INVESTIGATIONS ON THE ROLE OF THE RENIN-ANGIOTENSIN SYSTEM IN ASTHMA

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SUBMITTED FOR THE DEGREE OF

DOCTOR OF MEDICINE

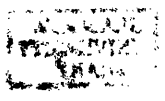
UNIVERSITY OF GLASGOW

DEPARTMENTS OF RESPIRATORY MEDICINE AND

PATHOLOGY, WEST GLASGOW HOSPITALS UNIVERSITY NHS

TRUST

FEBRUARY 1999



SUMMARY

Introduction

The renin-angiotensin system is activated in acute severe asthma although the mechanism for is unclear. Angiotensin II is a weak bronchoconstrictor, potentiates the effects of other bronchoconstrictors and can be formed by inflammatory proteases. *In vitro*, angiotensin II is a growth promoter for human airway smooth muscle cells. The insertion/deletion polymorphism of the ACE gene accounts for variation in ACE activity and the deletion allele may be more frequent in asthmatics. In view of these facts, further clarification of the role of the renin-angiotensin system in asthma is required.

Aims

This thesis examines the factors causing activation of the renin-angiotensin system in acute severe asthma, the role of the ACE gene polymorphism, the putative effects of angiotensin II on the action of histamine in the human airway both *in vitro* and *in vivo*, the effects of angiotensin II on rat airway smooth muscle cell growth *in vivo* and seeks evidence for the existence of a local renin-angiotensin system in the human lung.

1. Mechanism of Activation of the Renin-Angiotensin System in Asthma

Clinical data were obtained from 40 acute asthmatic hospital admissions and correlated with plasma levels of renin and angiotensin II. The genotype for the ACE gene polymorphism was determined in this group, a group of 20 non-asthmatic acute medical hospital admissions and 78 healthy volunteers to allow comparison of allele frequencies and correlation with both ACE activity and angiotensin II levels.

Plasma renin and angiotensin II were elevated in some, but not all of the acute asthmatics and no correlation was found with any of the parameters measured. The DD homozygote for the ACE gene polymorphism had the highest ACE activity in all groups measured. There was a non-significant trend towards an increased frequency of the deletion allele in asthmatics compared with normal controls. An unexpected finding was the increased ACE activity for the DD and ID genotypes found in acute hospital admissions compared with healthy controls. The clinical significance of this is uncertain.

2. Effect of Angiotensin II on Histamine-Induced Bronchoconstriction

The effect of angiotensin II on histamine-induced contraction of human bronchial rings *in vitro* was examined by constructing concentration-response curves to histamine (10^{-9}M - $3x10^{-4}$M) in the presence and absence of sub-threshold doses of angiotensin II (10^{-7}M - 10^{-6}M). This was also studied *in vivo* in 8 asthmatic volunteers who had histamine bronchial provocation testing in a double-blinded fashion in the presence of infusions of placebo (5% dextrose) and angiotensin II (1 and 2 ng/kg/min).

Angiotensin II in subthreshold doses for contraction of airway smooth muscle had no effect on histamine-induced bronchoconstriction in the human airway neither *in vitro* nor *in vivo*.

3. Effect of Angiotensin II on Remodelling of Rat Airway & Vasculature

The effect of endogenous activation of the renin-angiotensin system by dietary sodium restriction and subcutaneous infusion of angiotensin II on the rat airways was compared to the mesenteric vasculature. DNA synthesis was measured by infusing the thymidine analogue bromo-2'-deoxyuridine subcutaneously then revealing those cells undergoing DNA synthesis using immunocytochemistry. Changes in the volume of the airway tissues were measured morphometrically.

Endogenous activation of the renin-angiotensin system in rats known to increase arterial medial DNA synthesis results in increased renin mRNA expression in the renal juxta-glomerular cells with increased plasma aldosterone and plasma renin activity and minor insignificant variations in blood pressure. However, no change was seen in DNA synthesis or morphometry of the airways.

Similarly, infusion of angiotensin II for two weeks in rats causes a rise in blood pressure and suppresses renal juxta-glomerular renin mRNA expression with a non-significant suppression of plasma renin activity. DNA synthesis increased in the mesenteric arterial media and there was a non-significant tendency towards the artery wall being thicker. No difference was found in DNA synthesis or morphometry of the airways.

4. Evidence for a Local Renin-Angiotensin System in the Human Lung

Samples of normal human lung were obtained from thoracotomy specimens and were examined by immunocytochemistry for renin and ACE, and by *in situ* hybridisation and Northern blotting for renin mRNA, angiotensinogen mRNA, ACE

mRNA and AT_1 receptor mRNA. *In situ* angiotensin II receptor localisation and autoradiography were also performed.

In human lung, ACE was identified in vascular endothelium however, no other components of the renin-angiotensin system could be found. In control rat lung tissue, angiotensin II receptors appeared to be present.

