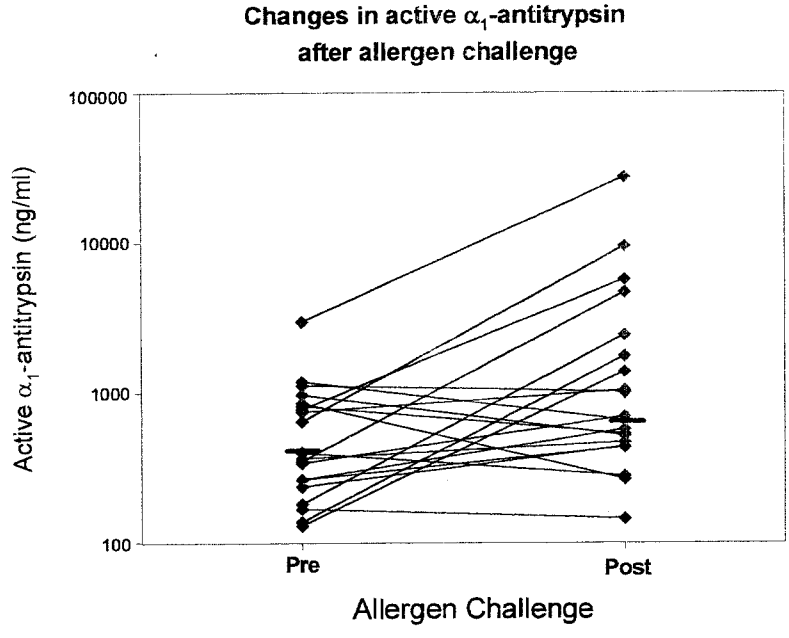

Figure 4.3 – Changes in *active* α_1-antitrypsin after allergen challenge. Mild asthmatics (n=20) underwent BAL before undergoing allergen challenge. 24 hours later they returned and underwent another BAL. BAL was then assayed for active α_1-AT. Individual patients are shown by a black diamond and group medians are indicated by a black bar. (before=377ng/ml, after=670ng/ml, p=ns) Although just under half of the patients had increased levels of active α_1-AT after allergen challenge, this was not significant. Repeated measures ANOVA was used to determine statistical significance.

Allergen challenge leads to a series of changes in inflammatory mediators and cells in the airways. It is possible that the rapid influx of α_1-AT into the lung may be counterbalanced by an equally swift inactivation, either by proteinases, oxidation of the active site methionine or simply by complexing with its substrate, elastase. To assess this we measured the levels of active α_1-AT before and after allergen challenge. Median levels of active α_1-AT were 377ng/ml prior to allergen challenge and 670ng/ml 24 hours later (Figure 4.3). Despite an almost two fold increase in active α_1-AT, this failed to reach statistical significance, indicating that the increase in α_1-AT was accompanied by its rapid inactivation. Another possibility is that the increase in α_1-AT is accompanied by an increase in its target proteinase, elastase. If the majority of α_1-AT may in fact be bound to elastase in a binary complex it will still be detected by the immunoassay but not by the activity assay.

Levels of α_1-ACT rose significantly from 232ng/ml before allergen to 549ng/ml after allergen challenge. (Figure 4.4, $p<0.05$). This was not surprising as α_1-ACT is involved in the acute phase response.

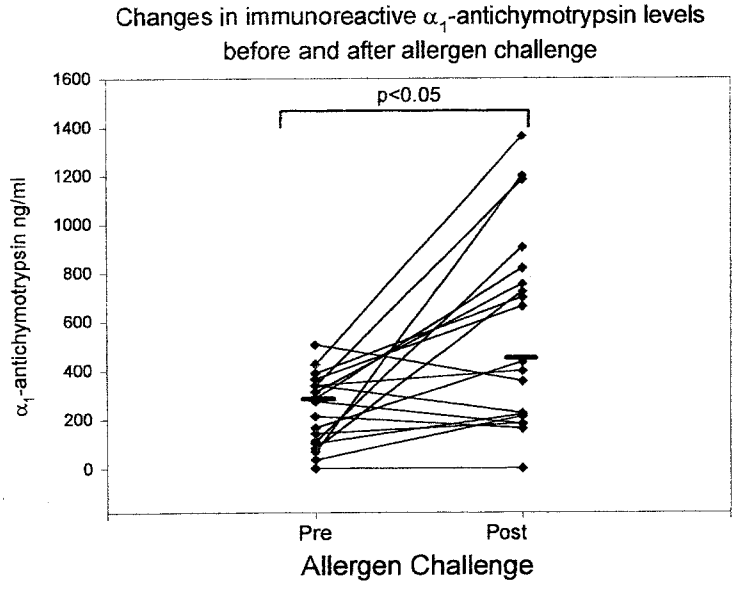

Figure 4.4 – Changes in immunoreactive α_1-antichymotrypsin after allergen challenge. Mild asthmatics (n=20) underwent BAL before undergoing allergen challenge. 24 hours later they returned and underwent another BAL. BAL was then assayed for total α_1-ACT. Individual patients are shown by a black diamond and group medians are indicated by a black bar. (before=272ng/ml, after=549ng/ml, $p<0.05$) Repeated measures ANOVA was used to determine statistical significance.

Allergen challenge results in significant increases in many mediator levels, including both SLPI and α_2-M (Table 4.1). Also shown are changes in albumin levels, total protein, total cells and neutrophil numbers.

	Pre Allergen Challenge	Post Allergen Challenge	Significance.
SLPI (ng/ml)	22	140	p<0.001 ***
α_2-M (ng/ml)	0.75	1.59	p<0.005 **
Albumin (μg/ml)	236	312	p=ns
Total protein (μg/ml)	264	562	p<0.001 ***
Total cells / ml	2.16	6.47	p<0.005 **
Neutrophils (cells / ml)	0.002	0.121	p<0.001 ***

Table 4.1. Changes in mediator levels in the asthmatic lung following allergen challenge. SLPI, α_2-M and albumin were all measured by ELISA, and total protein by BCA assay. There are significant increase in the inhibitors SLPI and α_2-M, and although albumin levels do not significantly increase total protein in the BAL more than double following allergen. Finally both the total number of cells/ml and neutrophil levels increase significantly following allergen. *(Thanks go to Dr Wendy Moore, Baltimore, USA, for the SLPI, α_2-M and cell count data)*

4.3.2 Effect of steroid on mediator levels at baseline compared to placebo

We examined the effects of a 6 week course of fluticasone propionate (250μg twice a day) compared to placebo. Although there were some fluctuations in serpin levels, (α_1-ACT especially), a 6 week course of steroid had no significant effect on either total inhibitory activity, total α_1-AT, active α_1-AT or total α_1-ACT. At first glance steroid appears to be vastly upregulating SLPI production in the lung (Medians were 8.95ng/ml pre steroid and 78.87ng/ml post steroid, see Table 4.2) However, closer inspection reveals that in 4 out of the 10 patients we actually observe a decrease in SLPI levels (See Table 4.2). Thus, the apparent increase in SLPI secretion does not quite reach significance. Does steroid have a general anti-inflammatory effect at baseline? One indicator of an anti-inflammatory action of fluticasone would be a reduction in the amount of plasma proteins in BAL such as albumin, in conjunction with a decrease in leukocyte number. Although we observe an apparent blunting of the plasma influx (albumin mediator levels drop from 190μg/ml to 122μg/ml following 6 weeks of steroid) this is not significant. Levels of α_2-M, another plasma protein appeared to be blunted, but again this was not significant (median levels were 0.54μg/ml compared to 0.35μg/ml post steroid). Due to low n numbers (some as low as 5) it is extremely difficult to draw any conclusions concerning white cell influx. However, total

cell numbers (n=9) do not change significantly following steroid. In fact, the only significant effect of the 6 week course of steroid is an increase in PD20 (3.45 compared to 19.90 following steroid, $p<0.05$, figure 4.5). Here is some evidence then, that steroid is having a beneficial therapeutic effect on asthmatic patients at baseline.

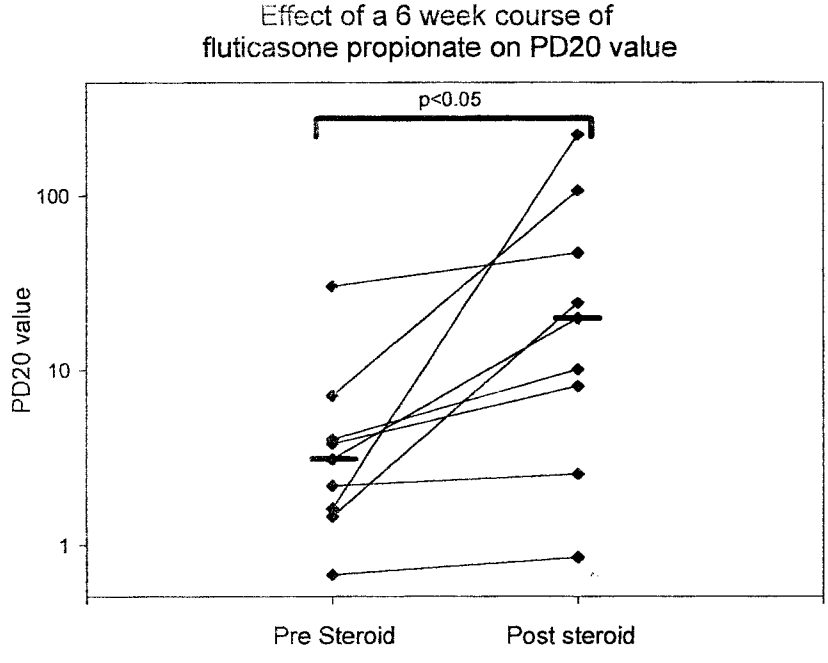

Figure 4.5 – Effect of fluticasone propionate on the PD20 value in asthmatics. Mild asthmatics (n=9) underwent a 6 week course of the steroid fluticasone. Their PD20 value was assessed both before steroid and after the treatment. Following the course PD20 levels rose significantly from a median of 3.8 to 19.9. ($p<0.05$) individual patients are shown by a black diamond and medians indicated by a black bar. Repeated measures ANOVA was used to determine statistical significance. *(Thanks go to Dr Wendy Moore, Baltimore, USA, for the PD20 data)*

	Pre Steroid	Post Steroid	Significance
Total inhibitory activity (AU)	1344	1617	p=ns
α_1-AT (ng/ml)	710	631	p=ns
Active α_1-AT (ng/ml)	304	385	p=ns
α_1-ACT (ng/ml)	325	157	p=ns
SLPI (ng/ml)	8.9	78.8	p=ns
α_2-M (ng/ml)	0.54	0.35	p=ns
Albumin (µg/ml)	190	122	p=ns
Total Protein (µg/ml)	293	296	p=ns
Total Cells / ml	2.1	1.9	p=ns
Neutrophils (cells/ml)	0.0018	0.0042	p=ns

Table 4.2. Changes in mediator levels in the asthmatic lung flowing a 6 week course of the steroid fluticasone proplonate. α_1-AT, α_1-ACT, SLPI, α_2-M and albumin were all measured by ELISA and total protein by BCA assay. Although there are some fluctuations in mediator levels, particularly α_1-ACT and SLPI, these changes are not significant. *(Thanks go to Dr Wendy Moore, Baltimore, USA, for the SLPI and cell count data)*

4.3.3 Effect of steroid on mediator levels following allergen challenge

We went on to investigate the effect of allergen challenge on asthmatic patients that had been receiving the aforementioned steroid therapy. Prior to steroid there was a significant increase in total inhibitory activity following allergen challenge. However, post steroid treatment and allergen, this increase was not observed. Levels were 1617AU pre allergen and only 1808AU post allergen challenge, data summarised in table 4.3.

Figure 4.6 shows the effect of allergen challenge on α_1-AT levels prior to treatment. The median value after allergen *and* steroid rose from 631ng/ml before challenge to 2688ng/ml ($p<0.05$). This increase in α_1-AT did not deviate significantly from observations made *prior* to steroid following allergen.

Levels of active α_1-AT following steroid and allergen challenge rose from a median of 385ng/ml to 757ng/ml although this was not a significant increase (Data summarized in Table 4.3). However, levels of α_1-ACT significantly rose from 157ng/ml prior to allergen following steroid to 324ng/ml after allergen.

The effect of fluticasone propionate and allergen challenge on other mediators is summarized in table 4.3.

