Thesis submitted for the degree of M.D.

University of Glasgow

ENDOTHELIN-1 AND THE USE OF INDUCED SPUTUM TO INVESTIGATE ITS ROLE IN AIRWAY DISEASES

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ABSTRACT

Endothelin (ET)-1 is a 21-amino acid peptide which has been the subject of intense interest since its discovery in 1988. It has a number of properties which may be important in physiology and pathophysiology, including potential relevance to airway diseases. A putative role for ET-1 in asthma has been proposed, and we sought to examine this further, as well as to extend our investigations to other respiratory diseases, including chronic obstructive pulmonary disease and cystic fibrosis. The technique of sputum induction has been developed recently as a non-invasive method of obtaining airway secretions for analysis, and we have applied this to the investigation of the role of ET-1 in diseases involving the airway. We have demonstrated for the first time that ET-1 is a highly potent bronchoconstrictor with a prolonged duration of action when administered by aerosol in asthma, and that asthmatics exhibit bronchial hyperreactivity to ET-1 compared with non-asthmatic subjects, but we found no evidence of an acute inflammatory airway response at 30 minutes or 4 hours following ET-1-induced bronchoconstriction in asthma, assessed by analysis of cell counts and soluble mediators in induced sputum. The bronchoconstrictor activity of ET-1 was not potentiated by an infusion of angiotensin II in stable asthmatics, despite animal evidence of potentiation, although the possibility of such interaction remains in acute severe asthma, where plasma angiotensin II levels are elevated. We did not find increased levels of ET-1 in induced sputum in mild asthmatics compared with non-asthmatic subjects, nor was there a fall in sputum ET-1 comparing samples obtained during acute severe asthma with those obtained in convalescence, although sputum and saliva levels of ET-1 are greater than plasma ET-1, suggesting local production within the respiratory tract. Examination of sputum ET-1 following allergen challenge in asthma showed a trend towards an increase in sputum ET-1 after allergen challenge, with a relationship between the increase in sputum ET-1 and the extent of sputum eosinophilia, suggesting a relationship between asthmatic airway inflammation and ET-1 release. Sputum ET-1 is increased in smokers without lung disease, and in subjects

with chronic obstructive pulmonary disease (COPD), with a trend towards a fall in sputum ET-1 comparing acute exacerbation with convalescence. Finally, we have demonstrated a marked increase in sputum ET-1 in patients with cystic fibrosis compared with healthy subjects. We conclude from this series of studies that there is continuing evidence for a role for ET-1 in a number of diseases affecting the airway, and speculate that drugs which oppose the action of ET-1 may have a role in the treatment of these conditions.

ACKNOWLEDGEMENTS

I am indebted to my supervisor Professor Neil Thomson for his guidance and unfailing enthusiasm throughout this project. His input has been considerable, thoughtful and timely, and his ability to listen, consider and advise has guided me through many of the uncertainties of clinical research. I am grateful for his involvement in the conception, design, analysis and presentation of the work, but his example in clarity of thought and purpose applied with kindness and good humour extends well beyond the immediate project, and will remain with me. My research colleagues Drs. Ken Dagg, Stuart Little and Rekha Chaudhuri have been involved in a number of the studies, and have been unstinting in their involvement, offering advice, support and coffee as required. Lorna Thomson, our Research Co-ordinator has mixed enthusiasm with practical help, kindness and sound advice throughout, and this work could not have been carried out without her. Kirsten Macleod in the Department of Immunology at the Western Infirmary has been involved throughout in the laboratory processing of samples, under the supervision of Dr Charlie McSharry. They have been involved in the design and execution of the studies, and in the preparation of manuscripts and presentation of work. I am grateful to both for their expertise, advice and cheerful flexibility. Dr J.J. Morton, Director of the isotope laboratory in the Department of Medicine at the Western Infirmary, Glasgow performed the endothelin-1 and angiotensin II assays and Ms Wendy Fallon of sterile pharmacy in the Western Infirmary was involved in the blinding and randomisation of the studies, and preparation of methacholine for inhalation and angiotensin II for infusion. The work presented here was supported by grants from the National Asthma Campaign (UK) and a Research Fellowship from Chest, Heart & Stroke Scotland. I am grateful to both of these organisations, and to those who support them, and I trust that their investment has not been squandered. Finally, my wife Audrey has been supportive, kind, interested, encouraging and patient in equal measure, for which I am deeply grateful.

DECLARATION

I am the sole author of this thesis and I have personally consulted all the references listed. The work was undertaken by myself in the Departments of Respiratory Medicine, Gartnavel General Hospital and the Western Infirmary, Glasgow. Laboratory processing was performed by my colleagues Kirsten Macleod and Dr Charlie McSharry in the Department of Immunology, Western Infirmary, Glasgow, with some assays being performed by Dr J. J. Morton, Department of Medicine, University of Glasgow.

This thesis has not previously been submitted for a higher degree.

ABBREVIATIONS

ET	endothelin
FEV_1	forced expiratory volume (in 1 second)
SGaw	specific airways conductance
PC	provoking concentration
$Sp0_2$	oxygen saturation (assessed by pulse oximetry)
BAL	bronchoalveolar lavage
Ang II	angiotensin II
BHR	bronchial hyperresponsiveness
SD	standard deviation
SEM	standard error of the mean
IQR	interquartile range

PUBLICATIONS ARISING FROM THIS THESIS

1. The role of endothelin-1 in asthma.
   GW Chalmers and NC Thomson.
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2. Endothelin-1-induced bronchoconstriction in asthma.
   GW Chalmers, SA Little, KR Patel, and NC Thomson.
   American Journal of Respiratory and Critical Care Medicine 1997; **156**: 362-388

3. Sputum cellular and cytokine responses to inhaled endothelin-1 in asthma.
   GW Chalmers, KJ Macleod, LJ Thomson, SA Little, KR Patel, C McSharry and NC Thomson.
   Clinical & Experimental Allergy 1999 *in press*

4. Effect of infused angiotensin II on the bronchoconstrictor activity of inhaled endothelin-1 in asthma.
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5. Endothelin-1 levels in induced sputum samples from asthmatic and normal subjects.
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   Thorax 1997; **52**: 625-627

6. Sputum endothelin-1 is increased in cystic fibrosis and chronic obstructive pulmonary disease.
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CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 - ENDOTHELINS

1.1.1 HISTORICAL BACKGROUND AND CLASSIFICATION

Endothelin (ET) was first isolated from porcine aortic endothelial cells by Yanagisawa and co-workers (1) in 1987, based on a report of a previously unidentified vasoconstrictor factor present in a conditioned medium of cultured bovine endothelial cells, published 2 years earlier by Hickey *et al* (2). In 1980, Furchgott *et al* (3) had demonstrated the presence of an endothelial-derived, locally acting, non-prostaglandin relaxing factor (later identified as nitric oxide), and the discovery of this and other relaxant factors, prompted a search for counterbalancing constrictor substances. Hickey demonstrated that one of these substances produces vasoconstriction lasting for more than 60 minutes, in contrast to the brief actions of other substances produced by the endothelium, and within a remarkably short time period in 1987, Yanagisawa and colleagues had purified and sequenced the propeptide (preproendothelin) and cloned and pharmacologically profiled the 21 amino-acid peptide which was later designated endothelin-1. Analysis of human genome sequences subsequently revealed the presence of three distinct genes for ET, which encode the three distinct isopeptides, designated ET-1, ET-2 and ET-3 (4), comprising the human endothelin family. ET-1 was found to be the most potent vasoconstrictor peptide known, and since it is the major endogenously produced endothelin, ET-1 has been the focus of the majority of the research work in human systems, with less being known about physiological and pathophysiological roles for ET-2 and ET-3. At an early stage of research, autoradiographic studies demonstrated the presence of ET-1 binding sites not only in the cardiovascular system, but also in a range of other organs, including lung, adrenal gland, kidney, brain, intestine and others (5), suggesting a wide variety of function in different organ systems. Assay systems to measure ET-1 in plasma were developed

soon after its identification, and it became apparent that circulating ET-1 in healthy adults is present at very low levels (6), which were not sufficient to elicit constriction of vascular smooth muscle. Like prostacyclin and nitric oxide, ET-1 appears not to be stored in granules in cells (7), but produced by rapid induction of ET-1 messenger RNA (mRNA), with synthesis and secretion of ET-1 within minutes in response to certain stimuli (8). In addition, despite its prolonged duration of action in a number of tissues, ET-1 has a short plasma half life, and is extensively cleared by the lungs during first passage (9). Studies using vascular endothelial cell culture reveal that 75% of ET-1 secretion from endothelial cells is towards the vascular smooth muscle side of the cell, and away from the vascular lumen (10), and similar studies using tracheal epithelial cells suggest that the proportion secreted basally may be greater than 90% (11). These data suggest that ET-1 acts not primarily as a circulating hormone, but rather in a paracrine fashion, although plasma levels may rise with disease activity in some conditions, including chronic heart failure (12), renal failure (13), and acute severe asthma (14).

1.1.2 ENDOTHELIN-1 - BIOSYNTHESIS, RECEPTORS AND CLEARANCE

Structure and Biosynthesis

Each endothelin is a product of a separate gene which codes for a large precursor protein mRNA, with the gene for ET-1 being on chromosome 6 (15). The preproendothelin-1 gene is 203 amino-acids long, and is processed to the 39 amino-acid prohormone big endothelin-1 (big-ET-1) which is secreted and circulates in plasma **(fig 1.1)** (16). Big-ET-1 is pharmacologically much less active than ET-1, and is cleaved between positions 21 and 22 by endothelin converting enzymes (ECEs), (a group of metalloproteinases), resulting in the generation of ET-1, the more active 21 amino-acid peptide (17). ET-1 differs from ET-2 and ET-3 by 2 and 6 amino acids respectively **(fig 1.2)**, and although the synthesis of ET-2 and ET-3 are less well characterised, ET-1 synthesis is taken as a likely template for the processes involved. Endothelial cell production of preproET-1 is known to be affected by a number of factors, including haemodynamic shear stress, hypoxia, transforming growth factor-ß (TGF-ß), tumour necrosis factor-α (TNF-α), thrombin, erythropoetin, bradykinin, angiotensin II, vasopressin and phorbol ester (which stimulates protein kinase C) (18). Endothelins exhibit close structural homology to a group of cardiotoxic peptides (sarafotoxins) isolated from the venom of the burrowing asp *Atractaspis engaddensis*, which similarly have 21 amino-acids, and have complete identity with endothelins at nine of these residues, including four crucial cysteine residues which form intrachain disulphide bonds (19). These compounds have been of considerable value in the study of the function of endothelins, and the structural homology, along with that of endothelins in other mammalian species, is taken to suggest a degree of genomic conservation consistent with an essential role for endothelins in the host (20). The disulphide bonds present in the ET molecule appear to be important for high-affinity binding to one class of ET-receptors (ET_A), but less so for the other main class of receptor (ET_B) (5).

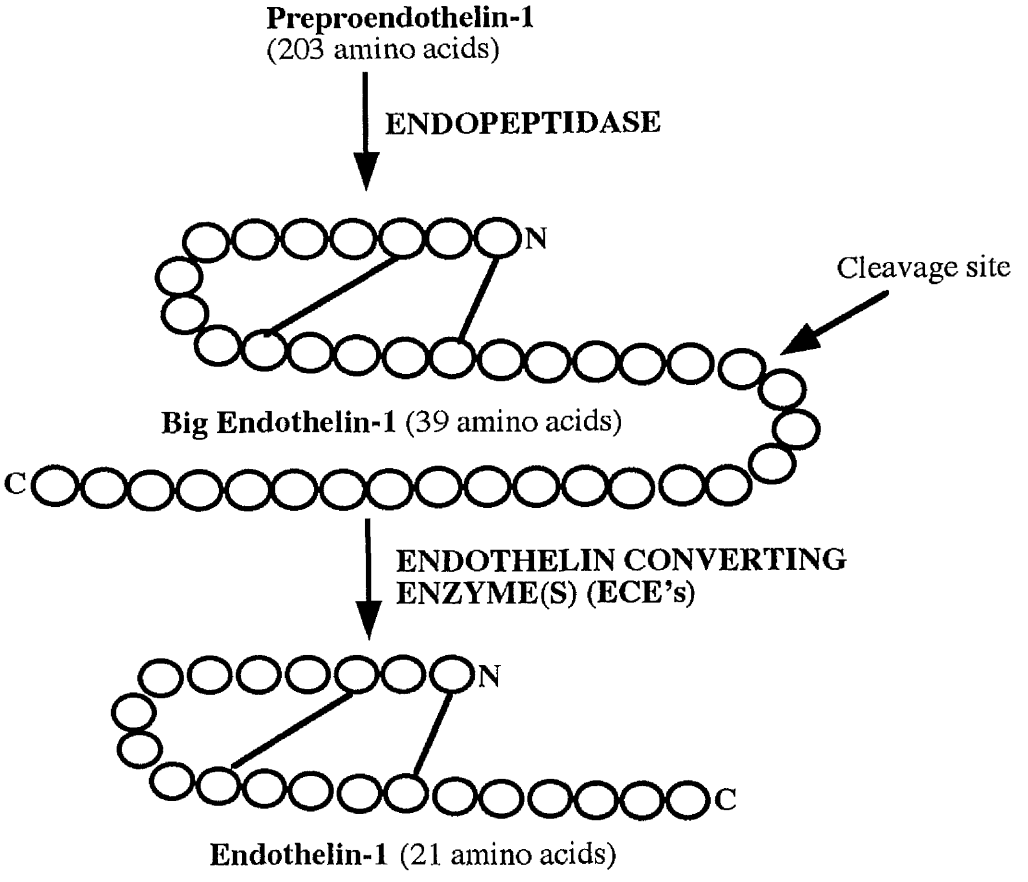

Figure 1.1
Biosynthetic pathway for endothelin-1 (adapted from Yanagisawa 1988)

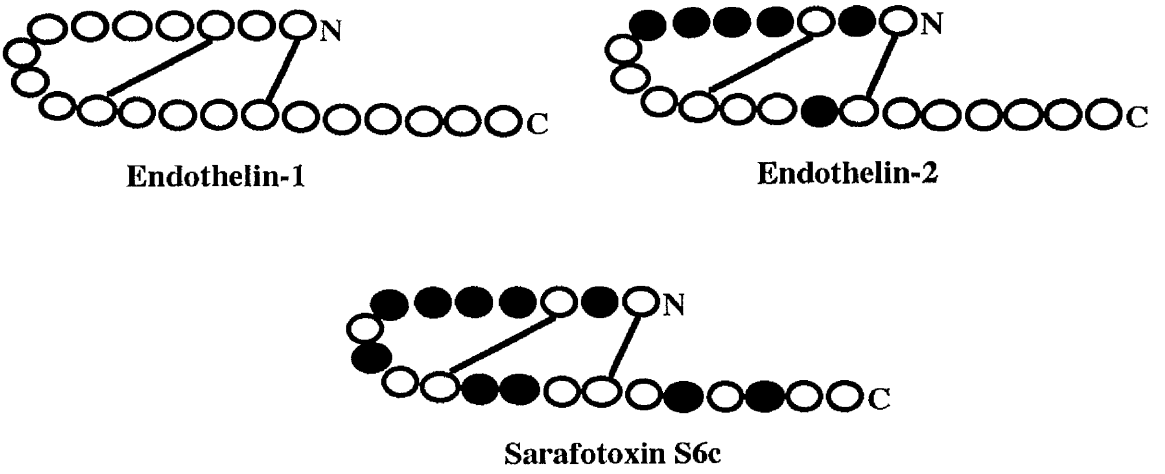

Figure 1.2
The structure of endothelin-1, endothelin-2 and the snake venom sarafotoxin S6c (an endothelin B receptor agonist). Differences in amino acid structure between the peptides are marked by shaded amino acid residues.

Receptors

Once the three isoforms of ET had been cloned and characterised, it became apparent that there were saturable, high-affinity membrane binding sites for these peptides, which were characterised initially on the basis of rank order of potencies of either binding or function. ET-1 was found to be significantly more potent than ET-3 in stimulating contraction of vascular smooth muscle in isolated tissues, and increasing blood pressure *in vivo* (4, 21), while ET-1 and ET-3 produce comparable transient vasodilation responses *in vitro* and *in vivo* (22), suggesting the presence of two receptors, which were designated ET_A and ET_B receptors (23). The discovery of specific ET receptor antagonists allowed the identification of receptor subtypes (sarafotoxins containing Glu^9 for example, are potent, highly selective ET_B antagonists (24)), and DNA cloning identified the two different types of receptors, one with isotype selectivity ET-1 $\geq$ ET-2 > ET-3 (25), and another with binding specificity ET-1 = ET-2 = ET-3 (26), which to conform with previous pharmacological characterisation were called ET_A and ET_B, respectively. The existence of a third receptor subtype (ET_C receptor) which selectively responds to ET-3 has been demonstrated in *Xenopus laevis* dermal melanophore (27), but to date there is no evidence of mammalian ET receptor with ET-3 specificity, nor has genomic analysis by Southern blotting of human DNA revealed any other genes closely related to the human ETA and ETB receptor genes (28). It is possible that the amphibian receptor is simply a species variant of the ET_B receptor, or that if such a receptor exists in human cells, it has a markedly different structure to that of the other receptors. There is tentative evidence for the existence of a subtype of the ET_B receptor in human bronchus (29), based on functional and binding characteristics, but no direct confirmation of its presence or structure at present.

Clearance from plasma

ET-1 is rapidly cleared from plasma, with the lung being responsible for about 50% of the clearance in man (30), and the estimated plasma half-life of ET-1 is around 3.5 minutes (31). Animal studies suggest that clearance is mainly achieved through specific binding in the pulmonary vasculature (32), which can be inhibited by the prior administration of an ET_B-selective antagonist (33), suggesting that the ET_B receptor may act as a "clearance receptor", which may be important in removing ET-1 from the circulation. The contribution of specific receptors to clearance of ET-1 in man has not been clearly defined. In addition, a number of enzymes capable of degrading ET-1 have been described, including deamidases, neutral endopeptidases, and other peptidases (18). Degradation of ^{125}I-labelled ET-1 has been demonstrated by activity of metalloproteinases and serine proteinases in rat lung and kidney extract, with metalloproteinases being more important in pulmonary than renal degradation (34). It appears therefore, that ET-1 is removed from the circulation by binding to its receptors, internalisation and uncoupling, followed by enzymatic degradation, resulting in low levels of free and intact ET-1 in serum, as is confirmed by plasma measurements. It is worth noting however, that the prolonged duration of vasoconstrictor activity of ET-1 following I.V. administration is not adequately explained on the basis of its pharmacokinetic properties. ET-1 binding to its receptor results in rapid internalisation and inactivation of the receptor-ligand complex, but this appears to be followed by externalisation of new or recycled receptors (35), suggesting that maintenance of contraction may be dependent on ET-1 binding to a supply of unoccupied newly externalised receptors.

1.1.3 PHYSIOLOGICAL PROPERTIES OF ET-1

The presence of binding sites for ET-1 has been demonstrated in a wide variety of body tissues, including vasculature, respiratory system, neuroendocrine system, central nervous system, gastrointestinal tract and the reproductive and fetoplacental system, with evidence for local synthesis and activity in each case. This brief summary of the physiological properties of ET-1 concentrates on cardiovascular and pulmonary activity, along with the role of ET-1 as a mitogen and co-mitogen in respiratory tract cells with mention of other systems where direct effect on these systems is suggested. The specific properties of ET-1 which could relate to asthma are discussed in greater detail in section 1.2.

Cardiovascular system

As mentioned above, ET-1 causes prolonged contraction of vascular smooth muscle after initial transient vasodilation, and it is a potent pressor agent, producing a prolonged rise in blood pressure in animals (1) and man (31) following I.V. administration. Local administration produces slow onset, prolonged local arterial constriction in the human forearm model (36), with similar effects on venous tone (13). Animal studies suggest that the pressor response is due to an increase in total peripheral resistance (37), with others reporting additional chronotropic (38) and inotropic (39) activity. Although pressor activity appears to be mainly mediated by ET_A receptors on vascular smooth muscle cells (25), the activation of ET_B receptors probably also contributes to vasoconstriction (40). Under physiological conditions endogenous production of ET-1 contributes to the maintenance of vascular tone (41), and this activity appears to be balanced by the release of nitric oxide (NO) and other vasoactive agents (42) produced, like ET-1, by vascular endothelial cells. ET-1 is a potent coronary vasoconstrictor, with a putative role in myocardial infarction/ischaemia (43), and ET-1 also produces vasoconstriction in a wide range of vascular beds and organ systems. Renal vascular resistance rises with ET-1 infusion, with a decrease in renal plasma flow and glomerular

filtration rate (44), and hepatic vasoconstriction also occurs (45). Interest in the cardiovascular activity of endothelins has grown exponentially, with thousands of publications, and phase 1 and phase 2 trials of endothelin antagonists in man. The detailed role of ET-1 in the cardiovascular system is beyond the scope of this thesis, although specific aspects will be referred to as appropriate in other sections.

Respiratory system

The first evidence of a non-vascular site of synthesis of ET in the lung was demonstrated by *in situ* hybridisation techniques, which demonstrated the presence of large quantities of ET mRNA in rat fetal lung bronchioles (46). Airway epithelial cells (47), endothelial cells, macrophages (48) and neuroendocrine cells (49) can all synthesise endothelins, and airway epithelial ET-1 synthesis is increased by thrombin (50) and various cytokines, including interleukin (IL)-8, TNF-α and TGF-ß, with big-ET-1 synthesis increased by IL-1, IL-2, IL-6 and insulin-like growth factor (IGF)-1 (51). Autoradiographic and binding studies have revealed high-affinity binding sites in human lung, localised largely to airway and vascular smooth muscle (52, 53), and parasympathetic ganglia (54), but also present in other tissues, including peripheral airways (55), airway epithelia (56) and alveolar septa (54). In addition to its vasoconstrictor activity, ET-1 is one of the most potent bronchoconstrictors yet isolated (57), with all three endothelins producing slowly developing and long-lasting contractions in human isolated bronchi (58). The mechanism by which ET-1 produces bronchoconstriction is not clear, and while in animal bronchi this effect is at least partially mediated by cyclo-oxygenase metabolites (59), this does not appear to be the case in human bronchial preparations where contractions to ET-1 are unaltered by indomethacin, and in contrast to many other bronchoconstrictor substances do not require activation of protein kinase C to produce a mechanical response (60).

A study by Pons *et al* (61) comparing the bronchopulmonary effects of ET-1 administered intra-arterially and by aerosol in the guinea-pig isolated lung found that

although both routes of administration produced bronchoconstriction, by contrast with intra-arterial ET-1, aerosol administration did not produce a rise in pulmonary perfusion pressure, and the effects were not mediated by arachidonic acid metabolites. In human isolated bronchus, contraction appears to be mediated by the mobilisation of intracellular calcium, rather than the influx of extracellular calcium (58, 62). The bronchoconstrictor activity of all three endothelins is significantly augmented by the removal of the bronchial epithelium (63), suggesting that the epithelium has a function in regulating the contractile activity of endothelins, possibly by the release of an epithelial-derived relaxant factor (64), such as nitric oxide (NO). There is evidence of a negative feedback loop in human bronchial rings, where ET-1 activation of ET_A receptors leads to epithelial release of NO, modulating the contractile response to ET-1 (65). Animal studies have also examined the effects of ET-1 administration *in vivo* on airway function with intravenous (66) and aerosol administration (67) producing dose-dependent bronchoconstriction in guinea-pigs without evidence of inflammatory reactions or epithelial damage (57), and confirmed the findings in the isolated guinea-pig lung (61) that aerosol administration of ET-1 produced bronchoconstriction without a rise in mean arterial blood pressure (67). The use of receptor antagonists suggests that in contrast to the vascular system (including the pulmonary vascular system (68)) where the ET_A receptor is the principal receptor mediating vasoconstriction (25), bronchoconstriction of human airways *in vitro* by ET-1 is mediated primarily by the ET_B receptor (68), while other actions such as proliferation of airway smooth muscle are ETA-dependent (69).

The potential contribution of ET-1 to airway diseases has been addressed in section 1.3 for asthma, and data relating to chronic obstructive pulmonary disease and cystic fibrosis is presented in the relevant chapters, since there is very little other work on the potential role of ET-1 in these conditions.

Mitogenic activity

ET-1 is known to possess mitogenic activity for a number of cell types, both alone and as a co-mitogen. It stimulates growth of vascular (70) and bronchial (71) smooth muscle cells along with bronchial epithelial cells (72) and a fibroblast cell line (73). An *in vivo* study of an 18 day continuous intravenous infusion of ET-1 in guinea-pigs resulted in thickening of the airway smooth muscle, with a doubling in proliferating cell nuclear antigen in the ET-1 treated group compared to controls (74). ET-3 failed to induce proliferation in either airway smooth muscle (75) or airway epithelial cells, suggesting that the mitogenic activity of ET-1 may be mediated via the ET_A receptor, a theory supported by the fact that this activity was inhibited by BQ-123, a selective ET_A antagonist, in a dose-dependent manner (72). Although the mitogenic potency of ET-1 alone is low compared to other mitogens, it can significantly potentiate the proliferative activity of other mitogens (76), with co-mitogenicity in airway smooth muscle having been demonstrated with platelet-derived growth factor (PDGF)-BB (75, 77), PDGF-AB (78), and epidermal growth factor (EGF) (69). Stimulation of fibroblast growth by ET-1 is potentiated by transforming growth factor (TGF)-ß, EGF, insulin and PDGF (79). Some of these growth factors may also increase the gene expression and production of ET-1 (including TGF-ß, EGF, insulin and PDGF (76)), and ET-1 can induce the expression of TGF-ß and PDGF-A chain (80) in vascular smooth muscle cells, mitogen activated protein (MAP) kinases and DNA synthesis in tracheal smooth muscle cells (81), and fibronectin (Fn) - which has potent fibroblast chemotactic activity - in bronchial epithelial cells (82). Mitogen-activated protein (MAP)-kinases are believed to regulate a number of early events in mitogenesis, and are essential for fibroblast proliferation (83). Although the role of ET-1 in pathophysiological processes involving cellular proliferation remains to be clarified, it appears to have a role in a number of mitogenic processes, and it is postulated that in airway diseases this may contribute to the development of structural changes in the airway with resultant airflow obstruction.

1.2 - POTENTIAL ROLE OF ET-1 IN ASTHMA

The question of whether ET-1 contributes to the pathogenesis of asthma may be addressed by examining a number of other questions in turn. The potential role of ET-1 in the pathogenesis of cystic fibrosis and COPD will be discussed in the relevant chapters.

- **1.2.1 Do the actions of ET-1 resemble clinical features of asthma?**
- **1.2.2 Is there evidence of increased synthesis and release of ET-1 in asthma?**
- **1.2.3 Are ET receptor numbers, localisation and subtypes altered in asthma?**
- **1.2.4 Does blocking the actions of ET-1 improve asthma?**

1.2.1 - DO THE ACTIONS OF ET-1 RESEMBLE CLINICAL FEATURES OF ASTHMA?

ET-1 has a number of biological activities which may mimic features of asthma (**figure 1.2.1**)

Actions of ET-1 which may contribute to asthma

- Bronchoconstriction
- Bronchial hyperreactivity
- Pro-inflammatory activity
- Airway remodelling
- Neuromodulation
- Mucous secretion
- Airway permeability

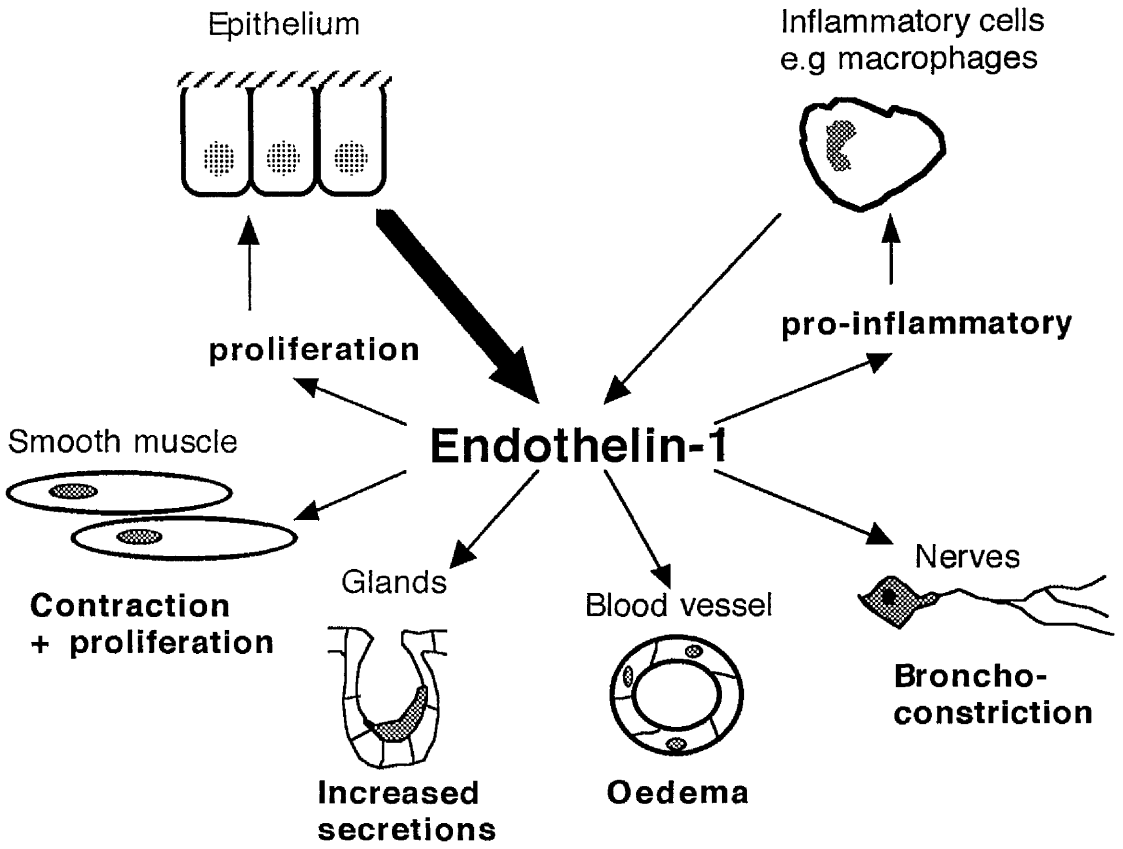

Figure 1.2.1
Potential actions of endothelin-1 in the airways

Bronchoconstriction

Our own group and others have demonstrated that ET-1 produces prolonged and potent contraction of tracheal and bronchial smooth muscle *in vitro* in the human (58, 60, 84), and in several animal species (53, 85) and in animal studies administration of ET-1 by aerosol produces a similar effect *in vivo* (67). Prior to the data contained in this thesis there were no reports of the direct administration of ET-1 to human airways *in vivo*. There is debate about the mechanism of action of ET-1 in the airways, with animal studies suggesting that contractile activity is at least in part mediated by arachidonic acid metabolites (in particular thromboxanes (86)), leukotrienes and histamine (87), but this has not been confirmed by studies using human bronchi (60), where ET-1 appears to act directly on smooth muscle, without the involvement of acetylcholine, leukotrienes, histamine or platelet activating factor (88). While evidence suggests that bronchoconstriction is primarily mediated by the ET_B receptor in the human airway, in contrast to vasoconstriction, which is primarily ET_A receptor-mediated, in both the bronchi (89) and the vascular system (90) blockade of both receptors is required to abolish contraction. Epithelial disruption is a feature of the inflammation associated with asthma (91), and it has been demonstrated that epithelial removal significantly enhances the contractile activity of ET-1 in the human isolated bronchus (63), raising the possibility that epithelial disruption contributes to contractile activity of ET-1 in asthma. Apart from direct action on bronchial smooth muscle, ET-1 is known to have other effects which could lead to muscular or non-muscular bronchial narrowing. ET-1 acts on glandular ET receptors in human nasal mucosa, inducing lactoferrin and mucous glycoprotein release (92), induces cytokine production (GM-CSF, IL-6 and IL-8) in human bronchial epithelial cell culture (93), and potently stimulates release of TNF-α, IL-1ß, and IL-6 from monocytes and monocyte-derived macrophages (94), all of which could contribute to airway obstruction.

Bronchial hyperreactivity

ET-1-induced bronchoconstriction in guinea-pigs is not associated with the development of bronchial hyperresponsiveness (95), and contrary to expectations, airway hyperreactivity to methacholine was markedly enhanced, rather than attenuated, in heterozygous ET-$1^{+/-}$ knockout mice (96). An additional effect of ET-1 knockout on nitric oxide synthesis was postulated, and it is difficult to be sure if this finding represents an incidental characteristic of ET-1 knockout mice or a true association between decreased ET-1 production and bronchial hyperresponsiveness. Subjects with increased bronchial reactivity to inhaled distilled water (and methacholine) have increased immunostaining for ET-1 on bronchial biopsy (97), but the contribution of airway ET-1 to bronchial hyperresponsiveness in asthma is not clear.

Airway remodelling

A proportion of asthmatics will develop a degree of irreversible airflow obstruction, thought to be due to in part to structural changes in the airway, with accumulation of collagen, fibronectin and specialised cells known as myofibroblasts beneath the bronchial epithelium (see section 1.3.1). There is some evidence that ET-1 may contribute to this process in that bronchial epithelial cells cultured from atopic asthmatic subjects and exposed to allergen induce differentiation of airway fibroblasts into myofibroblasts, and this process appears to be associated with an increase in ET-1 production (98). ET-1 has mitogenic properties, with animal studies indicating a role in the proliferation of cultured airway smooth muscle cells (71) and airway epithelial cells (72). Acting alone however, ET-1 has only modest mitogenic activity (99), but it is able to significantly potentiate the mitogenic activity of other growth factors (69), and it induces the expression of pro-fibrotic factors including tumor necrosis factor (TNF)-α (100) and fibronectin (82). These effects are predominantly mediated by the ET_A receptor (69). ET-1 also attenuates apoptosis in cultured human smooth muscle cells (101), which could contribute to abnormal cell growth. The potential role of ET-1 in contributing to airway remodelling remains to be explored.

Pro-inflammatory activity

In vitro data shows that ET-1 promotes the inflammatory activity of neutrophils (20) and macrophages (102), and increases the expression of adhesion molecules on endothelial cells. Additionally, ET antagonists have been shown to inhibit airway inflammation in animal studies (103, 104) in response to a number of stimuli. The production from mononuclear cells from asthmatics of the proinflammatory cytokines TNF-α (100) and IL-1ß (105) are increased by ET-1 *in vitro*, but there is very little *in vivo* or human data. ET-1 causes mast cell activation in the presence of IL-4 (106), and stimulates the release of IL-6 from human monocytes (107). There is animal evidence of a greater role for the ET_A receptor than the ET_B receptor in mediating proinflammatory activity in the lung (108, 109) and that ET receptor blockade can attenuate airway inflammation induced by antigen (103) and other stimuli (110).

Neuromodulation

ET_B receptors have been identified in pulmonary parasympathetic ganglia and submucosal nerve plexuses (111), and binding for radiolabelled ET-1 is present in parasympathetic ganglia and paravascular nerves in animals, while in humans, ET_A receptors are present in nerves associated with small bronchi (112). In functional studies, prejunctional ET_B receptor activation has been shown to potentiate cholinergic nerve-mediated contraction in human bronchial preparations (113), suggesting that ET-1 might exert a neurally mediated bronchoconstrictor effect, in addition to smooth muscle contraction.

Mucus secretion

As mentioned above, ET-1 immunoreactivity can be localised to mucus glands in the airway, and ET-1 is known to increase mucus secretion in human nasal cells *in vitro* (92) and *in vivo*, with more symptoms and secretions in subjects with allergic rhinitis (114), but no increase in histamine production. In the sheep, ET-1 inhibits tracheal mucus velocity (a marker of mucociliary clearance) (115), which could contribute to

bronchial obstruction. There is evidence that the increase in mucus glycoprotein secretion in response to ET-1 is inhibited in the presence of epithelial cells, suggesting that epithelial cells may release an inhibitory factor (116). The impact of ET-1 on mucus production in asthma has not been examined.

Airway permeability

Airway wall edema is a feature of asthma, along with airway inflammation and microvascular leakage. Intravenous administration of ET-1 in the rat induces extravasation of plasma proteins in the bronchi (117), a process which may be a result of the ability of ET-1 (and ET-3) to increase the formation of interendothelial gaps (118).

It is clear that endothelins (and ET-1 in particular) have a number of actions which could contribute to clinical features of asthma, and this has stimulated interest in its potential contribution to the disease, leading to studies assessing the relationship between the production and release of ET-1 in asthma.

1.2.2 - IS THERE EVIDENCE OF INCREASED SYNTHESIS AND RELEASE OF ET-1 IN ASTHMA?

Acute severe asthma

Modest increases in plasma levels of ET-1 have been observed in acute severe asthma (119, 120), with a relationship between plasma levels and severity of airflow obstruction, and a reduction in plasma ET-1 in response to treatment (120). While this is suggestive of a role for ET-1 in acute severe asthma, there is only one case report of increased levels of ET-1 in BAL from a patient with status asthmaticus (121). Since ET-1 in the airways is thought to primarily to act locally in an autocrine or paracrine fashion, information on airway levels of ET-1 may be of greater importance than plasma levels in assessing its contribution to the pathophysiology of acute severe asthma.

Chronic Asthma

Several studies have now demonstrated increased production and release of ET-1 in stable asthma. Staining for immunoreactive ET-1 in bronchial biopsies is increased in asthma (122), and this increase is abolished in subjects receiving inhaled corticosteroids (123). ET-1 in BAL is increased in steroid-naive asthmatics compared to controls (124), and in symptomatic but not asymptomatic asthmatics, although exposure of cells from asymptomatic asthmatics to inflammatory mediators *in vitro* resulted in an increase in ET-1 production (125). Similarly, cells from asthmatics expressing the low affinity receptor for IgE respond to IgE by increasing ET-1 production (126), suggesting that atopy may contribute to ET-1 production. Others have demonstrated that in isolated epithelial cells from asthmatic patients there is increased expression of the precursor of ET-1, preproendothelin, and increased ET release (47). Despite the observation in one study of a relationship between BAL ET-1 and % predicted FEV_1 (124), the relationship between detection of ET-1 and severity of airway obstruction is not consistent (122), and in the case of nocturnal asthma, the opposite relationship has been described (127).

The influence of asthma therapy on ET-1 release does however suggest a link between severity of asthma and ET-1 activity.

Influence of asthma therapy

The ability of corticosteroids (inhaled or oral) to decrease the synthesis of ET-1 is thought to result at least in part from a reduction in ET-1 mRNA production by epithelial cells (128), although their influence on the expression of proinflammatory cytokines may also influence ET-1 production. ET-1 is released from a variety of cells in response to proinflammatory cytokines, including IL-1α, IL-1β, TNF-α (129), IL-8 and TGF-β (51) whose expression may be reduced by corticosteroids. The β2 agonist bronchodilator salbutamol does not alter ET-1 expression (125), and in atopic asthmatic children, specific allergen immunotherapy did not significantly reduce plasma ET-1, but did reduce ET-1 production from stimulated blood mononuclear cells (14). While steroids are known to reduce expression of proinflammatory cytokines and inflammatory cell recruitment, reduction in ET-1 production may be another mechanism by which steroids influence symptoms in asthma.