Conclusion

The renin-angiotensin system is activated in some acute asthmatics but the mechanism for this remains unclear. It does not correlate with β-agonist administration as hypothesised. The deletion allele of the ACE gene polymorphism may be more frequent in asthmatics but larger study numbers are required to confirm this. Angiotensin II has no effect on histamine-induced bronchoconstriction despite its known potentiation of methacholine and endothelin-1. Although angiotensin II has mitogenic effects on human airway smooth muscle cells *in vitro*, it has no effect on airway remodelling in the rat but increases arterial smooth muscle cell DNA synthesis. Finally, the presence of ACE in pulmonary vascular endothelium has been confirmed and angiotensin II receptors may be present in rat lung but no evidence has been found of the other components of the renin-angiotensin system in the human lung.

Thus, the renin-angiotensin system has a minor involvement in asthma with borderline clinical relevance in some asthmatics, but is not a major contributor to the pathophysiology of asthma.

For my wife, Lorna and our family.

ACKNOWLEDGEMENTS

I would like to thank the following people for their invaluable contributions to the work of this thesis:

Mr. Gordon Clark from the Department of Clinical Pharmacology, Ninewells Hospital, Dundee for performing the plasma salbutamol assays, Dr. Ian Morton for performing the renin, angiotensin II and endothelin-1 assays and Dr. Charlie McSharry for performing the IgE, histamine, ECP and tryptase assays. Dr. Richard Spooner and Dr. Caroline Jagger for performing the ACE activity assays and ACE genotyping and for providing data from a West of Scotland control population. Dr. Ian McKay for his advice and assistance with statistical analysis of the acute asthma data.

The medical and nursing staff of the Acute Medical Receiving Unit, Western Infirmary, Glasgow and of the Respiratory Unit, Gartnavel General Hospital for accommodating me during patient recruitment and assessment. Mr. Alan Kirk, Consultant Cardiothoracic Surgeon, Western Infirmary, Glasgow for his help in identifying patients undergoing thoracotomy and lung resection.

Dr. Jane Nally for instruction in the techniques of the *in vitro* bronchial reactivity studies and Dr. Rob Clayton for his assistance in performing six of the *in vitro* bronchial reactivity cases. Ms. Dawn Farmer and Ms. Wendy Fallon from Pharmacy Department, Western Infirmary, Glasgow for the sterile preparation and blinding of the solutions for infusion.

Dr. Chris Kenyon, University Department of Medicine, Western General Hospital, Edinburgh for setting up the rat experiments under the auspices of his Home Office licence and for his assistance with organ retrieval from the animals. Mr. Niall Whyte for performing the analysis of the mesenteric vasculature from the rat experiments and permitting the data to be included in my thesis.

Mr. Rod Ferrier and Mr. Ian Downie for their patient instruction, guidance and assistance with histological processing and staining techniques and immunohistochemistry. Mr. Ferrier also provided instruction and assistance with molecular techniques including riboprobe preparation, *in situ* hybridisation, RNA extraction and Northern blotting.

Dr. Jim McQueen for his instruction and assistance in the techniques of iodination of saralasin, angiotensin II receptor labelling and autoradiography. Dr. Mark

McJury, Clinical Physicist, for digitising the autoradiographs and providing the luminosity values.

Dr. Kevin Lynch, University of Virginia School of Medicine, for kindly gifting the renin mRNA to allow riboprobe preparation. Dr. Eleanor Davies and Dr. Kathleen Curnow, INSERM Unit 36, College de France, Paris, for providing the AT_1 receptor and ACE mRNA for riboprobe preparation.

The Departments of Medical Illustration in Glasgow Royal Infirmary and the West Glasgow Hospitals University NHS Trusts for producing copies of my illustrations and Mr. Peter Kerrigan for assistance with pathological photographs.

Dr. Evelyn Millar for her ground-breaking work on the renin-angiotensin system in asthmatics and advice in performing the clinical infusion studies.

Professor David Stott for allowing me the time to continue the writing of this thesis during my training in Medicine for the Elderly.

I must also thank the volunteers who took part so willingly in my work and gave so freely of their time during the course of the studies.

A special thank you is due to my colleagues during my time spent in research, namely Dr. Ken Dagg and Mrs. Lorna Thomson, for their help, encouragement and companionship which made my time with them so enjoyable and left me with many memories to cherish.

Finally, I would like to thank my mentors: Dr. George Lindop for his fostering of my interest in pathology and basic science research, for his continued support and stimulating discussions of this work and willingness to provide constructive criticism.

Professor Neil Thomson for kindling my interest in Respiratory Medicine as a student, for providing me with the opportunity to perform research within the Asthma Research Unit, for his support throughout the course of the work and the subsequent preparation of this thesis.