	Pre steroid		Post steroid	
	Pre Allergen	**Post Allergen**	**Pre Allergen**	**Post Allergen**
Total inhibitory activity (AU)	1344	2908*	1617	1808
Total α_1-AT (ng/ml)	710	4535 *	631	2688 *
Active α_1-AT (ng/ml)	304	1017	385	757
Total α_1-ACT (ng/ml)	325	663 *	157	324 *
SLPI (ng/ml)	8.95	163 *	78.87	9.77 *
α_2-M (ng/ml)	0.54	1.85 *	0.35	0.74 *
Albumin (μg/ml)	190	147	122	371 *
Total protein (μg/ml)	293	580 *	296	395
Total cells / ml	2.17	9.27 *	1.98	5.46 *
Neutrophils (cells/ml)	0.002	0.133 *	0.042	0.403 *

Table 4.3 The effect of steroid on mediators in the lung following allergen challenge. A comparison between pre and post steroid. An asterisk indicates a significant change compared to pre allergen. α_1-AT, α_1-ACT, SLPI, α_2-M, and albumin were measured using ELISA and total protein measured using a BCA assay. *(Thanks go to Dr Wendy Moore, Baltimore, USA, for the SLPI, α_2-M and cell count data)*

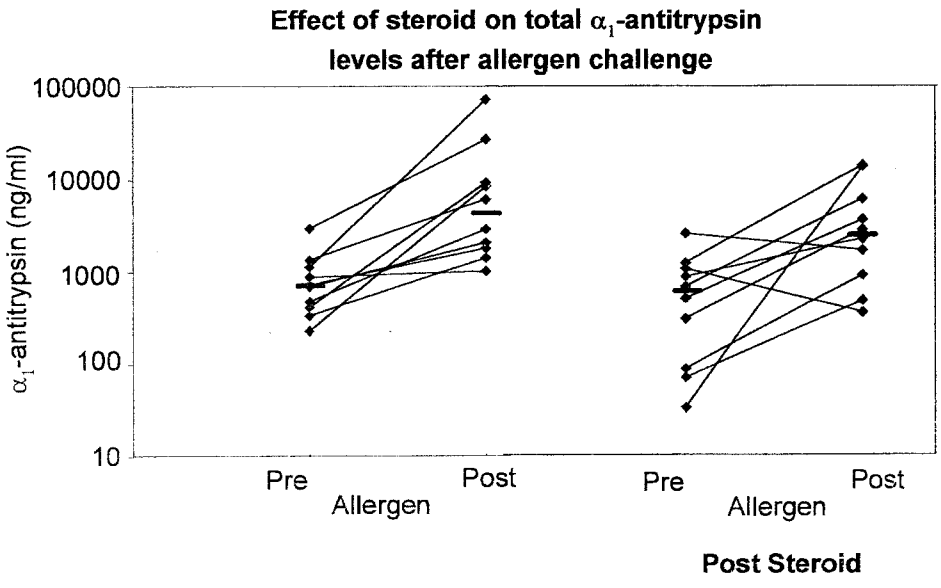

Figure 4.6 – Effect of steroid on total α_1-antitrypsin levels after allergen challenge. Mild asthmatics (n=10) underwent a 6 week course of the steroid fluticasone and BAL was then assayed for immunoreactive α_1-AT before and after an allergen challenge. Individual patients are shown by a black diamond and group medians indicated by a black bar. After allergen challenge, α_1-AT significantly increases, both prior to (before=710ng/ml, after=4535ng/ml, $p<0.01$), and after steroid treatment.(before=631ng/ml, after=2688ng/ml, $p<0.05$) Repeated measures ANOVA was used to determine statistical significance.

The increase in α_1-AT following allergen challenge may represent an influx of the plasma derived protein, local synthesis or a combination of the two. Since albumin is believed to be the main constituent of plasma we chose to examine the molar ratio of α_1-AT /albumin reasoning that this may indicate selective accumulation of α_1-AT (Figure 4.7). If the ratio at which *different* plasma proteins enter into the lung can be assumed to constant, then the ratio of α_1-AT to albumin should remain constant, but only if the majority of α_1-AT in the lung is plasma derived. Medians before steroid rose from $4.8x10^{-3}$ before challenge to $24.7x10^{-3}$ 24 hours after allergen challenge ($p<0.05$). This suggests that α_1-AT accumulates selectively in the airways following allergen challenge either through local synthesis, or by secretion from migratory cells, entering the lung after the allergen challenge. However, post steroid the medians increase only slightly

and not significantly from $6.7x10^{-3}$ to 9.4 $x10^{-3}$. As levels of albumin increase significantly following steroid and allergen, this distortion in the α_1-AT /albumin ratio suggests that steroid is inhibiting the production of locally produced α_1-AT following allergen challenge.

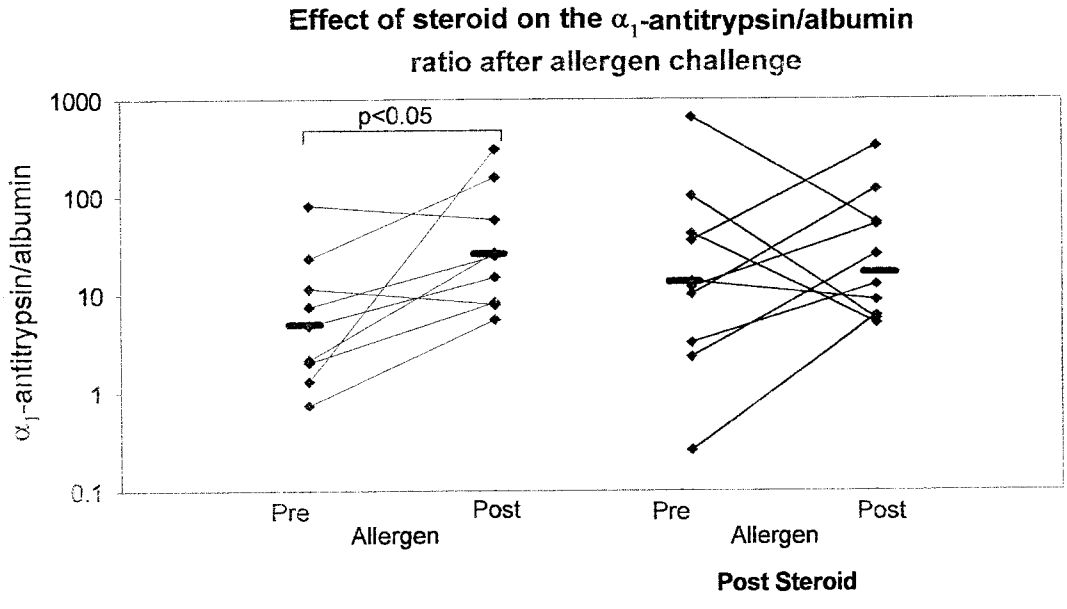

Figure 4.7 – Effect of steroid on the α_1-antitrypsin/albumin ratio after allergen challenge. Before the steroid treatment, the α_1-AT/albumin ratio increases after allergen challenge from $4.8x10^{-3}$ to $24.7x10^{-3}$. ($P<0.05$) Following the 6 week steroid course, this trend was abolished. Individuals are shown by a black diamond and medians by a black bar. Medians are $6.7x10^{-3}$ before challenge and $9.4x10^{-3}$ after challenge. Repeated measures ANOVA was used to determine statistical significance.

4.4 Discussion

In the majority of subjects after allergen challenge, the levels of α_1-antitrypsin increased significantly. This can be interpreted in three ways; as a rapid and major influx of plasma proteins into the lung, caused by increased capillary permeability and inflammation due to the allergen challenge, by the upregulation of locally produced serpin by the lung epithelia or other cells types native to the lung, or by an inflammatory response brought on by the process of bronchoscopy itself. This is an important factor that must be taken into consideration when examining this data. Subjects underwent BAL prior to allergen challenge as a control, and 24 hours later underwent a second BAL to examine the effect of allergen. It is very difficult to assess what effect the process of BAL would have on the lungs of these subjects, but an observed mild inflammatory response would not be surprising. However, we must take this data at face value if we wish to make any kind of interpretations.

There is data to support the first hypothesis concerning an influx of plasma proteins, as we observe an increase in BAL of the plasma based inhibitor α_2-M. However we did not find an increase in albumin following allergen challenge. The data seems to contradict itself, but is supported by findings made by Liu et al, 1991, in which a similar dose of ragweed gave no evidence of plasma influx in asthmatics. Although they did not measure α_2-M, they did observe a significant increase in total cell number similar to data observed in this study. This apparent lack of plasma influx following allergen challenge was also observed in a study conducted by Shaver et al 1995, in which no significant changes in albumin levels were observed following ragweed inhalation. This discrepancy between the significant increase in the plasma-based protein α_2-M and the non significant changes in the plasma-based protein albumin following allergen is best interpreted as a selective accumulation of antiproteinases in response to allergen. There *is* evidence of an inflammatory response in the lungs of these asthmatic patients. Significant increases in total cell number, neutrophil numbers and total protein in BAL following allergen challenge are all evidence for inflammation in the lung.

Although most subjects had an increase in total α_1-antitrypsin following allergen, not all had increased levels of *active* α_1-antitrypsin. This non-significant increase suggests that not all of the asthmatics have the ability to cope with the α_1-AT inactivation following allergen challenge. If the majority of α_1-antitrypsin is active as it enters the lung, then we can assume that the α_1-antitrypsin is becoming rapidly oxidised, clipped or complexed with elastase released from migrating neutrophils (which significantly increase following allergen challenge). Another important thing to note is the difference between chymotryptic inhibitory activity between these subjects and those in the previous chapter inhaling ozone. Total chymotryptic inhibitory activity in these asthmatics appears to be significantly higher that then asthmatics in the previous chapter. This can partly be explained by a difference in the volume of saline distilled into the lungs during the lavage procedure in these two trials. The previous trial used a total of 180mls of saline for the process of BAL, whereas this trial used just 80ml, half the original volume. A more concentrated BAL sample would result in an apparent increase in total inhibitory activity. However, if this was the case, then α_1-AT should also have increased in these subjects. However, this is not the case as baseline levels of α_1-AT are 710ng/ml for this study and 804ng/ml for the ozone study, not a significant difference. It is therefore difficult to interpret this apparent increase in total inhibitory activity in this asthmatic group compared to the previous sample group.

The 6 week course of fluticasone propionate appeared to have no effect on any of the variables we were measuring in BAL compared to placebo, with the exception of the PD20 value. Every single individual had an increase in his or her PD20 values indicating a clear therapeutic benefit to steroid treatment. Other such benefits have been observed in previous studies including a significant increase in morning peak expiratory flow rate in asthmatic children following fluticasone treatment (MacKenzie et al, 1993) and numerous other therapeutic benefits (Kerrebijn 1990 and Reed 1990). There are a number of studies (Nocker et al, 1999, Schleimer 1993, Williams and Yarwood 1990) that indicate glucocorticoids such as fluticasone propionate have an effect of reducing the microvascular permeability and plasma influx, but we have not observed such an effect in thus study. Levels of plasma proteins such as albumin and α_2-M do not significantly decrease *or* increase following steroid treatment. It seems then, that in this study

fluticasone is only having *some* of the clinical benefit that has been observed in earlier trials. However, although the dose of fluticasone given in this trial was as high as, or higher than doses in previous trials, the length of treatment was slightly lower. Previous data studies used a 12-week (Nocker et al, 1999) and an 8-week time course (Llewellyn-Jones et al, 1996), whereas the patients in this study had a 6-week treatment of flutica7sone. It is difficult to say if an increased time course for treatment would have had more beneficial effects, but this is certainly one factor that could be contributing to our observations.

Generally, the effect of fluticasone on mediator levels and reducing inflammation following allergen challenge was minimal in this study. Total inhibitory activity did not significantly increase following allergen challenge compared to placebo, even though levels of α_1-AT and α_1-ACT significantly increased following allergen (similar to the pre-steroid data). It is not clear why fluticasone propionate would be having the effect of reducing total inhibitory activity, especially if the drug was having an anti-inflammatory effect and reducing white cell influx. However, in this study steroid does not appear to have any anti-inflammatory effects following allergen challenge. Neutrophil levels significantly rise, as do albumin levels, suggesting fluticasone is not reducing plasma influx *or* blunting the migration of leukocytes. This supports the findings made by Ek et al (2000), in which they examined the effect of fluticasone on reducing the inflammatory response produced by another allergen, organic dust. Their findings suggested that fluticasone propionate has no major effects on airway inflammation induced by the inhalation of allergen.