Bronchial challenge testing

Studies in sensitised animals suggest a role for ET-1 in response to allergen in the airway, where immediate allergic responses (characterised by bronchoconstriction) can be modified by antagonism of the ET_B receptor (109), while late allergic responses (characterised by airway inflammation) are inhibited by ET_A or combined ET_A/ET_B receptor antagonists (103, 110). In rats there is a relationship between changes in ET-1 in BAL in response to allergen and the increases in BAL total cell count, eosinophils and neutrophils (110). The influence of allergen challenge in asthmatics on ET-1 production is not so clear. Using a bronchoscopic technique of segmental allergen challenge, Redington *et al* (130) unexpectedly found lower levels of ET-1 in allergen challenged bronchial segments compared with saline challenged segments in steroid-naive asthmatics, 10 minutes after the introduction of allergen, while a Japanese study

(English abstract) found the opposite effect in both early and late allergic responses measuring ET-1 in induced sputum after allergen challenge (131). The reasons for this difference are not known, although differences in timing of sample and method of sampling may be important. As mentioned above however, a reduction in BAL ET-1 has been observed during symptoms in nocturnal asthma (127), and studies examining ET-1 mRNA expression may help to clarify this area. Other forms of bronchial challenge have also been examined, with increased immunostaining for ET-1 demonstrated in biopsies from subjects who were sensitive to the bronchoconstrictor effects of hypertonic saline (97).

1.2.3 - ARE ENDOTHELIN RECEPTOR NUMBERS, LOCALISATION AND SUBTYPES ALTERED IN ASTHMA?

While the ET_A and ET_B receptor types are recognised in many species (with clear evidence for another receptor subtype only in non-mammalian species (132)), there is considerable species variation in the relative proportions of the 2 receptors in the lung, with human studies indicating that the ET_B receptor constitutes around 70% of total number in peripheral lung (112), and between 60 and 90% of the total number in the airway smooth muscle (84, 89). Receptors are also found in other sites including submucosal glands, nerve ganglia and plexuses, and blood vessels. While the ET_B receptor appears to be primarily responsible for mediating bronchoconstriction in the human lung (84), there is also evidence of bronchoconstrictor activity in the presence of ET_B blockade, and blockade of both receptors is required to antagonise the bronchoconstrictor activity of ET-1 in human bronchi (89). Studies in asthmatic human lung show no alteration of receptor subtypes, and no increase in ET receptors in asthmatic airways (84) or peripheral lung (112), with a slight decrease in sensitivity to bronchoconstriction by the ET_B agonist sarafotoxin S6c (84) in asthmatic lung. Despite this, increased production of ET-1 in the presence of normal receptor numbers is likely to result in ET-1 induced bronchoconstriction in asthma.

1.2.4 - DOES BLOCKING THE ACTIONS OF ET-1 IMPROVE ASTHMA?

Antagonism of the activity of ET-1 can be produced in a number of ways. Specific antagonists of ET_A and ET_B receptors exist, along with combined ET_A/ET_B receptor antagonists. In addition, it is possible to inhibit the activity of endothelin-converting enzymes (ECE's), and since other metalloproteases can cleave ET precursors, dual ECE and neutral endopeptidase inhibitors are also being developed. Animal and human *in vitro* studies suggest that ET receptor antagonists can attenuate both direct (89, 133) and neurally mediated (113) ET-1-induced bronchoconstriction, reduce allergen-induced (103, 109) and experimental (110) airway inflammation, and oppose co-stimulation by ET-1 of human airway smooth muscle proliferation (69), and although less information is available about ECE inhibitors, early evidence suggests they can reduce ET-1 release from tracheal epithelial cells (134, 135). Many of these studies and others testify to the ability of these compounds to modify actions of ET-1 which might contribute to asthma, but as yet no studies have been published examining their effect in asthmatic subjects. Immediate effects on bronchoconstriction and inflammatory responses are of interest, and longer term effects on airway remodelling in chronic asthma may have even greater potential. These studies may answer important questions, not only about the contribution of ET-1 to the pathophysiology of the disease, but more importantly about the potential therapeutic benefits of ET-1 antagonism in asthma.

1.2.5 SUMMARY

There is evidence that ET-1 has a number of properties which could contribute to asthma, with particular interest in its bronchoconstrictor, (co)-mitogenic and pro-inflammatory activities. ET-1 is present in BAL from asthmatics at concentrations greater than those found in non-asthmatic subjects, and conventional anti-asthma therapy such as inhaled glucocorticoids can reduce ET-1 levels in BAL. There is very little data on the effects of ET-1 *in vivo* in asthmatics, and data is presented in subsequent chapters to try to address some of the questions associated with this area. There is as yet no data on the effect of endothelin antagonists in asthmatic subjects, and we have been unable to obtain trial drugs for this purpose, despite approaches being made to a number of companies.

1.3 AIRWAY DISEASES AND THE ASSESSMENT OF AIRWAY INFLAMMATION

Airway diseases, predominantly asthma and chronic obstructive pulmonary disease (COPD) are among the world's most prevalent diseases. The prevalence of asthma has been increasing in a number of countries in the world, and it affects as many as 5% of the population in some western countries, with a higher incidence in children. Similarly, COPD is a common cause of morbidity and mortality, and COPD is now among the most common causes of death in the world, largely as a consequence of the prevalence of cigarette smoking. Both asthma and COPD are identified by the presence of characteristic symptoms and functional abnormalities, with airflow obstruction being the essential hallmark of both diseases. In asthma, airflow obstruction is characterestically reversible, while in COPD irreversible airflow obstruction is the norm when assessed over time, although some patients with chronic asthma who do not smoke may develop irreversible airflow obstruction. In both diseases lung inflammation is found, which is likely to be the underlying cause of the functional abnormalities observed.

1.3.1 ASTHMA

Although an exact definition of asthma is elusive, asthma is characterised by airflow obstruction which is variable over time, generally reversible to bronchodilator drugs, and which is associated with bronchial hyperresponsiveness to a number of bronchoconstrictor substances, such as histamine, cold air or cholinergic agonists (e.g. methacholine). With the exception of occupational asthma, the risk factors for adult and childhood asthma are broadly similar, and although there is a genetic link with the presence of parental asthma or atopy as the strongest determinants, genetic factors do not explain the majority of the heritability of asthma (136), and in general the heritability of surrogates for asthma such as atopy and IgE is greater than that observed for asthma itself. Reports of ethnic factors demonstrate a variable effect, but no substantive differences in asthma prevalence were demonstrated in a study of 3 ethnic populations within the UK (137). Other factors including *in utero* and neonatal factors may influence the likelihood of developing asthma, and environmental factors including exposure to cigarette smoke during childhood (138), personal smoking and dietary factors may play a role.

Airway inflammation in asthma

The central role of airway inflammation in the pathophysiology of asthma has been increasingly recognised, and bronchial biopsy studies have shown an increase in eosinophils, T lymphocytes and mast cells in the large airways of patients with asthma, with more recent information also suggesting the presence of inflammation in the small airways (139) and in the lung parenchyma (140). Studies have demonstrated airway inflammation in asthma even in subjects with newly diagnosed asthma (91) and in those with only intermittent symptoms (141), although to a lesser extent than those with persistent or more severe disease.

Cells involved in airway inflammation in asthma

Eosinophils - Immunohistochemical staining of bronchial biopsies identifies increased numbers of eosinophils in asthma (142), which is also observed in bronchoalveolar lavage (143) and induced sputum (144), and electron microscopic features along with markers of eosinophil activation would suggest that these eosinophils are in an activated state. The actual role of the eosinophil in asthma remains to be fully explained, since there is no specific method to counteract the recruitment and activation of eosinophils in the human, but the consistent association between relative eosinophilia and asthma strongly supports a pathophysiological role for the cell. Correlations have been reported between the severity of asthma assessed by clinical scoring and BAL eosinophils, with a stronger correlation for alveolar than bronchial eosinophils (145), and similar relationships have been reported between BAL eosinophilia and lung function parameters in asthma (146). Increases in eosinophil numbers following challenge with allergen have been demonstrated in BAL (147) and induced sputum (148), and a decrease in eosinophil numbers is generally seen in response to inhaled or oral corticosteroids (149, 150), although exacerbation of asthma is not invariably associated with eosinophilic inflammation (151), and some subjects have persistent airway eosinophilia despite regular treatment with inhaled corticosteroids (152). Although eosinophilic airway inflammation is characteristic in asthma, it is not specific to asthma, since airway eosinophilia has been reported in a number of conditions including COPD (153), Churg-Strauss syndrome, polyarteritis nodosa and interstitial lung diseases.

T-lymphocytes

The T lymphocyte is the most abundant nonconstituent cell in the airway epithelium (91) and there is evidence of increased T lymphocyte activation in asthma (154) which correlates with disease severity (155), even if studies have not consistently shown increased numbers of T cells in the asthmatic airway, with some finding no difference in T cell numbers or CD4+/CD8+ ratios in asthmatics compared with normals (142, 154), while others including electron microscopy have reported an increase (91). It has been

increasingly recognised that the T lymphocyte may have a central role in orchestrating the inflammatory process in asthma through the local generation and release of cytokines such as interleukins (IL)-3, IL-4, IL-5 and granulocyte macrophage stimulating factor (GMCSF), in a manner analogous to that of the murine Th2 subpopulation of CD4+ T cells. *In situ* hybridisation studies on BAL cells from asthmatics have demonstrated increased mRNA expression for the above cytokines along with IL-2, but not for interferon gamma (IFN-γ), consistent with the presence of an activated Th2-like population in asthma (156).

Mast cells

Mast cells comprise a very small proportion of the cells in BAL or sputum, although increased numbers have been recovered in asthmatics compared with non-asthmatics, with greater spontaneous and stimulated histamine release in asthma. In contrast to the small percentage in BAL, tissue mast cells are abundant and are activated (157). Mast cells have the capability to secrete cytokines, and several which are relevent to asthma have been identified stored within airway mast cells in a preformed state, including IL-4, IL-5, IL-6 and tumour necrosis factor (TNF)-α (158).

Macrophages

Bronchial macrophages are hypodense in asthma (159), and have increased spontaneous release of IL-1 (160), and although the majority of studies using BAL or induced sputum do not suggest increased numbers of macrophages or other mononuclear phagocytes in asthma compared with control subjects, there are increased numbers in the submucosa. T cell regulatory activity is an important part of macrophage function, and there is increasing evidence that the mononuclear phagocyte system plays a central role in the regulation of local T cells, through the release of soluble mediators such as cytokines, arachidonic acid metabolites and reactive oxygen species, and also through antigen processing and presentation (161).

Airway epithelial cells

In addition to their protective and structural function, bronchial epithelial cells have important metabolic funtion, being able to generate ET-1 as discussed in preceding sections, along with lipid mediators such as prostaglandins, and cytokines including IL-6, IL-8 and GMCSF on stimulation (162), as well as the inducible form of nitric oxide synthase (163).

Inflammatory mediators in asthma

A variety of mediators have been implicated in asthma, but since each mediator may have a number of effects on different cells, and may interact with other mediators in different ways, the role of individual mediators may be hard to assess. Mediators such as cysteinyl leukotrienes and other arachidonic acid metabolites, histamine, cytokines, adhesion molecules, growth factors, monoxides, oxidants and peptides may all contribute to the initiation and maintenance of asthmatic airway inflammation, and interest has focussed on those mediators which can be modified by treatment, in particular corticosteroids, and more recently the leukotriene antagonists, since there are relatively few specific antagonists of proposed asthmatic inflammatory mediators.

Structural changes in asthma

It is recognised that some patients with asthma have persistent impairment of lung function, without reversibility, either in terms of spontaneous variability or reversiblity to bronchodilators, and there is evidence of an increased rate of decline in FEV_1 in asthma, even in the absence of a smoking history (164). Relative thickening of the airway wall was reported in some very early studies in fatal asthma, and subsequent studies using bronchial biopsy techniques have confirmed the presence of thickening, particularly in the subepithelial layer of the bronchial wall (165). The use of electron microscopy has indicated that the lamina reticularis is greatly increased in depth, and immunohistochemical analysis suggests that this thickened layer is largely composed of collagen types III, V and to a lesser extent I and fibronectin (166). A specialised

network of fibroblastic cells possessing contractile apparatus have been identified, and have been termed "myofibroblasts", which are present in normal subjects, but increased in asthma, and it has been shown that the number of these cells correlates with the degree of collagen thickness, suggesting that these cells are responsible for the deposition of collagen (167). More recent data derived from high resolution computed tomography (HRCT) of the lungs suggests the presence of bronchial wall thickening in asthmatics of varying severity (168), and even in a study which did not confirm this finding, there was a relationship between bronchial wall thickness and airway reactivity (169), suggesting that airway remodelling has direct functional consequences in asthma. The clinical relevance of airway remodelling is further highlighted by the fact structural changes can occur early in the course of the disease as illustrated by their presence in young children (170), and there is evidence that the potential to reverse airflow obstruction and measures of bronchial responsiveness is impaired in patients in whom cessation of therapy with inhaled corticosteroids was delayed (171). Further work is needed to examine the effect of corticosteroids on structural changes in the airway, since only some studies have demonstrated reversal of airway collagen deposition (172) or remodelling (173), while others have not shown a similar effect (149, 174).

1.3.2 CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Like asthma, it has been difficult to provide an exact definition of COPD, although essential components are the presence of airflow limitation, and the fact that this limitation is not variable over short periods of time, with most attempts at definition specifying no significant variability in airflow limitation over a period of several months (175). Differentiation between severe chronic asthma and COPD on the basis of lung function tests can be difficult however, since asthmatics may exhibit irreversible airflow obstruction, while patients with COPD may have some reversibility to bronchodilator therapy, and in symptomatic terms, the presence or absence of cough and sputum, nocturnal or diurnal wheeze, partial response to bronchodilators or a family history of chest disease are unhelpful in differentiating chronic asthma and COPD (176). Both asthma and COPD are associated with airway and lung parenchymal inflammation, with consequent structural changes, although there are some differences in the nature and site of this inflammation and its consequent structural alterations which may be helpful in distinguishing the conditions, which may of course co-exist. The single most important cause of COPD is cigarette smoking, which dominates over all other aetiological factors, and which has a dose response relationship to the risk of developing COPD (177), and the best guide to prognosis is the rate of decline of the FEV_1 over time (175), which is accelerated in smokers who are susceptible to the development of COPD. Two major interactive hypotheses exist for the development of the tissue damage which is seen in COPD, namely a protease-antiprotease imbalance, and an oxidant-antioxidant imbalance. Cigarette smoke disturbs the normal balance between these processes, and results in unchecked protease and oxidant activity, which in turn leads to tissue damage by a variety of mechanisms.

Airway inflammation in COPD

Studies using a range of techniques have shown infiltration of the bronchi and bronchioles by inflammatory cells. In bronchial biopsies, T lymphocytes, in particular CD8+ cells are predominant (in contrast to the CD4+ cells found in asthma), and the CD8+ cell numbers show a significant relationship with decline in lung function (178). In contrast, BAL and induced sputum are characterised by increased numbers of neutrophils (179, 180), which are not reflected in the subepithelial area in bronchial biopsies, but increased numbers are found in bronchial glands (181) and in the epithelium (182). Eosinophils are present, particularly in exacerbations, but they do not appear to be activated and do not degranulate (153), and may not be active participants in the airway inflammation in COPD.

Inflammatory markers in COPD

Pro-inflammatory cytokines have been measured in bronchial secretions in COPD, and markers such as IL-8 and TNF-α (180) may have a role in neutrophil recruitment and activity. Similarly, increased levels of adhesion molecules which may be involved in neutrophil adherence and migration have been demonstrated in the circulation and BAL (183), and many studies have examined for evidence of protease and oxidant acitivity, which can influence inflammatory as well as structural processes in the lung.

Structural changes in COPD

In COPD structural alterations of the epithelium include atrophy, squamous metaplasia and qualitative and quantitative abnormalities of ciliated cells, and mucous galnd hyperplasia and enlargement are hallmarks of chronic bronchitis (184). Peribronchiolar and peribronchial fibrosis may occur, and abnormalities commonly extend to the lung parenchyma, where there may be disruption of the normal alveolar architecture, resulting in emphysema.

1.3.3 THE ASSESSMENT OF AIRWAY INFLAMMATION

As discussed above, airway inflammation is fundamental to a number of airway diseases including asthma, and recurrent exacerbations of symptoms are generally associated with exacerbation of airway inflammation. Clinical assessment of these conditions still relies principally on clinical findings and lung function, but there is increasing interest in more direct assessment of airway inflammation in the hope that this will clarify the impact of the type and severity of airway inflammation on disease outcome and treatment. This section will focus on asthma, but similar principles are applicable to the assessment of other airway diseases including COPD, although with different results in terms of the nature an extent of the inflammation. Assessment of airway inflammation is possible in fatal asthma at post mortem, and in less severe disease by bronchoscopic techniques, sampling of peripheral blood (and in some cases urinary markers) and more recently through the use of induced sputum and analysis of exhaled air.

Methods of assessment of airway inflammation other than induced sputum

Post mortem studies

Early studies assessing airway inflammation in asthma and COPD were based on post-mortem findings, and while fatal cases clearly represent an important subgroup of very severe disease, their relevance to mild cases and the evolution of the disease processes is not clear. Post-mortem studies have however contributed to the understanding of the pathophysiology of asthma, and demonstration of significant tissue eosinophilia, disruption of the bronchial epithelial cell layer and mucous plugging are hallmarks of severe asthma (185), although there is evidence of heterogeneity of cellular infiltration even in fatal asthma (186). Thankfully, asthma is only rarely a fatal condition, and therefore post mortem studies will rarely be able to provide important pathophysiological information about more mild asthma, and certainly cannot provide information on the

time-course of changes in airway inflammation in response to airway challenge or treatment.

Bronchoscopic studies

The use of fibreoptic bronchoscopy, which can be carried out safely subject to certain guidelines (187, 188), allows sampling of airway cells and mediators by bronchoalveolar lavage (BAL) along with histological studies using biopsy and bronchial brushing, and in addition controlled provocation of inflammation may be carried out using allergen challenge.

Bronchoalveolar lavage - While the technique of BAL has been widely used in the study of asthma and other airway diseases, there are limitations to its use, in particular the dilutional effect of BAL, with estimates suggesting that 2% of the BAL sample originates in the airway, with the remainder being return of the lavage fluid. Studies using BAL have confirmed the presence of eosinophilia with activation and degranulation of the eosinophils, and have shown associations between eosinophil numbers and eosinophil granule protein concentration and markers of disease severity such as symptom scores and measures of lung function (145, 146) and bronchial hyperreactivity (143). T cell numbers are relatively low in BAL, so a number of techniques have been used to try to increase the information obtained, including the detection of activation markers such as CD25 which suggests activation of CD4+ cells in atopic asthma (154) and both CD4+ and CD8+ cells in intrinsic asthma (155). BAL studies have also examined the effect of allergen challenge on airway cells and mediators, either using inhaled allergen, or using a segmental allergen challenge which involves treating one bronchial segment with allergen, and another with a sham challenge as a control (189).

Bronchial brushing - Bronchial brushing is a particularly effective way to study bronchial epithelial cells, and some studies have reported its use in asthma (190). It is a

complementary method which may be used along with BAL and biopsy in the study of airway inflammation.

Bronchial biopsy - Although some of the work done to establish the inflammatory basis of asthma was done using the rigid bronchoscope (e.g. (191, 192)), the advent of the flexible bronchoscope has allowed much wider use of bronchial biopsy samples in the study of asthma and other airway diseases. It has become apparent that some of the changes observed in fatal asthma accord with those observed in much more mild asthma, with evidence that airway inflammation characterised by eosinophilia and infiltration by activated T lymphocytes is present even in newly diagnosed asthma (91), but also evidence, even in mild asthma, that there is thickening of tissues adjacent to the epithelial basement membrane, with deposition of fibroblasts and myofibroblasts (166, 167). Bronchial biopsy samples taken before and after treatment demonstrate the efficacy of inhaled corticosteroids in reducing inflammation (193), although the effect on subepithelial fibrosis is less clear. The ability to assess morphology is a significant advantage of biopsy studies, and biopsies are generally taken as the standard against which other methods of sampling are judged, although there are obvious disadvantages relating to the invasive nature of the procedure.

Peripheral markers of inflammation

Peripheral markers of airway inflammation such as inflammatory cell counts in peripheral blood or plasma (or urine) levels of inflammatory mediators have been assessed as surrogate markers of airway inflammation. In general, the markers chosen relate to cells which are pertinent to airway inflammation and may be directly released by these cells.

Eosinophils - Until relatively recently, interest has centred on the eosinophil and its principal cationic proteins, in particular eosinophilic cationic protein (ECP), secreted during activation of eosinophils. Peripheral eosinophilia may be present in asthma (194), and reduce in response to inhaled steroid therapy (195), but peripheral eosinophilia is not specific to asthma. Similarly ECP may be of value in assessing

airway inflammation in asthma (196), and is elevated after allergen (197) and exercise (198) challenge in adult asthmatics, but elevation is not invariable, there is a wide scatter of results in most studies and the measurement of ECP is subject to a number of confounding factors, including the release by eosinophils of ECP after blood has been sampled. Elevated serum ECP may also be found in patients with atopic dermatitis (199), which may limit its utility in patients with co-existent disease.

T cells and cytokines - Peripheral blood T cell activation correlates with the severity of asthma (200), and circulating CD4+ T cell activity is reduced by glucocorticoid therapy (201). Numerous reports have been published of levels of circulating T-cell cytokines in asthma, with studies reporting levels of IL-5, IL-4, GM-CSF being of obvious relevance to the known pathogenesis of asthma. There are however limitations to interpretation of these findings, in that relatively little is known however about the factors which might influence the passage of these cytokines into the circulation, and the local and systemic degradation and clearance of cytokines secreted in the bronchial mucosa, either in health or disease. In addition since cytokines are primarily local messengers, it would seem important to know the relationship between local production and functionally relevant circulating levels of cytokine, but such data is not yet known, and conversely it is recognised that a cytokine may have an important local role without being detectable in significant quantities in the circulation.

Mast cells and neutrophils - Although histamine levels may rise during early allergic reactions and during exercise, levels may be modified by the numbers of circulating basophils (202), and mast cell tryptase is not a useful marker for asthma. Circulating neutrophils are primed or activated in asthma, with evidence of increased chemotactic activity in response to a number of stimuli (203, 204), and in acute severe asthma (205). These markers are not however routinely used in the clinical or experimental assessment of airway inflammation.

Other markers in peripheral blood

Adhesion molecules, eicosanoids (also in urine) and reactive oxygen species have been studied, with evidence of some relevance to asthma, but further work is needed to clarify their relationship to airway inflammation and their utility as markers for clinical monitoring in asthma.

Exhaled air

There has been substantial interest in the measurement of markers of inflammation in exhaled air, with the prospect that this could provide a completely non-invasive method of assessing airway inflammation. Exhaled nitric oxide (NO) is increased in asthma (206, 207), probably due to upregulation of inducible nitric oxide synthase (iNOS), and levels of exhaled NO are related to bronchial hyperresponsiveness (208), asthma exacerbation (209) and airway inflammation following allergen challenge (210). Exhaled NO is reduced by treatment with inhaled steroids (211), but not by β-agonist bronchodilators, suggesting that exhaled NO may be a marker of airway inflammation. Similarly, levels of another monoxide, carbon monoxide (CO) have been shown to be elevated in asthma, with a reduction following treatment with corticosteroids (212), and further work has shown an association with increased expression of the stress protein heme oxygenase-1 which catabolises heme to bilirubin, free iron and CO (213). It is possible to measure other potential markers of inflammation in exhaled air, and this is an active area of research, but further work is need to assess the utility of these markers in the assessment of asthma.

1.3.4 INDUCED SPUTUM

The analysis of sputum in lung diseases is not a new phenomenon, but although a proportion of asthmatics may be able to produce spontaneous sputum samples on request, the cell counts and viability of cells in such samples is often too low for analysis, and there is greater contamination of samples with squamous epithelial cells (214). This limits the utility of spontaneous sputum samples in asthma (and even more so in healthy subjects) and has led to interest in sputum induction as a non-invasive method of assessing airway inflammation. The technique of sputum induction using hypertonic saline administered by aerosol was first reported in 1958 with the aim of obtaining sputum for cytology in suspected lung cancer (215), but it has only recently been extended to include the assessment of airway inflammation. The mechanism by which hypertonic saline induces sputum is not known although proposals include osmotic effects (216), an increase in mucociliary clearance (217) and stimulation of glandular secretions.

Methodology

The methodology and validity of sputum induction as a technique has been a topic of particular interest, with studies reporting the use of a number of techniques. Sputum induction has been shown to be safe in asthmatics of varying severity (218), including severe asthma (219), if necessary with variations in strength of hypertonic saline and time of inhalation, and the technique is now accepted as being valid, reproducible (220-222) and responsive. There are subtle differences in the method of inducing sputum between groups, some using increasing concentrations of hypertonic saline (generally 3, 4 and 5%) with others, including our own group, using a fixed concentration of saline (3%), and there are also minor differences in the time periods over which sputum is induced. Choice of technique does not seem to significanlty alter the differential cell count in the sputum obtained (223). Sputum induction is superior to the collection of spontaneous sputum in its greater applicability, greater cell viability and sputum yield in

both asthma (214) and COPD (224). There has been lively debate about the best method of processing of samples, either by analysis of the whole sputum sample or by analysing only the viscid portion of the sputum, which is taken to reduce salivary contamination. We have adopted the latter technique, and our findings for salivary levels of ET-1 presented in chapter 6 indicate that salivary contamination may have consequences other than simply dilution of the sample and an increase in squamous epithelial cells. Studies have compared the methods, and current evidence suggests that although sputum selection may provide more viable cells, including eosinophils, and increased concentrations of mediators (225), both methods allow differentiation between health and disease (226). The general consensus is that although there are differences between the two techniques and the results are not therefore interchangeable, the utility of the sputum induction when performed in a standardised way, with appropriate monitoring and safety precautions is not influenced by the choice of processing method (223). Standardisation and consistency in method of sputum induction and processing of samples would allow direct comparison of results from different groups, but despite calls for such standardisation (227) no agreement has yet been reached.

Conformity with other measures of airway inflammation

The composition of induced sputum in asthmatics has been assessed in comparison with other direct measures of airway inflammation, such as bronchial wash (BW), bronchoalveolar lavage (BAL) and bronchial biopsies, and also in comparison with some non-invasive markers of airway inflammation. Current evidence suggests that sputum contains higher absolute numbers of eosinophils and neutrophils, but fewer lymphocytes and macrophages than BW or BAL (228-231). There is agreement that eosinophil proportions are similar comparing induced sputum with BW, but not all studies have reported a significant relationship with BAL eosinophils (228, 229, 231). The relationship between cell numbers and proportions comparing induced sputum with bronchial biopsies is less clear (228, 232). Comparison of induced sputum composition with non-invasive markers of airway inflammation would suggest that sputum

eosinophilia is a better marker of asthma than peripheral blood eosinophil counts (233) or ECP levels (234), while there is a relationship between sputum eosinophilia and exhaled NO in asthma (208).

Responsiveness to intervention

Experience to date would suggest that induced sputum is able to reflect changes in the degree of airway inflammation in asthma in response to a variety of factors. Exposure to allergen (148, 235) has shown increases in eosinophils and metachromatic cells in sputum, and conversely, responsiveness of induced sputum parameters to treatment has been demonstrated in asthma over a range of severity (236-238), including responsiveness to relatively subtle changes in treatment regimens (237, 239).

1.3.5 GENERAL COMMENTS ON METHODS OF ASSESSING AIRWAY INFLAMMATION

Airway inflammation and airway function

There is a tendency to assume that the assessment of airway inflammation in asthma offers direct information about symptoms, prognosis and response to treatment, and *vice versa*, but in fact the relationship of airway inflammation to airway function and its clinical patterns is not yet clear (240, 241). Current treatment strategies in asthma are directed towards the control of symptoms of the disease and lung function (242), partly on the assumption that these are correlates of airway inflammation, but there is evidence that patients who appear to be clinically controlled may still have airway hyperresponsiveness (AHR) and persistent airway inflammation (243, 244). Relationships have been described between airway inflammatory cells and AHR assessed by methacholine, and it has been suggested that assessment of AHR should be a component of clinical evaluation in asthma (152), although it is important to note that airway inflammation is not the only contributor to AHR (245), and that some studies have demonstrated a dissociation between AHR and airway inflammation in asthma (246, 247). If it is true that airway inflammation contributes to airway remodelling, then it may be necessary to incorporate some measure of airway inflammation into treatment protocols, either directly, or via some surrogate marker, for example AHR or non-invasive markers such as exhaled gases. There is evidence that AHR may act as a marker of the risk of exacerbation in asthma (248), and that treatment aimed at controlling AHR rather than symptoms alone can reduce the exacerbation rate, with associated reduction in inflammatory cells in bronchial biopsies (249). Interestingly, the same study demonstrated a greater reduction in the reticular layer thickness beneath the epithelium in bronchial biopsy specimens comparing the group whose treatment was aimed at controlling AHR with the group whose treatment was aimed at controlling symptoms (249), supporting a presumed association between AHR, airway inflammation and airway remodelling. Overall, a link between symptoms, AHR, airway

inflammation and response to therapy seems intuitive, but regardless of the method of assessing airway inflammation, it should be recognised that each of these "measures" of asthma may reflect subtly different components of the disease, giving complementary rather than identical information about disease activity and prognosis.

Bronchoscopic methods

Although generally safe and well tolerated, bronchoscopy is invasive and carries some degree of risk, which although small, may limit the utility of the technique in clinical management. The degree of risk which is acceptable will vary according to the patient group under investigation, such that a degree of risk which might be tolerable in lung cancer could be intolerable in a disease such as asthma which has a very low death rate, and similarly intolerable in healthy or patient group volunteers. In general, ethical permission can be obtained for bronchoscopic studies in patients with mild asthma, and for a small number of healthy volunteers, and while some studies have involved repeated samples, the nature of the procedure would make time-course studies difficult, and the expense and need for specialised equipment would prevent bronchoscopy being used for larger studies of airway inflammation. Bronchoscopic studies are however likely to remain the "gold standard" against which other methods of assessing airway inflammation are measured.

Induced sputum

There has been some concern that sputum induction itself may act as an inflammatory stimulus, and studies have shown that in normal volunteers, a rise in sputum neutrophils is seen on repeat sputum induction at 8 hours after the first induction, which was sustained at 24 hours (250), with similar results in asthmatics who had repeat sputum induction at 24 hours (251). While this may be a limitation of the technique of sputum induction, there are important technical considerations which remain to be fully explored, such as the effect of the nebuliser output and type of nebuliser and the concentration of saline used, as studies differ in these respects, and although these data

should not affect results of a single sputum induction, there may be an effect on serial samples.

1.4 HYPOTHESES AND AIMS OF THESIS

The background to this thesis is found in the work published from our group reporting the *in vitro* activity of ET-1 in bronchial tissue. Our group and others had demonstrated that ET-1 has potent bronchoconstrictor activity in animal (85) and human (60) tissue *in vitro*, and demonstrated differences between the mechanism of bronchoconstriction of ET-1 in human compared with bovine tissue (60), and responses to bronchodilators of tissue pre-contracted by ET-1 (252). From this we hypothesised that ET-1 could have a role in bronchoconstriction in asthma, and were supported in this hypothesis by the published literature identifying increased immunostaining for ET-1 in asthmatic airways (122), and a relationship between BAL ET-1 and airflow obstruction in asthma (124). As part of a study from our group examining a role for angiotensin II in acute severe asthma, plasma ET-1 concentrations were measured, and were found to be elevated (119), providing further support for a role in acute severe asthma. The other component behind this work was an evolving interest in the use of the non-invasive technique of sputum induction in the assessment of asthma, and the desire to apply it in the measurement of novel mediators as well as in quantification of cellular inflammation in asthma.

Initial aims were therefore based along two lines of enquiry, the first being to confirm *in vivo* the bronchoconstrictor activity and potency of ET-1, and the second to examine the use of sputum induction to assess airway concentrations of ET-1. These two themes form the basis for the main subdivisions of the thesis, with the bronchoconstrictor activity of ET-1 being the focus of chapters 3, 4 and 5, while the use of sputum induction to obtain fluid for the measurement of ET-1 underlies the work presented in chapters 6 to 9. The initial hypothesis was based around asthma, but as outlined above, several of the properties of ET-1 could relate to other diseases affecting the airways, and we extended the hypothesis therefore to include cigarette smoking and COPD, and on

the basis of some speculative pilot work, also measured sputum ET-1 in cystic fibrosis (CF).

The results of these investigations should increase our understanding of the potential role of ET-1 in the pathophysiology of asthma, and provide the basis for further investigation of a previously unsuspected role in COPD and CF.

CHAPTER 2

GENERAL METHODS AND MEASUREMENTS

GENERAL METHODS AND MEASUREMENTS

2.1 SUBJECTS

Mild asthmatic subjects were recruited from the outpatient asthma and general respiratory clinics of the West Glasgow Hospitals University NHS Trust (Gartnavel General Hospital and the Western Infirmary, Glasgow) and patients presenting with acute severe asthma or COPD were recruited via the respiratory wards at Gartnavel General Hospital and the Acute Receiving Unit at the Western Infirmary, Glasgow, with the permission of the admitting consultant. Patients with cystic fibrosis were recruited from the West of Scotland Adult Cystic Fibrosis Unit, which is based at our institution. Healthy volunteers were recruited mainly from medical, nursing and ancillary staff in these hospitals. None of the asthmatic subjects were cigarette smokers. Further demographic data for each subject group is presented and discussed in the appropriate chapter.

2.2 LUNG FUNCTION AND BRONCHIAL CHALLENGE TESTS

Spirometry

Spirometry was performed using a Vitalograph Compact electronic pneumotachograph spirometer (Vitalograph, Buckingham, UK). The spirometer was calibrated regularly using a fixed volume syringe, and regular maintenance checks were performed. Subjects were requested to inhale to full inspiration and then exhale forcibly to full expiration, and the best of three readings was taken for each measurement. The commonest measurement used to assess airflow obstruction in the forced expiratory volume in 1 second (FEV_1), and values used are the best of three readings in each case. In most cases, FEV_1 is expressed as a percentage of age and height predicted values (253), with values below 70% in general indicating airflow obstruction. Many mild asthmatics however will have normal or near normal FEV_1 values, and in the confirmation of asthma in particular, greater reliance was placed on bronchial provocation testing than on FEV_1 values *per se*.

Peak Expiratory Flow

Peak expiratory flow rates (PEFR) were used to assess severity in the patients with acute asthma or COPD at the time of admission. Where used, PEFR are expressed as litres/minute, and the value stated is the best of three readings.

Constant volume body plethysmography

For the study described in chapter 3 measurements were made using a constant volume body plethysmograph (Erich Jaeger GmbH, Wuerzburg, Germany; "Masterlab v4.2"), with assessment of specific airways conductance (SGaw). SGaw is calculated using the software provided with the body plethysmograph, and in challenge testing, a fall of 35% in SGaw was used for analysis. Further details of the procedure employed in the study are given in chapter 3.

Bronchial provocation tests

Bronchial hyperresponsiveness is an increased tendency to respond to a variety of stimuli by bronchoconstriction, and is characterstic of but not exclusive to asthma. Asthmatics will in general respond to a bronchoconstrictor stimulus at a lower dose than non-asthmatics, and this characteristic can be used to support a clinical diagnosis of asthma. Since bronchial responsiveness is influenced by inhaled bronchodilator therapy and there is some diurnal variation, tests were performed at similar times of day, and subjects were asked to withhold short-acting $ß_2$ agonists (e.g. salbutamol/terbutaline) for 8 hours prior to testing, and long-acting $ß_2$ agonists for 24 hours. Slow release theophylline preparations were withheld for 48 hours if applicable, and inhaled corticosteroids were unchanged. (Although inhaled corticosteroids may alter bronchial hyperreactivity compared with values when not taking inhaled steroid, this effect is not an acute effect, and bronchial provocation tests performed while on a stable dose of inhaled steroid are comparable.) The technique of bronchial challenge testing has been described for histamine by Cockcroft *et al* (254), and is applicable to other bronchoconstrictor substances. In summary, a substance is administered by nebulisation in incremental doses (usually doubling doses) with measurement of lung function (e.g. spirometry or body plethysmography) at each dose. This allows a dose-response curve to be established, and a threshold response can be identified to allow quantification of the response, expressed as the provoking concentration (PC) of the bronchoconstrictor substance required to produce a given change in lung function, and calculated by linear interpolation. For example, asthmatics tend to have increased sensitivity to histamine, with a provoking concentration required to produce a 20% fall in FEV_1 ($PC_{20}FEV_1$) of $\leq$ 8mg/ml.

For the purposes of the studies described, bronchial hyperresponsiveness to methacholine (an acetylcholine analogue) was used as confirmation of asthma, and as an entry requirement for all studies, with a value for $PC_{20}FEV_1$ to methacholine of < 8 mg/ml. For screening purposes and for most of the studies, methacholine was

administered using a timed inhalation at each dose from the same Wright's nebuliser. The nebuliser contained 3ml of nebulisate and was driven by a continuous airflow of 9 l/min from a compressed air source of 50lbs/in (345 kPa), calibrated to deliver an output of 0.13 ml/min. Where greater accuracy of dosing was required, for example for administration of allergen (Chapter 7) or endothelin-1 (Chapters 3,4 and 5), the substance was administered using an air-driven dosimeter ('Nebicheck' PK Morgan Ltd, Gillingham, U.K.), calibrated to deliver 0.006ml/breath, and dose was incremented by increasing the number of breaths at each dosage interval. In each case, baseline FEV_1 was measured, and the first inhalation was of phosphate buffered saline, followed by spirometry at 30, 90 and 180 seconds, with the lowest post saline value taken as the baseline from which subsequent falls were measured. At 5 minute intervals, subjects received inhalations of doubling concentrations of methacholine (dose range 0.0625 to 8 mg/ml), and FEV_1 was repeated at 30, 90 and 180 seconds. Inhalations were continued until the FEV_1 fell by more than 20%, and the PC20FEV1 was then calculated by linear interpolation. At the end of each methacholine challenge, subjects received inhaled bronchodilator (from metered dose inhaler or nebuliser) to reverse the bronchoconstriction.

2.3 SPUTUM INDUCTION AND PROCESSING OF SAMPLES

Sputum induction procedure

For the studies where induced sputum samples were obtained, sputum induction was performed using a modification of the method described by Pin *et al.*(144) Briefly, after salbutamol 200 mcg was administered by metered-dose inhaler with large volume spacer, sputum induction was started using hypertonic (3%) saline administered via an ultrasonic nebuliser (Schuco International Ltd, London, U.K. or Medix Sonix 2000) over a period of 20 minutes. The subjects were encouraged to expectorate at any time throughout the procedure, and in addition inhalation was stopped every 5 minutes to allow expectoration, and to allow spirometry to be carried out. Prior to expectoration subjects were asked to rinse their mouth with water and to blow their nose to try to minimise contamination of sputum samples with saliva and nasal secretions. The sample was collected in a sterile container and was transferred to the laboratory on ice as soon as possible, and in all cases in less than 2 hours. If FEV_1 fell by more than 20%, the procedure was discontinued.