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LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ACEI	Angiotensin-converting enzyme inhibitor
Ang.II / AII	Angiotensin II
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
APAAP	Alkaline phosphatase anti-alkaline phosphatase
APES	3-Amino-propyl-triethoxy-silane
ASMC	Airway smooth muscle cell
AT_{1-3}	Angiotensin II receptor types 1-3
BALF	Bronchoalveolar lavage fluid
BDP	Beclomethasone dipropionate
BP	Blood pressure
BrdU	Bromo-2'-deoxyuridine
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complimentary deoxyribonucleic acid
COX-2	Cyclo-oxygenase 2
DAB	Diamino-benzidine
DAG	Diacyl glycerol
DD	Deletion homozygote of the ACE gene polymorphism
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ECP	Eosinophilic cationic protein
EDAC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epidermal growth factor
ET-1	Endothelin-1
ET_{A-B}	Endothelin-1 receptor type A-B
FEV_1	Forced expiratory volume in one second
FiO_2	Inspired oxygen concentration
FVC	Forced vital capacity
GM-CSF	Granulocyte-monocyte colony stimulating factor
H_{1-3}	Histamine receptor type 1-3
ID	Heterozygote of the ACE gene polymorphism
IgE	Immunoglobulin E

IGF	Insulin-like growth factor
II	Insertion homozygote of the ACE gene polymorphism
IL_{1-5}	Interleukin 1-5
IP_3	Inositol triphosphate
LTA-D_4	Leukotriene A-D_4
M_{1-3}	Muscarinic cholinergic receptor type 1-3
MCh	Methacholine
MOPS	4-Morpholine propane sulphonic acid
mRNA	Messenger ribonucleic acid
n	Number of observations
NANC	Non-adrenergic non-cholinergic
NSB	Non-specific binding
PBS	Phosphate-buffered saline
$PaCO_2$	Partial pressure of arterial carbon dioxide
PaO_2	Partial pressure of arterial oxygen
PC_{20}	Concentration of bronchoconstrictor producing 20% fall in FEV_1
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGI_2	Prostacyclin
prn	As required
RAS	Renin-angiotensin system
RAST	Radioallergosorbant test
S	Salbutamol
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
Sm	Salmeterol
SSC	Standard saline citrate
TBS	Tris-buffered saline
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TOT	Total binding
TPA	Tissue plasminogen activator
VIP	Vasoactive intestinal peptide

1. INTRODUCTION

1.1 Asthma

Asthma is a chronic inflammatory disease of the airways characterised by the symptoms of cough, wheeze, dyspnoea and chest tightness. It can be classified as intrinsic, where no environmental cause can be identified, and extrinsic, where an identifiable environmental inducer is found (1). The physiological manifestation, bronchoconstriction, is caused by a number of factors: these include neural and chemical stimuli, inhaled bronchial irritants, stress, cold air and exercise.

1.1.1 *History of Asthma*

Asthma was first recognised 3,000 years BC in Ancient China with therapeutic use of the plant Ma Huang, a source of ephedrine, documented in 1,000 BC. The Ancient Egyptian Ebers papyrus refers to respiratory diseases including asthma in 1550 BC and around 100 AD in Ancient Greece, Aretaeus described asthma as "heaviness of the chest, difficulty breathing while walking on a steep road and wheeze" which worsened at night and tended to remit. Maimonides in 1190, advised the avoidance of humid air, the importance of maintaining an even temperament and avoiding excessive exercise and emotions. The importance of allergen avoidance was first noted by Cardamo in 1547 who advised the Archbishop of St. Andrews to remove his feather bedding (2).

In the seventeenth century, Van Helmont noted the association between wheezing and diet, season and dust exposure, Sir John Floyer identified the involvement of tobacco smoke and Thomas Willis described the pathology of asthma as "pneumonick" with obstruction of the bronchi by mucus and "convulsive" involving bronchoconstriction, also proposing the use of anti-spasmodics as a treatment (3).

Early asthma treatment included ipecacuanha, tobacco smoke, exercise, sea travel and strong coffee which was thought to help by preventing sleep but probably represents the first use of xanthines (2). In the late 1800's asthma was thought to be caused by vasodilatation, hence the use of vasoconstrictor agents such as cocaine, adrenaline, sympathomimetics and steroids, leading serendipitously to the discovery of modern bronchodilator therapy (4).

The use of steroids stems from early observations that stressful events with elevations of cortisol led to improvements in asthma such that physicians prescribed frequent cold bathing; an undoubtedly stressful experience! In 1900, Cohen was the first to treat asthma with steroids using dried bovine adrenal glands and noted their preventive effect but lack of efficacy in acute asthmatic attacks. However, it was many

years later before it was appreciated that glucocorticoids were responsible for this effect (4).

1.1.2 *Epidemiology*

Despite improved knowledge of the aetiology and pathophysiology of asthma, it remains a disease of increasing prevalence. The lack of a clear, globally accepted definition of asthma reflects the heterogeneity of the disease and its manifestations. Asthma can arise at any age and it is difficult to define its exact prevalence. In the United Kingdom, around 13% of children have a wheezing episode annually (5). A study from Tucson, U.S.A. revealed that 49% of children aged 0-6 years had wheeze, of which 20% were transient episodes. Those with persistent wheeze had a family history of asthma, raised IgE, atopy and positive skin prick testing (6). The International Study of Asthma and Allergies in Childhood (ISAAC) quotes a worldwide prevalence for wheezing in 13-14 year olds varying from 10% in Singapore up to 29% in Australia (7).