It was thought previously that the majority of α_1-AT in the lung after allergen challenge came from the plasma. We looked at albumin concentrations in the lung as a marker of plasma exacerbation. If the majority of α_1-AT in lung was indeed due to diffusion from the plasma, then the ratio of α_1-AT to albumin should remain constant after allergen. We observed a significant rise in the α_1-AT / albumin ratio after allergen challenge suggesting that there is another significant source of α_1-AT after allergen challenge. The observed increase in locally produced SLPI following allergen challenge makes the epithelial cells the prime candidate for local α_1-AT production. I hypothesize that the local production of serpins such as α_1-AT may be playing a more significant role in airway homeostasis than is currently believed.

Chapter 5

α_1-antitrypsin and α_1-antichymotrypsin production by human lung fragments

5.1 Introduction

Although the main site of action for α_1-AT and α_1-ACT is thought to be the lung, the majority of both inhibitors originates from the liver. The liver secretes these inhibitors into the plasma where they circulate before diffusing into the lung. However, the previous chapter suggested that local production of the serpins α_1-AT and α_1-ACT may play a significant role in lung homeostasis and recent papers had highlighted the fact that lung epithelial cells have the ability to secrete α_1-AT and α_1-ACT (Cichy et al 1997, Cichy et al 1995). Even if this microenvironment produces relatively little inhibitor, the surface area of the lung is so vast that the combined output could be significant. We therefore decided to investigate production of these inhibitors from both human lung tissue fragments and from primary broncho-epithelial cells. These two systems are complementary and both offer distinct advantages as experimental models to examine lung serpin synthesis. Lung epithelial cells are a more accurate model for the lung surface that is exposed to outside antigens etc. whereas the lung parenchyma is typically located in the underlying matrix of the lung. However, the epithelial cell model is limited in that it does not truly represent the mixed cell population that is found in human lung. The complex interactions that may occur, for example, between matrix and migratory cells would not occur in the epithelial cell model. However, these interactions are accessible through the lung fragments. This chapter will discuss the findings from our lung fragment model. Methods for the preparation of lung tissue are described in Chapter 2.4.1.

5.2 Results

5.2.1 – Production of α_1-AT and α_1-ACT by lung fragments over 24 hours.

Firstly it was important to determine relative amounts of inhibitor that were being produced by lung fragments. We looked at levels of α_1-AT and α_1-ACT that were produced by unstimulated lung fragments over 24 hours and the results are shown in figure 5.1.

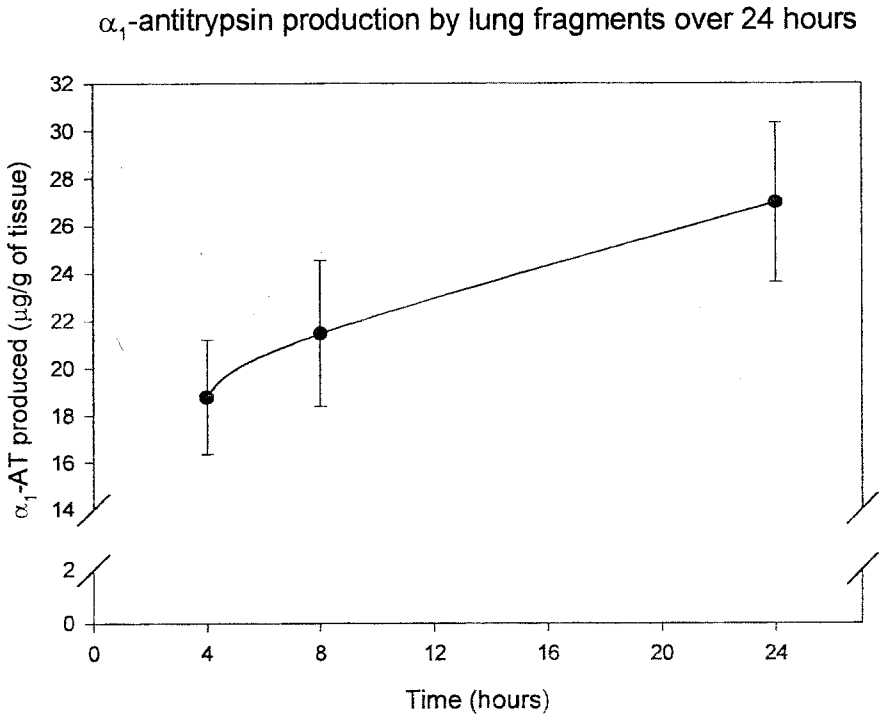

Figure 5.1 – Levels of α_1-AT produced by lung fragments over 24 hours. Lung fragments (n=9) were incubated for 24 hours and α_1-AT secreted into the medium measured using ELISA and then adjusted according to weight of fragments. Median levels rise to an average of just under 30μg/gm lung tissue after 24 hours.

As figures 5.1 and 5.2 show, lung fragments secrete significant amounts of both α_1-AT and α_1-ACT. Levels of both inhibitors are detectable after 4 hours rising to 30μg/gm lung tissue after 24 hours for α_1-AT, compared to 22μg/gm lung tissue for α_1-ACT. The decreased gradient of the line after 8 hours can be attributed to two things. Firstly, if the serpins are being released from pre-formed stores, these reserves could be used up rapidly in the first 4 hours and be heavily depleted by 24 hours. Secondly, the cells could be synthesising serpins at a constant rate but the lung tissue could be slowly dying, resulting in less viable cells to secrete the protein.

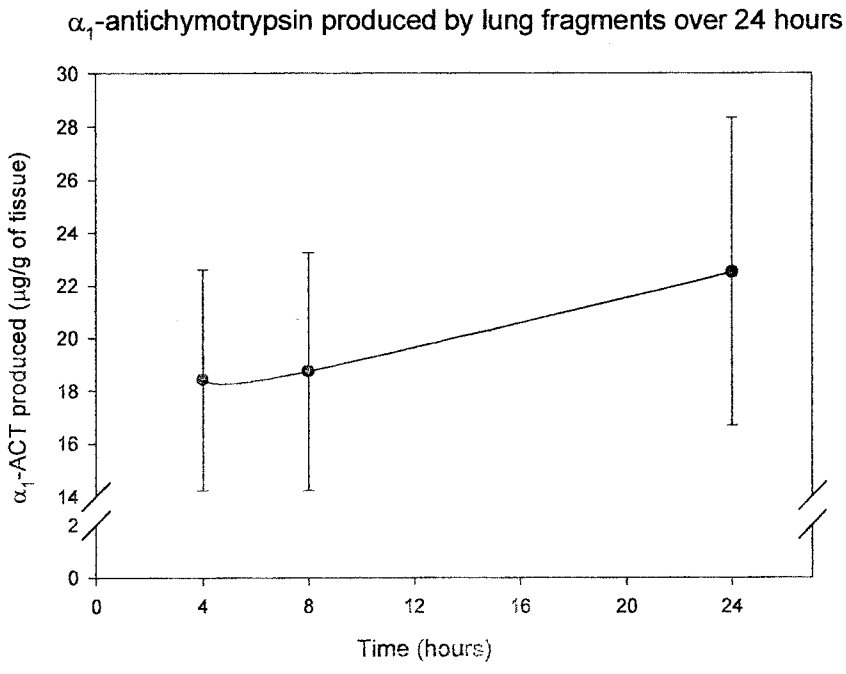

Figure 5.2 – Levels of α_1-ACT produced by lung fragments over 24 hours. Lung fragments (n=9) were incubated for 24 hours and α_1-ACT secreted into the medium is measured using ELISA and then adjusted according to weight of fragments. Median levels rise to over 22 µg/ gm lung tissue after 24 hours.

5.2.2 Protein inhibition by cylohexamide treatment.

There are two possible sources of inhibitors secreted from epithelial cells; both α_1-AT and α_1-ACT may be released from presynthesied intracellular stores or by *de novo* synthesis. To examine this we used cyclohexamide treatment followed by pulse chase radiolabelling. We incubated the fragments overnight with a concentration of 10µg/ml cyclohexamide before starting a time course the next day. We then used pulse chase techniques as described in chapter 2.4.5 to determine the relative levels of newly made inhibitor compared to that which was presynthesised. Initially we examined levels of total protein produced by the lung fragments following cyclohexamide treatment over 8 hours. This was to determine how effective this does of cyclohexamide was at inhibiting protein production. Our results are shown in figure 5.3.

Having shown that this level of cyclohexamide inhibited protein synthesis in the lung we then went on to examine the effect of cyclohexamide on levels of α_1-AT and α_1-ACT secreted from lung fragments over 24 hours. Results are shown in figures 5.4 and 5.5.

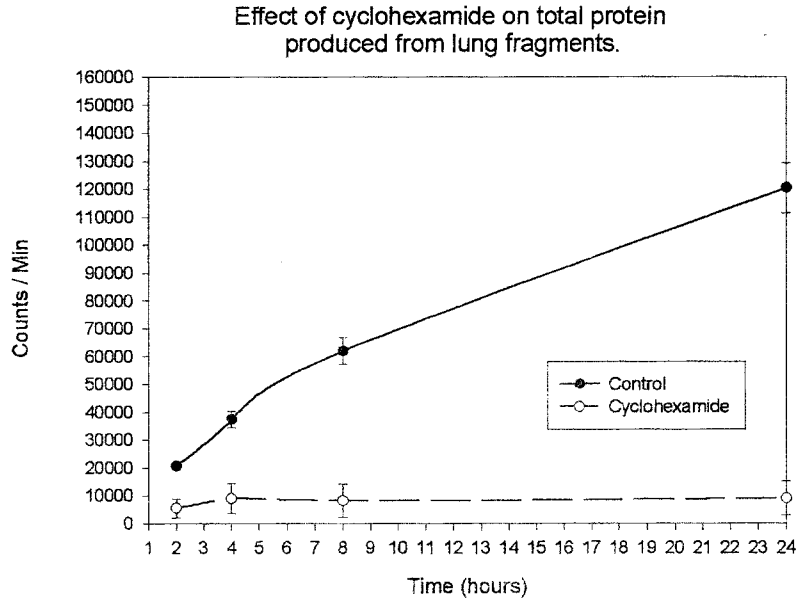

Figure 5.3 – Effect of cyclohexamide on total protein produced from lung fragments (n=3) Lung fragments were incubated with10μg/ml of cyclohexamide for 26 hours. Fragments were then resuspended in new medium and levels of total radiolabelled protein secreted was measured using a scintillation counter. Control levels rise to over 60,000 counts/min, compared to the cyclohexamide protein count which never rises above 10,000/min counts.

Initial observations suggest that cyclohexamide did not inhibit the *secretion* of α_1-AT or α_1-ACT from lung fragments into the medium. There was a slight decrease in inhibitor production after 2 and 4 hours, although after 24 hours, levels of both α_1-AT and α_1-ACT were comparable to control and this initial inhibition was not significant. Because total protein production is inhibited, this time dependent secretion of α_1-AT and α_1-ACT into the medium suggests either that we are observing the release of preformed inhibitors that have been stored intracellularly or that we are observing significant necrosis of lung cells after 24 hours and upon death these cells release α_1-AT and α_1-ACT into the medium.

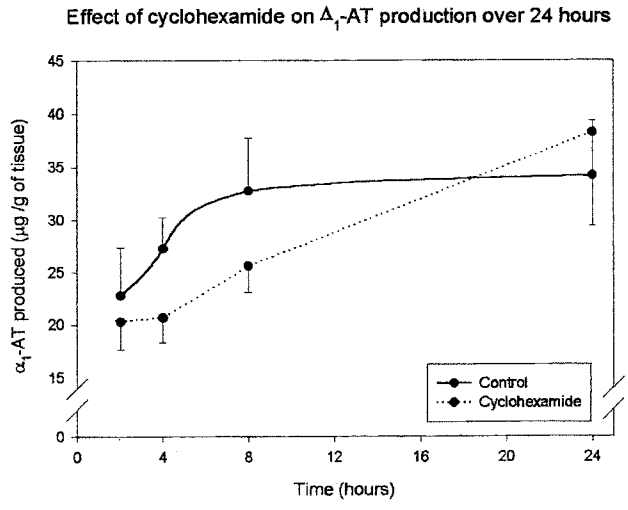

Figure 5.4 – Effect of cyclohexamide on α_1-AT produced by lung fragments over 24 hours. Lung fragments (n=3) are treated with cyclohexamide for 16 hours before being placed into fresh medium for 24 hours. Levels of α_1-AT secreted are measured using ELISA and adjusted for lung fragment weight. Although there appears to be an initial blunting of total α_1-AT production, after 8 hours, levels are comparable to control after 24 hours.