Processing of sputum samples

Samples were processed without the laboratory staff being aware of the clinical information relating to the individual subject, and the procedure followed was similar to that described by Popov *et al* (255). This procedure is diagramatically represented in **figure 2.3.1**. Saliva samples (where appropriate) were stored at -20°C and sputum samples were transferred to a Petri dish and the volume and macroscopic characteristics of the whole sample were recorded. Sputum plugs were selected, if necessary using inverted microscopy, to minimise salivary contamination (256) and were split into two Eppendorfs. One was treated with fresh dithiothreitol (DTT) (Sigma U.K. Ltd) in a balanced salt solution and the other was centrifuged (13,000 rpm for 15 minutes) without prior treatment with DTT for endothelin assay, and the resulting supernatant stored at -70°C. Following incubation with DTT for 20 minutes the DTT-treated samples

were filtered through 50μm mesh (R Cadoch & Sons, London U.K.) to remove residual mucus clumps and a total cell count was made using a white cell counter (CBC5, Coulter Electronics Ltd. U.K.). The sample was centrifuged (3,000 rpm for 4 minutes), the supernatant removed and stored at -70°C prior to assay and the remaining pellet (re-suspended at $1x10^6$ cells/ml) was cytocentrifuged (500 rpm for 5 minutes) using a Shandon centrifuge. Differential cell counts were made from the resulting slides using Giemsa staining, and are expressed after exclusion of squamous epithelial cells which are taken to represent salivary contamination (256). Criteria were established for rejection of samples if viability was <40%, or squamous cells >20% although in fact no samples were rejected on these criteria (viability was typically >70% and squamous cell count around <10%). The bronchial epithelial cell count was very low in all samples (typically <2%).

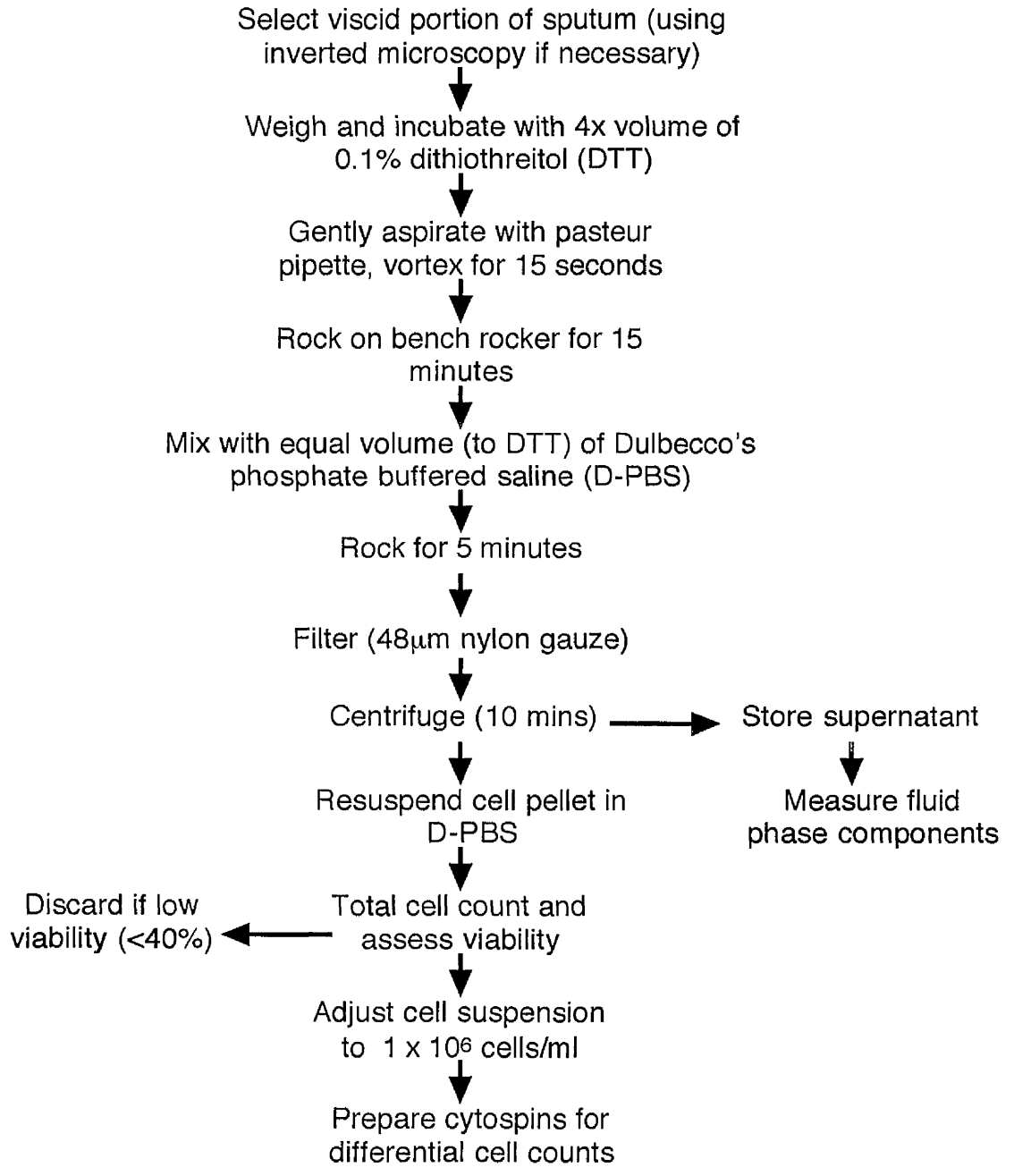

Figure 2.3.1

Laboratory processing of sputum samples
(adapted from Pavord et al, Thorax 1997; 52: 498-501)

2.4 ASSAYS

Endothelin-1 assay

ET-1 assay was performed by Dr J.J. Morton in the radionuclide laboratory of the Department of Medicine, Western Infirmary Glasgow, using a commercially available radio-immuno assay (RIA) (Nichols Institute Diagnostics Ltd, San Juan Capistrano, CA, U.S.A.) This assay has 100% sensitivity for ET-1, with some cross-reactivity for other ET species (67% for ET-2, 84% for ET-3, and much lower cross-reactivity for the precursor molecules big-ET-1/2/3). Our own results from reverse-phase high-performance liquid chromatography (RP-HPLC) however, suggest that ET-1 is the dominant species in sputum samples, with no signal being detected for ET-2 or ET-3 (see below). The lower limit of detection of the RIA is c. 2 pg/ml in plasma, and assays were run in duplicate, with the mean value used for analysis. We found the coefficient of variation of the assay to be 11.4% for sputum samples. Sputum samples for ET-1 assay were not treated with the reducing agent DTT, since there was concern that DTT might alter the ET-1 assay, based on a pilot assay using a small number of samples, as outlined below. Samples for ET-1 assay were thawed and pre-extracted using C-18 columns prior to RIA. We performed plasma ET-1 assay on a sub-group (n=9) of patients both before and after sputum induction, and found that there was no difference in plasma ET-1 due to the procedure itself (data not shown).

Effect of DTT on ET-1 assay

To examine the effect of DTT on ET-1 measurements, assays were performed on ET-1 control samples along with DTT, and we found that DTT altered the ET-1 standard curve with apparent elevation of ET measurements, when using pure ET-1 standards. This effect was not apparent with DTT treated clinical samples as opposed to control ET-1 samples, but to avoid potential confounding factors we modified the process to allow sputum plugs to be assayed without prior treatment with DTT. The mechanism for this possible interference is not known, but since RP-HPLC did not suggest an alteration in

the ET-1 molecule following treatment with DTT, it is presumed that any interference might be with some component of the assay system rather than the ET-1 molecule itself. Due to an assay failure, with resultant loss of DTT-free samples, ET-1 assay was performed on DTT-treated samples from acute severe asthmatics and patients with acute exacerbation of chronic obstructive pulmonary disease presented in chapters 6 and 8 respectively. No comparison is made between these data and non-DTT-treated samples.

Recovery of ET-1 from sputum samples

In order to assess recovery of ET-1 from sputum samples, sputum samples were "spiked" with a known concentration of ET-1, and assay techniques performed in the usual way. We found that recovery averaged 68%.

Other assays

Assays were perfomed on induced sputum samples for a variety of other mediators, including eosinophilic cationic protein, interleukin-1ß, nitrite and albumin. The details of these assays are contained in the relevant chapters.

Reverse-phase high performance liquid chromatography

Reversed phase high performance liquid chromatography (RP-HPLC) was carried out on a number of saliva and sputum samples to identify the presence of ET-1 and confirm the findings of the RIA. RP-HPLC was performed by Dr Brian McGinn at Thistle Research, Glasgow UK. Dried samples from C-18 column extraction were dissolved in 0.1ml water/acetonitrile (9:1) with 0.05% trifluoroacetic acid (TFA), and applied to a Vydac Peptide & Protein C18 HPLC column 4.6 x 250mm. The column was eluted with a 20-50% gradient of A: 0.05% TFA in water and B: 0.05% TFA in acetonitrile over 30 minutes at a flow rate of 1 ml/min. During the gradient elution, 10 x 1ml fractions were collected between 15 and 25 minutes, since a calibration analysis using synthetic ET-1 showed its elution point to be 21.1 minutes. Following elution, fractions were dried down for radio-immunoassay (RIA).

2.5 DATA HANDLING AND STATISTICAL ANALYSIS

Statistics were performed on an Apple Macintosh desktop computer (Apple Computer Inc., Cupertino, CA, U.S.A.) using a statistical software package (Minitab Statistical Software, Minitab Inc., State College, PA, U.S.A.). Non-parametric statistics (Mann Whitney-U test) were used to compare cell counts and concentrations of mediators, and values are expressed as median (interquartile range) unless stated otherwise. PC_{20} and PC_{15} values were calculated by log-linear interpolation and are expressed as geometric mean and range. The geometric mean is the antilog_{10} of the mean of the logs_{10} of the PC values, and it is used because the dosage intervals in the bronchial challenge tests are doubling rather than linear. Analysis of variance (ANOVA) was used to compare blood pressure, pulse rate and pulse oximetry data in ET-1 inhalation studies.

2.6 ETHICAL APPROVAL

Ethical permission for each study was obtained from the Glasgow West Ethical Committee prior to commencement of the studies. Written information was given to all subjects, and written informed consent was obtained from all subjects.

2.7 DRUGS

Endothelin-1 - Dried purified ET-1 was obtained from Thistle Research, Glasgow UK for studies described in chapters 3 and 4, and from Calbiochem-Novabiochem (UK) Ltd. Nottingham, UK for the study described in chapter 5, reconstituted in each case using 0.9% saline prior to nebulisation, at a concentration of 0.2mg/ml.

Salbutamol - (Ventolin ®) Allen & Hanburys Ltd, Uxbridge UK. Metered dose inhalers or pre-prepared solution for nebulisation 2.5ml in normal (0.9%) saline.

Methacholine - for inhalational challenge (acetyl-ß methyl choline chloride) Sigma Chemical Company, Poole, Dorset UK, made up in phosphate buffered saline to pH 7.4. Methacholine was prepared by sterile pharmacy in our institution.

Angiotensin II - (Affiniti Research Products Ltd, Exeter, U.K.) prepared in 5% dextrose as a solution for intravenous administration at doses stated in chapter 5.

House dust mite allergen - (Allergy Therapeutics Ltd, Worthing, UK - previously SmithKline Beecham Pharmaceuticals PLC) *Dermayophagoides pteronyssinus* aqueous extract 1.2% standard preparation, used for chapter 7.

CHAPTER 3

INHALED ENDOTHELIN-1 INDUCES BRONCHOCONSTRICTION IN ASTHMA

3.1 INTRODUCTION

In view of reports from our own group and others of the highly potent bronchoconstrictor activity of ET-1 *in vitro* in both animal (85, 87) and human (60) bronchial preparations, we wished to examine the potential bronchoconstrictor effect of ET-1 in humans (asthmatic and non-asthmatic) *in vivo*. Bronchoconstriction is a central feature of asthma, and the highly potent bronchoconstrictor effects of ET-1 *in vitro* relative to other known bronchoconstrictor substances such as histamine and methacholine merit investigation in asthma, where increased airway ET-1 has been reported. In the guinea-pig, administration of ET-1 by intra-arterial infusion or by aerosol in isolated perfused lungs (61) or in anaesthetised and ventilated animals (67), produced dose-dependent bronchoconstriction, but there are no reports in humans of the *in vivo* bronchoconstrictor effects of ET-1. Interestingly, topical application of ET-1 to the nasal mucosa results in increased nasal secretions in healthy volunteers, with a greater increase in secretions in allergic rhinitis (114), suggesting increased upper airway sensitivity to the local effects of ET-1 in allergic subjects.

The objectives of the study were to examine the effects of inhaled ET-1 on lung function in asthmatics, in comparison with normal subjects, and to assess the *in vivo* bronchoconstrictor potency of ET-1 in comparison with methacholine.

3.2 PILOT WORK

Since there are no reports of aerosol adminstration of ET-1 in human subjects, and it was necessary to base this study on human *in vitro* data from our own laboratory, pilot data was obtained, primarily to establish a dose range for use in the study. Bronchial challenge tests were performed as stated below initially on one non-asthmatic subject and thereafter one asthmatic subject, using an ET-1 starting dose below that recorded to produce any contraction in human bronchial tissue *in vitro* (0.03 nmol), with doubling doses up to the starting dose outlined in the study below. No bronchoconstriction was observed in either subject, nor was there evidence of a pressor effect, nor any change in pulse oximetry. Two further asthmatic subjects were then recruited, and repeat bronchial challenge performed using the dose-range outlined below, with resultant dose-dependent bronchoconstriction. This dose range was then used for the study as a whole, and data from these subjects is included in the study (asthmatic subject numbers 1 and 2).

3.3 METHODS

Subjects

13 adult subjects were studied, sub-divided in to 8 asthmatics and 5 normal volunteers, with numbers limited by the high cost of ET-1 (roughly £125 per inhalation). Asthmatics all had current asthma with stable symptoms at the time of study, no history within the preceding month of respiratory infection, antibiotics or oral corticosteroid use and other than house dust mite, none of the subjects had been exposed to allergens to which they were allergic in the preceding month. Asthma was defined according to the ATS definition (257), baseline lung function was recorded, and non-specific bronchial hyperresponsiveness was established using a methacholine challenge test, with all the asthmatics subjects having a methacholine PC_{20} of less than 8mg/ml. Normal subjects had no history of breathlessness or wheeze, normal baseline spirometry ($FEV_1 \geq$ 70% predicted in the absence of symptoms) and had a methacholine PC_{20} of greater than 16mg/ml. The study was approved by the West Ethics Committee, West Glasgow Hospitals University NHS Trust, and each subject gave written informed consent.

Protocol

The study was performed in randomised single-blind fashion. Asthmatic subjects attended for methacholine screening test, followed by three visits for ET-1 (2 visits) or methacholine (1 visit) inhalations. Asthmatics withheld ß2-agonist therapy for 12 hours prior to each study visit. Normal subjects attended for screening methacholine challenge followed by a single visit for ET-1 inhalation. The interval between visits was not fixed, but was generally around 1 week for most subjects. On the study days, blood was sampled for plasma ET-1 assay before and after each inhalation, and oxygen saturation (Sa_{O_2}) recorded by pulse oximetry and non-invasive blood pressure were recorded at regular intervals through the inhalations. Both ET-1 and methacholine were administered using an air-driven dosimeter ('Nebicheck' PK Morgan Ltd, Gillingam, U.K.), calibrated to deliver 0.006ml/breath, with a doubling dose range for ET-1 of 0.96-15.36

nmol, and methacholine of 0.33-21.0 μmol. Dried purified ET-1 (Thistle Research, Glasgow, U.K.) was reconstituted in 0.9% saline prior to nebulisation, and methacholine was prepared by sterile pharmacy in our own hospital. The nebulisate was prepared by a second operator, with both patient and principal operator blinded to the contents of the nebuliser chamber. Measurements were made using a constant volume body plethysmograph (Erich Jaeger GmbH, Wuerzburg, Germany; "Masterlab v4.2"), with assessment of specific airways conductance (SGaw) and FEV_1 5, 10 and 15 minutes after each dose of study drug. The inhalation was stopped following a 15% fall in FEV_1 or following the maximum dose in the dosing schedule, whichever came first, and on all but 2 occasions albuterol (2.5mg nebulised) was given and measurements repeated after 10 minutes. On 2 occasions, albuterol was not given, and measurements were continued until the SGaw had returned to within 25% of pre-challenge value to assess the duration of bronchoconstriction in these patients (both asthmatic).

Laboratory processing and biochemical assays

Following venepuncture, blood was centrifuged at 3000 rpm for 10 minutes and the resulting supernatant frozen and stored for ET-1 assay. After thawing, plasma samples were pre-extracted using C-18 columns (Waters Ltd, Watford, U.K.) prior to ET-1 assay by radio-immuno assay (RIA) (Nichols Institute Diagnostics Ltd, San Juan Capistrano, CA, U.S.A.). The lower limit of detection is c. 2 pg/ml in plasma, and assays were run in duplicate, with the mean value used for analysis. To ensure that ET-1 was not altered by nebulisation, a sample of ET-1 was returned for ET-1 assay following nebulisation, and this confirmed that there was no loss of activity of ET-1.

Data handling and statistical analysis

Lung function values were calculated using the software linked to the constant volume body plethysmograph (Erich Jaeger GmbH, Wuerzburg, Germany; "Masterlab v4.2") and are expressed as percent of baseline value except where stated. Statistics were performed on an Apple Macintosh desktop computer (Apple Computer Inc., Cupertino,

CA, U.S.A.) using a statistical software package (Minitab Statistical Software, Minitab Inc., State College, PA, U.S.A.). Demographic factors and baseline and maximum fall in lung function parameters were analysed using parametric statistics (t-test) with correction for multiple comparisons, and pre- and post-inhalation plasma ET-1 values compared using non-parametric statistics (Mann Whitney-U test), with significance accepted at the 95% level in each case.

3.4 RESULTS

Patient demographics

Comparison of demographic factors reveals that the asthmatic group were older ($p<0.05$) on average than the normal group. SGaw and FEV_1 (absolute and % predicted) were lower in the asthmatic group than in normals (table 3.1) and there was no difference between asthmatics' baseline values of FEV_1, SGaw or $Sa0_2$ on the three study days (table 3.2).

Pulse oximetry

In both asthmatics and normals, pulse oximetry was normal prior to methacholine and ET-1 inhalations, and did not alter significantly during any of the inhalations, with a mean variation of less than 1% for all the inhalations (table 3.2, appendix 3.1).

Pulse and blood pressure - (Appendix 3.2)

There was no significant change in pulse rates for any of the inhalations, in either asthmatics or normals. In the asthmatic group mean (S.D.) blood pressure (systolic/diastolic) was 120 (9.6)/75 (10.0) mmHg (pre-) and 119 (8.2)/72 (9.4) mmHg (post-) methacholine inhalation; 121 (8.7)/80 (6.1) mmHg (pre) and 123 (11.8)/76 (5.9) mmHg (post) for the first ET-1 inhalation; 121 (8.3)/73 (7.4) mmHg (pre) and 121 (9.0)/73 (6.4) mmHg (post) for the second ET-1 inhalation. These represent percent changes of less than 4% in each case, and these changes were not statistically significant. In normal subjects mean (S.D.) blood pressure was 123 (6.8)/71 (2.8) mmHg (pre) and 113 (5.3)/73 (5.0) mmHg (post) ET-1 inhalation, and the fall in systolic blood pressure does reach statistical significance ($p=0.03$).

Plasma endothelin-1

Normal subjects had no change in plasma ET-1 across the ET-1 inhalation (median (IQR) pre- and post ET-1 challenge values of 2.2 (2.1-2.5)pg/ml and 2.0 (1.5-

2.5)pg/ml respectively). In asthmatic subjects there was no change in plasma ET-1 across the methacholine inhalation (median (IQR) pre- and post methacholine challenge values of 3.3 (2.1-3.9) pg/ml and 3.0 (2.0-3.5) pg/ml respectively), nor across either of the ET-1 inhalations (median (IQR) pre- and post ET-1 challenge values of 3.2 (2.9-4.5) pg/ml and 3.8 (2.8-4.6) for the first ET-1 inhalation, and 3.3 (3.0-4.0) and 4.0 (2.9-4.3) for the second ET-1 inhalation). Both pre- and post ET-1 inhalation values were higher in asthmatics than normals for both ET-1 inhalations, but this did not reach significance for the methacholine inhalation.

Specific airways conductance (Sgaw)

Individual dose-response graphs for asthmatic subjects are presented (figure 3.1) indicating that all the asthmatic subjects experienced a dose-related fall in SGaw over the dose range used, with mean (S.D.) fall in SGaw of 69.7 (15.9)% for methacholine, and 63.0 (11.8) % and 60.5 (14.9) % for the 2 ET-1 inhalations. The mean (S.D.) concentration required to produce a 35% fall in SGaw ($PC_{35}SGaw$) in asthmatics was 0.42 (0.18) μmol for methacholine, 5.15 (4.4) nmol for the first ET-1 inhalation, and 4.29 (2.5) nmol for the second ET-1 inhalation, suggesting that ET-1 is around 100 x more potent as a bronchoconstrictor in asthma than methacholine on a molar basis (range 30-350 x for individual patients). Treatment with albuterol (2.5mg nebulised) completely reversed bronchoconstriction in all cases. The correlation between methacholine $PC_{35}SGaw$ and ET-1 $PC_{35}SGaw$ was not significant (in contrast to $PC_{15}FEV_1$ below). None of the normal subjects experienced a significant fall in SGaw (mean (S.D.) fall in SGaw of 9.2 (10.6) %) even at the top of the dose range (15.36 nmol) of ET-1 (figure 3.2) (ET-1 $PC_{35}SGaw$ for normals is >15.36 nmol).

Forced expiratory volume in 1 second (FEV_1)

Individual dose response graphs for asthmatic subjects are presented in figure 3.1. Although the mean (S.D.) fall in FEV_1 was 20.1 (8.9) % for methacholine, 18.3 (6.9) % for the first ET-1 inhalation, and 16.25 (5.1) % for the second ET-1 inhalation (table 2), and all had a dose-related fall in FEV_1, 2 of the asthmatic patients had a fall in FEV_1 of less than our target of 15% following the maximum dose of ET-1 (15.36 nmol). Figures for provoking concentrations of ET-1 required to produce a 15% fall in FEV_1 ($PC_{15}FEV_1$) are therefore calculated using geometric extrapolation of the fall at the maximum dose for these 2 patients (3 ET-1 inhalations). Mean $PC_{15}FEV_1$ concentrations for the asthmatic group were 0.93 (0.66)μmol for methacholine, 10.52 (11.6)nmol for the first ET-1 inhalation, and 12.15 (8.12)nmol for the second ET-1 inhalation, again suggesting that ET-1 is roughly 100 x as potent a bronchoconstrictor in asthma as methacholine on a molar basis (range 25-200 x for individual patients). The

correlation between methacholine $PC_{15}FEV_1$ and ET-1 $PC_{15}FEV_1$ was strong (r=0.912 and 0.816 for each ET-1 inhalation) and statistically significant (p=0.002 and 0.014 respectively). None of the normals experienced any significant fall in FEV_1 with mean (S.D.) change of 0.4 (0.9) % from baseline (figure 3.2) (ET-1 $PC_{15}FEV_1$ for normals is > 15.36nmol).

Duration of bronchoconstriction

Duration of bronchoconstriction was not examined as a formal part of the study protocol, but in 2 asthmatic patients, albuterol was not given at the end of the inhalation of ET-1 on their final visit, and these patients serial lung function measurements were made until the SGaw had returned to within 25% of baseline values. In one patient this took 60 minutes, and in the other 90 minutes.

Repeatability

Repeatability of the two ET-1 inhalations was assessed using the method described by Bland and Altman (258) to establish a repeatability coefficient, by comparing the standard deviations of the differences in $PC_{35}SGaw$ values for each asthmatic patient for the two ET-1 inhalations (figure 3.3). This gives a repeatability coefficient (2x S.D. of the differences) of 8.47 nmol for a $PC_{35}SGaw$ evaluation, with 7 out of 8 values lying within this range (the value outwith this range was a difference of 8.93 nmol), suggesting reasonable repeatability for $PC_{35}SGaw$ estimation.

Table 3.1

Demographic factors, baseline lung function and treatment

No.	Age (years)	Sex	FEV_1 (litres)	FEV_1 % predicted	SpO_2 (%)	SGaw (s/kPa)	Treatment	Methacholine PC_{20} (mg/ml)
Normal volunteers								
1	29	F	3.52	110	98	1.62	none	>16
2	24	M	4.30	105	98	1.88	none	>16
3	29	F	3.11	104	98	2.31	none	>16
4	31	M	4.88	112	98	2.11	none	>16
5	21	M	4.85	107	96	1.84	none	>16
Mean	**26.8**	**3M 2F**	**4.1**	**107**	**98**	**1.95**		
(S.D.)	(4.2)		(0.8)	(3.4)	(0.9)	(0.3)		
Asthmatics								
1	49	M	3.20	86	98	0.67	A.	1.33
2	28	F	2.44	79	96	0.66	A.BDP200	0.33
3	35	M	3.04	78	98	0.88	A.BDP200	3.28
4	30	F	2.60	81	97	1.50	A.Sm.BDP400	0.33
5	34	M	2.92	83	97	0.83	A.BDP2000	3.21
6	39	M	3.92	84	97	1.19	A.BDP300	1.49
7	48	F	2.42	89	97	1.30	T.Sm.BDP2000	2.92
8	34	M	4.33	106	98	0.92	A.BDP1000	2.72
Mean	**37.1***	**5M 3F**	**3.1†**	**86†**	**97**	**0.99†**	**geometric mean**	**1.83**
(S.D.)	(7.8)		(0.6)	(8.9)	(0.7)	(0.3)	(range)	(0.3-3.3)

key * greater than normal volunteers
† less than normal volunteers
A. Albuterol 200µg as required
BDP Beclomethasone dipropionate total daily dose (µg)
T. Terbutaline 500µg as required
Sm. Salmeterol 100µg daily

Table 3.2
Baseline and percent maximum fall in lung function (FEV_1, specific airways conductance (SGaw)) and oxygen saturation ($Sp0_2$), and provoking concentrations to produce a 35% fall in SGaw (PC_{35}SGaw) for endothelin-1 and methacholine (20% fall in FEV_1 ($PC_{20}FEV_1$) for methacholine in normal subjects) for asthmatic (n=8) and normal (n=5) subjects. Figures expressed as mean (S.D.).

	Baseline		Max fall (%)	p value
ASTHMATICS				
Methacholine				
FEV_1 (litres)	2.9 (0.6)		20.1 (8.9) %	p=0.001
SGaw (*s*/kPa)	1.06 (0.4)		69.7 (15.9) %	p=0.002
$Sp0_2$ (%)	96.6 (1.3)		0.6 (0.7) %	N.S.
PC_{35}SGaw (µmol)		0.42 (0.18)		
Endothelin-1 (1)				
FEV_1 (litres)	3.05 (0.7)		18.3 (6.9) %	P=0.004
SGaw (*s*/kPa)	1.02 (0.3)		63.9 (11.8) %	p=0.000
$Sp0_2$ (%)	97.2 (1.2)		0.1 (1.1) %	N.S.
PC_{35}SGaw (µmol)		5.15 (4.4)		
Endothelin-1 (2)				
FEV_1 (litres)	3.01 (0.7)		16.3 (5.1) %	p=0.0006
SGaw (*s*/kPa)	1.08 (0.3)		60.5 (14.9) %	p=0.0006
$Sp0_2$ (%)	96.3 (1.3)		0.3 (0.7) %	N.S.
PC_{35}SGaw (µmol)		4.29 (2.5)		
NORMALS				
Methacholine				
FEV_1 (litres)	3.99 (0.9)		5.4 (7.3) %	N.S.
$PC_{20}FEV_1$ (µmol)		> 21.00		
Endothelin-1				
FEV_1 (litres)	4.07 (0.7)		0.4 (0.9) %	N.S.
SGaw (*s*/kPa)	1.95 (0.3)		9.2 (10.6) %	N.S.
$Sp0_2$ (%)	97.6 (1.3)		0.2 (0.8) %	N.S.
PC_{35}SGaw (µmol)		> 15.36		

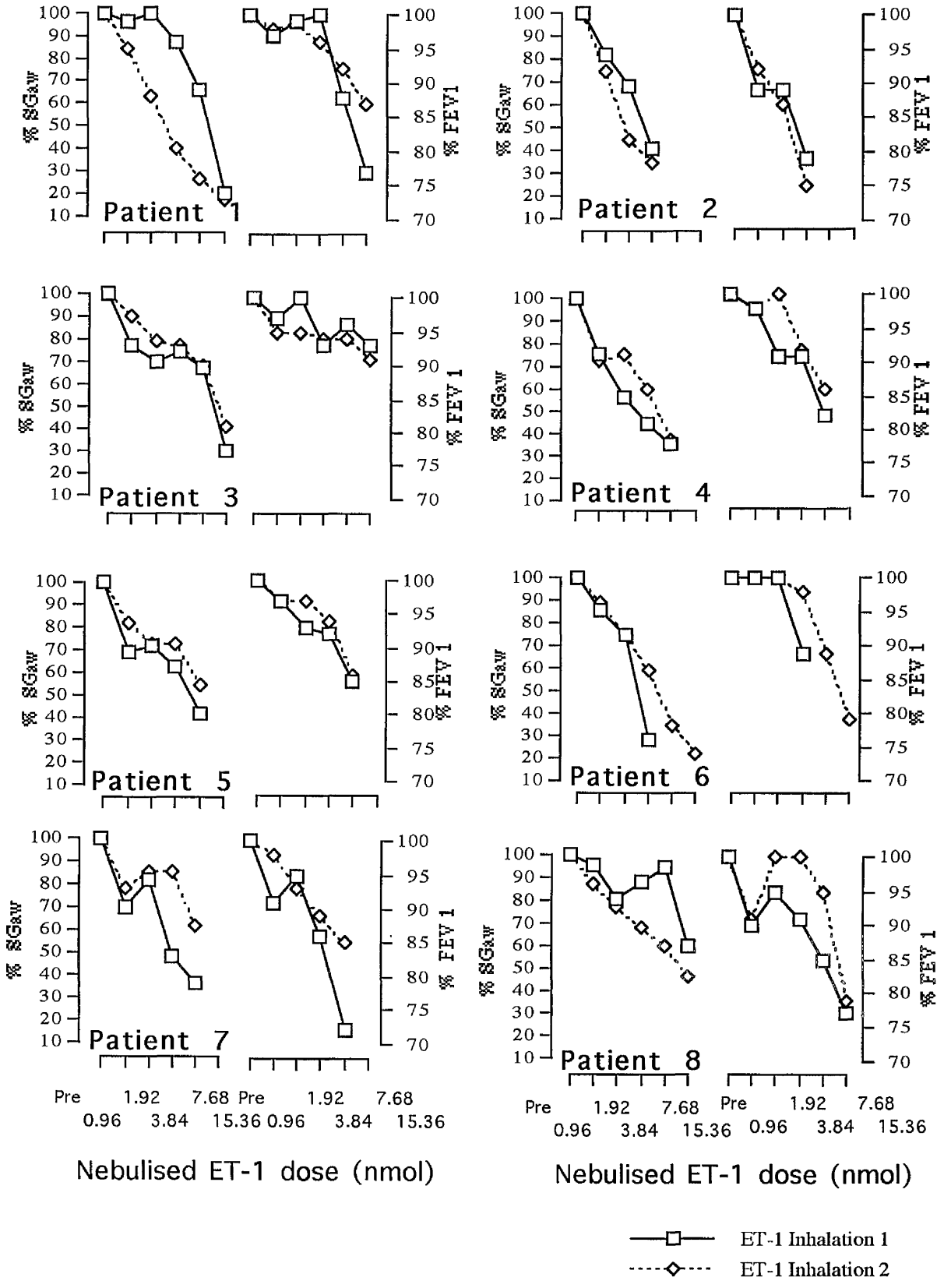

Figure 3.1
Individual percent change in specific airway conductance (SGaw) and FEV1 during two endothelin-1 inhalations in eight asthmatic subjects.

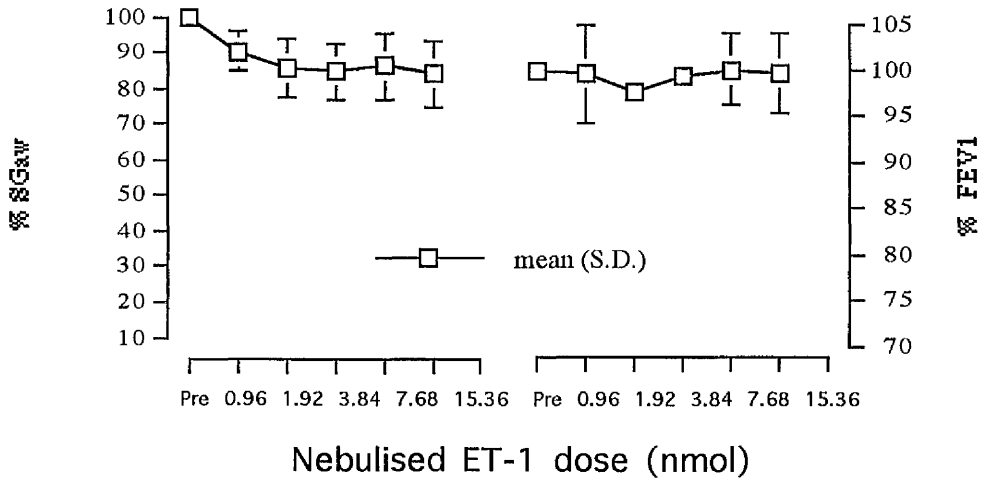

Figure 3.2
Mean (SD) percent change in specific airway conductance (SGaw) and FEV1 during endothelin-1 inhalation in five normal subjects.

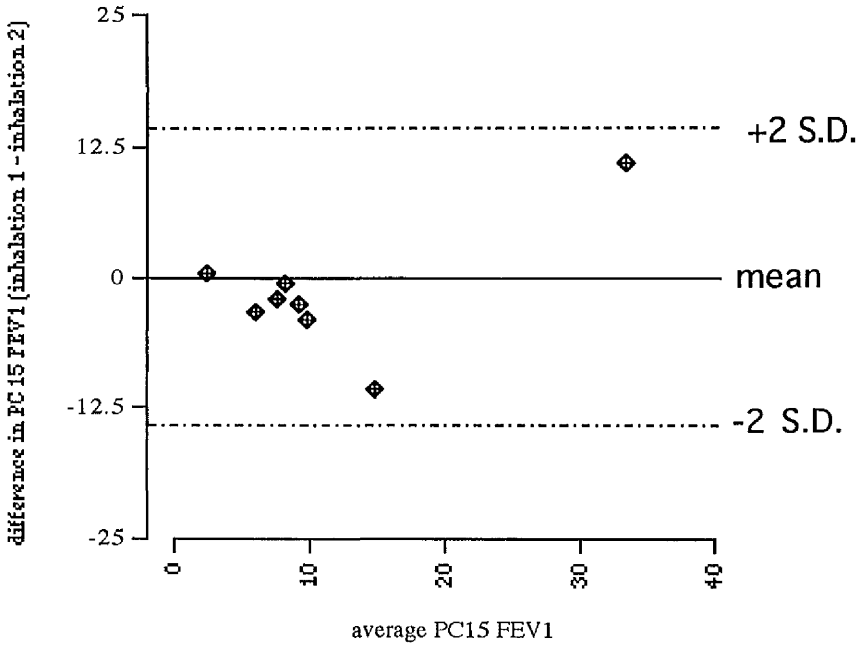

Figure 3.3
Bland-Altman plot of repeatability of bronchial reactivity to endothelin-1 in asthmatic subjects (expressed as ET-1 PC15FEV1). The coefficient of variability for FEV1 is 14.82 nmol, and for SGaw is 8.47 nmol.

3.5 DISCUSSION

We have demonstrated for the first time that endothelin-1 (ET-1) is a highly potent bronchoconstrictor in asthma, with a relative potency to methacholine of c.100 x on a molar basis, and that asthmatics exhibit bronchial hyperreactivity to ET-1 compared to normal subjects. Relatively rapid onset (<5 minutes) bronchoconstriction occurs in a dose-dependent fashion, and we conclude that ET-1 can be safely given by inhalation to asthmatics and normal subjects as a bronchial challenge test in a dose range of 0.96-15.36nmol. We observed no change in blood pressure, oxygen saturation or plasma ET-1 levels related to the inhalation of ET-1. Albuterol completely reverses ET-1-induced bronchoconstriction and the duration of bronchoconstriction was at least 60 and 90 minutes in the 2 patients formally assessed.

We observed bronchoconstriction within 5 minutes of ET-1 inhalation, which is in keeping with animal studies using aerosolised ET-1 where the maximal response was reached in around 4-5 minutes (67), and Henry *et al* (53) observed a similar time to reach maximal contraction (5-10 minutes) in human isolated bronchi. Henry *et al* expressed the contractile potency of ET-1 as the concentration needed to produce a contraction 30% of the maximum contractile response to 10μM carbachol (excitatory concentration "EC_{30}"), with a value of 35nM. While it is difficult to directly relate that figure to a dose given *in vivo*, allowing for the increased sensitivity we have observed in asthmatics, it is of similar order of magnitude and relative potency to methacholine as we found in this study expressed as ET-1 provoking concentrations for SGaw and FEV_1. The dose range used in this study was based on in vitro studies of the bronchoconstrictor response in human airways (60), along with pilot work on healthy volunteers starting with inhalations of significantly lower concentrations of ET-1 to establish overall safety of the technique. The nature of nebulisation even with a dosimeter means that although it is possible to be reasonably accurate in terms of the

dose nebulised, the effective dose reaching the lungs is much harder to assess, but is probably of the order of 20-30% of the doses stated.

We found increased plasma ET-1 in asthmatics compared with normals both before and after the 2 ET-1 inhalations (although with no change across the inhalations), but no significant difference on the day of the methacholine inhalation. This finding does not reflect our own previous experience with plasma levels (unpublished data) where we found no difference between mild asthmatics and normals, and although we are aware of one study which correlates plasma ET-1 with lung function (120), we presume pending further data that this finding is a consequence of the small numbers in each group. The lack of change in blood pressure during ET-1 inhalation is in keeping with animal studies using aerosolised ET-1 (67).