Persistence of childhood asthma into adulthood varies from 25% (8) to 72% in different studies (9). Those most likely to have persistent asthma have a low FEV_1, airway hyperresponsiveness and a personal or family history of atopy (10), while those with mild childhood asthma usually outgrow their disease. The increasing prevalence of wheezing may be due to increased sensitisation to inhaled allergens (11). Exposure to an increased allergen load, tobacco smoke and worsening air pollution have also been implicated. This may be compounded by lower rates of breast feeding, lower antioxidant intake, increased salt intake and reduced exposure to childhood infections (9).

1.1.3 *Aetiology*

Asthma is part of the atopic triad along with allergic rhinitis and eczema. In adults, wheezing is associated with low socio-economic status, smoking and a family history of atopic disease (12). A Norwegian twin study showed a relative risk of developing asthma as 17.9 for identical twins compared to 2.3 in non-identical twins, suggesting that genetic susceptibility accounted for 75% of the risk (13). Other studies have shown that traits for bronchial hyper-responsiveness and increased serum total IgE levels are co-inherited (14). The inheritance of asthma is probably polygenic (6) and environmental influences seem to be more important than genetic factors as supported by migrant studies, in-keeping with the increasing prevalence of the disease (9).

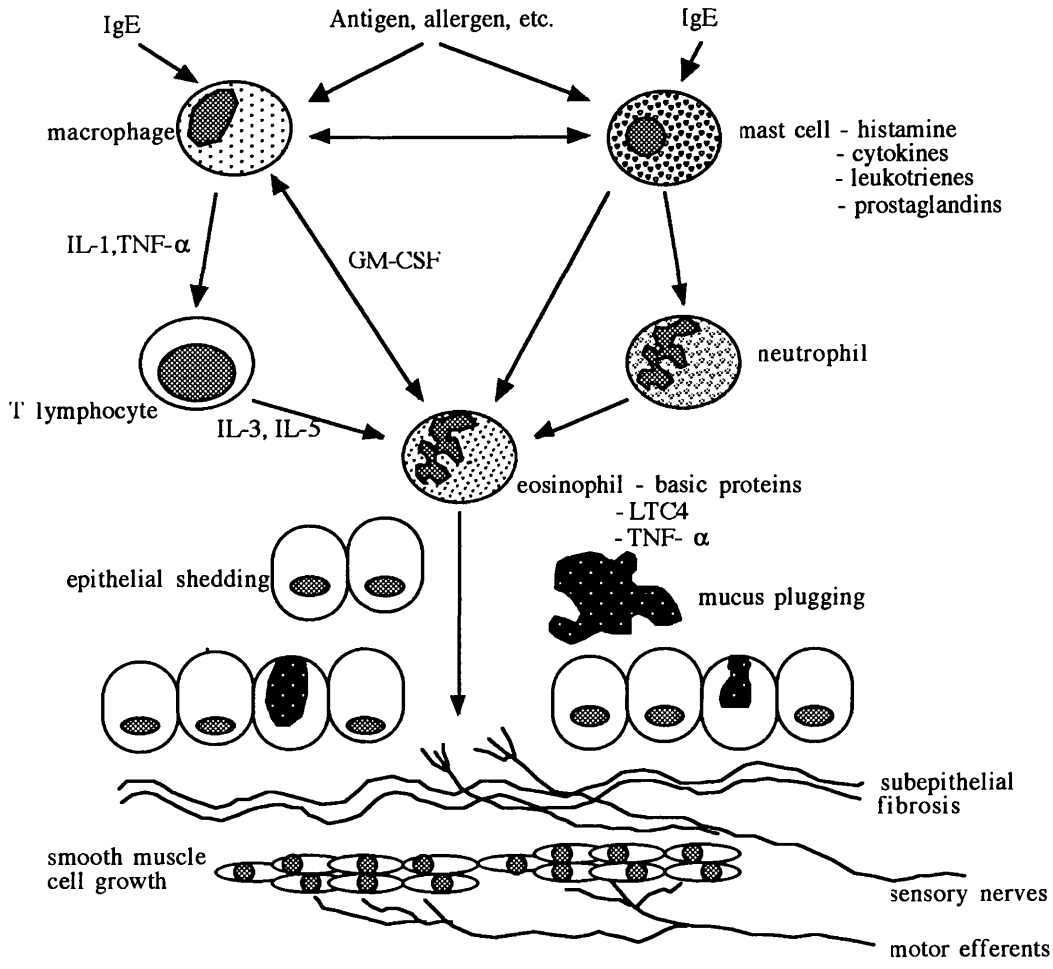
1.1.4 *Pathophysiology*

In asthma, airways are sensitised by exposure to inhaled allergens. This results in inflammation and bronchial hyper-reactivity, the hallmark of asthma. Attacks are triggered by exposure to allergens (e.g. house dust mite, cats, dogs, etc.), inhaled irritants (e.g. smoke, ozone, particulate air pollution), respiratory infections, exercise, stress and cold air. In acute asthma there is bronchoconstriction, microvascular leakage, mucosal oedema, vasodilation and mucus hypersecretion.