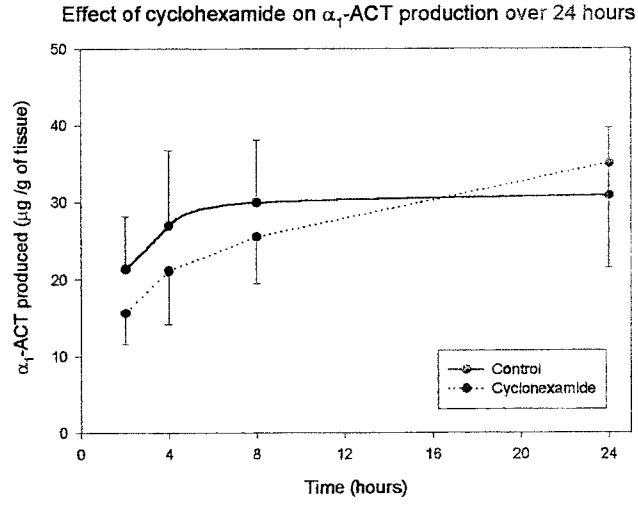

Figure 5.5 – Effect of cyclohexamide on α_1-ACT produced by lung fragments over 24 hours. Lung fragments (n=3) are treated with cyclohexamide for 16 hours before being placed into fresh medium for 24 hours. Levels of α_1-ACT secreted are measured using ELISA and adjusted for lung fragment weight. Similarly to α_1-AT, initially there appears to be a blunting of α_1-ACT levels, but after 24 hours levels are comparable to control.

5.2.3 Effect of fluticasone propionate on serpin levels secreted by lung fragments.

We then went on to examine the effect of the corticosteroid fluticasone propionate and several mediators on these fragments. Firstly we used three concentrations of fluticasone; these were 10^{-6} M, 10^{-7} M and 10^{-8} M and figure 5.6 shows the effect of fluticasone on levels of α_1-AT produced by lung fragments.

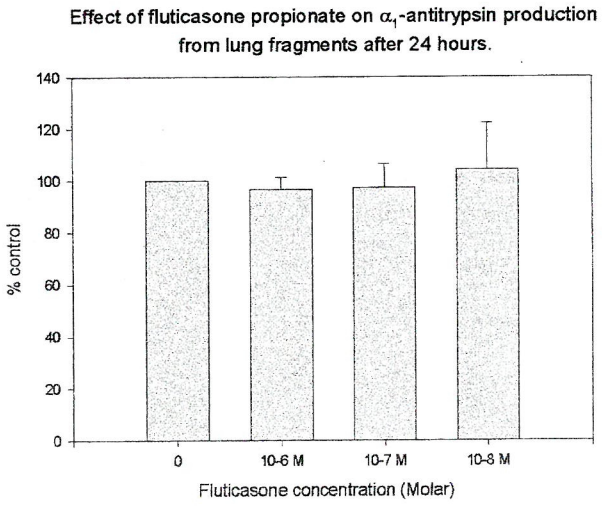

Figure 5.6 – Effect of fluticasone propionate on α_1-AT levels secreted from lung fragments over 24 hours. Lung fragments (n=6) were incubated with various concentrations of fluticasone propionate for 24 hours. Levels of α_1-AT secreted into the medium were measured using ELISA and adjusted for lung weight. Baseline levels of α_1-AT were 13μg/gm lung tissue after 24 hours for control.

There were no significant differences between the levels of α_1-AT secreted from lung fragments that had been exposed to *any* concentration of fluticasone compared to control. Figure 5.7 shows the effect of fluticasone on levels of α_1-ACT produced by lung fragments.

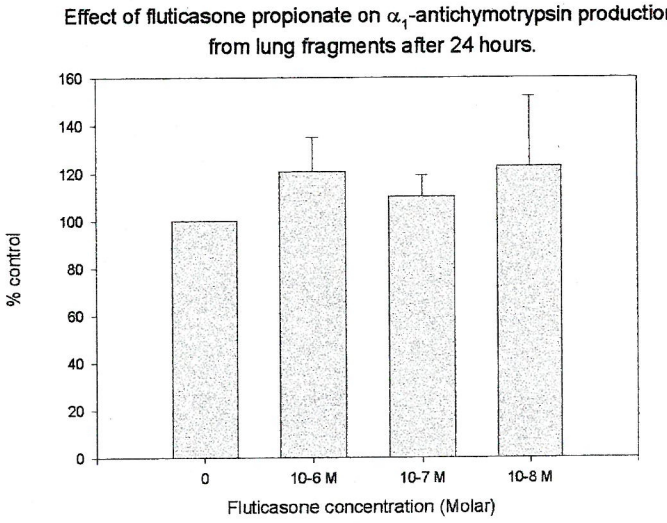

Figure 5.7 – Effect of fluticasone propionate on α_1-ACT levels secreted from lung fragments over 24 hours. Lung fragments (n=6) were incubated with various concentrations of fluticasone propionate for 24 hours. Levels of α_1-ACT secreted into the medium were measured using ELISA and adjusted for lung weight. Baseline levels of α_1-ACT were 7μg/gm lung tissue after 24 hours for control.

Although there is a slight increase in α_1-ACT levels produced by lung fragments that have been exposed to fluticasone, these variations are not significant. Indeed, no concentration of fluticasone causes levels of α_1-ACT to rise significantly above control values.

5.2.4 The effect of anti-IgE on serpins secreted from lung fragments

We used an antibody to immunoglobulin E to cross-link any IgE receptors present in the lung fragments such as those on mast cells. We were interested in the levels of serpins produced as a result of this cross-linking and incubated lung fragments for 24 hours with 50μg/gm of anti-IgE antibody. Our results are shown in figures 5.8 and 5.9. Levels of α_1-AT rise to 25μg/gm after 24 hours for anti-IgE compared to 15μg/gm for control.

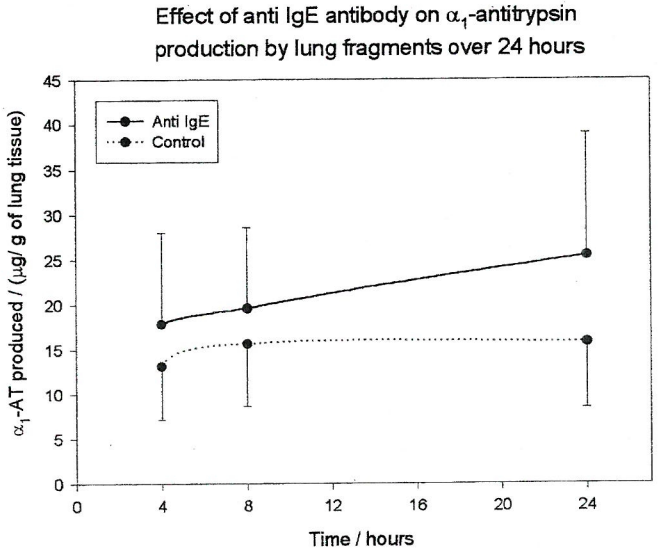

Figure 5.8 – Effect of anti-IgE on α_1-AT production from lung fragments after 24 hours. Lung fragments (n=4) were exposed to 50µg/ml of anti IgE antibody for 24 hours. Levels of α_1-AT secreted into the medium were measured using ELISA and adjusted for lung weight.

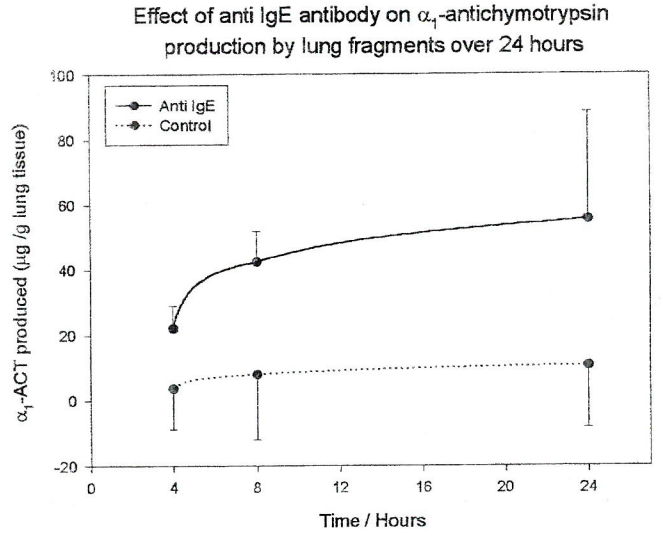

Figure 5.9 – Effect of anti-IgE on α_1-ACT production from lung fragments after 24 hours. Lung fragments (n=4) were exposed to 50µg/ml of anti IgE antibody for 24 hours. Levels of α_1-ACT secreted into the medium were measured using ELISA and adjusted for lung weight.

Again, levels of α_1-AT secreted by lung fragments were significantly higher than α_1-ACT (15µg/gm for control α_1-ACT compared to 5µg/gm for α_1-ACT). However, the effect of anti-IgE

was more pronounced on levels of α_1-ACT than for α_1-AT, with levels of α_1-ACT in the anti-IgE treated lung being double that of control.

5.2.5 – Effect of proteinases on serpin production by lung fragments

Previous work has shown that elastase has the ability to upregulate SLPI production in airway epithelial cells. We were interested in examining the effects of proteinases on lung fragments and whether exposure to proteinases such as elastase would result in an upregulation of α_1-ACT and α_1-AT. We exposed lung fragments to the *serine* proteinase porcine pancreatic elastase (PPE) but also used the *cysteine* proteinase papain as a control. We also inactivated PPE by exposure to PMSF for 15 minutes at room temperature. PMSF irreversibly binds proteinases, rendering them inactive, but keeping their protein structure intact.

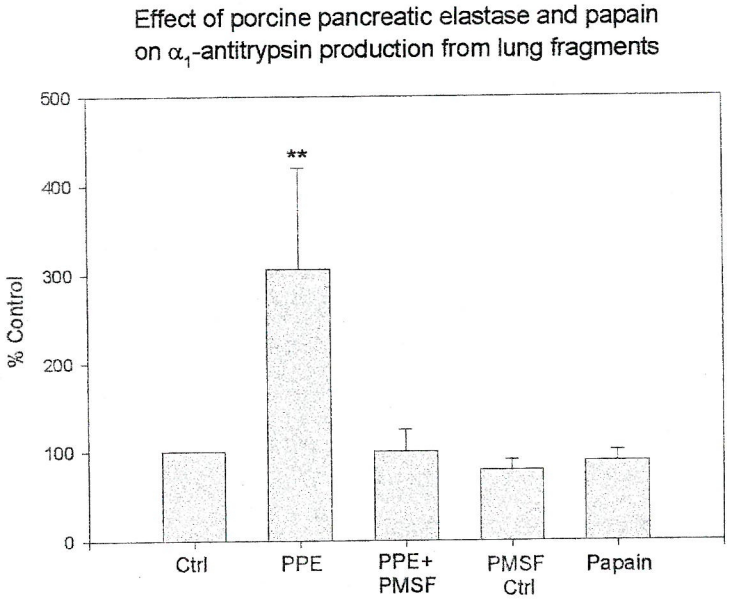

Figure 5.10 – Effect of the serine proteinase PPE, inactivated PPE and the cysteine proteinase papain on α_1-AT production from lung fragments after 24 hours. Lung fragments (n=5) were incubated for 24 hours with either 10μg/ml of PPE, 10μg/ml PMSF treated PPE, PMSF on its own (ctrl) or 10μg/ml papain. α_1-AT secreted into the medium was measured using ELISA and adjusted for lung weight. Data is shown as a percentage of control. Control release of α_1-AT was 27μg/gram lung tissue.