The contractile activity of ET-1 on bronchial smooth muscle has been assessed in animal models, and in human tissue *in vitro*. Aerosol administration of ET-1 in animal studies produces a dose-dependent bronchoconstriction, with no blood pressure response (67), no increase in bronchial hyperreactivity or histamine response (259), and no inflammatory reaction (57) and there is evidence from animal studies that the responses to intra-arterial and aerosolised ET-1 are mediated by different mechanisms (61). *In vitro* studies have confirmed the bronchoconstrictor activity of ET-1 in human bronchi (58), although in contrast to our findings *in vivo*, Goldie *et al* (55) found no difference between asthmatic and non-asthmatic bronchi in sensitivity to ET-1 *in vitro*, and in the same study demonstrated no increase in ET-1 binding sites in asthmatic bronchi (nor in asthmatic peripheral lung (112) in a study from the same group), suggesting that the bronchial hyperreactivity to ET-1 that we observed does not depend simply on increased ET-1 receptor numbers in asthma. In human isolated bronchi, ET-1 exerts its contractile effect mainly through the action of the ET_B receptor subtype (68) which is the most numerous receptor in this tissue, although interestingly, bronchial smooth muscle from asthmatic lung appears to be less sensitive to the contractile effects of a specific ET_B

receptor agonist, sarafotoxin S6c (84), presumably due to a decrease in receptor sensitivity. The above data, with our finding that asthmatics exhibit bronchial hyperreactivity to ET-1 *in vivo*, suggests that factors other than receptor numbers and sensitivity contribute to this increased sensitivity to ET-1 in asthma, since it would not be predicted from the *in vitro* receptor data. Epithelial disruption is a feature of the inflammation associated with asthma (91), and it has been demonstrated that epithelial removal significantly enhances the contractile activity of ET-1 in the human isolated bronchus (63), raising the possibility that epithelial disruption contributes to increased contractile activity of ET-1 in asthma. It has also been demonstrated *in vitro* that ET-1 may interact with other mediators implicated in asthma, with potentiation of its contractile activity, including prostaglandins (PG)-D_2, $PGF_{2\alpha}$, leukotriene D_4 (260), and angiotensin II (261), but other than the finding of increased nasal mucosal sensitivity to ET-1 in allergic rhinitis (114) and a rise in endothelin-like immunoreactivity 24 hours after allergen challenge in sensitised guinea-pig airways (262), there is no direct data on the contribution of airway inflammation to ET-1 activity. The absence of response to inhaled ET-1 in normals is striking, and supports the observation that an intact airway epithelium modulates the response of airway smooth muscle to ET-1 (64). While the mechanism for this is not known, an intact epithelium in normal subjects might form a physical barrier to ET-1 coming into contact with submucosal target cells, or increase clearance and metabolism of ET-1, either by binding and internalisation of ET-1 by ET_B receptors (33), or by enzymatic degradation. There is also evidence in non-asthmatic human tissue that ET-1 can act on ET_A receptors in bronchial epithelium causing release of relaxant factors including nitric oxide (65) which could attenuate the bronchoconstrictor effects of ET-1 in normal airways.

There is debate about the mechanism of action of ET-1 in the airways, with animal studies suggesting that contractile activity is at least in part mediated by arachidonic acid metabolites (in particular thromboxanes(86)), leukotrienes and histamine (87), but this has not been confirmed by studies using human bronchi (60), where ET-1 appears to act

directly on smooth muscle, without the involvement of acetylcholine, leukotrienes, histamine or platelet activating factor (88). Although this study was not designed to examine the mechanism of action of ET-1, the strong correlation that we observed between methacholine reactivity and ET-1 reactivity (at least for FEV_1 response) lends indirect support to the suggestion that ET-1, like methacholine, exerts its bronchoconstrictor effect directly on bronchial smooth muscle, rather than through a second mediator. This is not conclusive however, and ET-1 is known to have other effects which could lead to muscular or non-muscular bronchial narrowing. ET-1 acts on glandular ET receptors in human nasal mucosa, inducing lactoferrin and mucous glycoprotein release (92), induces cytokine production (GM-CSF, IL-6 and IL-8) in human bronchial epithelial cell culture (93), and potently stimulates release of TNF-α, IL-1ß, and IL-6 from monocytes and monocyte-derived macrophages (94), all of which could contribute to non-muscular airway obstruction. In addition, ET_B receptor activation has been shown to potentiate cholinergic nerve-mediated contraction in human bronchial preparations (113), suggesting that ET-1 might exert an additional neurally mediated bronchoconstrictor effect.

Further work is needed to clarify the mechanism of action of ET-1 in human airways, and in particular to examine the influence which the specific bronchial micro-environment associated with asthmatic airway inflammation might have on the increased sensitivity to ET-1 that we have observed in asthma. These data support a role for ET-1 in the pathophysiology of asthma, and provide a potential means of further exploring this role through the use of ET-1 in bronchial challenge testing.

CHAPTER 4

SPUTUM CELLULAR AND CYTOKINE RESPONSES TO INHALED ENDOTHELIN-1 IN ASTHMA

4.1 INTRODUCTION

Having established the technique and dose range for inhalational challenge testing using ET-1, and that ET-1 has bronchoconstrictor activity in asthmatics, we wished to examine other mechanisms by which ET-1 might contribute to asthma symptoms. The importance of airway inflammation in the pathophysiology of asthma is established, and we were interested in reports that ET-1 may pro-inflammatory activity in the airways. *In vitro* data shows that ET-1 promotes the inflammatory activity of neutrophils (20) and macrophages (102), and increases the expression of adhesion molecules on endothelial cells. Additionally, ET antagonists have been shown to inhibit airway inflammation in animal studies (103, 263) in response to a number of stimuli. The production from mononuclear cells from asthmatics of the proinflammatory cytokines TNF-α (100) and IL-1ß (105) are increased by ET-1 *in vitro*, and exogenous ET-1 has been shown to decrease NO production and inducible NO synthase (iNOS) expression in lung epithelial cells (264). Like NO, sputum concentratrions of nitrite and nitrate, which are stable metabolites of NO representing iNOS activity, have been shown to be elevated in asthma (265). We have therefore investigated the effect of inhaled ET-1 on cellular influx, and the concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-1ß, nitrite and albumin (assessed as a measure of airway epithelial permeability) in induced sputum obtained 30 minutes and 4 hours after inhalation of ET-1 or placebo in 10 subjects with clinically stable asthma.

4.2 METHODS

Subjects

10 non smoking asthmatic subjects participated in the study (table 1) All subjects had current asthma with stable symptoms at the time of study, no history within the preceding month of respiratory infection, antibiotics or oral corticosteroid use and other than house dust mite, none of the subjects had been exposed to allergens to which they were allergic in the preceding month. Asthma was defined according to the ATS definition (257), baseline lung function was recorded, and non-specific bronchial hyperresponsiveness was established using a methacholine challenge test, with all the asthmatics subjects having a methacholine PC_{20} of less than 8mg/ml. The study was approved by the West Ethics Committee, West Glasgow Hospitals University NHS Trust, and each subject gave written informed consent.

Protocol

The study was performed in randomised blinded fashion. Subjects attended for screening methacholine challenge, followed by two visits for either ET-1 (dose range 0.96-15.36 nmol) or placebo inhalation, having withheld ß2-agonist therapy for 12 hours prior to each study visit. Blood was sampled for ET-1 assay at the beginning of each visit, following the inhalation, and 4 hours after the inhalation. Both ET-1 and placebo were administered using an air-driven dosimeter ('Nebicheck' PK Morgan Ltd, Gillingam, U.K.), calibrated to deliver 0.006ml/breath. Dried purified ET-1 (Thistle Research, Glasgow, U.K.) was reconstituted in buffered 0.9% saline, and buffered saline was used as placebo. Non-invasive blood pressure and pulse oximetry were monitored throughout the inhalations. Unlike the previous study, where constant volume body plethysmography was used to assess changes in airway resistance, in this study spirometry was measured 1, 2, 5 and 10 minutes after each dose of ET-1/placebo. The inhalation was stopped following a 15% fall in FEV_1 or following the maximum dose in the dosing schedule, whichever came first. Following each inhalation,

salbutamol was given (200μg) by inhalation, and sputum induction was performed 30 minutes and again 4 hours after each inhalation.

Sputum induction, laboratory processing and biochemical assays.

Sputum induction was performed as outlined in chapter 2, the sample was collected in a sterile container and was transferred to the laboratory on ice as soon as possible, and in all cases in less than 2 hours. Laboratory processing of sputum samples is outlined in chapter 2. Criteria were established for rejection of samples if viability was <40%, or squamous cells >20% although in fact no samples were rejected on these criteria (viability was typically >70% and squamous cell count <10%). The bronchial epithelial cell count was very low in all samples (typically <2%). Plasma ET-1 was assayed using a radio-immuno assay (RIA) (Nichols Institute Diagnostics Ltd, San Juan Capistrano, CA, U.S.A.), with a lower limit of detection of 2 pg/ml in plasma, and assays were run in duplicate, with the mean value used for analysis. IL-1ß was assayed using enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, U.K.), TNF-α was assayed using an in-house ELISA using paired antibodies and cytokine standard from R&D Systems. Nitrite was measured by the Griess reaction (in-house assay) and albumin by radial-immuno diffusion (The Binding Site Ltd, Birmingham, U.K.).

Data Handling and Statistical Analysis

Statistics were performed using a statistical software package (Minitab Statistical Software, Minitab Inc., State College, PA, U.S.A.). Non-parametric statistics (Mann Whitney-U test) were used to compare cell counts and concentrations of mediators, and values are expressed as median (interquartile range) unless stated otherwise. PC_{20} and PC_{15} values were calculated by log-linear interpolation. Analysis of variance (ANOVA) was used to compare blood pressure, pulse rate and pulse oximetry data.

4.3 RESULTS

General

Patient demographics and airway responsiveness to methacholine and ET-1 are outlined in table 4.1. All the subjects completed the study, and airway challenge with ET-1 was well tolerated. Mean (S.D.) baseline FEV1 was similar on the two study days [ET-1 2.79 (0.6) litres, placebo 2.75 (0.7) litres]. ET-1 produced progressive, dose-dependent bronchoconstriction in all subjects, with all but one subject attaining a fall in FEV1 of $\geq$ 15% at or before the maximum dose used (15.36 nmol). The remaining subject (subject 3) achieved a 12% fall in FEV1, and PC_{15} is calculated by interpolation. Mean (SD) FEV1 at the end of the challenge test was 2.25 (0.5) litres for ET-1 and 2.94 (0.23) litres for placebo (p=0.016), and 4 hours after challenge testing was 2.9 (0.69) litres for ET-1 and 2.9 (0.76) litres for placebo. Comparing ET-1 with placebo inhalations, there was no change in blood pressure, oxygen saturation or pulse rate.

Sputum Cell Counts

Total sputum cell counts are given after exclusion of squamous epithelial cells, and expressed as cells per ml of sputum plug. Total cell counts were not different 30 minutes after challenge tests comparing ET-1 with placebo (median (IQR) 3.55 (1.6-11.4)x10^6 cells/ml vs 3.2 (2.3-6.4)x10^6 cells/ml respectively), nor at 4 hours following ET-1 compared with placebo inhalation (median (IQR) 3.7 (3.1-5.3)x10^6 cells/ml vs. 3.55 (2.2-4.8)x10^6 cells/ml respectively). Sputum cell differentials are presented in figure 4.1, and are expressed as % of total cell count after exclusion of squamous epithelial cells. The percent of lymphocytes in induced sputum was very low for both ET-1 (lymphocytes median (IQR) 0 (0-1) % at 30 minutes and 1 (0-1) % at 4 hours) and placebo inhalations (median (IQR) 0 (0-1) % at 30 minutes, and 0 (0-1) % at 4 hours) so this data has been omitted from figure 4.1. There was no significant difference in the sputum cell differentials comparing ET-1 and placebo, either at 30 minutes or at 4 hours, whether expressed as % of total cell count or as cells/ml of sputum, with the exception

that the sputum percent macrophage count (but not absolute macrophage numbers) was lower at 4 hours after placebo inhalation than at 30 minutes (median (IQR) 36 (16-53)% vs. 64 (32-79)% respectively. p=0.05). This reduction in % macrophages is largely explained by a nonsignificant rise in the % neutrophil count (figure 4.1).

TNF-α, IL-1ß, NO2 and Albumin

Figure 4.2 illustrates that we found no significant change in sputum concentrations of TNF-α, IL-1ß, nitrite or albumin comparing placebo with ET-1 challenge at either 30 minutes or 4 hours. Comparing concentrations of TNF-α, IL-1ß, NO2 and albumin at 4 hours compared with 30 minutes following ET-1 inhalation did not show any significant change.

Plasma Endothelin-1

There was no difference in plasma ET-1 comparing median (IQR) levels for ET-1 inhalation compared with placebo inhalation (2.5 (2.0-3.9) vs. 3.4 (2.0-4.7) pg/ml at baseline, 3.1 (2.0-4.2) vs. 3.0 (2.0-4.6) pg/ml at 30 minutes, and 2.8 (2.2-3.8) vs. 2.8 (1.9-3.0) pg/ml at 4 hours) respectively.

Table 4.1

SUBJECT CHARACTERISTICS

Subject No.	Sex	Age (years)	FEV1 % predicted	PC20 methacholine (mg/ml)	PC15 ET-1 (nmol)	Current treatment
1	M	34	113	2.72	9.54	S,Sm,BDP1000
2	F	38	100	0.84	3.58	S
3	F	48	75	2.92	21.70	T,Sm, BDP1000
4	F	38	71	1.41	12.07	S, BDP400
5	M	49	73	1.33	10.45	S
6	M	37	83	4.92	9.46	S, Bud800
7	M	40	76	1.49	6.69	S, BDP200
8	F	20	91	3.33	7.68	S, Sm, FP1000
9	F	46	81	0.27	1.17	S, BDP400
10	F	42	92	1.76	12.19	S
Mean (S.D.)	6F, 4M	**39.2** (8.4)	**85.5** (13.5)	**2.1** range (0.27-4.9)	**9.45** range (1.17-21.7)	

Key
- S Salbutamol 200 µg as required
- T Terbutaline 500 µg as required
- Sm Salmeterol 100 µg daily
- BDP Beclomethasone dipropionate daily dose in µg
- Bud Budesonide daily dose in µg
- FP Fluticasone propionate daily dose in µg

Table 4.2

Absolute sputum differential cell counts 30 minutes and 4 hours after placebo and ET-1 inhalations - expressed as median (IQR) cells x 10^6/ml of sputum plug.

	Macrophages ($x10^6$/ml)	Neutrophils ($x10^6$/ml)	Eosinophils ($x10^6$/ml)	Lymphocytes ($x10^6$/ml)
Placebo				
30 minutes	**1.83**	**1.09**	**0.09**	**0.0**
	(0.9-4.5)	(0.7-1.4)	(0.02-0.5)	(0-0.03)
4 hours	**1.14**	**1.36**	**0.12**	**0.0**
	(0.7-1.6)	(0.7-2.3)	(0-0.8)	(0-0.04)
ET-1				
30 minutes	**1.57**	**1.0**	**0.15**	**0.0**
	(0.9-3.3)	(0.6-2.2)	(0.05-0.5)	(0-0.03)
4 hours	**1.76**	**1.3**	**0.18**	**0.01**
	(1.0-2.4)	(0.8-2.2)	(0.05-0.9)	(0.-.04)

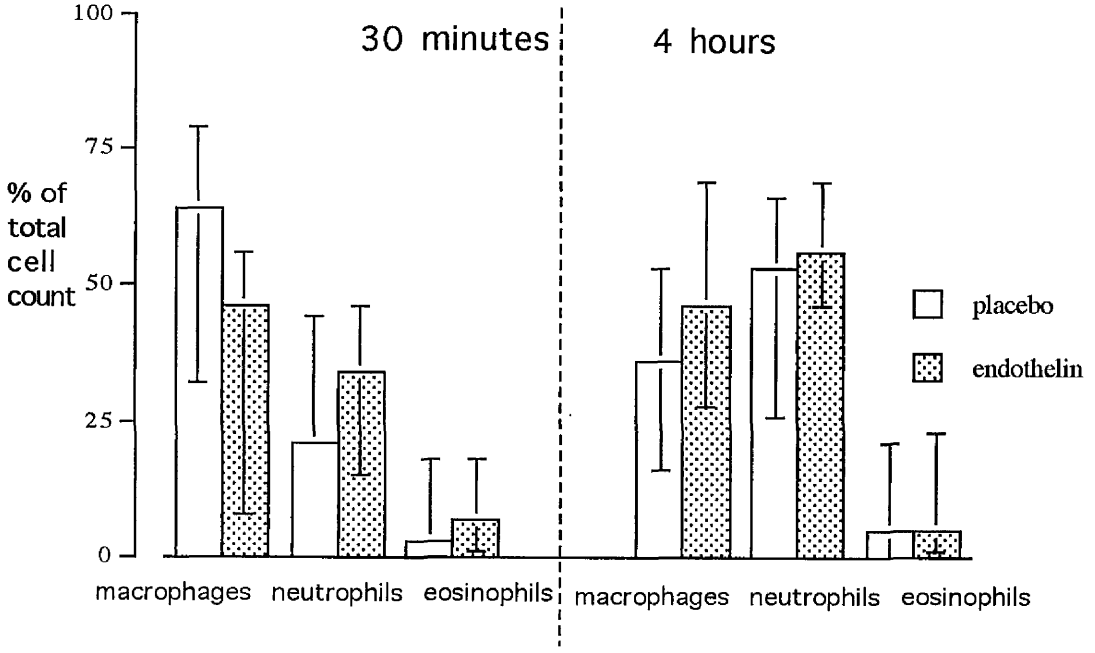

Figure 4.1
Percentage differential cell counts 30 minutes and 4 hours after placebo and endothelin-1 inhalations; values expressed as median (interquartile range).

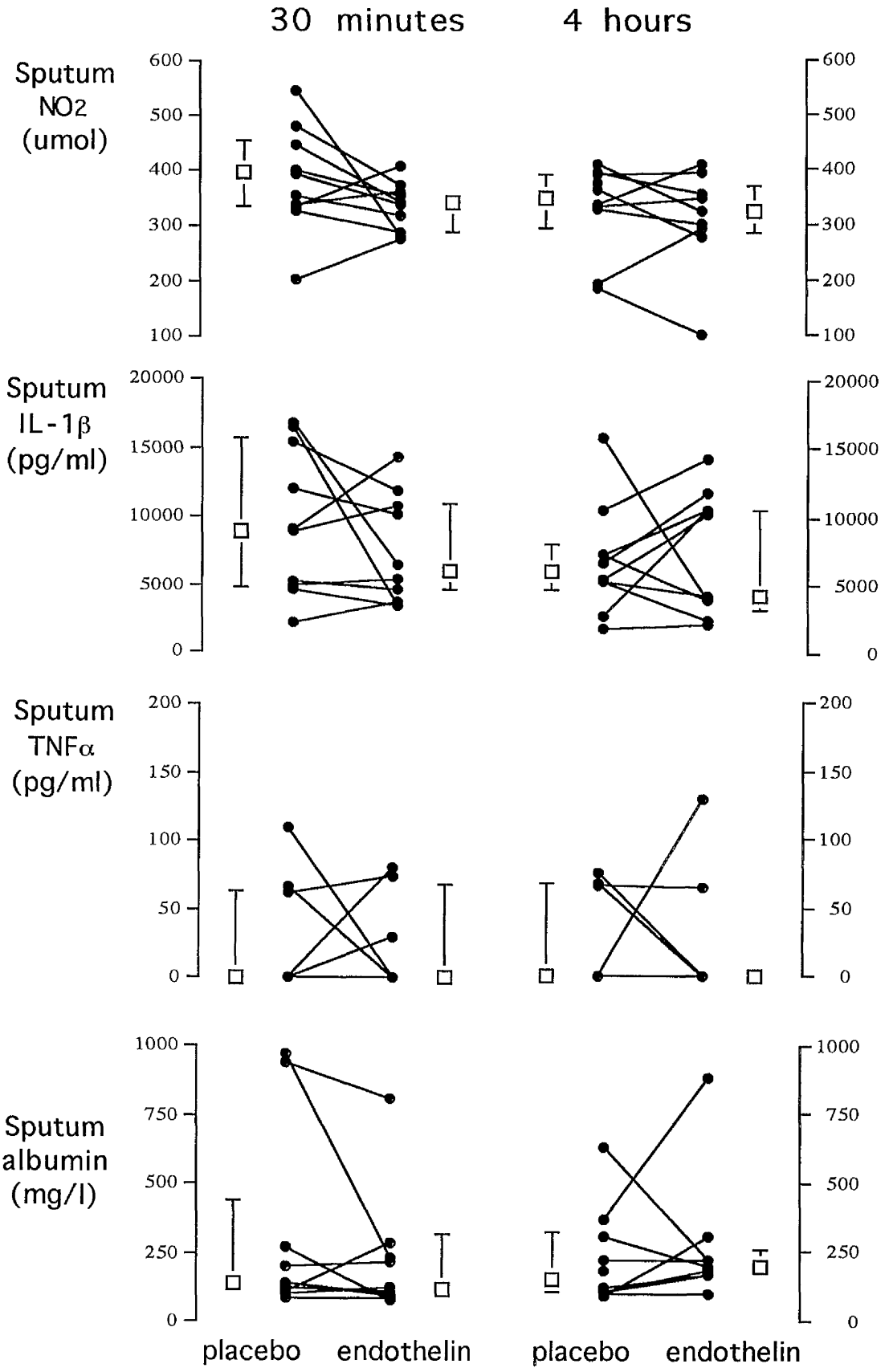

Figure 4.2

Sputum nitrite (NO2), interleukin (IL)-1β, tumour necrosis factor (TNF)α, and albumin, 30 minutes and 4 hours after endothelin-1 inhalation; median (IQR).

4.4 DISCUSSION

Using the non-invasive technique of sputum induction, we have shown that bronchoconstrictor doses of inhaled ET-1 in asthma do not provoke an acute inflammatory response as assessed by cellular influx, changes in the concentrations of the pro-inflammatory cytokines TNF-α and IL-1ß, nitrite production, or albumin extravasation at 30 minutes or 4 hours after ET-1 inhalation.

Our group of patients contained 7 subjects who were taking inhaled steroids (mean daily dose of 685 μg beclomethasone or equivalent). It has been shown that treatment with steroids decreases bronchial epithelial expression of ET-1 in asthmatic patients (123), and in a rat model of lung inflammation the increase in lung ET-1 content in response to the inflammatory stimulus (Sephadex) was reduced by pre-treatment with budesonide (266). While these data suggest that corticosteroids may decrease ET-1 production, there is no specific information on the impact of corticosteroids on the potential airway response to ET-1, and this cannot be inferred from the effect of steroids on ET-1 production. Although we did not observe any obvious differences in bronchoconstrictor or inflammatory responses between those patients taking or not taking inhaled steroids, the study was not designed to detect differences between these groups, and a possible effect of inhaled steroids cannot be excluded.

ET-1 is produced in response to a number of inflammatory stimuli both *in vitro* and *in vivo* , and ET-1 can also trigger the release of other locally acting mediators, including TNF-α, IL-1ß, IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (267), which are involved in inflammatory cell attraction and activation in the lung, some of which may also stimulate ET production. Endothelin receptor antagonists have been shown to inhibit lung inflammation induced by antigen in mice (103), and airway inflammation induced using dextran in rats (110), with some evidence that ET_B receptor-antagonists may inhibit the early allergic response, and ET_A antagonists the late

allergic response in sensitised guinea-pigs (109). These data however, only provide indirect evidence that ET-1 may be involved in the inflammatory process, and it is not clear whether ET-1 has direct pro-inflammatory activity in the airways.

In allergic rhinitis, while nasal administration of ET-1 increased symptoms and secretion weights, it did not increase secretion of albumin or histamine (114), and we found no increase in albumin secretion, suggesting that the bronchoconstriction induced by ET-1 in asthma is not associated with significant alteration in bronchial epithelial permeability. Chanez *et al* (100) have shown that BAL mononuclear cells from stable asthmatics (but not control subjects) respond to stimulation with ET-1 *in vitro* by increasing release of TNF-α. In cells from patients with unstable asthma in the same study, *in vitro* TNF-α release was already increased compared to control cells, and did not further increase on stimulation with ET-1. We did not observe an increase in sputum TNF-α following ET-1 administration *in vivo*, and the reason for this difference in TNF-α response to ET-1 not known. It has been shown that cytokines can alter cellular responsiveness to ET-1 (106), and clearly factors may be present *in vivo* which may modify the TNF-α response to ET-1.

There is increasing evidence that endogenous nitric oxide (NO) is involved in the pathophysiology of asthma (268). Similarly, increased levels of NO derivatives including nitrite have been demonstrated in induced sputum in asthma, with a relationship between levels of NO derivatives in sputum and eosinophil numbers (265). Regulation by ET_A receptors of ET-1-induced NO release in guinea-pig trachea has been described (269), but there appear to be species differences in the impact of ET-1 on NO production (264). We found no alteration in sputum nitrite concentrations in response to ET-1 inhalation in asthma. Sputum concentrations of the pro-inflammatory cytokine IL-1ß are increased in symptomatic asthma (270), and ET-1 is known to stimulate the production of IL-1ß in human monocytes *in vitro* (105). We did not observe an increase in IL-1ß in response to ET-1 inhalation in this study.

Overall, there appears to be uncertainty about a postulated pro-inflammatory rôle for ET-1 in the airways. Inter-species differences have been described in the mechanism of ET-1-induced bronchoconstriction (57, 60), but it is not yet known if such differences exist for pro-inflammatory activity. The route of administration of ET-1 may also be important, since intravenous or intra-arterial administration is associated with cellular accumulation and mediator release in the lungs in animal studies (61, 108, 271), whereas aerosolised administration of ET-1 in the guinea-pig does not result in airway edema, TxB2 release (61), nor in eosinophil accumulation in the lungs or bronchial hyperresponsiveness (95). Similarly, our own study using inhaled ET-1 in asthma does not show evidence of cellular influx or albumin secretion. While the reasons for this observation are not known, the ETB receptor is the most numerous ET receptor in airway smooth muscle (84), and it is possible that through its postulated function as a clearance receptor it could modify the ability of ET-1 to exert pro-inflammatory activity. In both its bronchoconstrictor activity and mitogenic activity, there is evidence for interactions between ET-1 and other humoral and cytokine factors, and more investigation is needed into the potential for such interactions in airway inflammation. The evidence for the ability of ET receptor antagonists to modify inflammatory processes in particular suggests that ET-1 may play a role in airway inflammation, even if not as a direct pro-inflammatory agent.

CHAPTER 5

EFFECT OF INFUSED ANGIOTENSIN II ON THE BRONCHOCONSTRICTOR ACTIVITY OF INHALED ENDOTHELIN-1 IN ASTHMA

5.1 INTRODUCTION

While a large number of bronchoconstrictor substances have been identified, there has been relatively little work reporting interactions between these substances. Having established that ET-1 is a potent bronchoconstrictor in asthma, we wished to examine a potential interaction between it and another endogenously produced peptide angiotensin II, based on previously reported work from our laboratory. Angiotensin II (Ang II) is a product of the renin-angiotensin system (RAS), and, like ET-1, it is a potent vasoconstrictor which also has bronchoconstrictor activity, causing contraction of isolated human bronchial rings (272). Our group has shown that the RAS is activated in acute severe asthma with increased plasma levels of Ang II (119, 273), but not in chronic stable asthma, and that infusion of Ang II in mild asthmatics to plasma levels found in acute severe asthma causes bronchoconstriction (273). Plasma ET-1 is also increased in acute severe asthma (120), and a reduction in BAL ET-1 following treatment with oral steroids and inhaled bronchodilators has been described (274).

Although the bronchoconstrictor potency of angiotensin II is modest, there is evidence of synergy in bronchoconstriction between Ang II and other bronchoconstrictors, including the acetyl choline analogue methacholine, and ET-1. Ang II potentiates methacholine-induced bronchoconstriction in human airway, both *in vitro* and in mild asthmatics *in vivo* (272), and potentiates ET-1-induced bronchoconstriction in bovine bronchial rings (261). We sought to extend this last observation, and our work on the bronchoconstrictor activity of ET-1, by examining the potential interactive effect of angiotensin II on ET-1-induced bronchoconstriction in mild asthmatics *in vivo*. Ang II infusion was used to simulate the elevation of plasma Ang II observed in acute severe asthma, but sub-bronchoconstrictor doses of Ang II were used to ensure that bronchoconstrictor effects were not simply additive to the effects of inhaled ET-1. ET-1 was given by inhalation as a bronchial challenge test. Interactions between potential mediators in asthma may provide important information about the pathophysiology of

the condition, and we felt that the *in vitro* bronchoconstrictor synergism between ET-1 and Ang II required investigation *in vivo*.

5.2 METHODS

Patients

8 mild asthmatics were recruited, with stable symptoms at the time of study and no history within the preceding month of respiratory infection, antibiotics or oral corticosteroid use. Asthma was defined according to the ATS definition (257), baseline lung function was recorded, and non-specific bronchial hyperresponsiveness was established using a methacholine challenge test, with all the asthmatics subjects having a methacholine PC_{20} of less than 8mg/ml. Long-acting β-agonist bronchodilators were withheld for 24 hours, and short acting β-agonist bronchodilators for 6 hours prior to each study visit. The study was approved by the West Ethics Committee, West Glasgow Hospitals University NHS Trust, and each subject gave written informed consent.

Protocol

Asthmatic subjects attended for methacholine screening test, followed by three visits for ET-1 inhalation, and infusion of angiotensin II (Affiniti Research Products Ltd, Exeter, U.K.) at either 1 ng/kg/min or 2 ng/kg/min or placebo in a randomised double-blind fashion. The interval between visits was not fixed, but was generally around 1 week. Randomisation and preparation of angiotensin II was carried out by sterile pharmacy in our institution. On attending the laboratory, spirometry was checked, with all subjects having a pre-challenge FEV1 of $\geq$ 70% predicted. After a period of rest (15 minutes) in a recumbent position, blood was sampled for baseline measurement of plasma angiotensin II, and the infusion of angiotensin II or placebo was started, using a syringe pump (IVAC P2000, IVAC Ltd. Hampshire, U.K.). After a further 30 minutes to reach steady state, another blood sample was taken, and the bronchial challenge with ET-1 was started. Dried purified ET-1 (Calbiochem-Novabiochem (UK) Ltd. Nottingham, UK) was reconstituted using 0.9% saline prior to nebulisation to a concentration of 0.2 mg/ml, and administered using an air-driven dosimeter ('Nebicheck' PK Morgan Ltd, Gillingham, U.K.), calibrated to deliver 0.006ml/breath, with a doubling dose range for

ET-1 of 0.96-15.36 nmol. Spirometry was checked 1, 3, 5, 10 and 15 minutes after each dose, and the challenge test was discontinued once a 15% fall in FEV_1 had been observed, or at the maximum dose in the dosing schedule, whichever came first. We have previously reported the use of ET-1 as a bronchial challenge test, and although we observed bronchoconstriction which persisted for up to 1 hour, the onset of bronchoconstriction was within 5 minutes in each case (275). A final blood sample for plasma angiotensin II was taken at the conclusion of the ET-1 challenge test (defined as a fall in FEV1 of $\geq$ 15% or on reaching the maximum dose in the dosing schedule). Pulse oximetry (Datex Ltd, Helsinki, Finland), non-invasive blood pressure, pulse rate, and spirometry (Vitalograph, Buckingham, U.K.) were monitored at regular intervals throughout the study visit, with the patient in a recumbent position throughout. Albuterol 200 μg was given, and spirometry repeated to ensure that the bronchoconstriction had been reversed.

Laboratory Processing and Assays

Plasma angiotensin II was assayed using an in-house assay which has previously been described (276), with inter-assay and intra-assay coefficients of variation of less than 10% in each case.

Data Handling and Statistical Analysis

Statistics were performed on an Apple Macintosh desktop computer (Apple Computer Inc., Cupertino, CA, U.S.A.) using a statistical software package (Minitab Statistical Software, Minitab Inc., State College, PA, U.S.A.). Demographic factors and baseline and maximum fall in lung function parameters were analysed using parametric statistics, plasma angiotensin II levels by non-parametric (Mann-Whitney U test) and values for provoking doses of ET-1 on each visit compared by analysis of variance (ANOVA) with significance accepted at the 95% level in each case. Provoking concentrations (PC values) of bronchoconstrictor substances are expressed as geometric mean (range). The

geometric mean is used because the scale of dose increase is non-linear, and it is obtained by calculating the antilog of the mean log PC values.

5.3 RESULTS

Demographics & lung function

The 8 subjects had a mean (SD) age of 37.1 (9.8) years, and spirometry showed a mean (SD) FEV_1 of 2.72 (0.5) litres (87.9 (12.8) % predicted). See table 5.1 for demographic factors and asthma treatment. All subjects had bronchial hyperreactivity with geometric mean (range) $PC_{20}FEV_1$ to methacholine of 1.8 (0.47-8.0) mg/ml. There were no differences in mean (SD) FEV_1 at baseline on each study day (placebo 84 (11) % predicted; angiotensin II 1 ng/kg/min 84 (15) % predicted; angiotensin II 2 ng/kg/min 80 (16) % predicted).

Plasma angiotensin II

Plasma angiotensin II concentrations (at baseline, prior to and on completion of each ET-1 inhalation) were median (inter-quartile range (IQR)) 8.1 (6.2-13.8), 11.25 (6.1-15.8) and 5.7 (4.1-9.0) pg/ml after placebo, 9.3 (6.8-10.8), 20.8 (12.2-25.4) and 15.3 (12.0-19.6) pg/ml after angiotensin II 1 ng/kg/min and 8.4 (6.3-11.5), 28.4 (18.4-51.3) and 23.8 (17.1-53.3) after angiotensin II 2 ng/kg/min respectively (figure 5.1 - presented as mean (SEM) for clarity). Plasma levels were not different to placebo at baseline for either angiotensin II infusion dose, and greater than placebo pre- and post-ET-1 inhalation for both angiotensin II doses.

Airway responses

Angiotensin II infusion *per se* did not result in bronchoconstriction at either 1 ng/kg/min (mean (SD) FEV_1 85 (13) % predicted) or 2 ng/kg/min (mean (SD) FEV_1 80 (15) % predicted). ET-1 inhalation produced dose-dependent bronchoconstriction in all subjects as previously demonstrated (275), with geometric mean (range) $PC_{15}FEV_1$ ET-1 of 3.20 (0.48-15.36) nmol for placebo, 3.76 (1.04-10.86) nmol for angiotensin II at 1 ng/kg/min, and 3.53 (0.64-11.3) nmol for angiotensin II at 2 ng/kg/min (figure 5.2 and

table 5.1), but there was no difference in bronchial responsiveness to ET-1 (ANOVA p.0.05) comparing placebo infusion with the 2 doses of angiotensin II.

Blood pressure, pulse and oxygen saturation

There were no differences between mean blood pressures at baseline on each of the three study visits (placebo, angiotensin II 1ng/kg/min and angiotensin II 2 ng/kg/min). The higher dose of infused angiotensin II resulted in an increase in mean (SEM) diastolic blood pressure compared with placebo prior to the ET-1 inhalation (82 (2.7) vs 73 (2.9) mmHg respectively), and an increase in both systolic and diastolic mean (SEM) blood pressure at the end of the study visit compared with placebo (SBP 136 (6.6) vs 117 (4.7), DBP 86 (2.4) vs 75 (2.1) mmHg respectively) (figure 5.3). Pulse rates and oxygen saturation measured by pulse oximetry did not alter significantly on any of the study days (Appendix 5.1).

Table 5.1

Demographics, treatment and bronchial responsiveness to endothelin-1

No	Age (years)	Sex	FEV1 (litres)	FEV1 % predicted	Methacholine PC20 (mg/ml)	Treatment	ET-1 PC15 placebo infusion (nmol)	ET-1 PC15 Ang II infusion 1 ng/kg/min (nmol)	ET-1 PC15 Ang II infusion 2 ng/kg/min (nmol)
1	48	F	2.20	81	2.92	T. BDP1000. Sm	4.68	3.05	4.57
2	43	F	2.31	87	1.76	S	10.40	6.20	11.30
3	27	F	2.96	94	0.47	S. BDP800. Sm	0.48	1.11	0.64
4	35	M	3.32	88	2.72	S. BDP1000. Sm	1.12	3.75	4.12
5	34	M	2.70	76	2.21	S. BDP1000	5.13	5.45	2.57
6	38	F	2.25	89	0.72	S. Flu1500. Sm	1.24	1.04	1.84
7	22	F	3.47	113	8.00	S. BDP400	15.36	8.16	11.30
8	50	M	2.63	71	1.33	S.	4.27	10.86	3.34
					(geometric)		(geometric)	(geometric)	(geometric)
Mean	**37.1**	**3M**	**2.72**	**87.8**	**1.8**		**3.20**	**3.76**	**3.53**
(SD/ range)	(9.8)	**5F**	(0.5)	(12.8)	(0.47-8.0)		(0.48-15.36)	(1.04-10.86)	(0.64-11.3)

Key T Terbutaline 500µg as required
S Salbutamol 800µg as required
BDP Beclomethasone dipropionate (daily dose in µg)
Flu Fluticasone propionate (daily dose in µg)
Sm Salmeterol 100µg daily

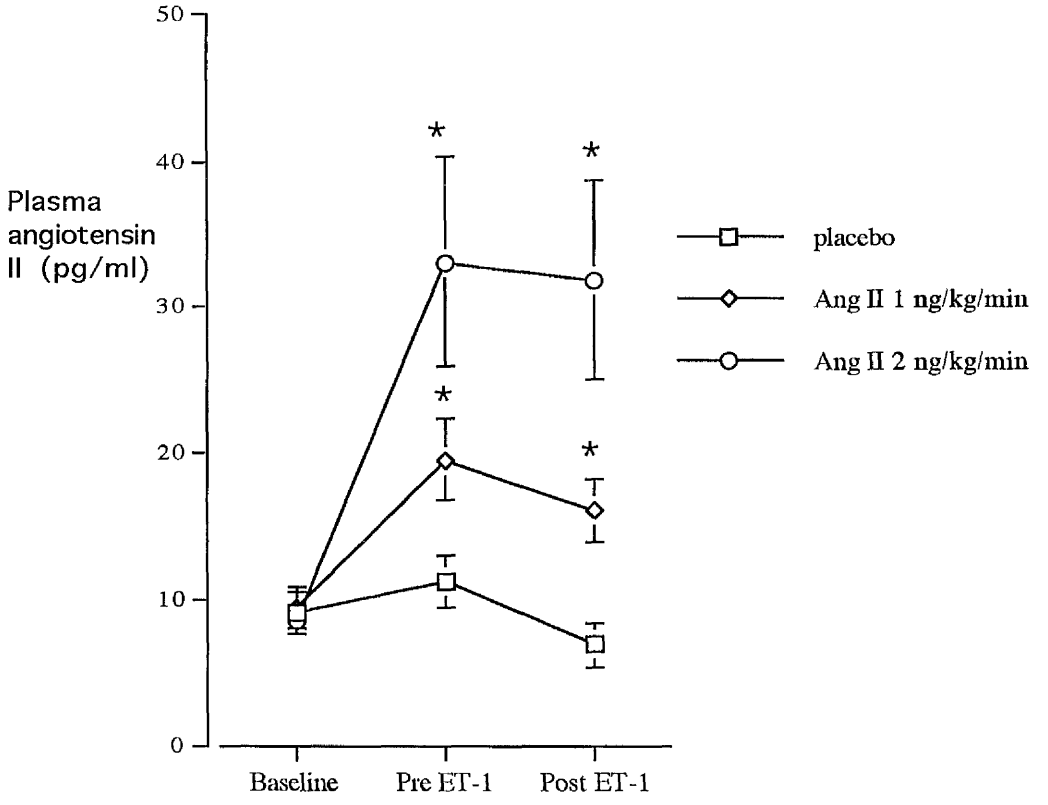

Figure 5.1
Plasma angiotensin II levels (mean (SEM)) at baseline, steady state infusion (pre-ET-1 inhalation) and post-ET-1 inhalation, during infusion of placebo, angiotensin II 1 ng/kg/min and angiotensin II 2 ng/kg/min.
* ($p<0.05$) compared with placebo value.