In atopic individuals there is overproduction of allergen-specific IgE. This binds to mast cells, basophils and to a lesser extent platelets, monocytes, eosinophils, lymphocytes and macrophages (1). Exposure of antigen to bound IgE causes mast cell degranulation with release of histamine, leukotriene C_4, prostaglandin D_2, interleukin-5, tryptase (15), granulocyte-monocyte colony stimulating factor (GM-CSF) and interleukin-3 (16) resulting in the bronchoconstriction of the early asthmatic response (17).

Eosinophils are key players in asthmatic inflammation and are involved in the late asthmatic response. Cytokines released from macrophages, T-lymphocytes, activated eosinophils and epithelial cells are responsible for the recruitment of eosinophils which adhere to vascular endothelium, then migrate into tissues where they are primed (17). Eosinophil degranulation releases basic proteins (including major basic protein, eosinophil cationic protein (ECP), eosinophil-derived neurotoxin and eosinophil peroxidase) and mediators (including platelet activating factor, leukotriene C_4, tumour necrosis factor-α, interleukin-5 and GM-CSF). ECP is increased in serum and broncho-alveolar lavage fluid in asthmatics (18). This results in increased capillary permeability, epithelial shedding and activation of neural reflexes via exposed sensory nerve endings (Figure 1).

Figure 1 *The pathological inflammatory processes occurring in the asthmatic airway.*



Adapted from Mac Cochrane *et al.* (19)

1.1.5 *Humoral Control of the Airways*

1.1.5.1 Histamine

Histamine has many actions in the airway and acts via three types of receptor (20). H_1-receptors mediate airway smooth muscle contraction, increased vascular permeability, increased cyclic GMP and synthesis of prostaglandin E_2 & $F_{2\alpha}$ (21). H_2-receptors increase airway mucus secretion, increase cyclic AMP and cause bronchodilatation in some species. H_3-receptors have an inhibitory effect on parasympathetic ganglionic neurotransmission, an inhibitory effect on mast cell histamine synthesis and may serve to limit unnecessary activation of neural reflexes by the physiological basal release of histamine from mast cells (22).

Asthmatics are 100-fold more sensitive than normals to histamine-induced bronchoconstriction and inhaled histamine bronchial provocation testing is used in the diagnosis of asthma. In addition to H_1-receptor mediated bronchoconstriction, histamine also stimulates irritant receptors and activates vagal afferent nerves eliciting vagal reflex bronchoconstriction (22). Histamine and methacholine (an acetylcholine analogue) have additive effects on bronchoconstriction and histamine selectively increases α_2-adrenergic responses in airway smooth muscle.

1.1.5.2 Endothelin-1

Endothelin-1, a potent vasoconstrictor, is increased in symptomatic asthmatics (23) and elevated in both plasma (24) and bronchoalveolar lavage fluid (25) in acute asthma. Endothelin-1 is also a potent bronchoconstrictor *in vitro* (26) and *in vivo* is 100-fold more potent than methacholine when inhaled by asthmatics (27). *In vitro*, endothelin-1 is a mitogen for airway smooth muscle in many species (28, 29) including humans (30, 31) and potentiates the mitogenic effects of other mediators (31, 32). Endothelin-1 is secreted by cultured human bronchial epithelial cells (23) and both type ET_A and ET_B endothelin-1 receptors are present on human airway smooth muscle cells *in vitro* (33) where ET_B receptors predominate in a ratio of around 2:1 (31). The ET_A receptor is thought to mediate growth promotion and the ET_B receptor is involved in bronchoconstriction (31).

1.1.5.3 Leukotrienes

Leukotrienes are implicated in the pathogenesis of asthma. They are synthesised by eosinophils, mast cells, macrophages, monocytes and basophils from arachidonic acid by the action of the enzyme 5-lipoxygenase to form LTA_4. This is subsequently converted to either LTB_4 by the action of LTA_4 hydrolase, or LTC_4 by the action of LTC_4 synthase. The role of LTB_4 in asthma is unclear but it is chemotactic for neutrophils and eosinophils. The cysteinyl-leukotrienes (LTC_4, LTD_4 and LTE_4) cause bronchoconstriction, mucus secretion from goblet cells and submucous glands, increase microvascular permeability, induce airway hyperresponsiveness and attract eosinophils. As bronchoconstrictors, they are 100-1000 times more potent than histamine. LTD_4 is the most potent and LTE_4 has the most prolonged activity (34).

Following allergen challenge LTC_4, LTD_4 and LTE_4 levels are elevated in broncho-alveolar lavage fluid from asthmatics (35). Elevated urinary LTE_4 levels (a metabolite of LTD_4) are found during acute exacerbations of asthma (36) and in aspirin-sensitive asthma (37). Leukotrienes may also play a role in remodelling of the asthmatic airway (38).

The most recent asthma treatments to be licensed are aimed at leukotrienes: these are cysteinyl-leukotriene receptor antagonists such as zafirlukast, montelukast and pranlukast, and 5-lipoxygenase inhibitors such as zileuton. These agents reduce symptoms, have additive effects to β_2-agonists, have a steroid-sparing effect and are of value in aspirin-sensitive and exercise-induced asthma (34).