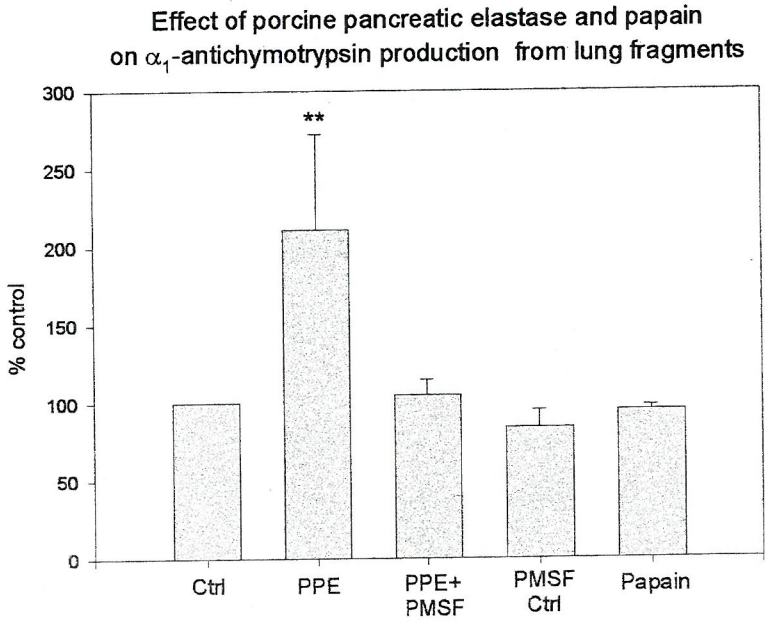

Figure 5.11 – Effect of the serine proteinase PPE, inactivated PPE and the cysteine proteinase papain on α_1-ACT production from lung fragments after 24 hours. Lung fragments (n=5) were incubated for 24 hours with either 10μg/ml of PPE, 10μg/ml PMSF treated PPE, PMSF on it own (ctrl) or 10μg/ml papain. α_1-ACT secreted into the medium was measured using ELISA and adjusted for lung weight. Data is shown as a percentage of control. Baseline levels of control were 22μg/gram lung.

Following PMSF treatment the PPE has less than 2% of pretreated activity. The effects of all these treatments are shown in figures 5.10 and 5.11. As shown, PPE triggers a significant response ($p<0.01$) in serpin secretion from lung fragments, resulting in a 300% rise and 220% rise in α_1-AT and α_1-ACT production respectively. However, this is only observed if the PPE is active. PMSF treated PPE shows no response, nor does the cysteine proteinase papain.

5.3 Discussion

The first important observation that we made was that human lung fragments have the ability to synthesize significant amounts of both α_1-AT and α_1-ACT. Over 24 hours, levels can rise to as high as 30μg/gm of lung tissue for α_1-AT and 22μg/gm for α_1-ACT. These inhibitors may be coming from a variety of sources, as lung fragments are a mixed cell population. They contain the main lung parenchyma i.e. the underlying tissue of the lung, as well as neutrophils, and some

macrophages. The majority of macrophages are washed out during the chopping process and so it is unlikely that these are a main contributor to the serpin levels observed. Secreted levels of α_1-AT and α_1-ACT are so high that it is also doubtful these serpins could be produced by the neutrophils alone. Levels of α_1-AT and α_1-ACT continue to rise up to 48 hours after incubation (data not shown) suggesting that there is actually a progressive source of inhibitor, most likely within the lung parenchyma itself.

Serpin levels continue to rise even after the application of cylohexamide, a substance that should inhibit total protein synthesis. There are several explanations for this. Several cell types have the ability to store serpins, and these may be responsible for the increase in serpin following cylcohexamide. Neutrophils are just one of the cell types that can store α_1-AT intracellularly (Paakko et al, 1996; Du Bois et al, 1991; Mason et al, 1991) Macrophages, monocytes and lymphocytes also express the α_1-AT gene, and can act as storage cells for this serpin (Mornex et al, 1986; Yuan et al 1992, Ikuta et al, 1982). Finally, the lung itself may be sequestering serpins, although there is no literature to support this hypothesis.

Data in the previous chapter had suggested that fluticasone propionate had no effect on serpin levels in the lung. In our lung fragment model, serpin levels neither increased nor decreased following a 24 hour incubation of fluticasone propionate. Although the lung fragments were only exposed to fluticasone propionate for 24 hours, compared to the 6 week course the asthmatic patients underwent, this data still supports observations made in the previous chapter. The fact that fluticasone has no effect on serpin levels is surprising. Not only is the corticosteroid-blinding gene located on the same cluster as the serpin genes (14Q32.1) but other studies have demonstrated that corticosteroid treatment results in a decrease in α_1-AT in sputum (Rollini and Fournier, 1997; Stockley et al, 1986).

The effect of anti-IgE was far more pronounced. Even after 4 hours of incubation with anti-IgE there was a significant increase in levels of both α_1-AT and α_1-ACT. After 24 hours, levels of α_1-AT had increased to 25μg/gm for anti-IgE compared to 15μg/gm for control. For α_1-ACT, levels had increased to 10μg/gm for anti-IgE treated fragments compared to just 5μg/gm for control. It is clear then, that the cross linking of IgE receptors in lung parenchyma triggers a

cascade of events that lead to the release of α_1-AT and α_1-ACT. The exact nature of these events is still unclear, but we can hypothesize that if mast cell IgE receptors were crosslinked then histamine would be released by the mast cells into the medium (Nissen et al 1998).

Lung epithelia exposed to proteinases secrete the inhibitor SLPI (Marchand et al, 1997), and therefore we exposed the lung fragments to the serine proteinase porcine pancreatic elastase. The effects were a significant increase in both α_1-AT and α_1-ACT. This suggests a negative feedback mechanism, by which the lung can protect itself from proteolytic attack. We did not look at SLPI levels secreted into the medium due to cost of assays, but this would have been a most interesting experiment. There is some speculation as to the exact role that SLPI plays in the lung. It has been suggested that SLPI is heavily involved in lung homeostasis (Kouchi et al 1993), and this was supported by the fact that SLPI is secreted by lung epithelia in response to proteolytic attack. However, we have demonstrated that lung fragments also secrete α_1-AT and α_1-ACT after proteinase exposure. We have demonstrated that the upregulation of α_1-AT and α_1-ACT in response to PPE is dependant on a proteolytic event, rather that the recognition of a certain structure by inactivating PPE with PMSF. After PMSF treatment, the inactive PPE had no effect on α_1-AT or α_1-ACT production by lung fragments. This would prove beneficial as once the proteinases have become inactive by binding to inhibitor, they are no longer a threat. If α_1-AT and α_1-ACT were produced in response to structure recognition there would be a surplus of inhibitor in the lung, distorting the delicate of balance of proteinase and inhibitor. A surplus of inhibitor would result in a loss of overall proteolytic activity resulting in an impaired host defence mechanism against inhaled pathogens etc. The likelihood is that elastase is acting on a proteinase activated receptor (PAR) on the surface of the epithelial cells. PAR-2 is present on a diverse range of human tissue including lung epithelial cells (Bohm et al, 1996). These PARs are cleaved by proteinases such as trypsin and elastase, and have numerous activities including lung fibroblast proliferation (Akers et al, 2000). It is easy to imagine a feedback mechanism whereas proteinases acting on PARs results in a series of events leading to serpin synthesis by lung epithelial cells. However, to date there is no evidence to prove that PAR activation leads to serpin upregulation. However, there *is* evidence of a specific serpin-enzyme complex receptor (SEC).

Once PPE has bound to α_1-AT, this complex has been shown to activate SEC receptors that are capable of modulating α_1-AT production (Perlmutter et al, 1990). This would explain the increase in serpin levels following PPE exposure.

This lung fragment model could be used to examine a variety of other stimuli, which could include the effect a variety of proteinases, other than just PPE, the effect of allergens and the effect of oxidative stress on the lung. However the biggest limitation of this model is the fact that the results are difficult to interpret due to the mixed cell population present. We have addressed this problem in the next chapter in which we examined serpin production from a single cell population, primary bronchoepithelial cells.

Chapter 6

Generation of α_1-antitrypsin and α_1-antichymotrypsin by lung epithelial cells.

6.1 Introduction

The lung is composed of a variety cell and tissue types, all of which come together in a structured and organised manner to make up an effective and working organ. Cell types in the lung that have the ability to produce the serpins α_1-AT and α_1-ACT include migratory cells such as the neutrophil and macrophage, but also the epithelial cells lining the surface of the lung. The previous chapter suggested that α_1-AT and α_1-ACT are secreted in large quantities by a mixed cell population extracted from the lower human lung parenchyma. The next logical step was to examine the production of these inhibitors in a single cell population which would narrow down the effects of different cell to cell interactions and give us a clearer insight into the effects of stimuli on one specific area of the lung. We examined the role of the bronchoepithelial cell in the production of these serpins, as this cell type is the first to encounter any external stimuli that may be inhaled. The bronchoepithelial cell also represents an entirely different area of the lung than was examined in the previous chapter. Lung tissue fragments from Chapter 5 were taken primarily from the underlying parenchyma which consists not only of white cell types, but also of a variety of matrix proteins and even blood vessels. The epithelial cells used in the following model comprised of a monolayer of homologous cells. In comparison to lung fragments, there is a wealth of information concerning epithelial cell serpin production. But the majority of research seems to be aimed at the effect of a well known inflammatory mediator, oncostatin M (OSM). In this chapter we examine the effect of different stimuli, including, but not limited to OSM on serpin production by primary bronchoepithelial cells.

It is important to note at this time the difficulty in comparing this model with the lung fragment model in the previous chapter. Initially we tried to conduct a cell count on the epithelial cells and express our data as α_1-AT produced per 10^{-6} cells. However, the cell count proved inherently difficult to achieve and upon conferring with several sources at Southampton General Hospital it was determined that a more practical way would be to lyse the cells and conduct a total protein count using the BCA assay. Therefore all values would be expressed in the α_1-AT / μg/ protein format. This study was developed after the lung fragment study in which values were expressed as α_1-AT/ μg lung tissue weight. It would be very useful to compare serpin secretion

from these two models directly, but this proved difficult, as the weight of a monolayer of epithelial cells is incredibly difficult to determine. Similarly, the initial experiments using our lung fragment model could not be repeated due to shortages of lung tissue and these fragments were no longer available to assay for protein content. To date, my research has found no method to accurately determine the total protein content of a given piece of tissue, other that using a BCA assay.

6.2 Results

Initially we examined levels of α_1-AT and α_1-ACT produced by bronchoepithelial cells after 24 hours. We also examined the effect of OSM on the production of these serpins. We chose to examine the effect of OSM as recent literature showed OSM had the effect of drastically upregulating serpin production. These initial experiments aimed to reproduce this data and to characterise our cell model. Figure 6.1 shows the effect of 10ng/ml OSM on serpin production by bronchoepithelial cells compared to control after 24 hours incubation.

Levels of both α_1-AT and α_1-ACT significantly increase ($P<0.005$) following incubation with oncostatin M for 24 hours. Initial observations revealed that levels of α_1-ACT were 11 times higher than levels of α_1-AT at baseline (levels were 0.84ng/ml for α_1-AT and 9.4ng/ml for α_1-AT $p<0.01$). This was surprising, as α_1-AT is believed to be the more important of the two inhibitors. 10ng/ml OSM significantly increases levels of both α_1-AT and α_1-ACT, although appears to affect α_1-AT levels more. Levels rose from 9.4ng/μg protein to 15.1ng/μg protein for α_1-ACT ($p<0.01$, a doubling of inhibitor) compared to a four fold increase in α_1-AT (level rose from 0.84 to 3.36, $p<0.005$).