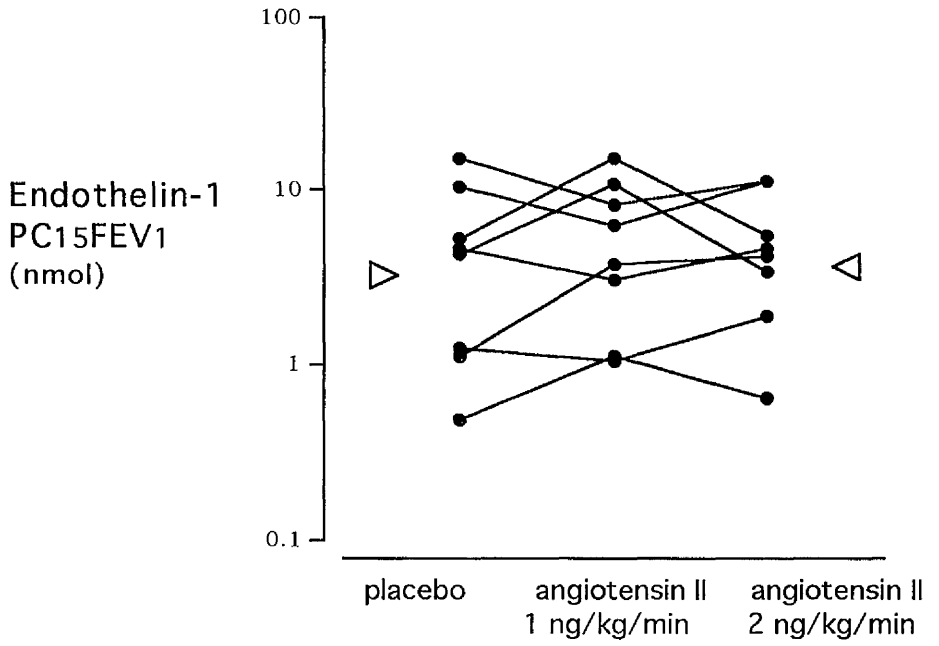

Figure 5.2
Provoking concentration of ET-1 required to produce a fall in FEV1 of 15% (PC15FEV1) during infusion of placebo, angiotensin II 1ng/kg/min and angiotensin II 2ng/kg/min. Log scale with geometric mean values shown for placebo and angiotensin II 2 ng/kg/min. There were no significant differences between bronchial reactivity to ET-1 on any of the study days.

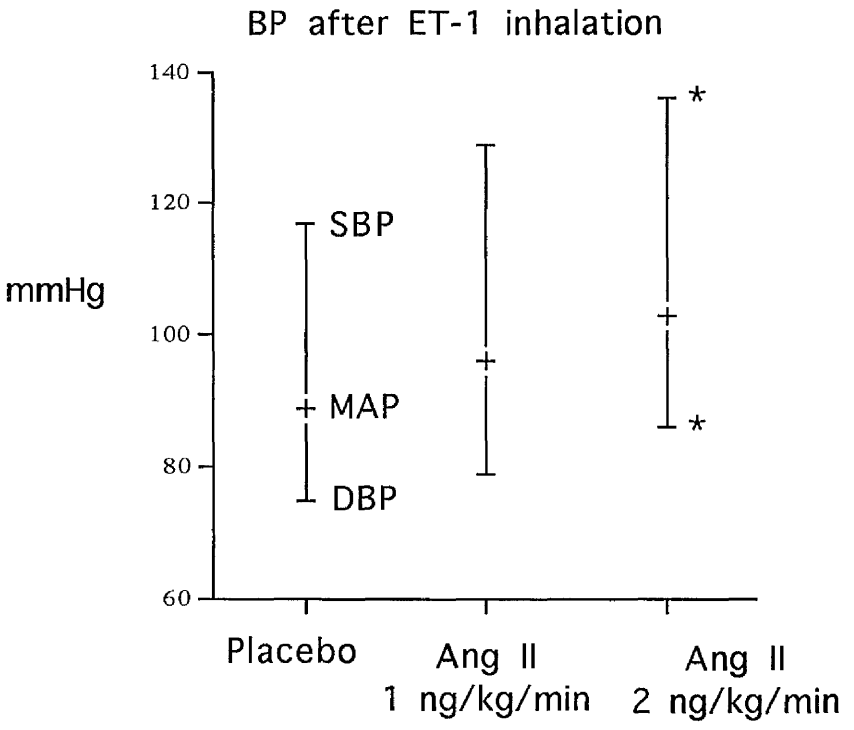

Figure 5.3
Non-invasive blood pressure comparing average values of systolic, diastolic and mean arterial pressure during infusion of placebo, angiotensin II 1 ng/kg/min and 2 ng/kg/min

5.4 DISCUSSION

We have demonstrated that inhaled ET-1 produces bronchoconstriction in mild asthmatics, but did not show any potentiation of this effect by infusion of sub-bronchoconstrictor doses of Ang II.

The lack of potentiation of ET-1-induced bronchoconstriction by Ang II was contrary to our expectations, having previously demonstrated potentiation by Ang II of ET-1-induced bronchoconstriction in bovine bronchial preparations *in vitro* (261). Similarly, our group has demonstrated potentiation by Ang II of methacholine-induced bronchoconstriction in both human bronchial rings *in vitro* and in asthma *in vivo* (272), although in contrast, there was no evidence of potentiation by Ang II of histamine-induced bronchoconstriction, either in human bronchi *in vitro* or *in vivo* in asthma (277), suggesting that potentiation of bronchoconstriction by Ang II may vary according to the spasmogen used.

ET-1-induced bronchoconstriction in human airways is mediated mainly by the ET_B receptor (68), but the exact mechanism of bronchoconstriction is not known in man. In contrast to animal tissues, ET-1 appears to exert bronchoconstrictor activity in human airways directly on smooth muscle (60), without the involvement of acetylcholine, leukotrienes, histamine or platelet activating factor (88), although there is evidence that ET_B receptor activation may potentiate cholinergic nerve-mediated contraction in human bronchial preparations (113). Interestingly, Ang II has also been shown to potentiate neural cholinergic bronchoconstriction evoked by electrical field stimulation (278) in rabbit airway smooth muscle. The finding that methacholine-induced bronchoconstriction is potentiated by Ang II in asthmatics *in vivo*, while histamine and ET-1-induced bronchoconstriction are not, may therefore suggest that potentiation of bronchoconstriction by Ang II is specific to cholinergic agents. In addition, while the *in vitro* component of the study by Millar *et al* (272) suggests that Ang II potentiates

methacholine bronchoconstriction post-junctionally, the influence of pre-junctional factors *in vivo* is not known, and may account for differences in potentiation between different bronchoconstrictors.

ET and histamine receptors in the airway are coupled to specific G proteins, with signal transduction in each case involving (among other pathways) stimulation of phospholipase C with subsequent synthesis of 1,4,5-inositol triphosphate (IP_3) and diacylglycerol (DAG) with activation of protein kinase C (279, 280). Similarly, cholinergic muscarinic receptors in the airways are also coupled to membrane phospholipid hydrolysis to form IP_3, but there are differences in the pathways involved (280), and cross-talk at the second messenger level between Ang II intracellular pathways and ET or cholinergic second messenger pathways may account for the differences in interactions between Ang II and ET or methacholine in the airways.

Ang II is a weak bronchoconstrictor, and the infusion doses of Ang II were deliberately chosen to fall below the levels required to produce bronchoconstriction *per se*. We found no bronchoconstriction in our subjects which could be attributed to the effects of Ang II alone, but it could be argued that potentiation of ET-1-induced bronchoconstriction might occur at higher plasma levels of Ang II. Potentiation of methacholine-induced bronchoconstriction in asthma by sub-bronchoconstrictor doses of Ang II was observed in a previous study from our group (272), in which an increase in bronchial responsiveness to methacholine was observed in 6 of 7 patients during infusion of Ang II at 2 ng/kg/min. Comparing plasma Ang II levels with this study showed that we achieved similar elevation in mean plasma levels of Ang II prior to the ET-1 inhalation, and elevated but slightly lower levels at completion of the study for both doses of Ang II. The reasons for this small difference are not clear, and while this could account for a difference in potentiation of bronchoconstriction, we observed no evidence of potentiation in those patients whose plasma Ang II levels exceeded the mean levels observed in the previous study. The median (IQR) peak plasma levels of Ang II

observed in this study (28.4 (18.4-51.3) pg/ml during infusion of Ang II 2 ng/kg/min) were lower than those reported in acute severe asthma (median 56 (12-109) pg/ml) (273) and there is therefore potential for interaction in acute severe asthma which could not be demonstrated in this study, if such interaction is dependent merely on the plasma concentration of Ang II. In the previous study showing potentiation by Ang II of ET-1-induced bronchoconstriction in bovine airways (261) the levels of Ang II used *in vitro* (10^{-7} or $3x10^{-7}$ M) were higher than plasma levels in our study at baseline (around $8x10^{-12}$ M) or at peak levels (around $3x10^{-11}$ M), and higher also than plasma levels in acute severe asthma (around $5x10^{-11}$ M) (273), and it is possible that this difference in concentration of Ang II accounts for the lack of interaction with ET-1 in asthma *in vivo*.

In conclusion, the role of the renin-angiotensin system in asthma is not fully understood, and in particular interactions between Ang II and bronchoconstrictors which may be implicated in asthma appear to be diverse, with potentiation of the effects of methacholine, but not histamine or ET-1 in asthma.

CHAPTER 6

INDUCED SPUTUM ENDOTHELIN-1 LEVELS IN ASTHMA

a) stable asthmatic and non-asthmatic subjects
b) acute exacerbation of asthma

6.1 INTRODUCTION

Having established the bronchoconstrictor activity of ET-1 *in vivo*, and demonstrated bronchial hyperreactivity to ET-1 in asthmatic subjects, we wished to examine whether we could use induced sputum to measure airway levels of ET-1 in asthma. ET-1 is produced by human bronchial epithelial cells, and histological studies show increased expression of endothelin in the bronchial epithelial cells of asthmatic patients compared with normal subjects (47). Similarly, elevated levels of endothelin have been reported in bronchoalveolar lavage in symptomatic and non-steroid treated asthmatic patients (124). Induced sputum has potential advantages over bronchoscopic techniques in that it is non-invasive, and can easily be repeated in an individual subject to compare parameters over time.

Since there have been no previous reports of sputum levels of ET-1, we first wished to assess whether or not ET-1 could be measured in induced sputum using a commercially available immunoassay, and sought to confirm this using reverse-phase high-performance liquid chromatography (RP-HPLC). We further wished to compare induced sputum ET-1 in asthmatic and non-asthmatic subjects, and sputum ET-1 levels in acute exacerbation of asthma compared with levels following treatment and recovery. This chapter is therefore a combination of pilot work using RP-HPLC to identify ET-1 in sputum, and 2 further studies, the first comparing sputum ET-1 measured by ELISA in asthmatic and non-asthmatic subjects (baseline study), and the second a study comparing sputum ET-1 in acute exacerbation of asthma and in convalescence (acute study).

6.2 METHODS

Subjects

(a) Baseline study

28 stable asthmatics and 9 non-asthmatic subjects who were non-smokers were studied. Asthma was defined by clinical symptoms, response to inhaled beta-2 agonist, and methacholine PC_{20} <8mg/ml. Normal subjects were asymptomatic, and had a methacholine PC_{20} >16 mg/ml, with normal baseline spirometry. The study was approved by the West Ethics Committee, West Glasgow Hospitals University NHS Trust, and each subject gave written informed consent.

(b) Acute study

9 subjects with a previously established diagnosis of asthma (2M, 7F) were recruited following admission to our unit with an acute exacerbation of asthma (5 non-smokers, 2 ex-smokers and 2 current smokers). Entry criteria were established for an age group of 18 - 75 years, with a known history of asthma (GP or hospital diagnosis). At the time of presentation, subjects were required to have a respiratory rate of >25/min, peak expiratory flow rate of <60% of predicted/usual and a pulse rate of >110 beats per minute. The study was approved by the West Ethics Committee, West Glasgow Hospitals University NHS Trust, and each subject gave written informed consent.

Protocol

(a) Baseline study

The study involved 2 visits to the laboratory, the first for methacholine challenge testing, and the second for sampling of blood and saliva and sputum induction, with spirometry monitored throughout the induced sputum procedure in the usual way.

(b) Acute study

Spontaneous sputum samples were obtained within 24 hours of admission to hospital in all cases (in most cases within 12 hours), and blood was sampled concurrently. Patients were managed according to clinical requirements (in all cases oral corticosteroid therapy was started or increased, and in 2 cases antibiotics were commenced), and a review visit was arranged for roughly 6 weeks after admission, or once symptoms had been stable for 3 weeks thereafter. At the review visit, spirometry was measured, blood sampled, and induced sputum performed following pretreatment with salbutamol 200μg, with spirometry monitored through the procedure.

Sputum Induction

This was performed as described in chapter 2.3. Additionally, in the baseline study saliva samples were obtained prior to sputum induction to measure salivary levels of ET-1. In a subgroup from the baseline study (n=9), blood was taken pre- and post-sputum induction to ensure no change as a result of the procedure.

Laboratory Processing

Sputum was processed as described in chapter 2.3, with the exception for the acute study mentioned under biochemical assays, with the laboratory staff unaware of the clinical details relating to the sample.

Reversed-phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC was carried out on saliva and sputum samples as described in chapter 2.4.

Biochemical Assays

Endothelin was assayed as described in chapter 2.4, but in the acute study, due to a failed assay on the first attempt, sputum ET-1 was not able to be measured on sputum which had not been treated with the reducing agent dithiothreitol (DTT), and this was repeated on DTT-treated samples. Sputum ET-1 levels are therefore not directly comparable between the

baseline and acute studies. Recovery of ET-1 from sputum was 88%. Eosinophilic cationic protein (ECP) was assayed using a radio immuno-assay (lower limit of detection 2 μg/l in plasma) (Pharmacia U.K. Ltd, Milton Keynes, U.K.).

Statistical Analysis

Non-parametric statistics were used to compare cell counts, ECP and ET-1 values using the Mann-Whitney U test, with differences in paired data analysed by subtraction followed by 1-sample Wilcoxon test. Significance was accepted at the 95% level. Power calculations suggest a power of >85% to detect a difference in sputum ET-1 of ± 1 S.D.

6.3 RESULTS - REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Presence of ET-1

RP-HPLC was performed initially on pure ET-1 standards to establish the elution point for ET-1 resulting in a retention time of 21.0 minutes. RP-HPLC was then performed on clinical samples under the same conditions, which confirmed the presence of ET-1 in saliva and sputum, with a single peak corresponding to the pure ET-1 standard in each case (figure 6.1), with elution points for saliva and sputum being 21.0 minutes and 21.1 minutes respectively.

Effect of dithiothreitol (DTT)

In order to examine the potential influence of DTT on the ET-1 molecule, an ET-1 standard was prepared in the presence of DTT, and as above for the pure ET-1 standard, the elution point was measured. This revealed a phase shift of 5 fractions for DTT-treated ET-1 standard, with an elution point of 25.5 minutes and fractionation was therefore shifted for the DTT-treated sputum sample. In fact, however, the elution point for the DTT-treated sputum sample proved to be identical to the saliva and sputum samples which were not treated with DTT, and to that of the pure ET-1 standard at 21 minutes (figure 6.1). There was a difference in magnitude of the peak in the DTT-treated sputum sample, measuring around twice that of the non-DTT-treated sputum sample.

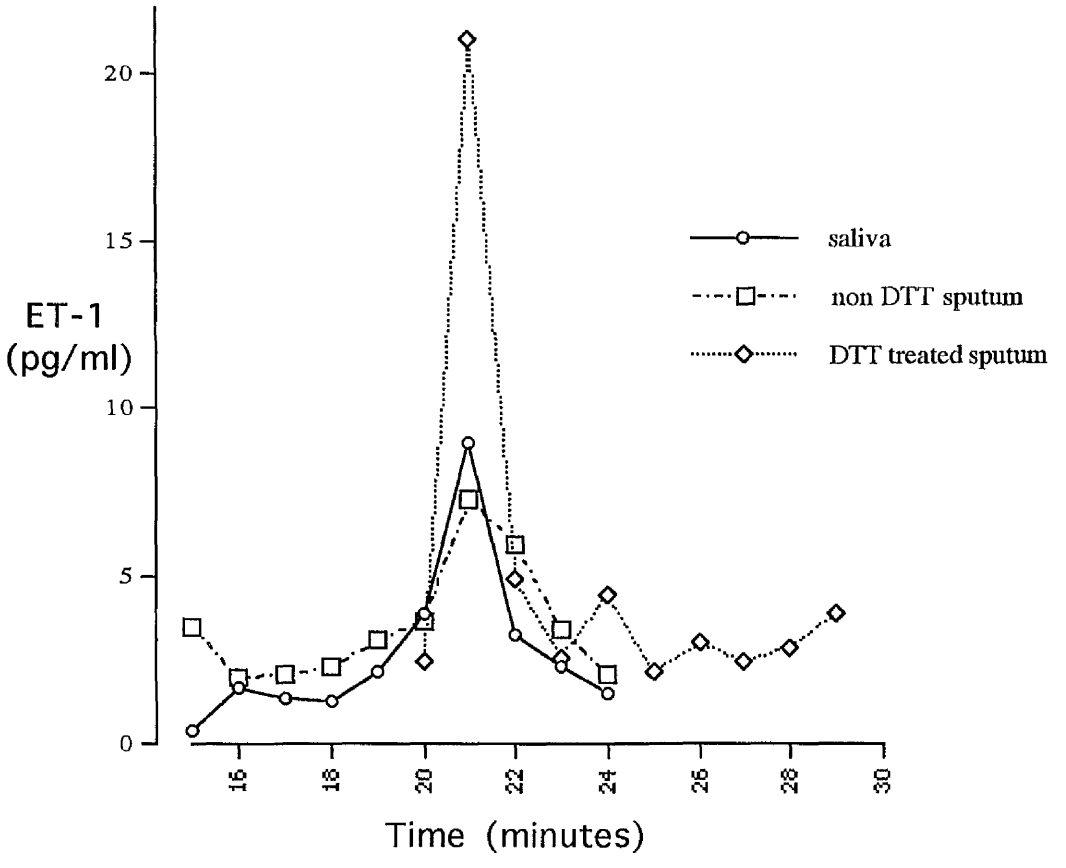

Figure 6.1
RP-HPLC retention time for saliva, DTT-treated sputum and non-DTT-treated sputum

6.4 RESULTS - BASELINE STUDY

Demographics

Demographic data is summarised in table 6.1. The asthmatic group was older (mean (S.D.) 41.4 (10) years vs. 31.8 (8.8) years), and had lower FEV_1 than normals, both as absolute values (2.99 (0.9) litres vs. 3.93 (1.08) litres) and percent predicted (84.0 (14)% vs. 99.2 (11.4) %). Mean daily inhaled corticosteroid dose was 400μg beclomethasone or equivalent in the asthmatic group.

Sputum cell counts

Sputum eosinophils were higher in asthmatics (median 3%) than normal subjects (median 0%), and no differences were seen in total cell counts, nor in macrophage, neutrophil or lymphocyte proportions (table 6.2)

Eosinophilic cationic protein

In asthmatics and normals, median (IQR) sputum ECP (129.0 (100-340) μg/l and 340.0 (134-710) μg/l respectively) was greater than serum ECP, with serum ECP greater in asthmatics than normals (9.0 (3-11) μg/l vs. 1.0 (0-7) μg/l. Saliva levels of ECP were assayed in a sub-group of the subjects (n=7), drawn from each of the groups at random to compare with sputum. In this group, saliva ECP (median (IQR) 10.0 (5-69) μg/l) was lower than sputum ECP (210 (26-460) μg/l).

Endothelin-1

Comparing median (IQR) plasma, saliva and sputum levels of ET-1 between each group showed no significant differences between asthmatics and normals for each fluid (median (IQR) ET-1: Asthmatics, saliva 23.8 (14.4-33.7) pg/ml; sputum 11.2 (9.4-18.9) pg/ml; plasma 3.6 (2.8-4.5) pg/ml: Normals, saliva 30.1 (20.1-43.4) pg/ml; sputum 15.5 (10.0-21.4) pg/ml; plasma 3.1 (1.7-4.8) pg/ml.) Plasma ET-1 did not alter as a result of sputum induction *per se* (subgroup n=9, data not shown). Pairwise analysis of asthmatic subgroups

revealed no differences between asthmatics taking inhaled steroids (n=16) and those taking inhaled ß2-agonists alone (n=12). There were significant differences within each patient group in the levels of ET-1 in the order saliva > sputum > plasma (figure 6.2 and table 6.3). We found no significant direct correlation between plasma or sputum ET-1 and the asthmatics' FEV_1 or methacholine PC_{20}.

Table 6.1

Demographic data - Baseline study

No.	Age (years)	Sex	FEV1 (litres)	FEV1 (%) predicted	Treatment	PC20 (mg/ml)
Normal volunteers						
1	30	M	3.78	76	none	>16
2	23	M	4.50	107	none	>16
3	45	F	2.54	96	none	>16
4	31	M	4.83	109	none	>16
5	25	M	4.66	110	none	>16
6	23	M	5.65	108	none	>16
7	33	F	2.41	90	none	>16
8	47	M	3.35	93	none	>16
9	29	M	3.66	104	none	>16
Mean	**31.8**	**7M 2F**	**3.93**	**99.2**		
(S.D.)	(8.8)		(1.08)	(11.4)		
Asthma						
1	36	F	1.33	49	S	0.04
2	37	M	3.04	81	S	4.4
3	45	M	3.47	83	S	4.21
4	39	M	4.21	81	S	0.19
5	46	F	1.97	84	S	1.27
6	23	M	4.64	104	S	7.4
7	30	F	3.47	113	S	7.8
8	47	M	2.80	71	S	1.33
9	37	F	2.62	93	S	0.84
10	34	F	3.09	103	S	6.21
11	31	M	3.55	85	S	1.29
12	29	F	2.34	75	S	0.33
13	30	M	4.86	102	S Bud 800	7.6
14	69	M	2.19	70	S Flut750	1.31
15	51	M	2.54	63	S BDP800 Sm	3.2
16	33	M	3.95	84	S BDP 400	2.65
17	57	F	2.31	86	S BDP800	0.34
18	48	M	3.32	92	S BDP400	4.9
19	48	M	4.02	83	S BDP1000 Sm	4.2
20	39	M	3.87	105	S BDP1000	0.26
21	34	M	3.05	83	S BDP400	3.28
22	38	M	2.80	72	S BDP800 Sm	0.62
23	47	F	2.23	88	S BDP400	6.86
24	54	M	2.50	77	S BDP1500	2.64
25	47	F	2.14	80	S Bud400	0.47
26	49	F	2.41	101	S BDP800	1.13
27	35	M	2.78	72	S Bud800	4.92
28	45	F	2.02	70	S BDP400	0.27
Mean	**41.4**	**17M 11F**	**2.99**	**84**	**geometric mean**	**2.9**
(S.D.)	(10.0)		(0.88)	(14)	range	0.04-7.8

key

PC20	Provoking concentration of methacholine producing a 20% fall in FEV1
S	Salbutamol 200 μg p.r.n.
Bud	Budesonide total daily dose in μg
Flut	Fluticasone propionate total daily dose in μg
BDP	Beclomethasone dipropionate total daily dose in μg
Sm	Salmeterol 100 μg daily

Table 6.2
Baseline Study - Sputum cell counts

	total cell count ($x10^6$/ml)	Macrophage (%)	Neutrophil (%)	Eosinophil (%)	Lymphocyte (%)
Normals	**8.5** (4.3-23.2)	**81.0** (65.0-90.0)	**19.0** (6.0-35.0)	**0.0** (0-0)	**2.0** (0-3.5)
Asthmatics	**6.25** (3.2-9.8)	**64.5** (50.8-78.8)	**23.5** (5.3-37.0)	**3.0*** (1.0-7.0)	**2.0** (1.0-5.0)

key values expressed as median (IQR)
differential counts expressed as % cell count after exclusion of epithelial cells
* greater than normals ($p<0.05$)

Table 6.3

Baseline study - Endothelin-1 and eosinophilic cationic protein in saliva, sputum and plasma/serum (Median (interquartile range))

	Endothelin-1 (pg/ml)			ECP (μg/l)	
	Saliva	Sputum	Plasma	Sputum	Serum
Normals	**30.1** (20.1-43.4)	**15.5** (10.0-21.4)	**3.1** (1.7-4.8)	**340.0** (134-710)	**1.0** (0-7.0)
Asthmatics	**23.8** (14.4-33.7)	**11.2** (9.4-18.9)	**3.6** (2.8-4.5)	**129.0** (100-340)	**9.0*** (3.3-11.0)

key * significantly greater than normal volunteers

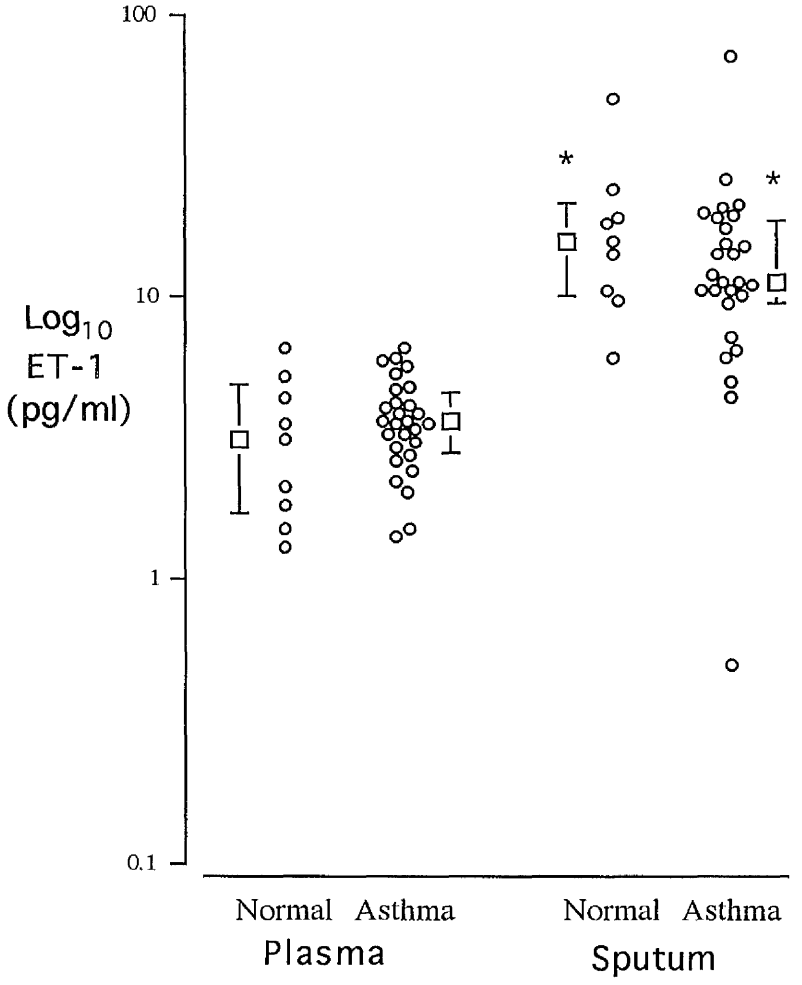

Figure 6.2
Baseline study
Plasma and sputum ET-1 in normals and asthmatics (median (IQR))
* greater than plasma value

6.5 RESULTS - ACUTE STUDY

Demographics

Demographic data is presented in table 6.4. The patients in this study were more severe asthmatics than in the baseline study, with a mean FEV_1 when stable of 71% predicted, but a range from 31% to 102%. All were prescribed inhaled corticosteroids prior to exacerbation, with a mean daily dose of beclomethasone (or equivalent) of 1900 μg/day, and 3 were prescribed maintenance oral corticosteroids (prednisolone dose range 1-20mg daily) prior to exacerbation. Mean (SEM) peak expiratory flow rate (PEFR) on admission was 32.6 (4.5)% predicted/best, and on recovery 75.0 (13.7)% predicted/best (p=0.004).

Sputum cell counts

Sputum cell counts are summarised in table 6.5. There was no significant difference between total sputum cell counts in exacerbation (median (IQR) 9.7 (7.5-40.4) cells $x10^6$/ml) and in recovery (median (IQR) 6.8 (5.2-17.4) cells $x10^6$/ml), and comparing paired data, the only significant change in differential cell counts comparing acute exacerbation and convalescence was a fall in total eosinophil numbers (p=0.04), and a rise in macrophage proportion (p=0.03), reflected by a non-significant rise in neutrophil proportion. There was significant neutrophilia both in acute exacerbation and in convalescence (median (IQR) 64 (50-82)% and 58 (21-81)% respectively), with a modest eosinophilia (median (IQR) 4 (2-18)% and 3 (1-10)% respectively).

Endothelin-1

There was no difference between plasma ET-1 in acute exacerbation (median (IQR) 4.1 (2.7-5.5) pg/ml) and plasma ET-1 in convalescence (median (IQR) 4.1 (2.6-4.7) pg/ml). Due to a failure of our initial assay, sputum ET-1 was measured in samples which had been pre-treated with DTT, and are therefore not directly comparable with values from other studies, including the baseline study. There was no difference between sputum ET-1 in

acute exacerbation (median (IQR) 4.75 (3.6-17.9) pg/ml) and sputum ET-1 in convalescence (median (IQR) 3.2 (1.7-27.6) pg/ml) (figure 6.3).

Table 6.4
Acute study - Demographic data

No.	Age (yrs)	Sex	FEV1 (litres)	FEV1 (% pred)	Prior inhaled steroid treatment (μg BDP/ equiv)	Additional asthma treatment
1	19	F	3.05	102	3000	S. Ef. NS. Bam
2	63	F	0.91	42	2500	S. NS. Ipr.Th.
3	49	F	0.79	31	2000	P20. S. NS. Ipr. NBud. Th
4	47	M	1.75	43	2000	P10. S. Sm. NBud
5	40	M	1.24	38	4000	P1. STb. NS. Ipr. Sm
6	37	F	2.87	99	800	S.
7	40	F	2.25	87	800	S.
8	58	F	2.09	101	1600	S. Sm. Tb. Th
9	35	F	2.59	95	400	S.
Mean	**43.1**	**3M**	**2.09**	**71**	**1900**	
(S.D.)	13.1	**6F**	(0.8)	(31)	(1160)	

Key

S.	Inhaled salbutamol 200μg as required
NS	Nebulised salbutamol 2.5 mg as required
Ef	Eformoterol 24μg daily
Bam	Bambuterol 20mg daily
Ipr	Nebulised ipratropium bromide 0.5mg as required
Th.	Oral theophyllines
NBud	Nebulised budesonide 2mg daily
Sm	Salmeterol 100μg daily
STb	Subcutaneous terbutaline 10mg/24hours
Tb	Terbutaline 0.5mg as required

Table 6.5
Sputum total and differential cell counts - Acute study
median (IQR)

	Total cell count	Macro-phage	Neutrophil	Eosinophil	Lympho-cyte
Cell numbers (cells x10^6/ml)					
Exacerbation	**9.70** (7.5-40.4)	**2.56** (0.8-5.9)	**6.28** (3.6-24.7)	**0.78*** (0.3-2.5)	**0.0** (0-0.04)
Convalescence	**6.80** (5.2-17.4)	**2.88** (1.2-6.3)	**3.75** (1.3-11.9)	**0.31** (0.08-0.8)	**0.0** (0-0)
Cell proportions (%)					
Exacerbation		**19.0*** (8.5-34.0)	**64.0** (50-82)	**4.0** (2-18)	**0.0** (0-0.5)
Convalescence		**37.0** (15-60)	**58.0** (21-81)	**3.0** (1-10)	**0.0** (0-0)

Key * $p<0.05$

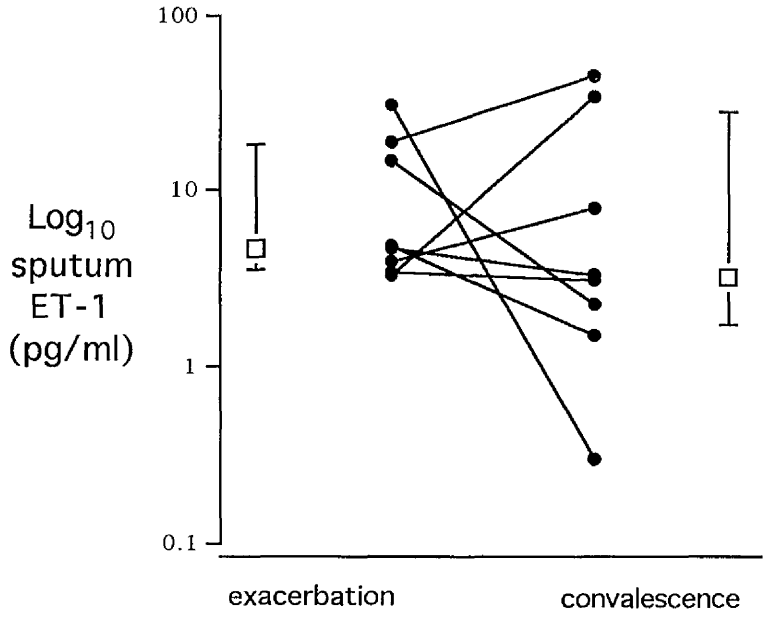

Figure 6.3
Acute study - Sputum ET-1 in exacerbation and convalescence in asthma

6.6 DISCUSSION

We report that it is possible to measure ET-1 by RIA, and confirm its identity by RP-HPLC in sputum obtained by sputum induction, and that saliva and sputum levels of ET-1 are significantly elevated when compared with plasma in stable asthmatics and non-asthmatics. We found no difference in sputum ET-1 levels between asthmatics and non-asthmatics, but unexpectedly found saliva ET-1 levels to be significantly higher than sputum levels in both groups. We are not aware of any previous study which has made this comparison, either in asthmatics or normal subjects. In the study examining sputum ET-1 levels in acute exacerbation of asthma, plasma and sputum ET-1 were not different comparing exacerbation and convalescence, although the possible influence of other factors on this finding will be discussed. The different components of this chapter will be discussed under separate headings.

Reverse-phase high-performance liquid chromatography

The pilot work examining using RP-HPLC was designed to confirm the presence of ET-1 in sputum and saliva samples, to allow us to accurately interpret the RIA results, since we were not aware of any reports of the measurement of ET-1 in sputum using RIA or any other assay technique. The results confirm that ET-1 is present in sputum and saliva. We extended this to examine the potential action of the reducing agent DTT on sputum samples, and although it is possible to speculate on the interpretation of this, this aspect was not pursued further. We found a difference in the elution points for DTT-treated pure ET-1 compared with DTT-treated sputum samples, and a difference in the magnitude of the ET-1 peak comparing DTT-treated and non-DTT-treated sputum. There are several possiblities which might explain aspects of this, although these remain speculations;

(1) Effect of DTT on disulphide bonds in ET-1 molecule.

DTT would be expected to disrupt the ET-1 molecule at its 2 disulphide bonds, but this process is pH-dependent, and differences in pH between the pure-ET standard (which was prepared at optimal pH for reduction) and the sputum sample might result in different

degrees of reduction or failure of reduction by DTT. This may account for the differences seen in the elution points of the DTT-treated ET-1 standard and the DTT-treated sputum sample. Alternatively, reduction may have taken place, but subsequent to RP-HPLC ET-1 was oxidised again to regain its original structure.

(2) Protective effect of DTT against oxidation

We found an increase in the magnitude of the signal for the ET-1 in the DTT-treated sputum sample compared with the non-DTT-treated sample. ET-1 has a methionine residue at position 7, which is sensitive to oxidation. This could result in impaired antibody binding in the RIA, with resultant underestimation of the ET-1 measurement. Sputum prepared in the presence of DTT may be protected from oxidation, with resultant higher values for ET-1 measured in this way.

Baseline Study

In the baseline study asthmatic patients were studied while their lung function was stable with minimal symptoms, not in exacerbation, and no bronchoconstrictor stimulus was given prior to sputum induction, to allow us to examine basal levels of ET-1. Differential cell counts show an elevation in eosinophils in the asthmatic group which is consistent with, but not as pronounced as some previous studies (246, 281), and in contrast to other workers, we did not find an elevation in sputum ECP in the asthmatic group compared to normals. We believe that this finding reflects a largely asymptomatic group of patients, the majority of whom have relatively mild asthma (overall mean daily dose of inhaled corticosteroid 400μg beclomethasone dipropionate or equivalent) since the methodology used, and commercially available assay kit were identical to other groups as far as can be ascertained. A relationship between FEV_1 (282), symptoms (283), and induced sputum eosinophil counts has been described in asthmatic patients, with lower eosinophil counts in asymptomatic patients, and patients with higher FEV_1. Since ECP is produced by activated eosinophils, we believe that the absence of an elevation in ECP is consistent with the median eosinophil count of 3% in our asthmatic group. Normal subjects were recruited who did not complain of symptoms of

breathlessness, and whose spirometry was within normal limits, to act as a control group for the asthmatic subjects.

The finding that ET-1 levels in saliva are higher than ET-1 in sputum is unexpected, and may suggest release of ET-1 either from the salivary glands or the epithelial cells of the oral mucosa. Animal studies have revealed staining for immunoreactive ET-1 in the rat parotid gland (284) and the presence of ET-1 in human saliva has been reported using fast protein liquid chromatography (FPLC) separation and RIA (285) although at levels around 6 times lower than we have observed. Differences in assays and laboratory processing can influence levels, but the reason for this discrepancy is not known. Little evidence is available regarding any potential physiological or pathological role of endothelin in saliva but one speculation on the basis of its known mitogenic activity is that it may be involved in the maintenance of mucosal integrity. Recovery of ET-1 (88%) from sputum samples using the technique of "spiking" the sample with a known quantity of ET-1 suggest that the levels of ET-1 measured by RIA following separation are a reasonable approximation of the concentration of ET-1 in the sputum supernatant.

In addition to questions about the physiological role of ET-1 in saliva, the finding of higher ET-1 in saliva than in sputum plugs, raises an important methodological issue regarding the technique of sputum processing. As mentioned in chapter 1.4, there are two principal methods of sputum induction, the first involving sputum plug separation, and the second involving the use of the whole sputum sample without prior separation of the viscid portion. Separation of sputum plugs is taken to minimise salivary contamination of sputum samples, and in keeping with this we found that ECP levels in sputum plugs were significantly higher than in saliva. While this study was not designed to test the hypothesis that plug separation allows distinction of supra-glottic and infra-glottic secretions, our data does suggest that separation produces qualitatively different samples from saliva, and allowed comparison of ET-1 levels in each fluid. Further, despite evidence from cell counts and measurement of ECP that selected sputum and entire sputum have similar diagnostic value in distinguishing

asthmatic from non-asthmatic subjects (226) the unexpected finding that ET-1 is present in saliva at higher concentration than in sputum suggests that it is incorrect to presume that salivary "contamination" will have no significant effect on the levels of relevant mediators in induced sputum.

The plasma levels of ET-1 that we report are consistent with other groups, who similarly found no increase in plasma endothelin in asthmatics with daytime (274) or nocturnal symptoms (127) other than in exacerbation (120).

Allowing as far as can be determined for dilution of BAL samples, our finding of sputum levels of ET-1 of around 10-15 pg/ml accord reasonably with levels found by other groups in BAL fluid (124, 274). Both these studies however report an increase in BAL ET-1 in untreated asthmatics which we did not observe in our asthmatic group (nor in the subgoup taking inhaled beta-2 agonists alone). In contrast to this, a fall in BAL ET-1 was observed at 0400 hours in the group of patients mentioned above with nocturnal symptoms (127), and it was postulated that this may reflect increased tissue binding of ET-1 in nocturnal asthma. Among the limitations of the use of bronchoscopy and BAL as a technique to investigate mediators in asthma is the possibility that the procedure may itself influence mediator levels. In addition, BAL is subject to uncertainty regarding the site of sampling, variable recovery of BAL fluid, and variable dilution of mediators. The reproducibility and validity of sputum induction has been demonstrated (221), and while other groups have demonstrated the presence of cytokines and soluble factors in induced sputum (e.g. Keatings *et al*(180)), it should be noted that samples obtained by sputum induction are not identical to those obtained by BAL (228, 230). A direct comparison of BAL ET-1 and induced sputum ET-1 in the same group would be necessary to exclude any difference caused by the techniques of obtaining and processing the samples, and it not possible to exclude such an influence on ET-1 levels in this study or those involving BAL.