1.1.5.4 Catecholamines

Circulating adrenaline may have a role in maintaining basal bronchomotor tone by reducing bronchial smooth muscle tone via β_2-adrenoceptors and prevents acetylcholine release from cholinergic nerves. Its role in acute asthma is unclear as circulating levels of adrenaline may or not be elevated (39). Following exercise, adrenaline increases causing bronchodilation in both normal and asthmatic volunteers, possibly antagonising the bronchoconstrictor effects of exercise in asthma (40). Noradrenaline and dopamine have no effect on bronchomotor tone in asthmatics (40).

1.1.5.5 Others

Atrial natriuretic peptide (ANP) causes natriuresis, diuresis, vasodilatation and inhibits aldosterone, angiotensin II and endothelin. ANP receptors exist on bronchial smooth muscle and ANP causes bronchodilation and protection against histamine- and distilled water-induced bronchoconstriction (40). Thyroid hormone excess worsens asthma control possibly by downregulating β-adrenoceptor responsiveness, altering arachidonic acid metabolism and weakening respiratory muscles (40). Progesterone may be beneficial for women with pre-menstrual exacerbations of asthma, but otherwise shows no association with pulmonary function. Oestrogen may have a steroid-sparing effect in post-menopausal asthma, but hormone replacement therapy has also been associated with an increased risk of late-onset asthma (40).

1.1.6 *Neural Control of the Airways*

There are three mechanisms of neural control of the airways: adrenergic control is mediated by circulating adrenaline released from the adrenal medulla and noradrenaline released from adrenergic neurones. In the airways, adrenergic neurones are sparse but innervate bronchial blood vessels, submucosal glands and may modulate neurotransmission in parasympathetic ganglia. Constriction of airway smooth muscle is mediated by α-adrenoceptors but this is not thought to have a major role in asthma. Bronchodilatation is mediated by β_2-adrenoceptors and it has been suggested that there may be a defect in β_2-adrenoceptor function in asthmatic airway smooth muscle (1). β_2-

adrenoceptors are also present on cholinergic neurones where they inhibit acetylcholine release (22).

Inhibitory non-adrenergic non-cholinergic (i-NANC) nerves cause airway smooth muscle relaxation. Nitric oxide is the bronchodilator neurotransmitter and antagonises acetylcholine *in vitro* (41). Excitatory NANC nerves may cause bronchoconstriction via the release of substance P (1).

Vagal parasympathetic nerves mediate cholinergic airway control and contribute to basal airway tone, bronchoconstriction following stimuli, ciliary movement and submucosal gland secretion. Nicotinic receptors mediate cholinergic neurotransmission within parasympathetic ganglia and facilitate the release of neuropeptides from sensory neurones (22). Muscarinic receptors mediate the majority of cholinergic effects in the airway.

Five types of muscarinic receptors have been cloned but the pharmacological effects are known for only three. M_3-muscarinic receptors predominate in proximal airways and mediate bronchoconstriction. M_2-receptors on pre-synaptic cholinergic nerve terminals provide negative feedback by inhibiting acetyl choline release. They may be defective in asthma. They also inhibit noradrenaline release from adrenergic nerves (22) and M_2-receptor function is also inhibited by eosinophil major basic protein (41) and influenza virus (22). M_1-receptors have a facilitatory effect on parasympathetic ganglia and inhibit noradrenaline release from sympathetic nerves (22).

Cholinergic activity is also inhibited by adrenergic agonists acting on inhibitory β_2-adrenoceptors on cholinergic nerves and by prostaglandin E_2. However, acetylcholine can augment histamine release after allergen challenge and promote leukotriene release *in vitro* (42).

1.1.7 *Modulation of Neurotransmission in the Airways*

Bronchoconstriction or bronchodilatation is the end result of a complex interaction of many influences on the neurones controlling the airways and also direct stimulation of airway smooth muscle. Many facilitatory and inhibitory influences act simultaneously, converging on a final common pathway such as an intracellular second messenger or an ion channel.

There are many potential sites for modulation of neurotransmission and the effects at these sites vary widely between species. In the airway wall, parasympathetic

ganglia are innervated by sympathetic nerves, sensory nerves and are susceptible to the actions of inflammatory mediators. Neurotransmitter release from pre- and post-ganglionic parasympathetic neurones, noradrenaline release from adrenergic neurones, neuropeptide release from C-fibres and modulation of sensory nerves can occur (22). The majority of the work in this field has been carried out on many different species *in vitro* and therefore may not be applicable *in vivo* to human asthmatics.

In parasympathetic ganglia, cholinergic neurotransmission may be inhibited by β_2-adrenoceptors, facilitated by M_1-muscarinic receptors and inhibited by H_3-histamine receptors (22). However, histamine can facilitate ganglionic neurotransmission in guinea pig airways *in vitro* via acetylcholine release (43). Prejunctional cholinergic neurones are inhibited by α_2-adrenoceptors, β_2-adrenoceptors, M_2-muscarinic receptors, vasoactive-intestinal peptide (VIP), PGE_2, nitric oxide and opioids (22), and potentiated by angiotensin II (44), thromboxane, substance P, neurokinin A, $PGF_{2\alpha}$ and PGD_2 (22).