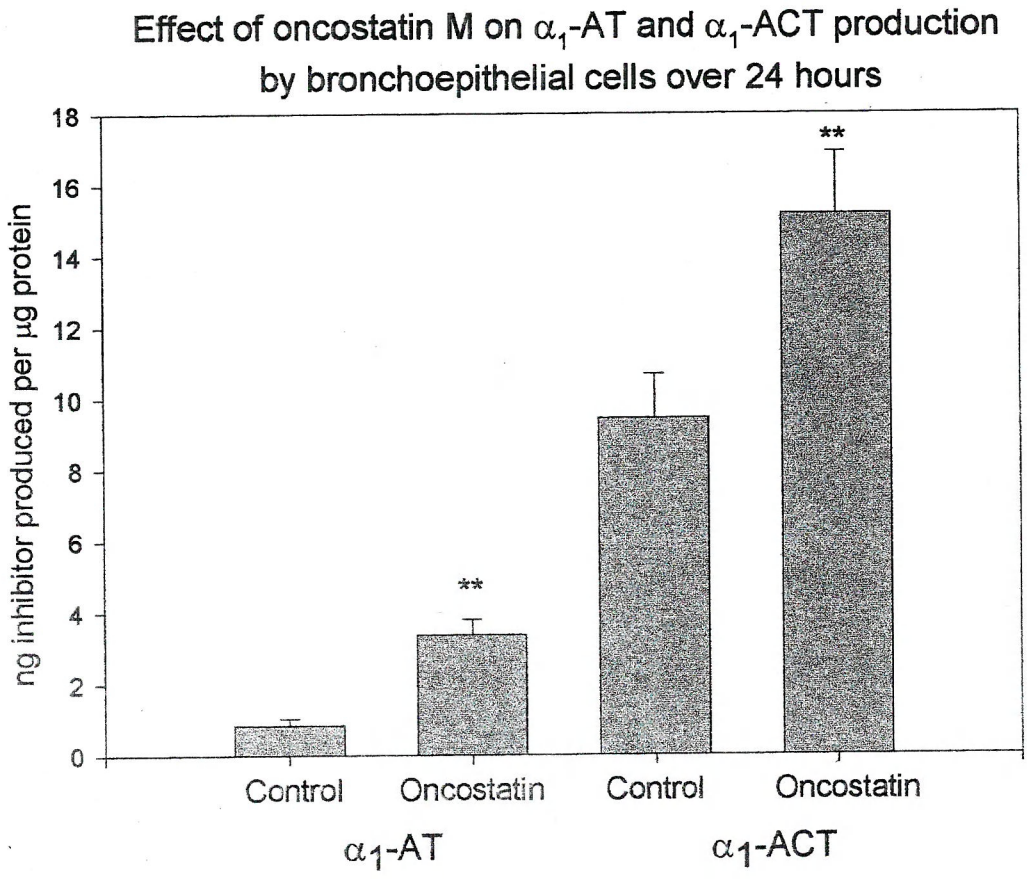

Figure 6.1 - Effect of 10ng/ml oncostatin M on α_1-AT and α_1-ACT production by bronchoepithelial cells after 24 hours compared to control. Levels are shown as amounts of inhibitor produced per µg of cell protein.

Having confirmed that our cell model was consistent with current literature we went on to examine the effects of a number of different mediators on serpin production by bronchoepithelial cells. This included fluticasone propionate, anti-IgE antibody, a number of different proteinases including porcine pancreatic elastase (PPE), papain, and finally Der p1. Our results are summarised in Table 6.1.

	α_1-AT (%Control)	α_1-ACT (% Control)
Oncostatin M (10ng/ml)	307 **	168 *
Fluticasone (10^{-6}M)	110	90
Anti-IgE anitbody	76	123
PPE (1μg/ml)	298 **	151 *
PPE (10μg/ml)	724 **	234 **
PMSF/PPE	420 **	100
PMSF Control	150	100
Papain (10μg/ml)	126	98
Der p1 (10μg/ml)	108	82

Table 6.1 – Effect of various mediators on serpin production by bronchoepithelial cells after 24 hours. Values are shown as a percentage of control. The effect of oncostatin M, fluticasone, anti-IgE antibody, porcine pancreatic elastase (PPE), PPE and poly PMSF, papain and Der p1 and all shown. Significant increases in inhibitor are indicated with an asterisk.

The serine proteinase PPE had a significant effect on both α_1-AT and α_1-ACT. Figure 6.2 and 6.3 shows the effect of this proteinase on α_1-AT and α_1-ACT production respectively, compared to control and to the other proteinases examined. It also appeared that the PPE was effecting inhibitor production in a dose dependent manner. PPE concentrations of 1μg/ml significantly increased both α_1-AT and α_1-ACT (levels rose to 300% and 151% to that of control, respectively) and 10μg/ml of PPE increased serpin production further to 700% and 250% to that of control for α_1-AT and α_1-ACT respectively. Although PPE upregulates both α_1-AT and α_1-ACT production, similarly to OSM, it appears to be having a more pronounced effect on α_1-AT levels. We went on to prove that the PPE induced upregulation of serpins was dependent on a preoteolytic event rather than by structure recognition. To achieve this we inactivated the PPE using the non-specific proteinase inhibitor PMSF. PMSF binds irreversibly to proteinases rendering them inactive, but retains their secondary and tertiary structure.

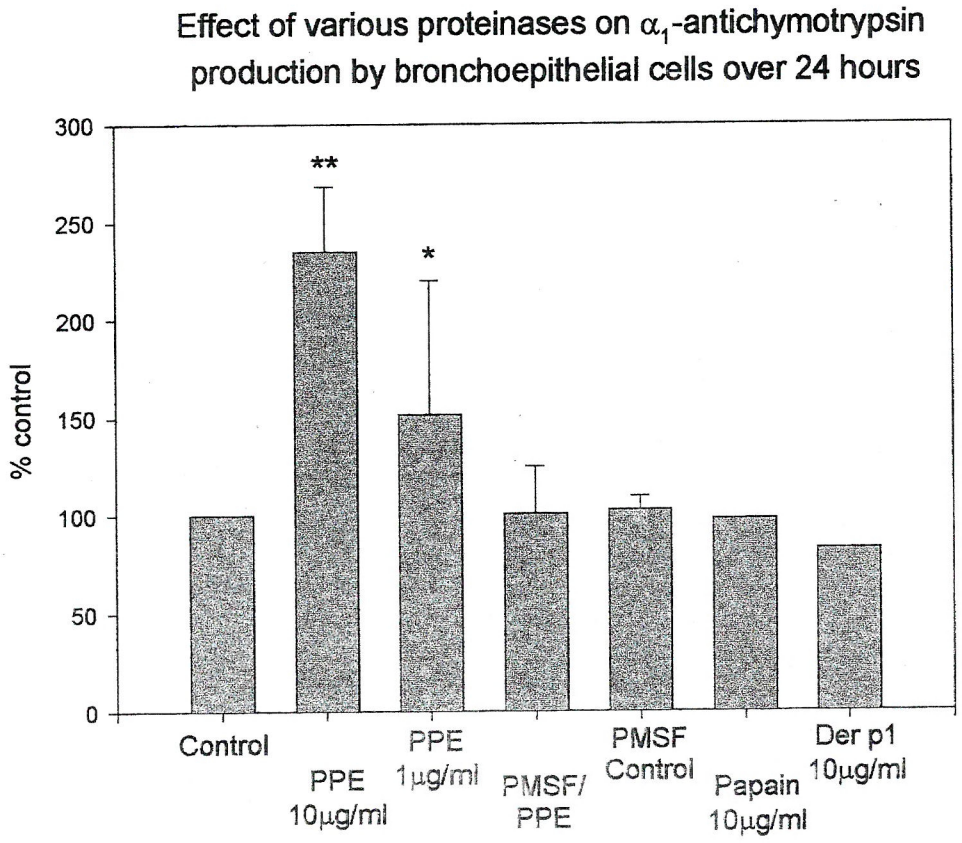

Figure 6.2 - Effect of various proteinases on α_1-AT production by bronchoepithelial cells over 24 hours. Bronchoepithelial cells were treated with a variety of proteinases for 24 hours. α_1-AT secreted into the medium was measured using an α_1-AT ELISA. Values are shown as a percentage of control, and significant increases in α_1-AT indicated by an asterisk.

We first showed that a 15-minute incubation at room temperature of a 2:1 molar ratio of PMSF to PPE resulted in the complete loss of proteolytic activity (Data not shown). PMSF treated PPE applied to epithelial cells brought about no increase in α_1-ACT levels after 24 hours compared to the 230% increase in α_1-ACT observed with active PPE (Figure 6.3). The effect of PMSF treated PPE on α_1-AT production appeared to be somewhat different.

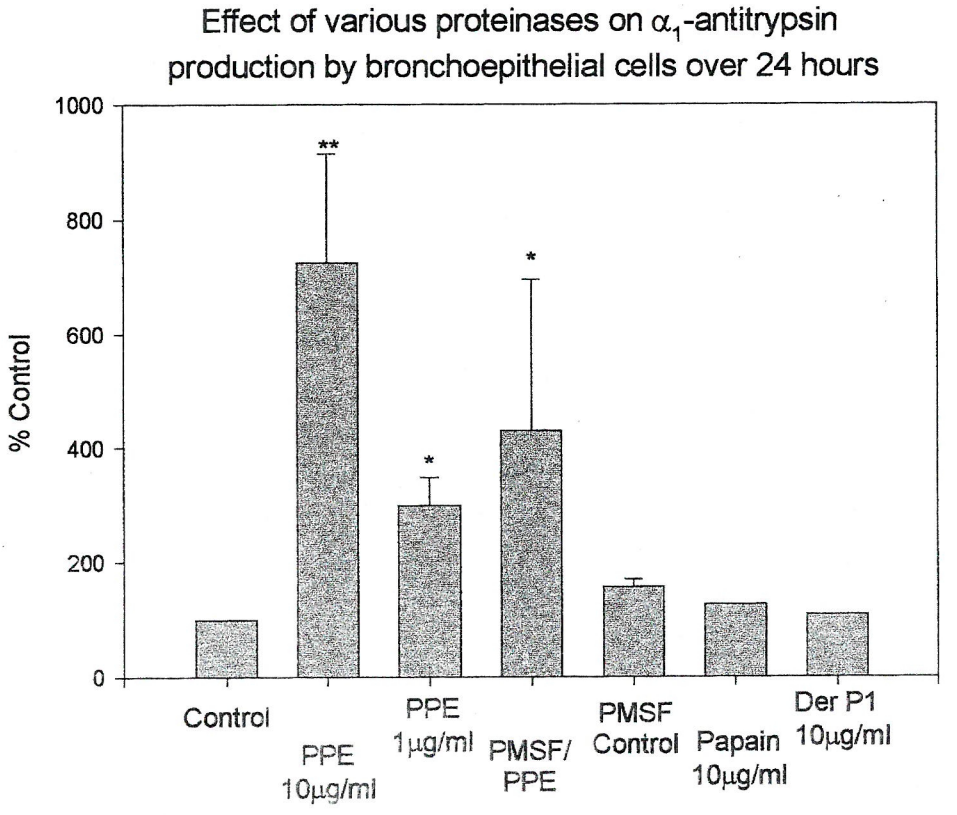

Figure 6.3 - Effect of various proteinases on α_1-ACT production by bronchoepithelial cells over 24 hours. Bronchoepithelial cells were treated with a variety of proteinases for 24 hours. α_1-ACT secreted into the medium was measured using an α_1-ACT ELISA. Values are shown as a percentage of control, and significant increases in α_1-AT indicated by an asterisk.

Although the PMSF/PPE treated cells secreted significantly lower levels of α_1-AT compared to the cells treated with active PPE, there was still a significant increase compared to control (levels of α_1-AT were 725% of control for active PPE treated cells compared to 420% of control for PMSF/PPE treated cells, figure 6.2). PMSF by itself had no effect on serpin production by our epithelial cell model.

Finally we examined the effects of different families of proteinases on our epithelial cell model. Our hypothesis was that only treatment with serine proteinases would result in the upregulation of serpins by bronchoepithelial cells. We therefore used the cysteine proteinase

papain (10μg/ml) as well as the dust mite derived antigen Der P1 that has been shown to have cysteine proteinase activity. The effect of these stimuli on serpin production is shown in figures 6.2 (α_1-AT secretion) and figure 6.3 (α_1-ACT secretion). A 24 hour incubation of papain had no effect on either α_1-AT or α_1-ACT production. Similarly, 10 μg/ml of Der P1 had no effect on serpin production after 24 hours.