The fact that ET-1 is present in sputum and saliva at levels higher than that in plasma suggests that ET-1 is produced or released locally within the respiratory tract. Endothelin immunoreactivity is present in airway epithelia and submucosal glands throughout the lung (56) in addition to airway smooth muscle and parasympathetic ganglia in the airways (54). Endothelin is secreted by bronchial epithelial cells in cell culture (52) and in the same cells isolated from biopsy samples from asthmatics (125). The presence of pro-endothelin-1 (56) and preproendothelin-1 have also been demonstrated in airway epithelial cells (47). Endothelin binding-sites are seen in lung parenchyma, airway smooth muscle and airway epithelia in decreasing order of density (56) and it is suggested that endothelin released from airway epithelial cells may act as a paracrine factor on airway smooth muscle producing bronchoconstriction. Other putative roles for endothelin, such as a neuromodulatory function based on its presence in parasympathetic ganglia require further investigation, but may be of relevance to asthma.

Acute study

A role for ET-1 in acute severe asthma was postulated originally in a case report of increased BAL ET-1 from a patient in status asthmaticus (121). Increased plasma ET-1 has been reported in acute severe asthma, with a relationship between plasma ET-1 levels and airflow obstruction (120). Although other studies examining ET-1 in acute severe asthma are lacking, there is evidence of increased BAL ET-1 in symptomatic versus asymptomatic asthmatics and that exposure of asthmatic bronchial epithelial cells to histamine and IL-1 induces endothelin synthesis and release, (125), although in contrast there was *reduced* BAL ET-1 in nocturnal samples from subjects with predominantly nocturnal asthma (127).

As mentioned above, the problems which we experienced with our initial ET-1 assay in the acute study meant that the assay was repeated using sputum supernatant following treatment with the reducing agent DTT. Although our results from RP-HPLC show a similar position for the elution point of DTT-treated ET-1, there were differences in magnitude of the levels compared with non-DTT-treated ET-1, and there is no definitive data to clarify any potential

effect of DTT on either the ET-1 peptide or on the RIA assay system. DTT is known to disrupt disulphide bonds, and the structure of the ET-1 peptide is known to contain 2 disulphide bonds (see chapter 1.1). It is not therefore possible to directly relate the values for ET-1 obtained in the acute study to those observed in the baseline study, and although it was possible to obtain measurements of ET-1 in DTT-treated sputum, the change in methodology adds a potentially confounding variable to analysis of the acute study.

Several studies have examined the influence of corticosteroids on ET-1 production, with *in vitro* evidence that corticosteroids decrease production of immunoreactive ET-1 (47) in asthmatic bronchial epithelial cells, and *in vivo* evidence that corticosteroids decrease ET-1 in BAL (274) and in bronchial biopsies (123) in asthma. In our study, all the subjects had received intravenous and/or oral corticosteroids by the time of sputum and blood sampling, and in some cases this had been started prior to admission to hospital with acute severe asthma. We cannot exclude the possiblity that ET-1 levels were reduced by the effect of coritcosteroids, resulting in our finding that there was no difference between induced sputum ET-1 levels on admission or in convalescence.

Contrary to expectations we did not find an increase in plasma ET-1 in acute exacerbation of asthma in the acute study, and the levels of plasma ET-1 found were lower than that observed in a previous study of acute asthma from our own group (119). The reasons for this are not known, although it is possible that blood samples were obtained earlier in the course of the admission in our previous study, or that patients had received more treatment (particularly oral corticosteroids) by the time of sampling.

Despite an improvement in mean PEFR from 32.6% predicted/best on admission to 75% predicted/best in convalescence, we found only modest changes in induced sputum inflammatory cell numbers or proportions. There was a slight fall in eosinophil numbers (although not eosinophil proportions), and a rise in macrophage proportions, but the most striking finding from the sputum cell counts was the significant degree of neutrophilia, both

on admission and in convalescence, without any reduction in neutrophil numbers or proportions comparing acute and convalescent sputum samples. Although cigarette smoking is associated with airway neutrophilia (286), only 2 of the subjects were current smokers, and 2 ex-smokers, and neutrophil numbers were not elevated only in smokers or ex-smokers. Other groups have reported significant neutrophilia in sputum (287) and BAL (288) in acute severe asthma but without follow-up data, suggesting that the neutrophil may have a role in airway inflammation in acute severe asthma. Our own data with follow-up sputum samples did not show any reduction in neutrophil numbers or proportions in recovery, suggesting an association between sputum neutrophilia and severity of asthma, not only in acute severe asthma.

Conclusions

The evidence presented above supports the observation of local production of ET-1 within the bronchial tract, and confirms that this can be assessed non-invasively using sputum induction. This data establishes a basis for the following chapters which explore a role for ET-1 in asthma and other airway diseases. Disappointingly, interpretation of the acute study is hampered by a technical failure in the original ET-1 assay, which limits its application in comparison with other data obtained.

CHAPTER 7

EFFECT OF IMMEDIATE ALLERGIC REACTION IN ASTHMATICS ON ENDOTHELIN-1 LEVELS IN INDUCED SPUTUM

7.1 INTRODUCTION

In view of the potent bronchoconstrictor activity of ET-1 in asthma, and having demonstrated that induced sputum can be used to obtain bronchial secretions for the measurement of ET-1, we were interested to use sputum induction to examine the potential contribution of ET-1 to allergen-induced bronchoconstriction in asthma.

Animal studies have demonstrated an early increase in ET-1 mRNA following experimental eosinophilic airway inflammation (289), a rise in BAL ET-like immunoreactivity following allergen provocation (262) and inhibition of antigen-induced airway inflammation in mice by ET receptor antagonists (290). Pro-inflammatory mediators are known to stimulate ET-1 release from airway epithelial cells (129), and ET-1 itself appears to have pro-inflammatory activity in the lung (105, 108). In the guinea-pig, ET_B selective antagonists inhibit the immediate response, while ET_A antagonists inhibit the late response (109), suggesting that ET-1 may have a role in both components of the airway responses to allergen.

We chose to perform sputum induction during the immediate allergic response, to examine if bronchoconstriction induced by allergen is accompanied by an increase in ET-1 release in the airways.

7.2 METHODS

Patients

12 asthmatic patients were recruited, all of whom had demonstrated sensitivity to house-dust mite allergen (skin test or RAST), and bronchial hyperresponsiveness with a provoking concentration of methacholine to produce a 20% fall in FEV_1 (PC_{20}) of <8mg/ml. Patient demographics are summarised in table 1. All subjects had current asthma with stable symptoms at the time of study, no history within the preceding month of respiratory infection, antibiotics or oral corticosteroid use and other than house dust mite, none of the subjects had been exposed to allergens to which they were allergic in the preceding month. Asthma was defined according to the ATS definition (257), baseline lung function was recorded, and non-specific bronchial hyperresponsiveness was established using a methacholine challenge test, with all the asthmatics subjects having a methacholine PC_{20} of less than 8mg/ml. The study was approved by the West Ethics Committee, West Glasgow Hospitals University NHS Trust, and each subject gave written informed consent.

Study Design

Bronchial challenge tests were carried out on three separate visits using placebo, methacholine (prepared by sterile pharmacy in house), or purified HDM allergen (SmithKline Beecham Pharmaceuticals U.K.) in a blinded, placebo controlled, randomised fashion. Challenge tests were administered using a dosimeter ("Nebicheck" - PK Morgan, Gillingham U.K.), with allergen dose expressed in breath units (BU - where 1 BU = 1 breath of 10^{-3} dilution) and were discontinued following a 20% fall in FEV_1 (with the exception of placebo). Plasma samples were taken before and after bronchial challenge tests, and sputum induction using 3% saline given by ultrasonic nebuliser ("Sonix 2000" - Medix Ltd, Lutterworth, U.K.) was performed 15 minutes after each challenge test, as previously described (291).

Sputum Induction and Laboratory Processing

Sputum induction and laboratory processing of sputum and plasma samples were performed as described in chapter 2.

Expression of Results and Statistics

Airway reactivity is expressed as provoking concentrations required to produce a 20% fall in FEV_1 (PC_{20}), with methacholine values in mg/ml, and allergen values in breath units (1 BU = 1 breath of 10^{-3} dilution). Sputum cell counts were expressed following exclusion of squamous cells, as these were taken to represent salivary contamination. Non-parametric statistics (Mann Whitney-U and Wilcoxon rank sum) were used to compare values, and results are expressed as median (interquartile range) unless specified otherwise, with significance accepted at the 95% level. Spearman rank correlation (r_S) was used where indicated. Statistical analysis was performed using a dedicated software package ('Minitab' - Minitab Inc., State College, PA, USA) on a Macintosh desktop computer (Apple Computer Inc., Cupertino, CA, USA).

7.3 RESULTS

Demographic Data

Demographic data including lung function, asthma therapy and airway responsiveness to methacholine and allergen are summarised in table 7.1. There was no correlation between methacholine and allergen responsiveness. All subjects demonstrated bronchial reactivity to methacholine (geometric mean (range) PC_{20} to methacholine of 1.47 (0.26 - 6.86) mg/ml) and house dust mite allergen (geometric mean (range) PC_{20} to allergen of 324 (71 - 860) BU).

Sputum Cell Counts

Sputum cell counts showed no significant differences between the challenge tests at this time point, with the exception of a slight but significant reduction in lymphocyte percentage following allergen challenge compared to placebo (table 7.2).

Eosinophilic Cationic Protein

Serum ECP did not alter across any of the challenge tests. Sputum ECP was greater than serum ECP in all cases, with no differences in sputum ECP comparing the challenge tests with placebo (table 7.2).

Endothelin-1

Plasma ET-1: Plasma samples taken before and after each inhalation (placebo, methacholine and allergen) showed no change in plasma ET-1 in relation to any of the challenge tests. Plasma ET-1 did not correlate with airway reactivity in either methacholine or allergen challenge tests.

Sputum ET-1: Median (IQR) sputum ET-1 was higher than plasma ET-1 in each case. Median (IQR) sputum ET-1 was 11.0 (4.9-19.6) pg/ml after placebo challenge, 19.2 (5.6-25.4) pg/ml after methacholine, and 19.8 (11.7-32.3) pg/ml after allergen. Assessing change in sputum ET-1 following allergen compared with placebo, there was

a trend towards an increase after allergen (median change +5.1 pg/ml) which did not reach statistical significance (p=0.071) (figure 1). Sputum ET-1 did not correlate with airway reactivity in either methacholine or allergen challenge tests, but the change in sputum ET-1 from placebo to allergen samples correlated strongly with the eosinophil count of the placebo sample (r_S=0.67, p=0.025) (figure 7.2).

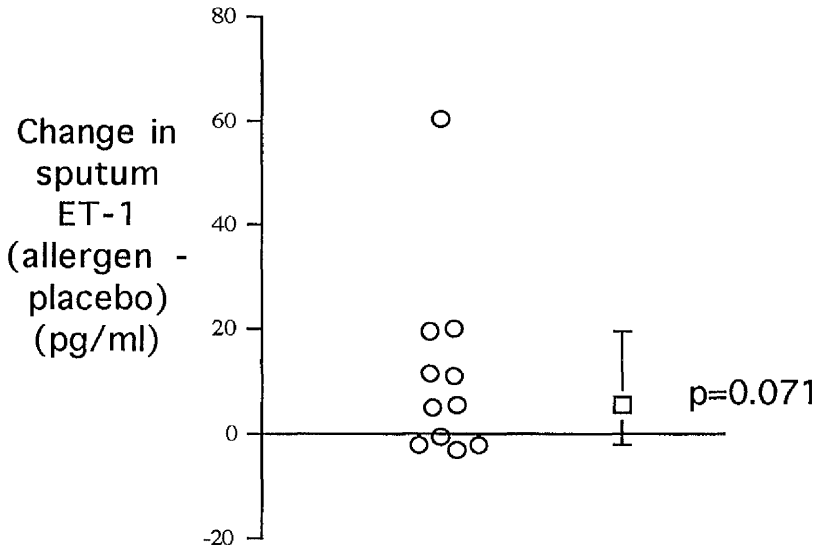

Figure 7.1
Change in sputum ET-1 (pg/ml) comparing allergen challenge with placebo challenge. Median (IQR) marked, with p value for comparison with "no change".

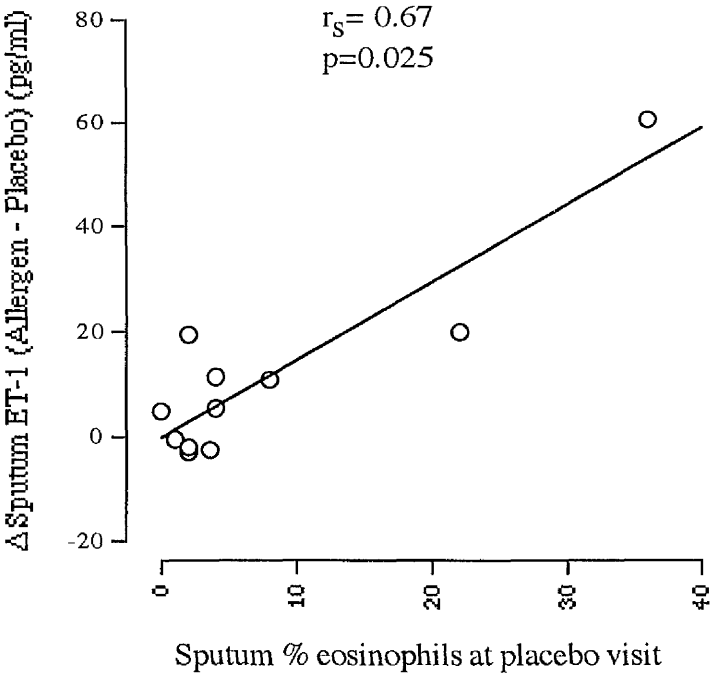

Figure 7.2
Relationship between sputum eosinophilia at placebo visit and the difference between sputum ET-1 (pg/ml) at allergen challenge and placebo challenge.

Table 7.1

Demographic data

No.	Age (years)	Sex	FEV1 (litres)	FEV1 (%) predicted	Treatment	Methacholine PC20 (mg/ml)	Allergen PC20 (BU)
1	37	F	2.62	93	S.	0.84	631
2	39	M	3.87	105	S. BDP1000	0.26	340
3	34	F	3.09	103	S.	6.21	233
4	34	M	3.05	83	S. BDP400	3.28	728
5	38	M	2.80	72	S. BDP800. Sm	0.62	747
6	47	F	2.23	88	S. BDP 400	6.86	272
7	31	M	3.55	85	S.	1.29	207
8	54	M	2.5	77	S. BDP1500	2.64	860
9	29	F	2.34	75	S.	0.33	562
10	47	F	2.14	80	S. Bud400	0.47	71
11	32	M	4.35	100	S. Sm BDP1600	1.81	130
12	34	F	2.68	98	S. Sm BDP500	5.70	197
						(geometric mean)	(geometric mean)
Mean	**38.0**	**6M 6F**	**2.93**	**88.3**	**825 µg daily**	**1.47**	**324**
(S.D.)	(7.6)		(0.7)	(11.4)	range	(0.26-6.86)	(71-860)

S. Salbutamol 200µg as required
BDP Beclomethasone dipropionate total daily dose in µg
Sm Salmeterol 100µg daily
Bud Budesonide total daily dose in µg

Table 7.2

Serum and sputum eosinophilic cationic protein (ECP) and differential sputum cell counts (expressed as percentage after exclusion of squamous epithelial cells).

	Serum ECP (µg/l)		Sputum ECP (µg/l)	macro-phages %	poly-morphs %	eosin-ophils %	lymph-ocytes %
	pre	post					
Placebo	**9.5** (6-10)	**10.0** (5-20)	**100** (67-149)	**53** (36-73)	**35** (8-60)	**4** (2-14)	**1** (1-2)
Methacholine	**5.5** (4-20)	**11.5** (5-40)	**125** (49-198)	**78** (27-85)	**18** (12-57)	**2** (0-8)	**1** (0-2)
Allergen	**8.5** (4-20)	**5.0** (4-11)	**125** (88-208)	**48** (44-68)	**41** (17-52)	**7** (2-13)	**0** (0-1)

7.4 DISCUSSION

Comparing allergen challenge with placebo challenge, there was a trend towards an increase in sputum ET-1 which approached significance, and a positive correlation between this change and sputum eosinophilia, suggesting that an increase in sputum ET-1 may occur in response to allergen challenge in those subjects with most marked airway inflammation. Methacholine challenge testing does not acutely alter ET-1 in plasma or sputum obtained by sputum induction.

Sputum induction is a relatively recent technique which allows bronchial secretions to be obtained non-invasively, and which allows repeated sampling of bronchial secretions, and which has been shown to be reliable and reproducible in the analysis of cells and soluble mediators in lung disease (220).

The role of ET-1 and ET-1 receptor blockade in antigen-induced airway responses have been examined in animal models, with evidence of a differential role for the two ET receptors, ET_A and ET_B. Rodent studies indicate that ET_A receptor antagonism results in a reduction in the airway cellular infiltration (103) and bronchoconstrictor response in the late allergic response, while the immediate bronchoconstrictor response was inhibited by ET_B antagonists (109). An increase in BAL ET-1 one day after experimental airway inflammation in rats has also been demonstrated, with a relationship between BAL ET-1 and total BAL cell count, BAL eosinophils and BAL neutrophils (110). In asthma, there is increased immunostaining for ET-1 in bronchial biopsies in stable asthmatics (122), and this has been shown to relate to the severity of airway inflammation and to bronchoconstrictor responses to distilled water (97). One study from Redington *et al* (130) has examined the influence of the bronchoscopic technique of segmental allergen challenge on ET-1 in BAL, and contrary to expectations found a reduction in BAL ET-1 10 minutes after challenge, compared with sham challenge with saline. It was speculated that allergen challenge may lead to increased proteolytic activity

with resultant breakdown of ET-1, although there is at present no direct evidence to support this. A similar finding has been observed in a study of BAL ET-1 in nocturnal asthmatics (127) in which a reduction in BAL ET-1 was observed during nocturnal symptoms, with similar speculations as to the mechanism. The influence on ET-1 production of the local anaesthetic used in bronchoscopy is not known, although there is potential for this to be a confounding factor in bronchoscopic studies, since bronchial epithelial cells are an important source of ET-1 in the airways. We did not find a reduction in sputum ET-1 following allergen challenge, and although not attaining statistical significance, there was a trend towards a rise in sputum ET-1. Our sampling point at 30 minutes was chosen to reflect the immediate allergic reaction, and was a little later than that of Redington *et al* (130), which would have represented the earliest phase of immediate allergic reaction. The time course of ET-1 generation in response to allergen challenge is not known, although it is known that ET-1 appears not to be stored in granules in cells, but produced by rapid induction of ET-1 messenger RNA (mRNA), with synthesis and secretion of ET-1 within minutes in response to certain stimuli (8).

In this study 8 of the 12 asthmatics were taking inhaled steroid therapy (mean daily dose 825 μg beclomethasone dipropionate or equivalent). It has been shown that treatment with steroids decreases bronchial epithelial expression of ET-1 in asthmatic patients (123), and although we did not observe such a difference comparing groups of asthmatics using induced sputum (291), it is possible that treatment could have an effect on the release of ET-1 in response to an appropriate stimulus. There was no clear difference between steroid-naive and steroid-treated subjects although the numbers are too small for formal analysis, and one steroid-naive subject did demonstrate a more pronounced rise in sputum ET-1 comparing allergen and placebo challenge.

As might be expected from our sampling time point, we found no immediate change in sputum total or differential cell counts or eosinophilic cationic protein (ECP). The immediate allergic response is primarily characterised by bronchoconstriction in the

absence of significant airway inflammation, while the late response is thought to be due in whole or in part to airway inflammation (292). Sputum induction has been shown to be useful in assessing airway inflammation in the late allergic response (148), but inflammatory cellular or mediator changes would not be expected in the immediate response. The absence of a rise in sputum ET-1 following methacholine challenge suggests that ET-1 is not involved in the bronchoconstrictor response to methacholine. This was primarily included as a "positive control" which would induce bronchoconstriction as opposed to the placebo challenge which was not bronchoconstrictor. On this evidence, ET-1 release is not associated with bronchoconstriction *per se*.

The relationship which we observed between sputum eosinophilia at baseline (taken from the placebo visit, since this did not involve an active airway challenge) and the change in sputum ET-1 following allergen challenge may suggest that those subjects with the most prominent airway inflammation respond to allergen challenge by increased ET-1 production or release. This is supported by *in vitro* data from a study by Endo et al (293) showing increased release of ET-1 from cultured epithelial cells following co-culture with eosinophils stimulated by IL-5 or GM-CSF, but not from unstimulated eosinophils. In additionET-1 synthesis by airway epithelial cells is upregulated by a number of proinflammatory cytokines (129), and as mentioned above, ET-1 is present in increased quantities in BAL from patients with symptomatic asthma (124). The immediate allergic reaction is accompanied by the release of histamine, prostaglandins and leukotrienes by activated mast cells (294), and both mast cells (295) and bronchial epithelial cells in some asthmatics (126) have the capacity to synthesise ET-1 in response to exposure to cross-linked IgE. There is insufficient information on the factors controlling ET-1 synthesis in the airways and their relationship to asthmatic airway inflammation, but it is possible to speculate that the influence of allergen on eosinophils, mast cells and possibly epithelial cells in the proinflammatory milieu of asthmatic airway inflammation might lead to increased ET-1 synthesis and release.

In conclusion, we have found that it is possible to use induced sputum to measure ET-1 following bronchial challenge tests, and that there is evidence for an increase in sputum ET-1 in the immediate allergic reaction at least in subjects with evidence of active asthmatic airway inflammation, and a trend towards an increase in the group as a whole. The influence of treatment on this response and the involvement of ET-1 in the late allergic reaction remain to be assessed.

CHAPTER 8

THE IMPACT OF SMOKING AND CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) ON SPUTUM ENDOTHELIN-1

a) smokers with and without airflow limitation
b) acute exacerbation of COPD

8.1 INTRODUCTION

The actions of ET-1 in the airway which prompted our investigations in asthma may be of relevance to diseases other than asthma, where bronchoconstriction, airway inflammation and airway remodelling are known to be a feature. Chronic obstructive pulmonary disease (COPD) is one such condition, with cigarette smoking as an obvious aetiological factor, and a number of the known properties of ET-1 could contribute to pathogenesis in COPD (figure 8.1). Like asthma, airflow obstruction is present in COPD, but unlike asthma this is not generally reversible by inhaled β-2 agonists. Similarly, airway inflammation is a feature of COPD, but whereas in asthma this is commonly (although not invariably) associated with a degree of airway eosinophilia, in COPD neutrophilia is predominant (180). There are no reports of airway production of ET-1 in COPD, and we wished to examine sputum levels of ET-1 in COPD, and the influence of exacerbation of COPD on sputum ET-1 levels. This chapter is therefore a combination of 2 studies, one baseline study examining sputum ET-1 levels in COPD compared with healthy smokers (baseline study), and the second a study examining sputum ET-1 in acute exacerbation of COPD and in convalescence (acute study).

Modest increases in plasma ET-1 have been reported following acute cigarette exposure in healthy smokers (296), but a pathological study did not find increased immunoreactive ET-1 staining in bronchial epithelial cells from smokers (297). There are however no reports of baseline levels of induced sputum ET-1 in smokers, so we chose to compare healthy smokers with smokers with COPD.

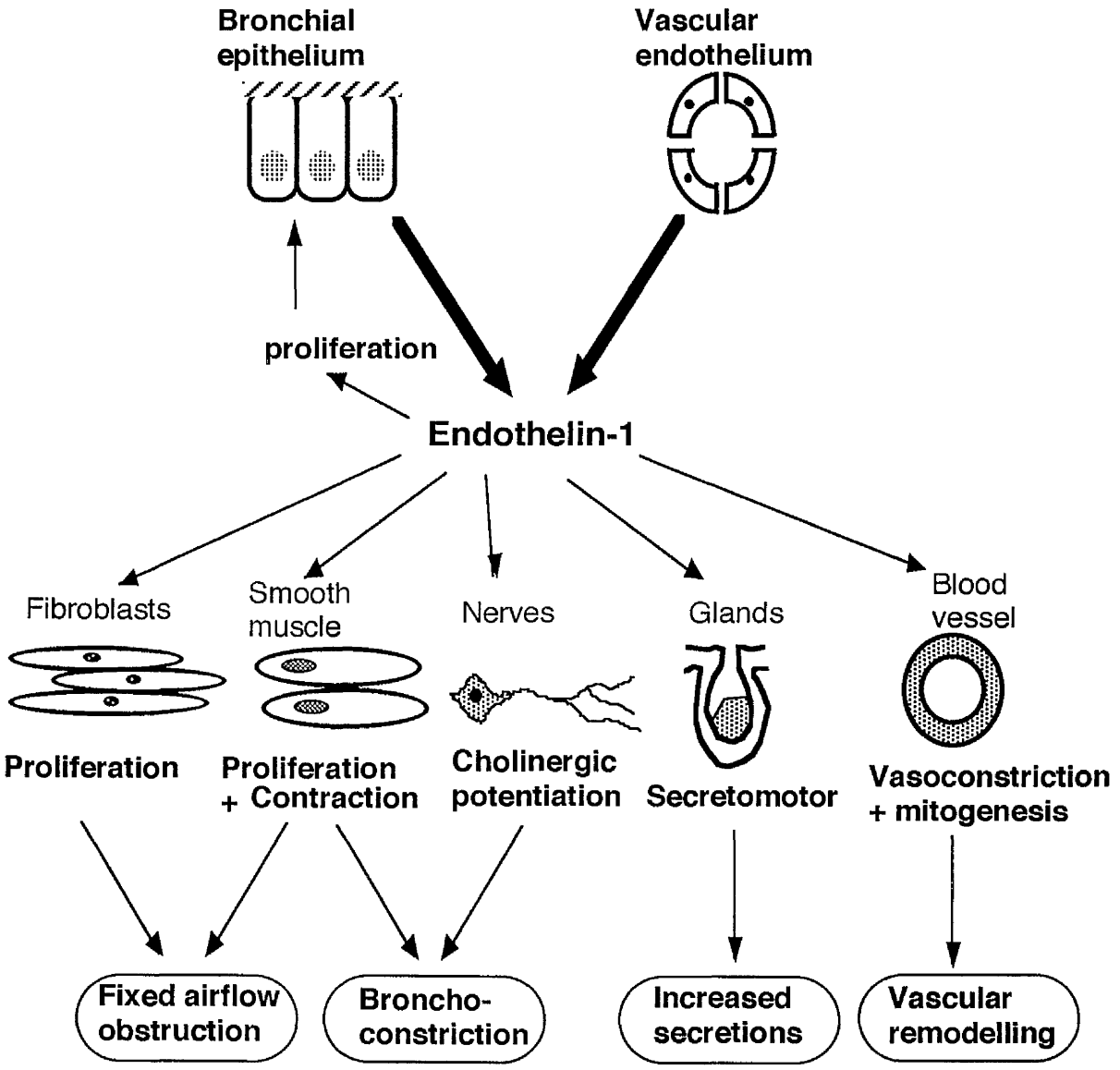

Figure 8.1
Potential actions of endothelin-1 in COPD

8.2 METHODS

Subjects

Baseline Study

10 healthy smokers (3 male, 7 female) without symptoms of lung disease and 9 smokers with COPD (2 male, 7 female) were studied. Smokers were asymptomatic and COPD patients had stable symptoms at the time of the study, and none had suffered a respiratory infection, or had received antibiotics or oral corticosteroids in the month preceding the study visits. Smokers had no history of wheeze or shortness of breath, and baseline lung spirometry was within normal limits (taken as greater than or equal to 70% of predicted FEV_1 in the absence of symptoms). The study was approved by the West Ethics Committee, West Glasgow Hospitals University NHS Trust, and each subject gave written informed consent.

Acute study

10 subjects with established COPD (6M, 4F) were recruited following admission to our unit with a stated diagnosis of an acute exacerbation of pre-existent COPD (5 current smokers and 5 ex-smokers). Entry criteria were established for an age group of 45 - 85 years, with a known history of COPD (GP or hospital diagnosis), and a smoking history. At the time of presentation, subjects were required to have 3 or more of the following 5 criteria to suggest an acute exacerbation - increased cough, increase in sputum production, increased dyspnoea, fever (>38°C), and new chest X ray abnormalities. The study was approved by the West Ethics Committee, West Glasgow Hospitals University NHS Trust, and each subject gave written informed consent.

Protocol

Baseline study

The study involved a single visit to the laboratory for blood sampling, salivary sampling and sputum induction, with spirometry monitored throughout the induced sputum

procedure in the usual way. All subjects were pre-treated with salbutamol 200μg prior to sputum induction.

Acute study

Spontaneous sputum samples were obtained within 24 hours of admission to hospital in all cases (in most cases within 12 hours), and blood was sampled concurrently. Arterial blood gases were sampled as part of clinical management. Patients were managed according to clinical requirements (in all cases oral steroid therapy was started or increased, and antibiotics given), and a review visit was arranged for roughly 6 weeks after admission, or once symptoms had been stable for 3 weeks thereafter. At the review visit, spirometry and pulse oximetry were measured, blood sampled, and induced sputum performed following pretreatment with salbutamol 200μg, with spirometry monitored through the procedure.

Sputum induction

This was performed as described in chapter 2.3. Additionally, in the baseline study saliva samples were obtained prior to sputum induction to measure salivary levels of ET-1.

Laboratory Processing

Sputum was processed as described in chapter 2.3, with the exception mentioned under biochemical assays, with the laboratory staff unaware of the clinical details relating to the sample.

Biochemical Assays

Endothelin was assayed as described in chapter 2.4, but in the acute study, due to a failed assay on the first attempt, sputum ET-1 was not able to be measured on sputum which had not been treated with the reducing agent dithiothreitol (DTT), and this was repeated on DTT-treated samples, with the result that the results are not directly

comparable between the baseline and acute studies. Assays were run in duplicate with the mean value used for analysis.

Statistical Analysis

Results are expressed as median and inter quartile range unless otherwise specified. Non-parametric statistics were used to compare cell counts and ET-1 values (Mann-Whitney U test), and parametric statistics used to compare demographic factors. Significance was accepted at the 95% level.

8.3 RESULTS - BASELINE STUDY

Demographic factors and lung function

Healthy smokers were younger on average than subjects with COPD (Mean (SD) 39.8 (9.0) years vs. 59.3 (7.4) years respectively), and had better lung function, whether expressed as mean (SD) absolute FEV_1 (2.63 (0.5) litres vs. 0.66 (0.1) litres respectively) or % predicted FEV_1 (89.1 (11.8) % vs. 26.9 (6.6) % respectively). Smoking history was greater for COPD patients (mean (SD) 50.9 (17.8) pack years) than for healthy smokers (mean (SD) 23.9 (16.2) pack years). Demographic data is presented in table 8.1.

Endothelin-1

Plasma ET-1

Plasma ET-1 was not different between healthy smokers and COPD patients (median (IQR) 4.35 (3.5-6.3) pg/ml vs. 3.3 (2.7-4.2) pg/ml respectively), but was greater in healthy smokers than that in healthy non-smokers (n=17) (median (IQR) 3.1 (1.7-4.4) pg/ml, p=0.042) (data for healthy non-smokers presented in chapter 9).

Sputum ET-1

As with plasma ET-1 there was no difference between healthy smokers and COPD subjects (median (IQR) 19.2 (15.3-25.6) pg/ml vs. 16.4 (6.8-38.2) pg/ml), but sputum ET-1 in both healthy smokers and COPD were greater than that observed in healthy non-smokers (n=17) (median (IQR) 6.0 (2.8-14.8) pg/ml, p=0.004 and p=0.04 respectively) (data for non-smokers presented in chapter 9) (figure 8.2). There was no significant relationship between sputum ET-1 and lung function in either healthy smokers or COPD subjects (Spearman rank correlation r_s=0.1 and 0.36 respectively, p>0.05) (Appendix 8.1).

Saliva ET-1

Median (IQR) salivary ET-1 was 29.9 (22.1-38.4) pg/ml for healthy smokers and 20.7 (16.3-42.3) pg/ml for COPD subjects, which were not significantly different, nor were either different from values observed in healthy non-smokers (median (IQR) 28.8 (18.3-34.5) pg/ml) (data for healthy non-smokers presented in chapter 9).

Sputum cell counts

Sputum cell counts are summarised in table 8.2. Total cell counts were higher in COPD (median (IQR) 12.4 (4.9-23.0) $x10^6$ cells/ml) than in healthy smokers (median (IQR) 3.7 (2.7-9.9) $x10^6$ cells/ml). There was sputum neutrophilia in COPD compared to healthy smokers, both in absolute cell counts (median (IQR) 9.7 (3.9-16.4) $x10^6$ cells/ml vs. 1.6 (0.5-5.4) $x10^6$ cells/ml respectively) and in relative cell counts (median (IQR) 78 (74-80) % vs. 38 (19-56) % respectively), with a consequent reduction in macrophage percentage (median (IQR) 2.6 (1.0-3.6) $x10^6$ cells/ml: 21 (18-24) %) in COPD compared to healthy smokers (median (IQR) 2.1 (1.3-3.3) $x10^6$ cells/ml: 53 (42-80) %). Absolute eosinophil numbers were higher in COPD (median (IQR) 0.15 (0-0.3) $x10^6$ cells/ml: 1 (1-2)%) than in healthy smokers (0 (0-0.04) $x10^6$ cells/ml: 0 (0-1) %). There was no difference in sputum lymphocyte numbers between the groups. Comparing these results with normal subjects presented in chapter 9 revealed no differences in sputum total and differential cell counts between normal subjects and healthy smokers, but a significant neutrophilia in the COPD group compared to normals (median (IQR) 0.81 (0.31-3.57) $x10^6$ cells/ml: 24 (9-40) %), and increased sputum eosinophils in COPD compared to normal subjects (median (IQR) 0 (0-0) $x10^6$ cells/ml: 0(0-0) %).

Table 8.1
Baseline study - Demographic factors, lung function and pulmonary therapy

No.	Age (years)	Sex	FEV_1 (litres)	FEV_1 % predicted	Cigarette pack years	Pulmonary therapy
Healthy smokers						
1	50	F	2.14	85	30	none
2	45	F	2.51	113	30	none
3	38	F	2.00	70	20	none
4	48	M	3.32	92	35	none
5	44	M	2.12	74	60	none
6	27	F	2.67	88	14	none
7	24	F	3.04	93	5	none
8	48	M	3.20	88	20	none
9	39	F	2.52	89	20	none
10	35	F	2.77	96	5	none
Mean	**39.8**	**3M**	**2.63**	**89.1**	**23.9**	
(S.D.)	(8.97)	**7F**	(0.5)	(11.8)	(16.2)	
COPD						
1	58	F	0.65	31	80	S: Sm: BDP 1000
2	64	F	0.53	25	60	S
3	56	F	0.74	30	40	S: Ipr : BDP 1000: Th
4	56	F	0.78	30	45	S: BDP 800
5	57	F	0.64	26	40	S: Sm: BDP 1000
6	67	F	0.83	34	50	S: BDP 800
7	73	M	0.42	16	50	S: Sm: BDP 1000
8	49	F	0.75	33	30	S: BDP 800
9	54	M	0.60	17	60	S: Sm
Mean	**59.3***	**2M**	**0.66†**	**26.9†**	**50.6***	
(S.D.)	(7.4)	**7F**	(0.1)	(6.6)	(14.7)	

key
* significantly greater than healthy smokers
† significantly less than healthy smokers
Ipr Ipratropium bromide 2mg daily nebulised
S Inhaled salbutamol 200µg prn
Sm Salmeterol 100µg b.d.
Th Theophylline 500mg daily
BDP Beclomethasone dipropionate daily dose in µg

Table 8.2

Baseline study - Sputum cell counts in healthy smokers and stable COPD (median (IQR))

	Total cell count	Macrophages	Neutrophils	Eosinophils	Lymphocytes
Healthy smokers					
cells x10^6/ml	12.4 (4.9-23.0)	2.09 (1.26-3.3)	1.61 (0.46-5.4)	0 (0-0.04)	0 (0-0.49)
percent		53.0 (42-80)	37.5 (19-56)	0 (0-1)	0 (0-4)
Stable COPD					
cells x10^6/ml	12.4 (4.9-23.0)	2.6 (1.0-3.6)	9.7 (3.9-16.4)*/**	0.15 (0.08-0.26)*/**	0.05 (0-0.10)
percent		21.0 (18-24)†/††	78.0 (74-80)*/**	1.0 (1-2)*	1.0 (01)
Healthy non-smokers					
(data from chapter 9)					
cells x10^6/ml	6.9 (2.4-11.3)	5.8 (1.5-7.3)	0.8 (0.3-3.6)	0.0 (0-0)	0.03 (0-0.2)
percent		81 (65-90)	19.0 (6-35)	0 (0-0)	2 (0-4)

Key
* greater than healthy non-smokers
*/** greater than healthy smokers
† lower than healthy non-smokers
†/†† lower than healthy smokers

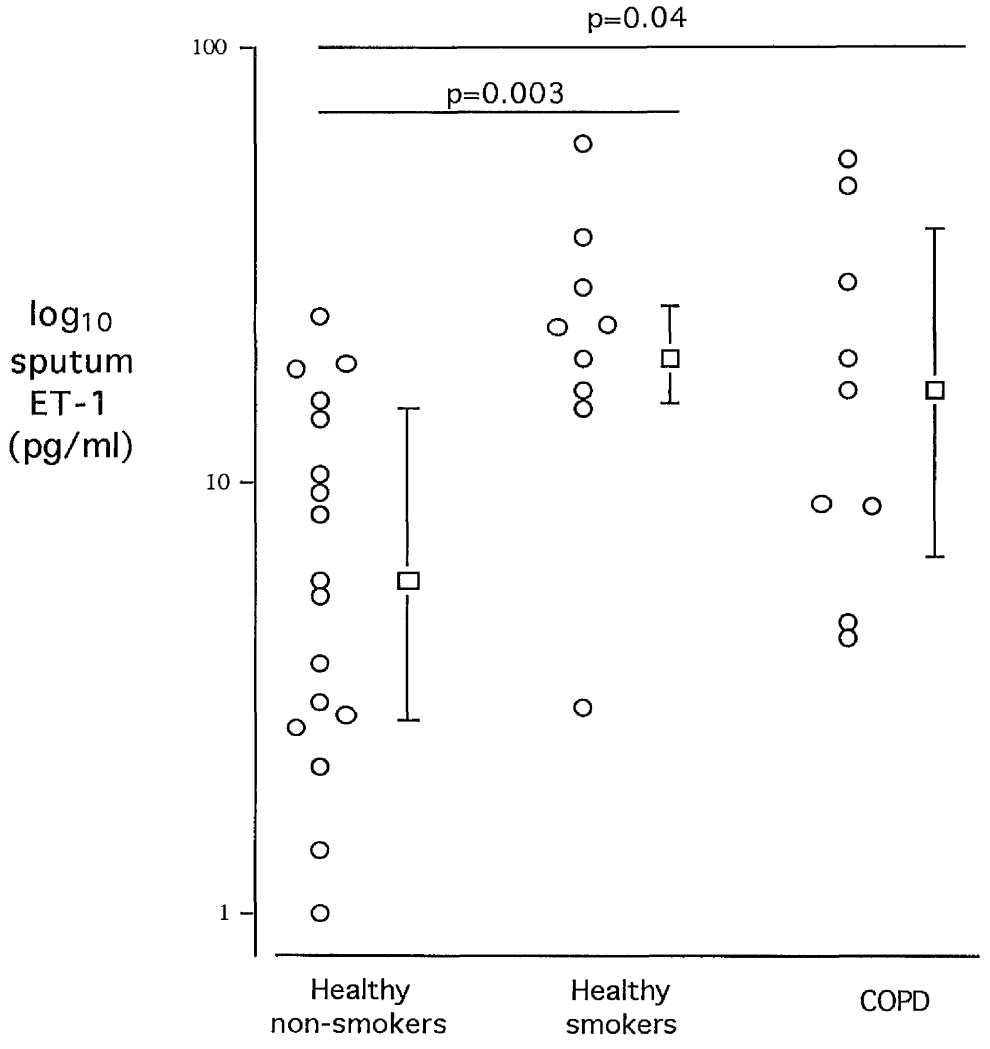

Figure 8.2
Sputum ET-1 in healthy non-smokers (data presented formally in chapter 9), healthy smokers and subjects with stable COPD (baseline study)

8.4 RESULTS - ACUTE STUDY

Demographic factors, lung function and blood gas analysis

The mean (SD) age of the patients was 68.3 (9) years, with a mean (SD) smoking history of 45 (24) pack years. Mean (SD) FEV_1 in convalescence was 0.77 (0.4) litres (29.0 (12)% predicted). Mean (SD) arterial p02 was 9.0 (1.9) kPa (the majority of arterial blood gases were taken with the patient receiving supplementary oxygen) and mean (SD) arterial pC02 was 6.9 (2.1) kPa. Demographic data is presented in table 8.3.