Co-transmitters can affect other neurotransmitters; neuropeptide Y amplifies sympathetic neuronal effects and VIP is an inhibitory co-transmitter with acetylcholine. Afferent sensory neurones may be sensitised by PGE_2, PGI_2, arachidonic acid, bradykinin, histamine and acetylcholine via nicotinic receptors, and inhibited by opioids (22).

The clinical relevance of neuromodulation in asthma remains to be clarified. In asthma, many different factors interact simultaneously with inflammatory mediators playing an important role, however *in vitro* studies examine individual factors in isolation. Further work on human airway tissue both *in vitro* and *in vivo* is required.

1.2. Airway Remodelling In Asthma

1.2.1. *Airway Remodelling*

Vascular congestion and local fluid exudation in acute inflammation, and connective tissue deposition and epithelial metaplasia in chronic inflammation, contribute to airway wall thickening (45). In chronic asthma, airway remodelling contributes to airflow obstruction by an increase in airway smooth muscle (46), increased volume of mucous glands (47), epithelial loss (48) and subepithelial fibrosis (49). Thickening of the subepithelial region is due to collagen deposition by myofibroblasts (49) beneath the basement membrane (50) and not thickening of the basement membrane as previously believed. This may be mediated by transforming

growth factor-β_1 (TGF-β_1) which is expressed by bronchial epithelial cells (51). These changes may be reversible as airway wall thickening in occupational asthma regresses if antigen exposure ceases (52).

Thickening of the airway wall results in greater narrowing of the lumen and even occlusion of the airway for a given contraction of smooth muscle (53). Asthmatic airways have fewer submucosal folds than normal airways resulting in a reduced load on the airway smooth muscle thus allowing greater luminal narrowing for a given contraction (54). In addition, exudation of plasma into the airway lumen increases the surface tension causing instability (55).

1.2.2 *The Functional Role Of Airway Smooth Muscle*

In normal human lungs there is relatively more smooth muscle in the peripheral than the central airways suggesting a regulatory function for airflow in the former region (56). In isolated canine lungs the site of maximal airway narrowing is the intermediate airways (57). A computer model of airway narrowing confirms that peripheral airways contribute most to airway resistance (58).

It is believed that bronchial smooth muscle is the most important functional component of airway wall thickening in asthma and contributes to airway hyper-responsiveness (53, 59, 60). Early pathological studies of asthmatic lungs found a 3-fold increase in bronchial smooth muscle compared with normals. Increased volume and muscle fibre number suggests hyperplasia as the main pathogenic mechanism (46, 61). In fatal asthma there is a general increase in airway wall thickness compared to lungs from asthmatics who have died from other causes. This is particularly true in smaller bronchi (60) where there is a significant increase in bronchial smooth muscle volume (47). Emerging evidence suggests that there are different patterns of airway smooth muscle thickening: Type I where hyperplasia occurs only in larger bronchi and Type II where hypertrophy occurs throughout the bronchial tree, increasing peripherally (62). In isolated canine lungs, smaller airways have greater reactivity to histamine than larger airways (63).

However, Bramley and colleagues (64) have shown a similar amount of lobar bronchial smooth muscle in asthmatic and normal airways, but a 3-fold increase in shortening in the asthmatic airway smooth muscle. Axial sections of 2nd to 4th generation large human airways show no evidence of increased airway smooth muscle in asthmatics but reveal a substantial component of extracellular matrix and connective tissue within the airway smooth muscle (65). Therefore excessive bronchoconstriction

in asthma is not solely due to excess agonist stimulation but may involve post-junctional factors within airway smooth muscle (66) and reduced load by decreased airway elasticity. Reduced elasticity may be due to destruction of extracellular matrix by proteases released from inflammatory cells (64). *In vitro*, collagenase treatment of human airway smooth muscle allows greater muscle shortening (67). Reduced subepithelial elastin may also contribute to decreased elasticity (64).

Adventitial thickening may facilitate uncoupling of the elastic recoil forces from surrounding parenchymal lung tissue permitting excessive smooth muscle shortening (53, 68). This could explain the discrepancies found between airway smooth muscle shortening *in vivo* and *in vitro* (69).

1.2.3 *Mechanism of Airway Smooth Muscle Growth*

In asthma, the mechanism of airway smooth muscle thickening remains controversial. There are similarities to arterial remodelling in cardiovascular disease (70) such as hypertrophy of vascular smooth muscle in hypertension. Indeed, physical stretching stimulates proliferation of airway smooth muscle cells *in vitro* (71). Furthermore it has been hypothesised that "overwork" of airway smooth muscle cells due to hyper-reactivity may result in hypertrophy (62) but there is no evidence to support this. Biopsies from human asthmatic bronchi show an association between airway smooth muscle proliferation *in vivo* and inflammatory cell counts from bronchoalveolar lavage fluid suggesting a role for inflammatory mediators and cytokines (72).

Several growth promoters have now been implicated in airway smooth muscle cell proliferation but much of this work has been carried out *in vitro* and it is not known if these results can be extrapolated to airway growth *in vivo*. Growth of airway smooth muscle cells may involve phenotypic modulation from contractile to synthetic phenotypes which has been demonstrated *in vitro*. The synthetic phenotype has greater mitogenic activity and may be more excitable (73).