6.3 Discussion

Our initial results demonstrated that primary lung epithelial cells have the ability to secrete detectable levels of both α_1-AT and α_1-ACT over 24 hours. This confirmed previous data from Venembre et al (1994), Cichy et al (1995, 1997) and Sallenave et al (1997) that showed epithelial cell lines have the ability to secrete α_1-AT and α_1-ACT. What was surprising was the *relative* amounts of inhibitor secreted by our bronchoepithelial cells. α_1-ACT levels were 10 times higher than α_1-AT levels (9.4ng/ml compared to just 0.84 ng/ml for α_1-AT). This interesting observation suggests that α_1-ACT may be playing a more central role in the lungs defence than is currently believed. The major inhibitor in the lung is believed to be α_1-AT, and yet our data clearly shows that it is α_1-ACT that is the dominant of the two serpins, at least in terms of local production. Unfortunately an accurate assay for measuring active α_1-ACT is not available and so it is difficult to determine what percentage of secreted α_1-ACT is actually active. What is not in doubt, however, is the effect of the inflammatory mediator OSM. A 10ng/ml dose of OSM drastically upregulated both α_1-AT and α_1-ACT in our epithelial cell model. Again this supports previous data showing an upregulation of serpin production in epithelial cells following OSM treatment (Cichy et al, 1995, Cichy et al 1997, Sallenave et al 1997). This is further evidence to suggest that our model is sound and that the difference in α_1-AT and α_1-ACT production is real. Anti-IgE, which resulted in a significant increase in both α_1-AT and α_1-ACT in whole lung fragments had no effect on our cell model. However, this is not surprising considering there is no evidence from literature to suggest that anti-IgE receptors are present on the surface of bronchoepithelial cells. Of all the stimuli we applied to our model only OSM and PPE induced any changes in serpin production after 24 hours. PPE actually had a more pronounced effect on

serpin production than OSM, and levels rose to 700% to that of control for α_1-AT and 300% compared to control for α_1-ACT. Although PPE appeared to have a more substantial effect on α_1-AT levels, it is difficult to believe that even this rise in α_1-AT could compare with resting levels of α_1-ACT. The increased expression of proteinase inhibitors by the lung, induced by exposure to proteinases has been observed before. SLPI transcription levels have been shown to increase following aerosolised elastase inhalation *in vivo* (Marchand et al 1997). We were unable to measure SLPI production in the cell supernatants and so had no way of studying the effects of proteinases on SPLI production in our cell model. However, this increase in serpin expression induced by PPE is indirect evidence to suggest that proteinase receptors are present on the surface of our epithelial cells. We know that the activation of these receptors must be a proteolytic event rather than the recognition of the particular conformation due to treatment with PMSF. Inactivated PPE resulted in the complete loss of any upregulation of α_1-ACT observed in the cell supernatants treated with active PPE. However, although PMSF treated PPE blunted the increase in α_1-AT levels, there *was* a rise and it *was* significant. Initially believed to be due to PPE not becoming completely inactivated by the PMSF we re-assayed for activity but found none. This observation of increasing α_1-AT levels by inactive PPE is difficult to interpret. Levels of inhibitor were so small in fact, that a modified ELISA standard curve had to be used. At these reduced levels, α_1-AT concentration becomes increasingly difficult to measure and interpret.

This phenomenon of increased serpin expression was limited to serine proteinases, of which the serpins are specific inhibitors. A similar dose of papain, a cysteine proteinase derived from plants, had no effect on serpin production whatsoever. Finally we looked at the effect of the house dust mite derived antigen Der p1 on serpin expression by epithelial cells. There is still some dispute over the interaction between Der p1 and α_1-AT. This antigen has been shown to have proteolytic activity (Hewitt et al, 1997), and can catalytically inactivate α_1-AT at the reactive centre loop (Kalsheker et al, 1996). However, α_1-AT has been shown to actually inhibit Der p1 activity (Hewitt et al 1995). We were interested to see if the combined effect of an antigen and proteolytic attack would result in any increase in serpin production. However, 24 hours of Der p1

treatment resulted in no observed increase in either α_1-AT or α_1-ACT levels compared to control. Der p1 is a cysteine proteinase (Chambers et al, 1997), and we have already demonstrated that another cysteine proteinase, Papain, had no effect on serpin production by epithelial cells. Clearly there is a substantial amount of work to be conducted in this area. Firstly, how significant is the observed difference between α_1-AT and α_1-ACT levels? Are there any mediators that induce an increase in α_1-ACT but not α_1-AT and vice versa? Our preliminary data suggests that this cell type does not contribute significantly to α_1-AT production and perhaps the majority of α_1-AT in BAL is derived from plasma and, perhaps more importantly, the lung parenchyma, underlying the epithelial cells.

Chapter 7

General discussion

7.1 General discussion

Asthma is a multifaceted disease that even in the 21st century is not entirely understood. New research into this disorder includes studying patients at the genetic level (Clarke et al, 2000) as well as developing new drugs to combat its devastating physiological effects (Cazzola et al, 2000). What is clear, however is that this disease involves extensive airway remodeling (Redington, 2000). This remodeling is due to a number of homeostatic proteinases, which have the potential to cause extensive lung damage if not kept in check by the lungs natural defence, the antiproteinases (Bernstein et al 1994). The area of research I have been concerned with involves possibly the most important proteinase inhibitor in the lung, α_1-antitrypsin (Carell et al 1982). More precisely I have investigated methods of inactivation of this inhibitor and its response to stress, such as allergen challenge. α_1-AT is very sensitive to inactivation by a variety of means. This can include polymerisation of the molecule (Lomas et al, 1992), cleavage of the reactive centre loop by non-target proteinases or by oxidation of the reactive center loop (Smith et al 1986). All of these render the protein inactive as an inhibitor in the lung. It has been hypothesized that one of the contributing factors to the lung damage associated with asthma, and a number of other lung diseases such as emphysema, could be a decrease in the natural antiproteinase shield (Gaillard et al 1993, Van Vyve et al 1994). A general inactivation of one of the lungs most important antiproteinases, α_1-AT, could contribute to a harmful lung environment (Gaillard et al 1992). There have been a number of studies comparing levels of antiproteinases such as α_1-AT in asthmatics to controls, and so far these reports are conflicting. Some state that levels of antiproteinases are impaired in asthma (Van Vyve et al 1994), whereas others state just the opposite (Vignola et al 1998). Although these two studies were conducted using a similar sample group of asthmatic patients, methods of α_1-AT detection were different. Van Vyve et al used a more accurate immunoradiometric method whereas Vignola et al used the older nephelometric assay. The differences observed may simply be a discrepancy in the measuring techniques.

It is also important to gain a deeper understanding of the sources of α_1-AT, as current thinking emphasizes that it is mainly plasma derived (Banda et al 1988). However, recent papers

have highlighted the fact that local production may be playing a more important role than originally believed (Venembre et al, 1994; Cichy et al, 1995; Cichy et al 1997). My initial research involved the development of several in house ELISA assays so we could examine the levels of total immunoreactive α_1-AT, active α_1-AT, α_1-ACT and albumin. We also developed a kinetic assay for detecting total chymotryptic inhibitory activity. We obtained a series of samples from a study investigating the effects of ozone exposure on asthmatic and normal subjects. The hypothesis was that asthmatics have a reduced anti-oxidant shield in their lungs, making them more susceptible to oxidative stress (Kelly et al 1999). The ozone may, in fact, have two consequences within the lung. Firstly, ozone provokes an inflammatory response, which leads to migrating cells such as the neutrophil to infiltrate the lung in large numbers (Kelly et al 1995). These cells can release cytotoxic agents, which not only have the ability to degrade the lung, but can also inactivate the very proteinase inhibitors that are in place to inhibit this reaction (Bernstein et al 1994). Secondly, ozone can react with macromolecules in the epithelial lining fluid to generate further oxidising agents that may inactivate these proteinase inhibitors (Smith et al 1986). In this study we investigated levels of total chymotryptic inhibitory activity, levels of total immunoreactive α_1-AT, as well as examining inflammatory cell counts. We found that the total inhibitory capacity in asthmatic BAL was not compromised compared to control subjects. In fact asthmatics actually had significantly higher levels of immunoreactive α_1-AT compared to controls. This supported the findings from an earlier study (Sibille et al, 1998), which showed elevated levels of α_1-AT in asthmatic BAL compared to control. This suggests that in the asthmatic lung there are adequate levels of proteinase inhibitors to deal with day to day fluctuations in proteolytic insult. However, we went on to show that asthmatics are not well equipped to deal with oxidative stress in the lung. Following ozone inhalation total inhibitory activity was severely compromised. This indirectly supported a previous study which demonstrated a reduction in α_1-AT following ozone exposure *in vitro* (Smith et al, 1987). There are no clinical studies in current literature that mimic the exact conditions of this ozone exposure study, however Devlin at al in 1996 using an ozone exposure that was twice as concentrated as this study actually demonstrated an increase in α_1-AT from asthmatic BAL. Our data points to further investigations, which would include

examining levels of anti-oxidants and perhaps other clinical trials involving other sources of oxidation such as nitrogen dioxide and sulphur dioxide as these stimuli result in similar mediator changes in BAL (Mohsenin and Gee, 1987, Johnston et al, 2000). The lung damage and remodeling seen in asthmatics may simply be a reflection of their living environment. Unless asthmatics living in highly polluted areas upregulated their antioxidant defences, they would certainly be more prone to oxidative damage than those living in rural, cleaner environments. To date there is no evidence of an increased antioxidant shield in asthmatic BAL.

There certainly appears to be a link between increased air pollution and increased asthma prevalence (Anderson et al, 1998; Yu et al, 2000) and it has been suggested airway epithelial cells may respond to oxidant air pollutants (particularly sulphur dioxide, ozone and nitrogen dioxide) by the activation of transcription factors such as NFκB (Barnes, 1995). This would result in the increased transcription of genes for cytokines such as IL-8 and other inflammatory enzymes. If we also consider that high levels of air pollution leads to decreased levels of α_1-AT in plasma, the combined effect could be devastating to the lung (Dziegielewska et al, 1993).

I have also examined the effect of allergen challenge and the effect of steroid on levels of inhibitors in the lung. We received samples from a group of asthmatics both before and after allergen challenge. We found that following allergen challenge levels of total immunoreactive α_1-AT increased dramatically and this supports previous data that demonstrate increased levels of α_1-AT in nasal secretions in atopic asthmatics compared to controls (Westin et al, 1999). To investigate the source of this α_1-AT further we re-plotted the data as a ratio of α_1-AT to albumin. We found there was a significant difference between α_1-AT and albumin ratios before and after challenge, suggesting that there is another important source of lung α_1-AT other than plasma. Our data indicated that the answer could lie with either local production by epithelial cells or from migrating leukocytes as both cell types have the ability to synthesise the serpin (Paakko et al, 1996; Cichy et al, 1997). Levels of α_1-AT correlated with both neutrophils and macrophages following allergen challenge, and so this suggested either that these migrating cells were the main source of α_1-AT or that these cell types stimulate the local production of α_1-AT. Levels of SLPI, an inhibitor exclusively secreted locally in the lung also increased significantly after

allergen. We have no direct evidence to suggest that allergen increases local α_1-AT production by epithelial cells as the correlation between inflammatory cells and α_1-AT may simply have been coincidental.

We also looked at levels of active α_1-AT and total chymotryptic inhibitory activity following allergen challenge. Although total α_1-AT levels increased significantly, levels of *active* α_1-AT or chymotryptic inhibition did not rise. This was potentially very exciting data, suggesting that although more α_1-AT is entering the lung (from whatever source), a greater percentage is becoming inactivated once it arrives. The most likely mechanism for this would be inactivation by neutrophil proteinases, as cell numbers increase drastically following allergen, and there is the potential to release significant quantities of cytotoxic agents which can inactivate α_1-AT (Damiano et al, 1988).

Following allergen, the sample group had been split into two, one group receiving placebo, the other receiving a course of the inhaled steroid Fluticasone Propionate. The subjects then underwent BAL before and after a further allergen challenge. This would enable us to examine the effect of steroid, if any, on baseline levels of α_1-AT, and on levels of α_1-AT following allergen challenge. We showed that steroid only had mild therapeutic effects on the asthmatic lung. PD20 value rose significantly in all patients, but these effects were not as marked as observations made by Llwellyn-Jones et al in 1996 or Nocker et al in 1999.

Following the steroid course *and* allergen challenge, levels of inflammatory cells increased as they did prior to steroid. Levels of albumin also increased dramatically after steroid and allergen. These pieces of evidence suggested that steroid did not actually reduce the inflammatory response following allergen challenge. However this can be explained by the fact that one of fluticasone propionates' main actions is the reduction of infiltrating eosinophils into the lung, rather that targeting plasma influx or neutrophil numbers. It is therefore not too that we observe no significant reduction in albumin levels or neutrophil number in the lung following allergen challenge and steroid.

Although we observed no comparative changes to levels of α_1-AT, active α_1-AT or chymotryptic inhibition following the steroid *and* allergen challenge, the correlation between

inflammatory cells and α_1-AT that was observed prior to steroid was abolished. This data is indirect evidence that the increase in α_1-AT observed after allergen challenge was not entirely explained by plasma influx, or by secretion by inflammatory cells. This narrowed down candidates for the increase in α_1-AT production following allergen to just one source, the epithelial cells. The distortion of the α_1-AT/albumin ratio observed following allergen challenge was also abolished following steroid. At first this suggested that steroid might be inhibiting the increase of *local* synthesis following allergen challenge. However, if you consider that steroid increased levels of *active* α_1-AT, and that this increase may be accounted for by an increase in local production before allergen, then any further increase in local α_1-AT production would be masked by the extra plasma derived inhibitor. Unfortunately, we had no evidence to suggest this is the case. However, evidence from this work suggests that the *local* production of α_1-AT may be playing a more important role in lung homeostasis than was originally believed.