Endothelin-1

Plasma ET-1 was not different comparing acute with convalescent visits (median (IQR) 5.5 (5.4-6.8) pg/ml vs. 5.2 (3.9-6.5) pg/ml. There was a mean reduction of 5.0 pg/ml in sputum ET-1 comparing convalescent with acute samples, but this was not significant (median (IQR) sputum ET-1 in exacerbation was 1.8 (0.6-12.4) pg/ml, compared with 1.0 (0-2.0) in convalescence). In 4 patients there was a clear reduction in sputum ET-1 from acute exacerbation to convalescence (figure 8.3), but there was no pattern of smoking history, sputum cell counts, lung function, co-morbidity or the presence or absence of arterial hypoxaemia which appeared to distinguish these patients. Comparing smoking and ex-smoking patients with COPD there were no differences in sputum ET-1 either in exacerbation or convalescence (Appendix 8.2).

Sputum cell counts

Total cell counts were not different comparing acute exacerbation with convalescence (median (IQR) 14.7 (9-42) $x10^6$/ml vs. 11.1 (8-23) $x10^6$/ml respectively). As would be expected in COPD, there was a significant sputum neutrophilia (median (IQR) sputum neutrophils 12.1 (5-36) $x10^6$/ml: 81.5 (73-90)% in acute exacerbation) with no significant reduction in neutrophilia in convalescence (median (IQR) 9.1 (6-19) $x10^6$/ml: 76.5 (69-82)%). There were no differences in sputum macrophages, eosinophils or lymphocytes comparing acute and convalescent samples (table 8.4).

Table 8.3

Demographic data, lung function and arterial blood gases (acute study)

Patient no.	Age (years)	sex	FEV_1 (litres)	FEV_1%	PaO_2 (kPa) (on admission)	$PaCO_2$ (kPa) (on admission)	Smoking pack years	Current /ex-smoker (years)
1	78	F	0.39	22	8.49	5.91	55	current
2	63	M	0.47	15	6.61	9.31	25	current
3	62	F	0.68	35	5.76	10.03	30	ex (2)
4	66	M	0.73	26	11.21	6.39	35	ex (1)
5	72	M	1.15	42	9.30	5.32	90	current
6	70	F	0.91	42	9.70	3.80	25	ex (12)
7	64	F	0.35	17	7.75	6.82	80	ex (10)
8	71	M	0.65	18	9.73	9.73	25	current
9	52	M	1.46	46	11.7	5.60	30	current
10	85	M	1.02	40	9.75	5.73	55	ex (25)
mean	**68.3**	**6M, 4F**	**0.78**	**30**	**9.00**	**6.86**	**45**	
(SD)	(9.2)		(0.36)	(12)	(1.88)	(2.10)	(24)	

Table 8.4

Acute study - Acute and convalescent sputum cell counts in COPD (median (IQR))

	Total cell count	Macrophages	Neutrophils	Eosinophils	Lymphocytes
Acute					
cells x10^6/ml	14.7 (8.6-42.0)	2.35 (1.2-6.4)	12.1 (5.1-36.1)	0.10 (0-0.3)	0 (0-0)
percent		17.5 (10-32)	81.5 (73-90)	1 (0-2)	0 (0-0)
Convalescent					
cells x10^6/ml	11.1 (8.1-23.2)	2.42 (1.6-3.6)	9.09 (5.9-18.8)	0.03 (0-0.2)	0 (0-0)
percent		22.0 (18-32)	76.5 (69-82)	0.5 (0-1.3)	0 (0-0)

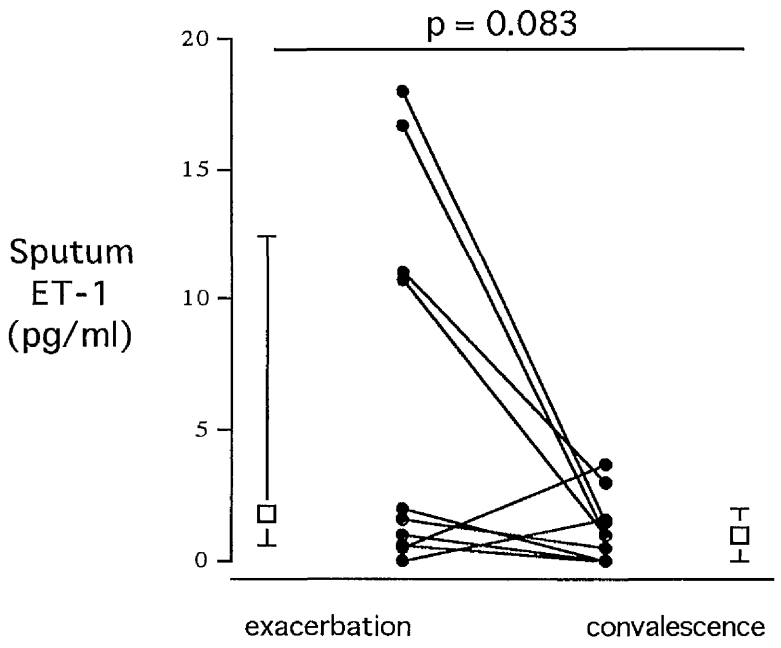

Figure 8.3
Sputum ET-1 in exacerbation and convalescence in COPD

8.5 DISCUSSION

We found no difference in plasma, sputum or salivary ET-1 levels comparing healthy smokers with smokers with established COPD. Both groups of smokers however had higher plasma and sputum ET-1 than healthy non-smoking controls, suggesting that cigarette smoke may result in increased ET-1 production. Sputum ET-1 levels were higher than plasma levels in both groups, suggesting local production of ET-1 within the respiratory tract. In the acute study, although some individuals had a reduction in sputum ET-1 from acute exacerbation to convalescence, there was no significant overall difference in sputum ET-1 comparing acute and convalescent samples. Methodological differences in sputum processing resulting from the loss of sputum samples in a failed assay mean that comparison between the studies is not possible.

The available data on the impact of cigarette smoke on ET-1 production is neither complete nor entirely consistent. An animal study (abstract only) has shown an acute dose-dependent increase in plasma ET-1 following experimental exposure to cigarette smoke in rats, which is sustained for several days after a single smoke exposure (298). In humans the effect is less pronounced, with a very modest rise in plasma ET-1 after acute smoke exposure, which is short-lived (around 10 minutes), related in magnitude to the "strength" (high tar vs. low tar) of the cigarette (296), and not related to nicotine *per se* , since the topical administration of nicotine by a transdermal nicotine patch did not increase plasma ET-1 in non-smokers (299). In contrast, although bronchial epithelial cells are known to produce ET-1, a study by Shokeir *et al* (297) examining samples from lobectomy/pneumonectomy specimens found no increase in ET-1 immunoreactivity comparing bronchial epithelial cells from smoking and non-smoking subjects. The same study examined a small number of samples from smokers with evidence of bronchial hyperreactivity to methacholine prior to surgery, and similarly found no increased immunostaining for ET-1 in this group. It is difficult however, to relate this pathological finding directly to the *in vivo* situation, since immunostaining is

not quantitative for ET-1 production, and it is possible that the determinant of airway ET-1 levels is not the number of cells which are positive on staining, but the amount of ET-1 which they produce. Additionally, the majority of samples used in the Shokeir's study were from patients with lung tumours, and an effect on ET-1 production at distant sites in the lung cannot be excluded, since increased ET-1 immunostaining has been reported in airway epithelium adjacent to tumours (300). In the acute study described in this chapter we did not find any difference in sputum ET-1 between current smokers or ex-smokers with COPD, suggesting that the effect of smoking on ET-1 production persists regardless of continuing smoking, at least in established COPD. More work is needed to establish the effect of smoking on ET-1 production, and the mechanisms by which it has any effect. We are not aware of any studies examining airway production of ET-1 in COPD, either by sputum induction or by bronchoalveolar lavage.

Cigarette smoke is a complex mixture of over 4,000 chemical compounds, which has the capacity to damage the bronchi in a number of ways including direct toxicity to the bronchial epithelium, oxidative damage, recruitment of inflammatory cells and increased epithelial permeability (301). Cigarette smoking results in airway inflammation, with cigarette-dose related increases in BAL neutrophils and macrophages, along with a number of pro-inflammatory cytokines including IL-8 (286), and in smokers with airway limitation, the extent of the airway limitation has been shown to be related to the extent of the airway inflammation (302), and to the smoking history (180). We found a significant sputum neutrophilia in both stable and acute COPD groups, although in these studies the extent of the neutrophilia did not correlate significantly with their lung function. While there is no evidence that neutrophils can synthesise ET-1 *per se*, neutrophils may be able to influence local levels of ET-1, since they can convert the ET-1 precursor big-ET-1 to the more biologically active ET-1 (303). Bronchial epithelial cells (52), neuroendocrine cells and human macrophages (48), can all synthesise ET-1 *de novo*, and the presence of increased neutrophil numbers and activity could increase available ET-1 by increasing conversion from big-ET-1 to ET-1.

In the acute study we did not observe any reduction in neutrophil numbers or proportion in response to treatment with antibiotics and oral corticosteroids, similar to a report of an elective trial of oral corticosteroids in COPD, where induced sputum eosinophilia was reduced if present, but no change was observed in neutrophils (304). Other studies in COPD have observed similar levels of sputum neutrophilia (180), and it has also been demonstrated that the cellular profile of sputum in COPD is not different comparing induced and spontaneous sputum samples (224). The studies described in this chapter include both spontaneous and induced sputum samples, and we did not observe differences in cell counts between these samples. Although sputum induction has been shown to be safe in severe asthma (218), bronchoconstriction can occur, and in the setting of an acute exacerbation of COPD with severe impairment of lung function we chose to use spontaneous sputum samples rather than induced.

The study examining sputum ET-1 in acute exacerbation of COPD did not demonstrate increased ET-1 in all patients, although a subgroup of patients did demonstrate a fall following treatment and recovery. One possible confounding factor in this is the effects of corticosteroids, either inhaled or orally administered, since corticosteroids are known to inhibit ET-1 production *in vitro* (47), and to diminish ET-1 in BAL (124) and ET immunoreactivity in bronchial biopsies (123) in asthma. Sputum samples in the acute study were obtained after oral corticosteroids had been started, albeit within around 12 hours in most cases, but since ET-1 is produced by rapid induction of ET-1 messenger RNA, (8), and is extensively cleared by the lungs during first passage (9), the effect of oral corticosteroids may have diminished airway expression of ET-1 in the airway prior to sampling.

The potential for interaction between ET-1 and nitric oxide (NO) also merits consideration in the context of cigarette smoking. Exhaled NO is reduced in smokers (305), but the mechanism for this is not yet clear. Cigarette smoke itself contains high

concentrations of NO, which may inhibit NO synthase (306), and smoke may inhibit NO synthase gene expression (307). Similarly, ET-1 can inhibit the expression of inducible NO synthase and NO release in rat bronchial epithelium (264), but it is also postulated that the bronchoconstrictor effects of ET-1 may be balanced to some degree by bronchodilator effects of NO (308), and there is animal evidence for NO release in response to ET_A receptor activation in the airway (309). In the vascular system, similar reduction of NO production has been observed in smokers, with enhanced vasoconstriction to exogenously administered ET-1 following acute smoking in long-term smokers (310). If the balance between ET-1 and NO is important in the maintenance of airway tone, the effects of cigarette smoke in reducing NO release while increasing ET-1 release could lead to bronchoconstriction, even at normal or modestly elevated levels of ET-1.

The observation of increased sputum ET-1 in cigarette smokers with or without airflow limitation requires further investigation, since the mechanism of the increase in ET-1 is not known. There is potential for interaction between ET-1 and NO, with possible potentiation of the bronchoconstrictor effects of ET-1. Exacerbation of COPD does not appear to be consistently associated with acute elevation of sputum ET-1, although this is seen in some patients, and may be influenced by concurrent administration of corticosteroids.

CHAPTER 9

SPUTUM ENDOTHELIN-1 IN CYSTIC FIBROSIS

9.1 INTRODUCTION

As discussed in previous chapters, the properties of ET-1 in the lung include bronchoconstriction, mitogenesis and pro-inflammatory activity, with resultant speculation about a potential role for ET-1 in airway diseases. The finding of a modest elevation of ET-1 in chronic obstructive pulmonary disease (COPD) has been described in chapter 9, and we speculated also that ET-1 might have a pathogenic role in cystic fibrosis, another condition which affects the airways. Cystic fibrosis (CF) is characterised by recurrent infection leading to the development of bronchiectasis, and by peribronchial and peribronchiolar fibrosis leading to airway remodelling with airflow obstruction. Like COPD, sputum neutrophilia is characteristic. While most patients with CF will over the course of their disease demonstrate a variable degree of reversibility to bronchodilators (311), progression to fixed airflow obstruction with associated secondary fibrosis is expected. Cell proliferation rates may be very high in CF airways (312), although the growth factors which may be associated with cellular proliferation in CF remain to be fully characterised.

The objectives of the study were to establish whether ET-1 could be measured in the sputum of CF patients, to compare the levels of ET-1 in sputum, saliva and plasma, and to compare levels of ET-1 between CF patients and normal subjects.

9.2 METHODS

Subjects

12 CF patients (7M, 5F) attending the West of Scotland Adult Cystic Fibrosis Unit were studied, in comparison with 17 healthy volunteers (10M, 7F) (table 9.1). None of the CF patients or healthy subjects were smokers. Informed written consent was obtained from all subjects, and the study was approved by the West Ethics Committee, West Glasgow Hospitals University NHS Trust.

Protocol

The study involved a single visit to the unit for consent and to obtain the samples of blood, sputum and saliva. Spontaneous sputum samples were obtained from the CF patients, and sputum induction using 3% saline was performed on the normal subjects.

Laboratory Processing

Sputum plugs were processed as outlined in chapter 2.3, with the exception that samples from CF patients were incubated for 10 minutes with recombinant DNAse (50μg/ml) (Genentech Inc., San Francisco, CA, USA) to reduce the viscosity of CF sputum due to its high DNA content (DNAse did not alter the ET-1 standard curve). Differential cell counts (Giemsa stained) are expressed after exclusion of squamous cells, which are taken to represent salivary contamination. The numbers of bronchial epithelial cells were in general very low (<2%) and these are excluded from cell counts. Endothelin-1 was assayed as described in chapter 2.4.

Statistical Analysis

Results are expressed as median and inter-quartile range unless otherwise specified. Non-parametric statistics were used to compare cell counts, ECP and ET-1 values (Mann-Whitney U test) using a software package (Minitab Statistical Software, Minitab

Inc, State College, PA, U.S.A.) on an Apple Macintosh desktop computer (Apple Computer Inc. Cupertino, CA, U.S.A.). Significance was accepted at the 95% level.

9.3 RESULTS

Patient Demographics

Comparison of demographic factors reveals that the CF patients were younger on average than the normal subjects, and CF patients had lower baseline spirometry than normals, with a mean (S.D.) FEV_1 of 44.2 (22)% predicted and 98.8 (9.66)% predicted respectively (table 9.1).

Sputum Cell Counts

Absolute and relative sputum cell counts are expressed after exclusion of squamous and bronchial epithelial cells and summarised in table 9.2. Absolute and relative sputum neutrophilia is present in both CF compared with normal subjects, with CF neutrophil counts exceeding those described in chapter 8 for COPD ($p<0.05$). There is a corresponding reduction in relative macrophage numbers in CF compared with normal subjects.

Endothelin-1

Assay

Recovery of ET-1 from sputum samples averaged 68% (n=2), and coefficient of variation for the ET-1 assay in sputum was 11.4%. DNAse had no effect on the ET-1 standard curve.

Plasma and saliva ET-1

Levels of ET-1 were examined in the plasma of all normal subjects and 10 patients with CF, and in saliva from 10 CF patients and a sub-group of 8 normal subjects (table 9.3). Plasma levels were mildly elevated in CF compared to normals (median (IQR) 5.3 (3.2-6.0) pg/ml vs 3.1 (1.7-4.4) pg/ml).There was no difference in median (IQR) salivary ET-1 between CF (21.0 (10.9-32.3) pg/ml) and normals (28.8 (18.3-34.5) pg/ml) although in both groups salivary ET-1 is greater than plasma ET-1.

Sputum ET-1

Sputum ET-1 was measured in all subjects, and was markedly elevated in CF compared to normals (median (IQR) 77.6 (29.0-122.8) pg/ml vs. 6.0 (2.8-14.8) pg/ml respectively) (figure 9.1). We found no direct correlation between the sputum ET-1 and FEV_1 in CF, nor between plasma and sputum ET-1. In CF sputum ET-1 is greater than in saliva, whereas in normals the converse is true, reflecting differences in sputum ET-1 rather than saliva ET-1.

Table 9.1
Demographic Data, Lung Function and Pulmonary Therapy (Summary data for normal volunteers)

No.	Age (years)	Sex	FEV_1 (litres)	FEV_1 % predicted	Cigarette pack years	Pulmonary therapy
Normal Volunteers (n=17)						
Mean	**31.8**	**10M**	**3.9**	**99.2**	none	none
(S.D.)	(8.8)	**7F**	(1.1)	(11.4)		
Cystic Fibrosis						
1	27	F	0.60	20	none	Ipr neb: Pred 20: DNAse
2	28	M	0.74	19	none	DNAse
3	22	M	0.99	24	none	DNAse
4	16	M	2.30	59	none	none
5	16	M	2.64	74	none	S: Bud 1200
6	23	F	1.70	54	none	none
7	36	M	0.70	21	none	S: BDP 1000: Ox
8	16	F	0.95	38	none	Terb.
9	17	F	2.05	64	none	S neb: Vol 16
10	18	F	1.50	53	none	Terb: Bud1600: Sm: DNAse
11	21	M	1.05	25	none	S: BDP 1000
12	19	M	2.92	79	none	Terb
Mean	**21.6***	**7M**	**1.5***	**44***		
(S.D.)	(6.1)	**5F**	(0.8)	(22)		

key * significantly lower than normals ($p<0.01$) † significantly greater than normals ($p<0.01$)

Ipr Ipratropium bromide 2mg daily nebulised
Pred Prednisolone daily dose in mg
S Inhaled salbutamol 200µg prn
Bud Budesonide total daily dose in µg
BDP Beclomethasone dipropionate daily dose in µg
DNAse recombinant human DNAse
Ox Oxitropium bromide 200µg tid
Vol Tablet salbutamol daily dose in mg
Sm Salmeterol 100µg b.d.
Terb Terbutaline 0.5mg prn

Table 9.2

Absolute and relative median (IQR) sputum cell counts for normal subjects and subjects with cystic fibrosis. Values expressed after exclusion of squamous and bronchial epithelial cells.

	Normals	Cystic fibrosis
absolute cell counts ($x10^6$/ml)		
total count	6.9 (2.4-11.3)	35.2 (16.0-53.6)*
neutrophil	0.8 (0.3-3.6)	30.8 (14.7-52.2)*
macrophage	5.8 (1.5-7.3)	2.1 (0.9-6.3)
eosinophil	0.0 (0.0-0.0)	0.0 (0.0-0.7)
lymphocyte	0.03 (0.0-0.2)	0.0 (0.0-0.3)
relative cell counts (%)		
neutrophil	19.0 (6-35)	92.0 (88-95)*
macrophage	81 (65-90)	6.5 (5-10)†
eosinophil	0 (0-0)	0.0 (0-1)
lymphocyte	2 (0-4)	0.0 (0-0.3)

key - * greater than normals † less than normals ($p<0.05$)

Table 9.3
Endothelin-1 in Sputum, Saliva and Plasma (median (IQR))

	Sputum	Saliva	Plasma
Normal	**6.0** (2.8-14.8)	**28.8** (18.3-34.5)	**3.1** (1.7-4.4)
Cystic Fibrosis	**93.6*** (59.6-142.6)	**21.0** (10.9-32.9)	**5.3*** (3.2-6.0)

key * significantly greater than normals

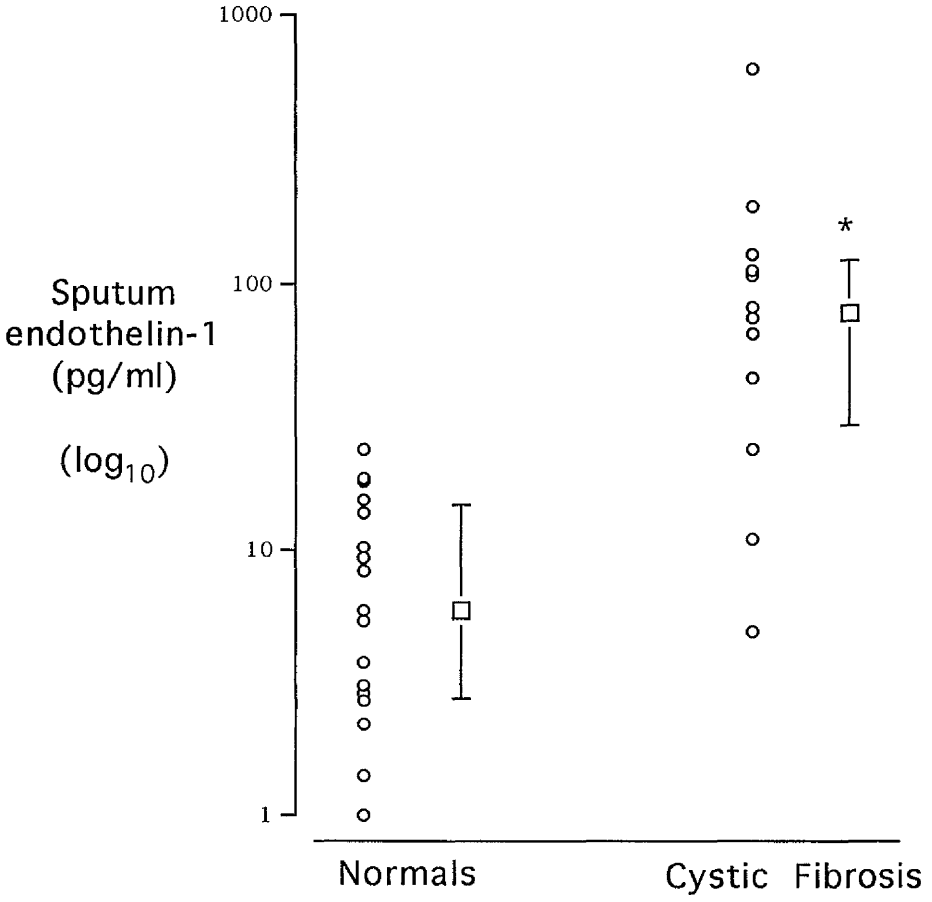

Figure 9.1
Sputum ET-1 in normal subjects and subjects with cystic fibrosis
* $p<0.05$

9.4 DISCUSSION

We have demonstrated for the first time that it is possible to measure ET-1 in the sputum of patients with CF, and that sputum levels of ET-1 are significantly elevated in CF compared with normal subjects. Plasma ET-1 is mildly elevated in CF compared to normals, but much less so than sputum ET-1. Sputum induction allowed us to obtain sputum from normal subjects for comparison. Our CF patient group was sampled during a period of relative stability of symptoms, but although they had a wide range of impairment of lung function (FEV_1 19-79% predicted), none had normal lung function, and as might be expected from their average age they represent a group with established disease. Differential cell counts show a prominent sputum neutrophilia in CF.

The presence of ET-1 in sputum and saliva at higher concentration than plasma in both groups suggests that ET-1 is produced or released locally within the respiratory tract, as demonstrated in other chapters for other patient groups, with an increase in production or release in the lower respiratory tract in CF compared to normal subjects. The factors leading to this increase in ET-1 in CF are not known. ET-1 is present in normal lung, and ET-1 immunoreactivity has been demonstrated in airway epithelia and submucosal glands throughout the lung (56) as well as in airway smooth muscle and parasympathetic ganglia in the airways (54). Release of ET-1 has been described from bronchial epithelial cells (52), neuroendocrine cells and human macrophages (48), and although human neutrophils can convert big-ET to ET-1 (303), there is no evidence that neutrophils can synthesise ET-1 *de novo*.. Increased ET-1 has been described in bronchial biopsy (122) and bronchoalveolar lavage (BAL) (124) in asthma, but we are not aware of any report of increased ET-1 in BAL, biospy or sputum in CF.

Spontaneous sputum was obtained from the CF subjects, but normal subjects do not expectorate spontaneous sputum, and sputum induction was therefore performed in these subjects. While it is accepted that spontaneous and induced sputum are not

identical in all respects, and that formal comparison of sputum ET-1 between spontaneous and induced sputum has not been reported, it has been demonstrated that total cell counts and measurement of soluble factors in sputum have good agreement between spontaneous and induced sputum (214), with better cell viability in induced sputum samples. The same study found increased numbers of squamous cells in spontaneous sputum, but this should be minimised by sputum plug separation to limit salivary contamination. The fact that sputum ET-1 levels were higher in sputum than saliva in CF suggests that salivary contamination is not responsible for the elevated levels of ET-1 in CF sputum. Sputum samples from CF subjects were treated with DNAse to facilitate processing of the unusually viscous sputum produced in CF, and while we did not use DNAse for normal samples, we tested for an effect of DNAse on the ET-1 assay, and found that DNAse did not alter the standard curve for the assay.

Saliva levels of ET-1 were greater than plasma levels in both groups, and although immunoreactivity to ET-1 has been demonstrated in rat parotid gland and human saliva (284), no physiological explanation for this has been offered.

ET-1 is a potent bronchoconstrictor in human bronchi in vitro (60), and we have previously demonstrated that inhaled ET-1 has bronchoconstrictor activity in asthma with bronchial hyperreactivity to ET-1 in asthmatics compared with normal subjects (Chapter 3) (275), supporting a putative role for ET-1 as a mediator in asthma. In CF, bronchial hyperresponsiveness to methacholine has been demonstrated in 50 to 77% of patients (313), and although the response to bronchodilators is less predictable than in asthma, many patients with CF are treated with bronchodilators (311), including 8 of the CF patients in this study. While the mechanisms of airway narrowing in asthma and CF may differ, the presence of a bronchoconstrictor with the potency of ET-1 at increased concentration in the sputum of patients with CF merits further investigation.

Activity of a number of inflammatory and growth factors has been described in CF lungs. Among other cytokines, tumour necrosis factor-alpha (TNF-α) and its receptor (314) are elevated in BAL fluid in CF. In normal lung tissue TNF-α increases the expression of ET-1 in bronchial epithelial cells (315), and TNF-α release is itself increased by ET-1 (100). ET-1 has been shown to be mitogenic for airway smooth muscle by a number of mechanisms both *in vitro* and *in vivo* (74) in animal studies. The precise nature of the various interactions between growth factors is by no means clear, nor are the cellular sources and identity of other potential mediators of peribronchial fibrosis and airway remodelling known. The presence of ET-1 in increased concentration in CF airways suggests that further investigation of its role in bronchoconstriction and airway remodelling is justified, and potential interactions of ET-1 with other growth factors in CF remain to be clarified.

CHAPTER 10

CONCLUSIONS

10 - CONCLUSIONS

10.1 PRINCIPAL FINDINGS

This series of studies is the first to demonstrate *in vivo* bronchoconstrictor activity of ET-1 in humans, and in particular asthmatics, confirming the presence of bronchial hyperreactivity to ET-1 in asthma, and the potency and prolonged activity of inhaled ET-1 as a bronchoconstrictor. Extending our exploration of the bronchoconstrictor activity of ET-1 in asthma *in vivo*, we found no evidence of an immediate inflammatory reaction following ET-1-induced bronchoconstriction in asthma, and no potentiation of ET-1-induced bronchoconstriction by another bronchoconstrictor peptide angiotensin II. We have demonstrated for the first time that ET-1 can be measured in sputum obtained by sputum induction, although we did not find a difference in sputum ET-1 levels comparing asthmatic and non-asthmatic subjects, nor any difference in sputum ET-1 comparing asthma in acute exacerbation and in recovery. Following allergen challenge we found a trend towards an increase in sputum ET-1 in those subjects who also had increased sputum eosinophil proportions, suggesting an association between airway inflammation and ET-1 release in response to allergen. Sputum ET-1 is increased in sputum from smokers and from subjects with COPD, with a trend towards a reduction in response to treatment in acute exacerbation of COPD, and sputum ET-1 is very markedly increased in patients with cystic fibrosis. These findings are both novel and exciting, and although this area of research is in its early stages, they suggest that ET-1 may have an important role in a number of airway diseases. The technique of sputum induction allows non-invasive measurement of ET-1, and demonstration of the use of ET-1 as an inhaled bronchial challenge test may provide the basis for initial exploration of the utility of ET-1 antagonists in asthma.

10.2 STRENGTHS AND WEAKNESSES INHERENT TO THE STUDIES AND IN RELATION TO OTHER STUDIES

The initial studies explored the bronchoconstrictor activity of ET-1 given by aerosol (chapters 3, 4 and 5). The first study demonstrated that ET-1 given by aerosol is a highly potent, reproducible bronchoconstrictor with a prolonged duration of action. The study was randomised, blinded, and had a positive (bronchoconstrictor) control. We used the technique of constant volume body plethysmography to make measurements of specific airway resistance (SG_{aw}), which is one of the most accurate methods available of assessing airway tone. This allowed us to measure accurately the changes in airway resistance which occurred as a result of inhalation of ET-1, and in addition to relate the increase in resistance to the decrease in FEV_1, which is a robust, but much less sensitive test of airway function. The use of the plethysmograph is however much more time consuming than simple spirometry, and having taken the dual end-points of changes in SG_{aw} and FEV_1 for the first study we were then able to use spirometry for subsequent studies. Reassuringly, we found ET-1-induced bronchoconstriction to be repeatable when comparing 2 challenge tests in the same subjects. Dose-dependent bronchoconstriction was observed in all the asthmatic subjects, although the high cost of ET-1 prevented us from extending the dose range high enough to observe bronchoconstriction in non-asthmatic subjects and therefore prevented quantification of the difference in sensitivity between asthmatic and non-asthmatic subjects. The cost of ET-1 together with the relative inefficiency of nebulisation also meant that we could only study limited numbers of patients for these studies, which in turn prevented exploration of certain aspects of the bronchoconstrictor activity of ET-1. We did not differentiate between atopic and non-atopic asthmatics, although it would have been interesting to do so, since a paper by Riccio *et al* (114) shows differences in the responses of the nasal mucosa to exogenous ET-1 in atopic rhinitis compared with normal subjects, but there is little data examining any relationship between atopy and ET-1 production or responses in atopic and non-atopic asthma (although genetic linkage studies have looked for

relationships between ET-1 gene polymorphisms and atopy and asthma (316)) In addition, although there were no apparent differences in bronchial reactivity to ET-1 in the three bronchoconstrictor studies between asthmatics grouped according to treatment, we did not specifically examine any effect which treatment with inhaled corticosteroids or other therapy might have on bronchial responsiveness to ET-1. While corticosteroids are known to affect the basal production of ET-1 from bronchial epithelial cells and macrophages (317), there is no data to indicate the effect of corticosteroid therapy on bronchial responsiveness to ET-1. Assessment of this could however be time-consuming, since effects of inhaled corticosteroids on bronchial hyperreactivity to other spasmogens can take several months to reach a plateau (318), although it would be interesting to combine this with an assessment of the effect of inhaled corticosteroids on ET-1 production in asthmatics over time. Other than in a research setting, the high cost of ET-1 would make it an unlikely choice for use in routine bronchial challenge testing, unless it were found to be significantly more disease-specific than currently available substances.

The second study examining airway cellular and mediator responses to ET-1-induced bronchoconstriction showed no acute change in sputum cell types, levels of the pro-inflammatory cytokines TNF-α and IL-1β, nitrite or albumin production at 30 minutes and 4 hours after bronchoconstrictor challenge with ET-1. The study was blinded, placebo controlled, and randomised for order. While it is tentatively possible to conclude from the data that ET-1 given by inhalation does not exert an immediate proinflammatory influence in the airways, this study is limited in a number of ways. One of the limitations of the use of induced sputum is the quantity of fluid available for analysis, particularly if the technique of separation of the viscid portion of the sputum ("plug separation") is employed, as we have done. While there are advantages in plug separation which are discussed in chapter 1.3, and reinforced by our finding of higher levels of ET-1 in saliva than in sputum in chapter 6, the available volume of sputum on which all analyses must be carried out may be less than 1 ml, and this is further reduced

by the processing involved in obtaining a differential cell count, with the result that only a limited number of assays or analyses can be carried out. Modern assay systems, particularly ELISA can operate on very small quantities of substrate, but most require at least 100 - 200 μl of sputum supernatant to ensure accuracy, and are routinely run in duplicate. As a result of this, it is possible to measure only a limited number of potential mediators, and in this study we were able to measure 4 parameters in addition to a differential cell count. These were chosen primarily to relate to known aspects of endothelin biology which could impact on asthma, but since work on the potential role of ET-1 in asthma is limited, the range of activity of ET-1 in relation to known mediators in asthma remains unclear, and the likely impact of administration of ET-1 on the local activity of markers and mediators such as eosinophilic cationic protein, IL-4, IL-5, GM-CSF and adhesion molecules for example, can only be the subject of speculation at present. The parameters measured were chosen carefully, but it is possible to argue that another combination of markers might give more information, or that other specific mediators might be a more accurate way of assessing similar processes. Similarly, the time points chosen to obtain sputum were intended to relate to a known property of ET-1, namely bronchoconstriction, rather than to all possible effects of ET-1. It is not known if ET-1-induced bronchoconstriction is associated with a late phase response such as that found with allergen, and in retrospect it would have been interesting to recall the patients after 24 hours to repeat sputum induction and spirometry at that time point, although that would introduce potential confounding factors, since repeat sputum induction has been shown to alter cellular proportions at 24 hours in both asthmatic and normal subjects (250, 251), with an increase in neutrophils characteristic (we found a trend towards an increase in neutrophils even at the 4 hour time point). It should be noted however that none of our patients reported symptoms suggestive of a late-phase response. Whatever the markers or time points chosen, this study represents a selection of individual responses at given time points, and cannot be used to suggest that ET-1 does not exert proinflammatory activity in the airways. Indeed, the weight of evidence

from studies using ET antagonists would suggest that ET-1 may have an active role in inflammatory processes in the airway.

The third bronchoconstrictor study reported data suggesting that the bronchoconstrictor activity of inhaled ET-1 is not potentiated by the presence of an infusion of angiotensin (Ang) II at sub-bronchoconstrictor levels. The study was placebo-controlled double-blind and randomised, with appropriate time intervals allowed for Ang II infusions to reach steady state, the patients remained recumbent throughout to prevent posture altering circulating Ang II levels, and the technique of ET-1 inhalation as a challenge test had been proved by prior work. The finding was contrary to our expectations based on previous *in vitro* work with ET-1 by Nally *et al* (261) from our own group, and although the data appears clear, this does merit investigation. The paper by Nally *et al* (261) reports potentiation by Ang II in bovine tissue, although some work was done by the same authors with a small number of human samples, apparently with similar results (unpublished - personal communication). Nonetheless, the possibility of species differences does exist, and since there is data to suggest that the mechanism of bronchoconstriction is different comparing humans with other species (60, 85), it is reasonable to speculate that interactions with other spasmogens may also differ between species. Potentiation by Ang II of bronchoconstriction induced by methacholine in the human both *in vitro* and *in vivo* has been reported by Millar *et al* (272), but no such interaction was found with histamine (319), suggesting that differences exist between spasmogens even within one species. The *in vitro* study by Nally *et al* (261) demonstrating potentiation of ET-1-induced contraction by Ang II was carried out using supraphysiological concentrations of Ang II, which might also account for differences between *in vivo* and *in vitro* findings. Direct comparison with the study by Millar *et al* (272) which showed potentiation by Ang II of methacholine-induced bronchoconstriction indicates that we achieved similar plasma levels of Ang II, suggesting that there was no deficit in the amount of circulating Ang II, although in both studies the plasma levels of Ang II are lower than that observed in acute severe asthma

(119), and lower than the *in vitro* studies. A clear effect on blood pressure was also observed in response to Ang II, confirming the biological activity of the Ang II being used. The study was carried out with identical methodology to Millar's study, which suggests that differences in results between ET-1 and methacholine are not due to methodological differences, lending credence to our findings, despite the *in vitro* data in bovine tissue with a contrasting result. Clearly, regulatory mechanisms may exist in the *in vivo* setting which are not active *in vitro*, and differences between laboratory and clinical data should not be unexpected. We studied a group of asthmatics with mild stable disease, and it would be interesting particularly to examine responses in patients with more severe asthma, since the presence of active airway inflammation might contribute to interactions between bronchoconstrictor substances which are not present in stable asthma.