Work with the lung fragment model enabled us to extend some of the observations made from previous chapters. We developed a well-defined lung fragment assay that provided new insights into the effect of different mediators on lung tissue. Mediators that had significant effects were the proteinase, porcine pancreatic elastase, and an antibody to IgE. This demonstrated to us that stress in the lung could bring about an increase in serpin synthesis that would combat the effects of inflammation. Unfortunately it was difficult to unambiguously assign roles to different cell types in this model due to the complex interactions that may occur between remnants of lung matrix, epithelial cells, fibroblasts, and even endothelial cells. To address this problem this we developed an epithelial cell model. We showed that the inflammatory mediator oncostatin M (Modur et al, 1997) brought about an increase in both α_1-AT and α_1-ACT that supported work conducted by Cichy et al in 1995 and Baumann and Gauldie in 1994. We went further to show that the proteinase, porcine pancreatic elastase, significantly upregulated both serpins after 24 hours, and that this was dependent upon an *active* proteinase. One potential target for this proteolytic activity is a member of the proteinase activated receptor (PAR) family expressed on the surface of the epithelial cells. PARs belong to the large superfamily of G-protein-coupled seven transmembrane domain receptors (Kawabata et al, 2000) and have the common property

of being activated by the proteolytic cleavage of their extracellular N-terminal domain. The new NH_2-terminus acts as a 'tethered ligand', which binds and activates the receptor itself (Vergnolle N, 2000). So far, 4 members of the family have been identified – three are activated by thrombin (PAR-1, PAR-3 and PAR-4) whereas PAR-2 is activated by trypsin and mast cell tryptase. PARs are present in a wide variety of tissues and participate in a number of physiological and pathophysiological phenomena, which includes platelet aggregation, inflammation and cardiovascular, digestive or respiratory functions (Kawabata, 2000). For example, PAR-2 activation induces calcium mobilization and eicosanoid production in enterocytes and PAR-1 and PAR-2 activation can alter gastrointestinal motility (Vergnolle, 2000). PAR-2 has also been shown to be present on human lung epithelial cells (Bohm et al, 1996). Recent studies have also shown that PAR-2 is present on human mast cells (Akers et al, 2000; D'Andrea et al, 2000) and this could also be a contributing factor to the observed increase in α_1-AT levels. PAR-2 expression has also been shown to be induced by inflammatory mediators (Nystedt et al, 1996), further evidence that supports the concept of PAR-2 involvement in the acute inflammatory response. Finally, PAR-2 activation has been shown to induce MMP-9 from airway epithelial cells in vitro (Vliagoftis et al, 2000), a significant indicator of its importance in lung pathophysiology.

Further work could be done by inhibiting PARs using PAR specific antibodies or peptide specific insertion in our epithelial cell and lung model *before* proteinases exposure. Would we still observe an increase in serpin secretion? One of the interesting stimuli that could be applied to our models would be the effect of oxidative stress. We could stimulate this using either hydrogen peroxide, or the more commonly used chloramine T. Due to time constraints we were not able to complete this set of experiments, but preliminary data (n=1) indicated that chloramine T resulted in an increase in serpin expression in the lung fragment model. The effect of chloramine T was not examined in the epithelial cell line.

This preliminary work does not support the data we have shown in chapter 3, where oxidative stress in the lung results in a *decrease* in serpin levels in asthmatics and no change in controls. However, there are fundamental differences between these two sets of results. The ozone work was an *in vivo* study whereas the chloramines T was *in vitro.* There are many

external factors to consider in the BAL ozone environment, including interactions with proteins such as antioxidants and existing serpins in the lung. Also, the concentration of Chloramine T that was used on the lung *in vitro* (10μg/ml) was significantly more concentrated than the ozone *in vivo* (2ppm) and so again, it is difficult to compare the two results.

We provided indirect evidence in chapter 4 that allergen challenge leads to an upregulation in local serpin production. In our epithelial cell model we used the allergen and cysteine proteinase Der p1 to mimic this. The asthmatics in our patient group were all ragweed sensitive and it is very doubtful that the original hosts of the epithelial cell lines were Der p1 sensitive. The fact that the lung fragments responded to anti-IgE whereas the epithelial cells did not, is not surprising. Bronchial epithelial cells *have* been shown to express high affinity IgE receptors on their cell surface, but only in asthmatics (Cambell et al). Due to the complex interactions that are ongoing in the human lung, it is very difficult to get an accurate model to assess serpin responses to various stimuli. Both models have drawbacks, and neither is complete in its own right. The process of BAL often results in an inflammatory response, which sometimes masks subtle changes in mediator levels. Until a less invasive method is developed for extracting mediators from lung is developed, it will be difficult, at best, to interpret some of the more subtle changes observed in BAL. Until then, researchers will have to rely on the limited clinical trials that take place in their specific area of research, and cell models, such as those that have been used in this thesis.

In conclusion, I have offered supporting evidence for several hypotheses in this thesis and these are summarized below:

- Asthmatics are less able to cope with oxidative stress in their lungs than control subjects. This may play a part in the lung damage and degradation associated with this disease.

- Allergen challenge leads to an increase in the serpins α_1-AT and α_1-ACT, and this increase may be brought about by the upregulation of these inhibitors in local lung tissue.

- The proteinase elastase leads to an upregulation of both α_1-AT and α_1-ACT in lung tissue and in lung epithelial cells. This upregulation of serpins is dependant upon an active proteinase.

What is most clear however from recent research and highlighted by my findings is that the lung surface has the capacity to secrete large amounts of α_1-AT and α_1-ACT, and it is likely this local production is providing a vital, but overlooked role in lung homeostasis.

i.i A1-AT immunoassay – further validation data

To further validate the α_1-antitrypsin ELISA we had developed we checked for crossreactivity with albumin and ovalbumin. The albumin control was essential because not only is albumin present in significant quantities in BAL, but is also the main candidate for contamination of any α_1-AT used to raise the antibody in the host rabbit. Ovalbumin was used as it is a SERPIN with a good degree of homology to α_1-AT and would show any crossreactivity for epitopes recognising protein motifs common to members of the superfamily. Figure i.i shows an SDS gel demonstrating no crossreactivity between our antibody and either albumin or ovalbumin. It also demonstrates that we are detecting plasma based α_1-AT is present in a single form, rather than polymerised or bound to any proteinase.

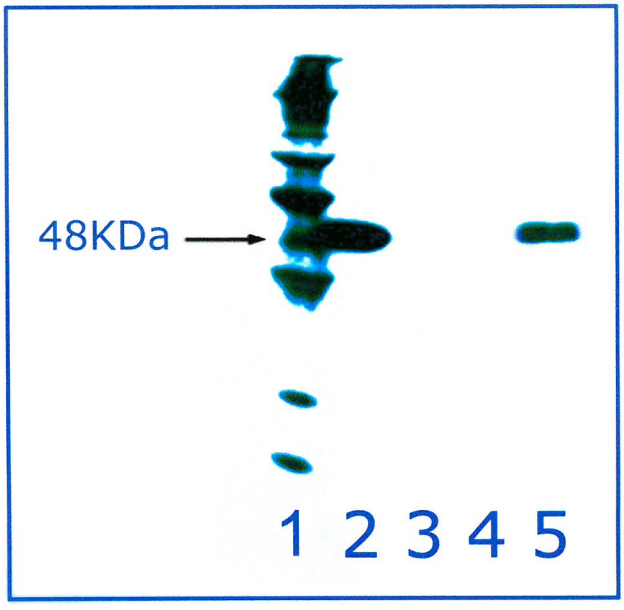

Figure i.i – Crossreactivity of the α_1-AT antibody against albumin, ovalabumin and human plasma. Column 1 shows molecular weight markers followed by 50μg/ml α_1-AT standard (2), albumin (3), ovalbumin (4), human plasma 1:500 dilution (5).

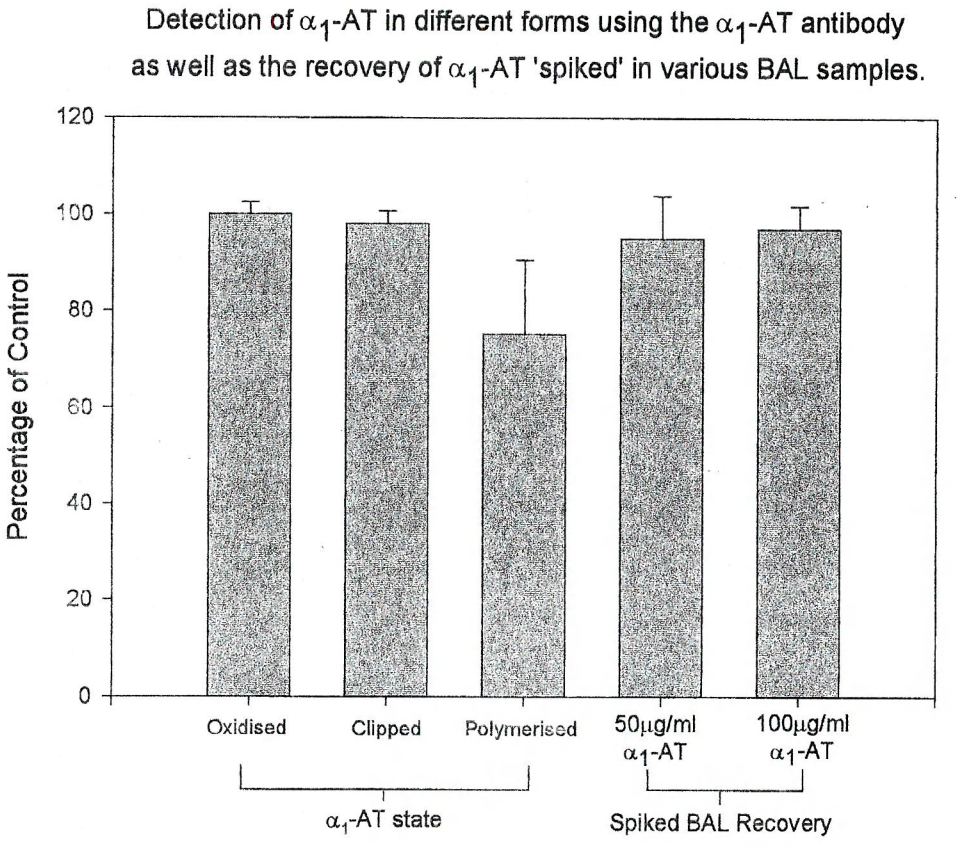

Fig i.ii – Graph showing the detection of α_1-AT in different forms using the α_1-AT antibody as well as the recovery of α_1-AT 'spiked' in a BAL sample. Results are shown as a percentage of control. 100% of oxidised α_1-AT, 98% of clipped α_1-AT and 75% of polymerised α_1-AT was detected in this ELISA. We also detected 95% and 97% of the original α_1-AT after 'spiking' BAL with 50µg/ml and 100µg/ml of α_1-AT respectfully.

To further validate this ELISA we oxidised, clipped and polymerised α_1-antitrypsin before placement in the assay. Oxidation was achieved by incubating with 10^{-4}M hydrogen peroxide for 30 minutes, α_1-AT was polymerised by heating at 65^0C for one hour and clipping was achieved by incubating an equimolar ratio of chymotrypsin with α_1-antitrypsin for 30 miniutes. We also 'spiked' a number of BAL samples with a known amount of α_1-antitrypsin (50 and 100µg/ml) in order to test the recovery of α_1-antitrypsin from BAL in this assay. Figure i.ii shows that the α_1-antitrypsin ELISA assay detected 100% of oxidised α_1-antitrypsin and 98% of clipped α_1-antitrypsin. However, the α_1-antitrypsin ELISA assay only detected 75% of the original α_1-antitrypsin after polymerisation. This may be because the polymers tend to

aggregate making accurate dilutions difficult. 'Spiking' our samples resulted in a recovery of >95% of α_1-antitrypsin. This verified the assays and proved that there was no external interference with the protein and that the α_1-antitrypsin antibody detected all forms of the protein.

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