The remaining studies (chapters 6 - 9) report the measurement of ET-1 in induced sputum (or in some cases spontaneous sputum), and although we found that ET-1 can be measured in sputum in a number of subject groups and that the measurement technique is sensitive to differences between groups, the application of the assay techniques to sputum is new. There are potential confounding influences, some of which are specific to ET-1, some to the use of sputum and some of which would apply to any measurement and processing techniques. As indicated in chapter 1.3, induced sputum is not identical to bronchial washing or BAL, and does not give the same information as bronchial biopsies in the assessment of airway inflammation and mediators. The technique is newer than some of the alternatives, and although much work has been done to establish its utility in the research setting, information is still accumulating about issues of methodology, processing of samples, and measurement of specific mediators. Our procedures for obtaining and processing the samples are widely used, and have been developed in collaboration with other centres, particularly that of the group under the direction of Professor F. Hargreave at McMaster University, Ontario, Canada, but there is as yet no universal agreement about methodology in this

area. In addition, there are methodological issues relating specifically to ET-1 measurement. The reducing agent dithiothreitol (DTT) is commonly used in the processing of sputum samples, but we found that DTT appears to have an effect the measurement of ET-1 by radio-immunoassay (RIA). The exact nature of this is not clear, since an effect was apparent with pure ET-1 mixed with DTT, but not so apparent with sputum samples mixed with DTT, and reverse-phase high performance liquid chromatography (RP-HPLC) did not suggest disruption of the ET-1 molecule by DTT. This problem merits further investigation, but we chose to process samples for ET-1 measurement in the absence of DTT, with the exception of those samples affected by an assay failure as stated in the relevant chapters, where we only had DTT-treated samples remaining (the results of which were not compared with results from non-DTT-treated samples). DTT is widely used in the processing of sputum, and although some authors have tested to see if it has an effect on the mediator being measured or on its assay, this is not universal. Sputum samples for ET-1 measurement were transported to the laboratory on ice, but we did not pre-treat the samples with protease inhibitors, since their effect on subsequent assays is not known, and we cannot rule out the loss of some ET-1 activity in transit and preparation time. Pathological samples do not allow easy quantification of the levels of ET-1 in bronchial biopsies, and PCR is at best semi-quantitative, so it is difficult to relate levels of ET-1 in sputum (or BAL) to tissue levels in health or disease. As mentioned in chapter 1, there is evidence to suggest that the majority of the ET-1 produced by tracheal epithelial cells is released basally, with only a small proportion being released luminally (11), and if the same is true in the bronchial epithelium, assay of bronchial secretions may under-represent the concentration of ET-1 being produced in the lung, and in addition, because of the relative proportions of basal and luminal release, a large change in basal secretion of ET-1 may be reflected only by a small change in the luminal concentrations of ET-1, rendering the assay of ET-1 in bronchial secretions relatively insensitive to changes in local production of ET-1. The influence of disease on the polarity of ET-1 release has not been explored, and the extent to which this might alter is unknown, and although it is assumed that the proportion of

ET-1 released luminally will be consistent relative to the total productions, this is not yet known. These questions are speculative, and other influences such as epithelial damage in disease could in fact amplify changes in sputum or BAL, but further investigation of the factors influencing ET-1 release and measurement is warranted.

Chapter 6 reports the measurement of sputum ET-1 in mild stable asthmatics compared with normal subjects (baseline study), and in acute severe asthma in exacerbation and in convalescence (acute study). Asthmatics were identified on the basis of symptoms and bronchial hyperreactivity in the baseline study, and entry criteria established for the acute study to try to ensure the presence of acute severe asthma. In the baseline study we performed ET-1 assay on blood, saliva and sputum samples, and were able to show differences between these three fluids, indicating local production in the upper and lower respiratory tract. We also confirmed the presence of ET-1 in sputum samples by RP-HPLC. The finding of higher levels of ET-1 in saliva than in sputum supports the use of sputum plug separation in the processing of induced sputum. The fact that we did not find a difference in sputum ET-1 between asthmatic and non-asthmatic subjects is in contrast to the findings of other investigators using BAL (124, 274), although elevation of BAL ET-1 is not invariable in asthma, an a study in nocturnal asthma reported a decrease at 4 am (127). The reasons for this difference are not known, and while it could be argued that induced sputum is somehow less effective at obtaining bronchial secretions that BAL, the levels of ET-1 which we observed in sputum were of a similar order of magnitude as those found in BAL allowing for dilutional effects in BAL, and data presented elsewhere in this thesis show that we have been able to measure quite high levels of ET-1 in sputum. While we could have strengthened this observation by the addition of BAL samples in the same group of patients, that would have involved the use of bronchoscopic techniques, for which we did not seek ethical permission. In the acute study we did not find a difference between sputum ET-1 in acute exacerbation and sputum ET-1 in convalescence, but the findings presented in the acute study are weaker than the baseline study in a number of ways, both avoidable and unavoidable. Firstly,

delays in presentation and a preference by both patients and their General Practitioners to treat asthma exacerbations out of hospital meant that some patients included in the study had presented acutely to the accident and emergency services, while others had been treated at home for several days prior to admission. Even after they had been admitted to hospital, the acute admissions pattern in our institution meant that the majority of patients were not admitted directly to the care of the respiratory team, and together these factors made it impossible to control for the duration of the exacerbation prior to admission to hospital. Similarly, the fact that patients were not admitted directly to the respiratory team meant that we could not obtain sputum samples immediately on admission, although all samples were obtained within 24 hours of admission. Secondly, although entry criteria for the study were set to try to correctly identify patients with exacerbation of asthma, we could not control for treatment of the exacerbation prior to the sputum sample being obtained, and in particular this meant that we could not obtain samples prior to the administration of oral corticosteroids, which were given to all the patients in the study. It is known that the production of ET-1 is downregulated by glucocorticoids (317), and it is not possible to exclude an effect on sputum ET-1 which is the result of treatment with oral corticosteroids, masking an effect of the exacerbation *per se*. Thirdly, as stated above, we suffered a failure of the original ET-1 assay on the samples which were not processed using DTT, and were therefore forced to repeat the assay using DTT-treated samples. It is not possible to retrospectively quantify the effect of DTT on the assay system (if any), and this means that we are not able to compare the results from the acute study with other measurements of sputum ET-1. ET-1 concentrations in the convalescent samples were lower than those observed in the baseline study, but we cannot tell if that is a result of the use of DTT, or if it results from differences in the patient groups, or differences in treatment, particularly the acute use of oral corticosteroids. The fourth main difficulty with the acute study is common to any study of acute severe asthma, namely that the patient group who present with acute severe asthma often represent a specific subgroup of asthmatics whose asthma is by nature unstable, difficult to manage, and subject to frequent alterations in therapy,

particularly oral corticosteroids. Convalescent samples were obtained 6 weeks after admission or once "stable" for at least 3 weeks thereafter, but stability in this group of asthmatic patients is relative, and for some patients it would be more accurately described as "the absence of acute exacerbation". In addition, all of the patients were taking inhaled or oral corticosteroids, even in recovery, which could have influenced sputum ET-1 concentrations. The acute study was a carefully performed, serious attempt to examine a possible role for ET-1 in acute severe asthma, but it was subject to avoidable and unavoidable limitations, and has fallen short of providing definitive data on the subject.

In chapter 7 we report the influence of allergen challenge on sputum ET-1 concentrations in stable asthmatics. The study was placebo controlled, randomised and performed in a single blind manner, since the operator had to monitor dose increments in the administration of allergen. An important aspect of the study design is the timing of the sputum sample, and as with many aspects of study design, several options could have been chosen. The release of ET-1 is not based on storage of ET-1 in secretory granules, but rather on rapid upregulation of protein synthesis with synthesis and secretion of ET-1 within minutes in response to certain stimuli (8). The study was designed to examine the possible contribution of ET-1 to bronchoconstriction in an allergic model of asthma, and therefore the sputum sample was obtained a relatively short time after the allergen challenge (30 minutes). It is recognised that sputum obtained at this time point will not reflect inflammatory changes resulting from allergen exposure, and this is reflected in the cellular data from this study, in which the only significant difference in sputum cell counts comparing placebo and allergen was a slight reduction in lymphocytes following allergen challenge. Animal work published after the study had started examining experimental eosinophilic airway inflammation suggests that although there is rapid upregulation of ET-1 mRNA, a rise in BAL ET-1 is not seen until 3 hours after the inflammatory stimulus, and peaks at 24 hours (289), suggesting that ET-1 may play a greater role in the induction and maintenance of airway inflammation than in the acute

bronchoconstrictor response in this context. If this animal data were reflected the situation in asthma, a change in design might have resulted in different results in this study, but would also have reflected a different component of the response to inhaled allergen. In retrospect, repeat sputum induction at 12 or 24 hours might have helped to examine both immediate bronchoconstrictor response and any potential later inflammatory response, but would have been subject to the confounding factor of repeated sputum induction, as discussed earlier. We attempted to use the polymerase chain reaction (PCR) to demonstrate ET-1 mRNA expression from samples obtained in this study, but experienced technical difficulties, and are not able to present this data. PCR data if available would have strengthened the observations reported. Despite this, a rise in sputum ET-1 was observed in some patients, with a relevant correlate in terms of airway inflammation, and there is *in vitro* experimental data to support a mechanism for the observed increase in ET-1 through the effects of activated eosinophils (293). The study design could have been different, perhaps with "positive" results, but we are confident that the findings are sound, although limited to the acute situation.

Chapters 8 and 9 present data on the levels of sputum ET-1 found in smokers with and without airflow limitation, acute and convalescent COPD and in cystic fibrosis (CF). These studies are exploratory in nature, since there is little background data on the presence or role of ET-1 in these conditions, and the studies were motivated by speculation based on the known physiological properties of ET-1 and the pathophysiology of the conditions concerned. The considerations discussed above about the use of sputum induction to assess airway ET-1 apply here also, although there are some specific questions relating to the conditions studied.

In the studies examining ET-1 in smokers and COPD we examined a mixture of spontaneous and induced sputum, for reasons of practicality and also safety in acute exacerbation of COPD, and although there is evidence that the cellular profile of COPD sputum does not differ between induced and spontaneous samples (224), information is

lacking about any influence on soluble mediators in sputum. There was no obvious difference in the COPD studies comparing induced with spontaneous samples comparing either sputum ET-1 or cell counts, but the studies were not designed to answer this question. The samples were of course processed in the same way, and assays performed identically, regardless of the method of obtaining the sample. It is not possible to assess on the basis of the data obtained whether those patients who were able to provide spontaneous samples (particularly for the acute study) somehow represent a different sub-group of COPD from those who did not, although our experience in acute severe exacerbation was that sputum production was almost universal, with very few patients failing attempted recruitment on the basis of failure to provide a sputum sample. In the baseline study it was not practicable to match subjects on the basis of age and total smoking history, and the COPD group were older and had smoked more than the healthy smokers. However, the amount of cigarettes smoked per day is comparable in the 2 groups, and it is possible that similar levels of sputum ET-1 result from similar recent smoking history rather than from the cumulative effect of cigarette smoking. We have not examined the acute effect of cigarette smoking on sputum ET-1. Similarly, since the COPD group were receiving treatment and the healthy smokers were not, the effect of treatment could influence sputum ET-1 levels. In the COPD group, 7 of the 9 patients were taking regular inhaled steroids, and as discussed above this could influence sputum ET-1 concentrations, with the possibility that ET-1 production is increased in COPD over and above the increase related to smoking, but suppressed to these levels by the influence of inhaled steroids. As discussed in relation to the acute asthma study in chapter 6, difficulties with an assay meant that we had to use sputum treated with DTT for analysis of the acute COPD study, making it impossible to compare values with stable COPD, and as in the acute asthma study, treatment was initiated prior to the sputum sample being obtained, which could have influenced sputum ET-1 levels. Despite these limitations, the acute study demonstrated a trend towards a fall in sputum ET-1 between exacerbation and convalescence. More work is needed to clarify the effect

of smoking and any additional effect of COPD on airway production of ET-1, but these studies lay the basis for further work in this area.

We demonstrated a marked increase in sputum ET-1 in cystic fibrosis (CF) compared with healthy subjects, using spontaneous samples of sputum. In some respects this study is a break from the theme of the work presented here, since asthma and COPD are traditionally characterised as "airway diseases", while CF is recognised to consist of a variety of pathological processes, not confined to the airway. In practice, however, involvement of lung tissue other than the conducting airways is present in both asthma and COPD, and variable airflow obstruction is a common feature of CF (311), although there are of course substantial differences between the conditions and their causes. This study was the result of speculation about the potential for ET-1 to contribute to both bronchoconstriction and airway remodelling in CF, and followed from some pilot work in which the possibility of very high levels of ET-1 in sputum was raised. It is a descriptive study, reporting the presence of ET-1 in sputum, but in the absence of pathological examination it is only possible to speculate about the source of ET-1 in CF. The study group represent a population with a range of disease, although it includes those with advanced disease, and it would be interesting to examine sputum ET-1 levels in children with more mild disease, to try to establish if the release of ET-1 is secondary to lung damage, or a primary contributor to the process. We used the enzyme DNAse to allow measurements to be made with the extremely viscid sputum produced in CF, and although we demonstrated that this does not appear to influence ET-1 assays, more work is needed to establish methodology for the use of sputum in CF.

10.3 POSSIBLE MECHANISMS AND IMPLICATIONS FOR PATHOPHYSIOLOGY AND TREATMENT

Asthma

The bronchoconstrictor activity of ET-1 in asthma has been established, with the demonstration of a potent, dose-dependent and prolonged effect following the administration of exogenous ET-1. The studies examining the bronchoconstrictor effect of ET-1 do not give direct information about the mechanism of action, although it is possible to speculate that ET-1 may act as a direct bronchoconstrictor on the basis of the relationship between bronchial reactivity to ET-1 and methacholine in asthma. It is not yet known if the bronchoconstrictor action of ET-1 is specific to asthma since we were not able to provoke bronchoconstriction in normals with the dose range used, and further work will be needed to examine responses to exogenous administration of ET-1 in normals and in subjects with other airway diseases. If the bronchoconstrictor activity were specific to asthma, then despite the high cost of ET-1 there would be the potential to use it as a diagnostic test, perhaps initially for research rather than clinical purposes. Demonstration of the activity, potency and duration of bronchoconstriction of ET-1 in asthma strongly supports a potential role for ET-1 in the pathophysiology of the disease, quite apart from the other potential actions of ET-1 which could contribute to asthma. In the studies examining induced sputum ET-1, although we did not find elevated levels in stable asthmatics compared with normal subjects, the concentrations of ET-1 which we measured in sputum were at least as high (and in most cases higher) than the doses required to produce bronchoconstriction when given exogenously in asthma, even when the inefficiencies of nebulisation are taken into account. This would suggest that subject to regulatory mechanisms, the levels of ET-1 present in the airway even in stable asthma are capable of exerting bronchoconstrictor activity. Asthmatic airways have increased sensitivity to the bronchoconstrictor effects of ET-1, and in this setting, even normal levels of ET-1 may have unwanted effects, and investigation of the potential use of ET antagonists as bronchodilators or inhibitors of bronchoconstriction in asthma is justified.

ET-1-induced bronchoconstriction does not appear to be associated with an early inflammatory response in the airways as assessed by sputum induction up to 4 hours after administration, but several animal studies have demonstrated the ability of ET-1 antagonists to modify inflammatory responses in the airway (290, 320), and more work is needed to clarify the mechanisms of this action, to examine the possible pro-inflammatory effects of inhaled ET-1 at later time points and to investigate the possibility that ET antagonists might have anti-inflammatory activity in asthma. We did not find potentiation of ET-1-induced bronchoconstriction by Ang II in asthma, although sub-bronchoconstrictor doses of Ang II were deliberately chosen, with resultant plasma levels of Ang II which were lower than that observed in acute severe asthma (273), so it is not possible to rule out an effect in acute severe asthma which we did not demonstrate in our study. We have shown that it is possible to use the non-invasive technique of sputum induction to obtain fluid to measure ET-1 in asthmatic and non-asthmatic subjects, with higher concentrations of ET-1 in sputum and saliva than in plasma, suggesting local production in the respiratory tract, in keeping with evidence that ET-1 tends to have predominantly autocrine or paracrine activity. We did not find an increase in sputum ET-1 in acute severe asthma, nor therefore a fall in response to treatment, although this study was limited as discussed above, and it is difficult therefore to draw conclusions about any role that ET-1 might have in the induction or maintenance of acute exacerbations of asthma. Although not all asthmatics had an increase in sputum ET-1 in the acute phase following allergen inhalation, in those in whom there was a rise, this was related to the extent of the asthmatic eosinophilic airway inflammation, suggesting a link between asthmatic airway inflammation and ET-1 production or release. The relationship between ET-1 and eosinophilic airway inflammation has been explored in animal studies (110, 289, 321) which support a link between ET-1 production and asthma, and which provide further incentive to explore the role of ET antagonists in asthma. The mechanism whereby eosinophilic airway inflammation is related to ET-1 production is not yet clear, although animal data indicates that eosinophils activated by IL-5 or GM-CSF can stimulate the release of ET-1 from airway epithelial cells (293),

and since these cytokines and others are clearly relevant to asthma, a similar mechanism may exist in asthma. Overall, our investigations support a role for ET-1 in the pathophysiology of asthma, although many aspects of its role and the mechanisms of action remain to be further clarified, and it seems likely that some aspects of the action of ET-1 in the lung will have greater bearing on asthma than others. Further work is need to clarify the influence of asthma treatment (in particular corticosteroids) on ET-1 synthesis and release, since we did not find any difference in sputum ET-1 between steroid-naive and steroid-treated asthmatics. Our group of steroid-naive asthmatics were however very mild, with few ongoing symptoms, and there is evidence for increased ET-1 expression in symptomatic compared with asymptomatic asthmatics (125). Similarly, bronchial biopsy studies have shown increased ET-1 immunostaining in steroid-naive asthmatics compared with steroid-treated asthmatics (123), and there is *in vitro* evidence that steroids reduce ET-1 expression in bronchial epithelial cells and macrophages (317). Bronchodilator drugs such as salbutamol do not appear to reduce ET-1 expression in asthma (125), and the general consensus that inhaled (or oral) corticosteroids should be included in the treatment of symptomatic asthma (242) may have potential additional benefits through the influence of corticosteroids on the production of ET-1 in asthma. At present it is only possible to speculate on the potential therapeutic role of ET-1 receptor antagonists or ET converting enzyme inhibitors in asthma, but the data so far would support pharmaceutical research in this direction.

Smoking and chronic obstructive pulmonary disease

We found increased sputum ET-1 in cigarette smokers without symptoms of lung disease, and in smokers with established COPD, suggesting that cigarette smoking may increase ET-1 production or release in the airways, although the mechanism for this is not known. Cigarette smoke has the capacity to damage the bronchi in a number of ways including direct toxicity to the bronchial epithelium, oxidative damage, recruitment of inflammatory cells and increased epithelial permeability (301), in addition to structural changes. Cigarette smoking is also associated with bronchial hyperreactivity, with

smokers demonstrating airway reactivity to methacholine (322) as well as to cigarette smoke itself (323). We have not explored the potential bronchoconstrictor effects of ET-1 in smokers, although it would be interesting to see if there is bronchial hyperreactivity to ET-1 in the absence of asthma. While the short term effects of ET-1 in smokers lungs is of interest, perhaps of greater importance are the potential results of increased ET-1 production and release in the long-term. In particular, the properties of ET-1 could play a role in the pathophysiology of COPD, through its mitogenic and profibrotic actions as well as potential proinflammatory actions, and more speculatively, mitogenesis and co-mitogenesis could also contribute to the pathogenesis of lung cancer, a condition where local ET-1 expression has been described (300). Smoking cessation is the only treatment which has been shown to be of value in the prevention of COPD, and although there is a need to explore the value of drugs which could modify the processes resulting in COPD (including corticosteroids and potentially also drugs influencing ET-1 production and action) smoking cessation crucial. Much more information is needed on the mechanism by which smoking alters ET-1 production, and its role in pathogenesis of lung disease before studies could be planned to investigate therapeutic modification of the harmful effects of cigarette smoke by ET antagonists or ECE inhibitors.

Cystic fibrosis

We are not aware of any other work examining the role of ET-1 in CF. The levels of sputum ET-1 observed are markedly greater than in healthy subjects, and raise a number of questions about the potential contribution of ET-1 to the disease. As mentioned above, we do not know if the increase in ET-1 observed is secondary to lung damage, or is involved in the pathophysiology of CF. Several actions of ET-1 could contribute to pathophysiology in CF, including bronchoconstriction, mitogenesis, fibrogenesis, proinflammatory action and stimulation of mucus secretion, but at present we can only speculate about the likely contribution of each of these to CF. CF is characterised by neutrophilic airway inflammation, although there is evidence of eosinophil activation (324), with elevated levels of a number of proinflammatory cytokines (314), some of

which (in particular IL-1 and TNF-α) may be involved in the upregulation of ET-1 expression (317). An important additional component of airway disease in CF is the presence of infection or colonisation by bacteria, and although it is known that bacterial endotoxin can stimulate ET-1 production *in vitro* (129), little is known about the effects of bacteria or bacterial products on ET-1 production *in vivo* in general, or in CF in particular. Although the observation of markedly increased ET-1 in CF provokes questions and speculation, the main requirement at this stage is to pursue further studies to try to establish the relationship between CF and ET-1, and hopefully thereafter to investigate possible therapeutic modification of the effects of ET-1 in CF if appropriate.

10.4 UNANSWERED QUESTIONS AND FUTURE RESEARCH

Although a number of studies have identified cells capable of producing ET-1 in the airway (principally airway epithelial cells and macrophages), the contribution of disease to ET-1 production requires further exploration, in terms of direct release of ET-1 from structural and inflammatory cells, autocrine regulation of that release, the effects of airway inflammation on ET-1 release from other cells, and the effects of treatment on ET-1 production and release. A number of techniques would be applicable in this area, including polymerase chain reaction, immunocytochemistry and cell culture techniques, and although most experience has been gained with biopsy samples, PCR and cell culture are applicable also to cells obtained by sputum induction, which would allow non-invasive assessment. A study examining BAL and induced sputum ET-1 in the same group of asthmatics would allow direct comparison between the 2 methods of assessment, and would be helpful in characterising the utility of each method in the assessment of airway levels of ET-1. The studies presented here have concentrated on the acute effects of ET-1 in the airway, but it may be of even greater importance to try to examine the long-term activity of ET-1 in asthma, with particular reference to airway remodelling. A longitudinal study examining sputum ET-1 along with lung function, biopsy samples and possibly some non-invasive measure of remodelling such as high-resolution CT scanning would be ideal, but extremely difficult to perform in view of the time period involved, and perhaps more realistic in the short-term would be a cross-sectional study comparing sputum ET-1 with measures of airway remodelling.

We have demonstrated the bronchoconstrictor activity of ET-1 in asthma, but we were not able to pursue this with non-asthmatic subjects beyond the dose range specified, to determine if the action is specific to asthma, or perhaps more likely, if asthmatics are simply more sensitive to the bronchoconstrictor effects than non-asthmatics, in keeping with other inhaled bronchoconstrictors. The main obstacle to such a study is the high cost of ET-1 (partly due to the inefficiencies of nebulisation), and the protocol would be

identical to that of the studies presented here. Although we found that all the asthmatic subjects experienced a fall in lung function within the dose range used, this study was of necessity performed in a small number of patients, and it would also be of interest to extend the study to include larger numbers of asthmatics encompassing a range of asthma severity, and also subjects with borderline bronchial hyperreactivity to other agents such as histamine or methacholine, to establish the dose range for ET-1 as a bronchoconstrictor and the range of responses in asthmatic and non-asthmatic subjects. The effect of asthma treatment on ET-1-induced bronchoconstriction could also be examined. The duration of bronchoconstriction induced by ET-1 was prolonged at around 60-90 minutes, but this was only formally examined in 2 asthmatic subjects, and larger numbers would be needed to confirm this. Although none of our asthmatic subjects reported symptoms suggestive of a late-phase response, we did not examine for this, and in combination with a study examining the duration of bronchoconstrictor activity it would be possible to test for such a response. As mentioned above, we did not distinguish between atopic and non-atopic asthma in our bronchoconstrictor studies, and this would also be possible, as would further examination of potential interactions between ET-1 and other airway mediators. The study examining interactions between ET-1 and Ang II could be extended to a more severe group of asthmatics, and although it would not be possible to perform such a study in acute severe asthma, it would be possible to compare responses before and after reduction of inhaled corticosteroid therapy to a level at which symptoms become apparent. In addition, it would be interesting to explore the bronchoconstrictor action of ET-1 in other lung diseases, in particular COPD and CF, since we have observed elevated levels of ET-1 in sputum in these conditions. Bronchial hyperreactivity in the presence of increased release of ET-1 might suggest a role for ET-1 in the pathophysiology of these conditions, with prospects for pharmacological modification of the effects of ET-1 in COPD and CF.

Examination of airway responses to inhaled ET-1 could be extended by increasing the number of mediators studied, and by extending the assessment to include other time

points, in particular to assess whether ET-1-induced bronchoconstriction is associated with a late-phase response, and similarly, it would be of value to extend follow up after allergen challenge to measure ET-1 at a later time point, to assess any role that ET-1 might have in the late-phase allergic response, and to compare the results with animal data (103, 289). The limitations of the study examining sputum ET-1 in acute severe asthma would suggest that this could be re-examined, with attempts made to obtain samples as early in the exacerbation as possible, and ideally prior to the administration of oral corticosteroids. While there are practical difficulties with this, the question will remain unanswered until these factors are controlled for.

It is not clear whether the effect of smoking on ET-1 levels is an acute effect only, or a more chronic upregulation of ET-1 production. It would be possible to perform a study in smokers comparing induced sputum following a short period of abstinence from smoking, with a samples obtained after acute cigarette smoking, and in the light of the data from vascular studies indicating that short-term smoking enhances ET-1-induced vasoconstriction (310), it would be of interest also to examine the impact of smoking on bronchial reactivity to ET-1 in asthmatic and non-asthmatic smokers. Similarly, it would be interesting to compare sputum ET-1 in COPD patients treated with inhaled steroids with those taking only inhaled bronchodilators, current and ex-smokers with COPD, and to compare COPD patients with and without respiratory failure, since circulating ET-1 is increased by hypoxaemia (325).

The finding of elevated sputum ET-1 in CF is a novel, but isolated finding, which will require confirmation in larger and better characterised groups of CF patients, ideally representing a range of severity of the disease, to see if there is a relationship between sputum ET-1 and disease severity or prognosis. Any relationship between ET-1 and the presence or absence of active infection should be sought, and in view of its prognostic significance, it would be of interest to relate sputum ET-1 levels to colonisation with the organism *Burkholderia cepacia*, which is a common but adverse finding in CF.

One of the most significant areas for future research into the role of ET-1 in airway diseases is the development of ET receptor antagonists and converting enzyme inhibitors. Although clinical trials have been initiated in other diseases, we have had no success in obtaining these compounds to assess their potential role in the treatment of airway diseases. Drugs which modify the production or action of ET-1 may have bronchodilator activity, even in non-asthmatics if ET-1 is involved in the maintenance of basal bronchial tone, and in asthma since airway concentrations of ET-1 may be sufficient to provoke bronchoconstriction either because of increased production or heightened bronchial reactivity to ET-1. A placebo-controlled study comparing lung function measurements before and after administration of the drug would be easy to perform, perhaps using body plethysmography as the most accurate method of assessing airway tone. ET receptor antagonists and enzyme inhibitors may also have the ability to inhibit bronchoconstriction in asthma, which could be studied using a non-specific bronchoconstrictor such as methacholine, ET-1 given by inhalation as presented above, and also using allergen challenge to assess any contribution of ET-1 to allergen induced bronchoconstriction or hyperresponsiveness, as seen in animal models (263). Using sputum induction or bronchoscopic techniques, it would also be possible to use this model to assess the ability of these drugs to influence airway inflammation in response to allergen challenge, to compare with animal work in this area (103), and following from this, further exploration of potential suppression of asthmatic airway inflammation could be assessed using sputum induction. Any effects on airway remodelling would require longer studies to assess, although there is animal work to support this potential (74), and it may be that there is a greater role for drug development in the area of remodelling than bronchodilation for example, since currently available bronchodilators are not only cheap, but also very effective. Drugs could be administered either orally, or ideally by inhalation or nebulisation, although some pilot work would be required to ensure no loss of activity by the inhaled route. The choice of drug is also of interest, since although *in vitro* work suggests that ET_B receptors may primarily

mediate bronchoconstriction (68), there is animal evidence that blockade of both ET_A and ET_B receptors is necessary to completely abolish ET-1-induced bronchoconstriction (89). By contrast, other actions of ET-1 such as proliferation of airway smooth muscle (69) and proinflammatory activity appear to be predominantly ET_A dependent (108, 109).

The potential for the use of ET-1 antagonists or enzyme inhibitors in other conditions such as COPD or CF remains to be established, although the pulmonary vascular activity of ET-1 in COPD has been the source of much interest, and clinical studies have been initiated with an orally available ET-1 receptor antagonist in hypoxaemic COPD.

In summary, the results of this thesis provide evidence for a putative role for ET-1 in the pathophysiology of asthma, with initial evidence of a potential role in the processes affecting the airways in cigarette smoking, COPD and CF. We believe that our results support further studies in order to increase our understanding of the role of ET-1 in the airway, and speculate that in the future, specific pharmacological targeting of ET-1 may form the basis of a novel approach to therapy in asthma and in other conditions affecting the airway.

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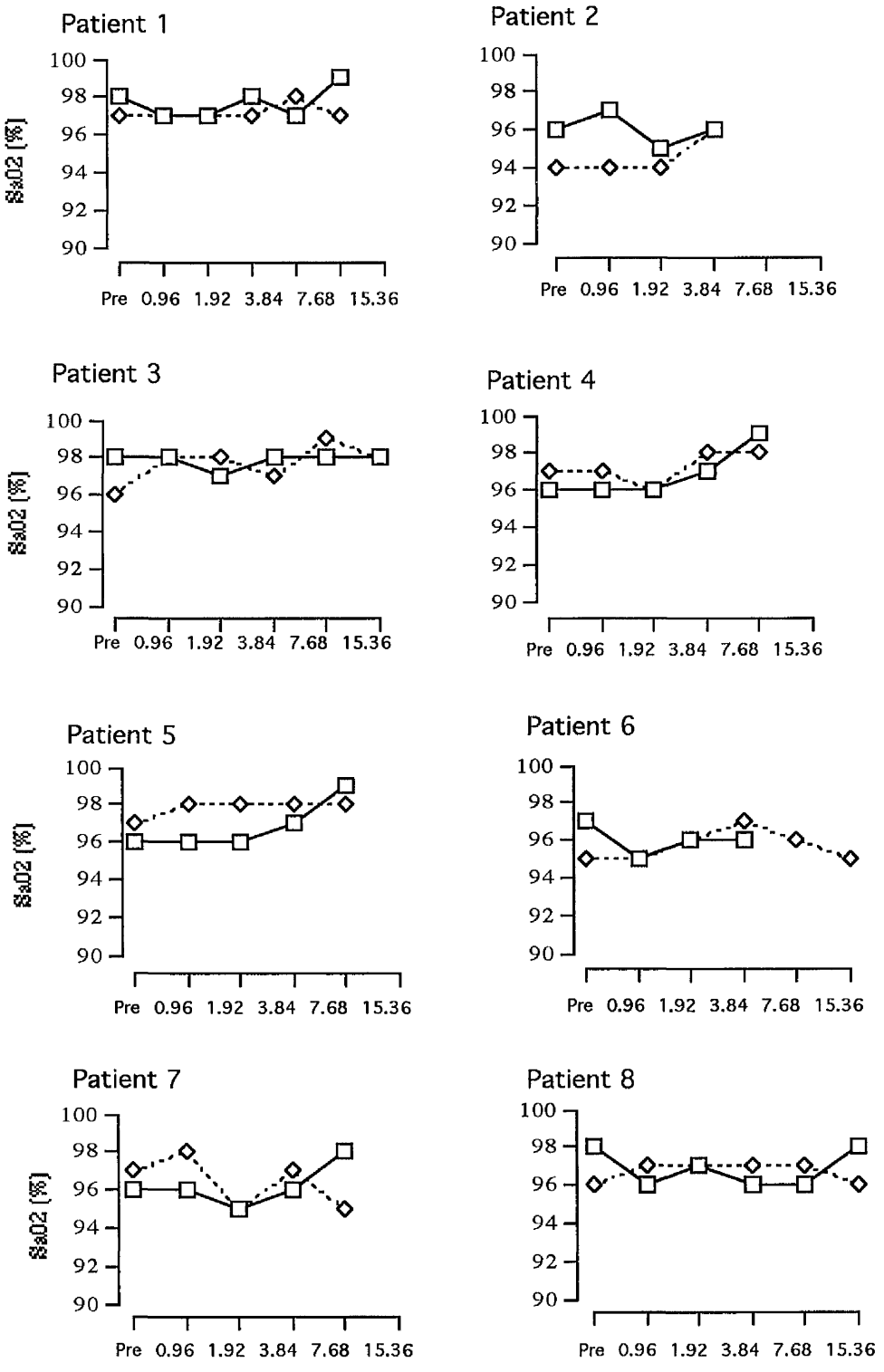
Appendix 3.1

O_2 Saturation during ET-1 inhalation



Appendix 3.2 Blood pressure and pulse before and after ET-1 inhalation

ASTHMA

	BP - pre	Pulse - pre	BP - post	Pulse - post	BP - pre	Pulse - pre	BP - post	Pulse - post
1	117/64	60	125/77	58	121/72	62	110/65	67
2	127/81	74	142/84	77	135/78	87	139/69	88
3	124/87	72	127/81	58	121/76	54	128/82	70
4	105/67	81	104/69	86	115/58	77	116/66	75
5	124/81	55	123/78	55	110/67	55	122/71	57
6	135/41	87	132/78	90	116/77	75	117/78	74
7	119/86	55	111/69	72	118/79	78	115/80	68
8	121/83	59	121/69	59	131/79	62	121/74	60
Mean	**122/80**	**70**	**123/76**	**69**	**121/73**	**69**	**121/73**	**70**

NORMALS

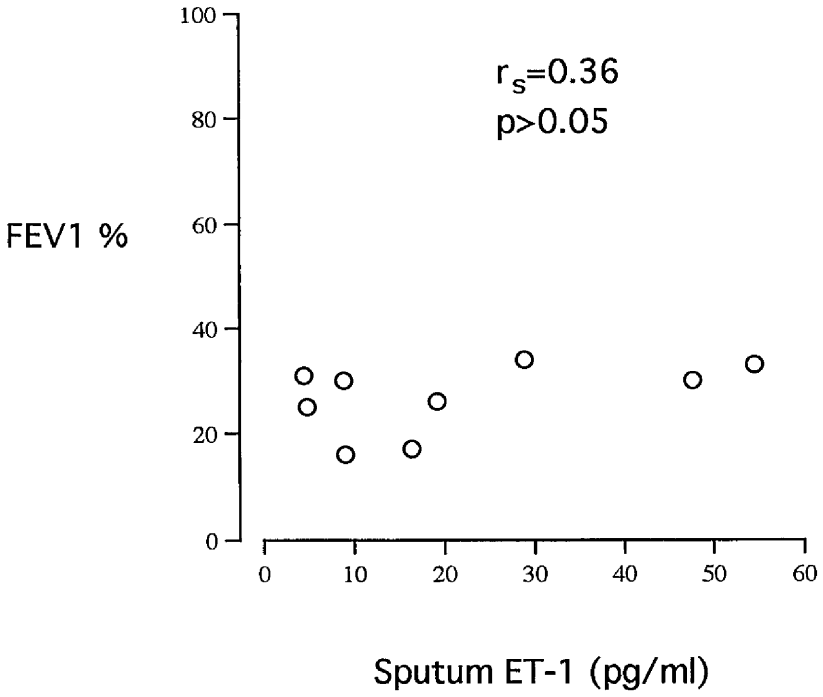
1	118/71	61	116/78	58
2	125/66	78	116/75	81
3	114/73	65	104/68	55
4	129/72	63	111/76	63
5	129/72	57	116/67	48
Mean	**114/66**	**65**	**104/67**	**60**

Appendix 5.1 - Pulse and oxygen saturation before and after infusion of placebo / Ang II 1ng/kg/min / Ang II 2ng/kg/min

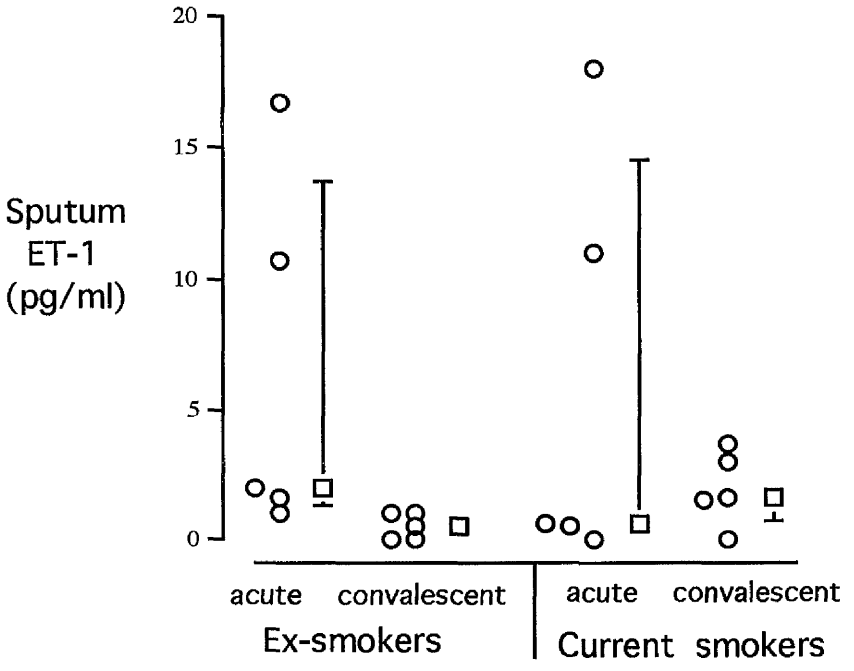
	Placebo				**Ang II 1 ng/kg/min**				**Ang II 2 ng/kg/min**			
	Pulse		SpO2		Pulse		SpO2		Pulse		SpO2	
	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post
1	78	64	96	94	80	75	96	95	68	74	97	97
2	88	81	96	94	85	82	96	95	87	77	95	95
3	74	64	95	94	65	68	97	96	78	76	99	98
4	51	61	97	95	60	60	97	97	58	60	95	96
5	60	56	96	94	51	49	97	96	47	46	97	97
6	65	62	97	97	66	64	96	97	69	64	97	96
7	69	72	98	98	68	63	97	97	70	74	98	97
8	65	67	97	95	64	66	97	95	62	61	97	97
Mean	**69**	**66**	**97**	**95**	**67**	**66**	**97**	**96**	**67**	**67**	**97**	**97**

Appendix 7.1 - Plasma ET-1 before and after inhalation of placebo, methacholine and allergen

	Placebo inhalation		Methacholine inhalation		Allergen inhalation	
	pre	**post**	**pre**	**post**	**pre**	**post**
1	4.7	3.5	4.0	4.3	3.9	4.4
2	3.4	2.8	3.8	3.0	2.3	2.9
3	2.2	0.1	3.5	2.3	2.2	3.6
4	2.6	2.9	3.0	2.9	2.6	3.1
5	3.6	3.9	7.3	5.2	2.9	3.6
6	4.6	6.2	3.1	3.7	3.1	3.9
7	5.9	5.9	3.0	3.5	2.9	4.6
8	4.1	4.7	6.5	6.9	4.2	4.3
9	3.6	6.9	3.0	3.2	4.1	3.1
10	3.0	3.2	2.8	3.0	3.4	3.5
11		2.6				1.9
12		0.5				0.5
Mean	**3.8**	**3.6**	**4.0**	**3.8**	**3.2**	**3.3**



Appendix 8.1
Relationship between FEV1 (% predicted) and sputum ET-1 in stable COPD (Spearman rank correlation - not significant)



Appendix 8.2
Sputum ET-1 in acute exacerbation and in convalescence, comparing ex- and current cigarette smokers - no significant differences between groups