1.2.4 *Promoters of Airway Smooth Muscle Cell Growth*

As discussed above, in chronic asthma many inflammatory cytokines, growth factors and peptides affect cell growth: these are summarised in Table I. Several of these growth promoters have been identified in airway tissues: Platelet-derived growth factor (PDGF) is the most important circulating mitogen (74) and occurs in bronchial epithelial cells (51) and in interstitial monocytes and macrophages. PDGF β-receptors

are present on mononuclear interstitial cells, the baso-lateral membrane of epithelial cells (75) and have been identified on sheep airway smooth muscle cells *in vitro* (76).

Endothelin-1 has been found in cultured human asthmatic bronchial epithelial cells and the endothelin-1 receptor has been identified on human (23) and sheep airway smooth muscle cells with the ET_A receptor subtype predominating (29). Circulating GM-CSF is elevated in bronchoalveolar lavage fluid from asthmatics (77), is present in asthmatic bronchial epithelial cells (78) and can stimulate fibroblast growth *in vivo* in rats (79). Eosinophils can stimulate human fibroblast growth *in vitro* (80). Interleukin-1β is elevated in asthmatic broncho-alveolar lavage fluid (81) and stimulates guinea pig airway smooth muscle cell growth *in vitro* (82, 83). Both insulin-like growth factor (IGF) and TGF-β_1 are expressed in bronchial epithelium in normal and asthmatic airways (51).

Several growth promoters are known to have synergistic effects on cell growth *in vitro*. Endothelin-1 has synergistic effects with PDGF and epidermal growth factor (EGF) *in vitro* (31, 32, 84). Bradykinin and LTD_4 potentiate the effect of EGF on human airway smooth muscle cells *in vitro* (31).

Table I *Promoters of airway growth.*

Mitogen	Cell Type	Experiment	Species	Reference
PDGF	ASMC	*In vitro*	Sheep	(76)
Leukotriene D_4	ASMC	*In vivo*	Rat	(59)
Leukotriene D_4	ASMC	*In vitro*	Human	(85)
Endothelin-1	ASMC	*In vitro*	Rabbit	(86)
Endothelin-1	ASMC	*In vitro*	Sheep	(29)
Endothelin-1	ASMC	*In vitro*	Human	(33)
Histamine	Fibroblasts	*In vitro*	Human	(87)
Histamine	ASMC	*In vitro*	Dog	(88)
Tryptase	ASMC	*In vitro*	Dog	(89)
T lymphocytes	ASMC	*In vitro*	Human	(90)
β-Hexosaminidase	ASMC	*In vitro*	Cow	(91)
Interleukin-1β	ASMC	*In vitro*	Guinea pig	(83)
EGF	ASMC	*In vitro*	Guinea pig	(92)
EGF	ASMC	*In vitro*	Cow	(93)
EGF	ASMC	*In vitro*	Human	(94)
Thromboxane A_2	ASMC	*In vitro*	Rabbit	(28)
Thrombin	ASMC	*In vitro*	Human	(95)
IGF-1	ASMC	*In vitro*	Rabbit	(96)
IGF-1	Fibroblasts	*In vitro*	Human	(97)
TNF-α	ASMC	*In vitro*	Human	(98)
Substance P	ASMC	*In vitro*	Rabbit	(99)
Serotonin	ASMC	*In vitro*	Dog	(100)
Angiotensin II	ASMC	*In vitro*	Human	(101)
Physical stress	ASMC	*In vitro*	Dog	(71)
Hyperoxia	ASMC	*In vivo*	Rat	(102)
Hyperoxic BALF	ASMC	*In vitro*	Rat	(103)
Isocyanates	ASMC	*In vivo*	Human	(52)
Inhaled allergen	ASMC	*In vivo*	Cat	(104)

Abbreviations: PDGF - Platelet-derived growth factor; ASMC - Airway smooth muscle cell; EGF - Epidermal growth factor; IGF - Insulin-like growth factor; TNF - Tumour necrosis factor; BALF - Bronchoalveolar lavage fluid

Only a very limited number of studies have used *in vivo* models to assess cell growth within the airways. In sensitised cats, repeated allergen challenge causes histological changes similar to those found in human asthmatics with increased airway smooth muscle (104). Similar experiments in rats result in increased airway smooth muscle and bronchial responsiveness which correlates in the larger airways only (59). Occupational exposure to inhaled isocyanates results in structural changes in the airway wall which are reversible upon avoidance of further antigen exposure (52).

Exposure of rat airways to hyperoxia (FiO2 >95%) results in an increase in the epithelial and airway smooth muscle cells with a significant increase in DNA synthesis and bronchial hyper-responsiveness (102). Bronchoalveolar lavage fluid obtained from hyperoxic rat airways also stimulates rat airway smooth muscle cell growth *in vitro* (103).

1.2.5 *Inhibitors Of Airway Smooth Muscle Growth*

Several inhibitors of airway smooth muscle cell growth are listed in Table II.

Table II *Inhibitors of airway smooth muscle growth.*

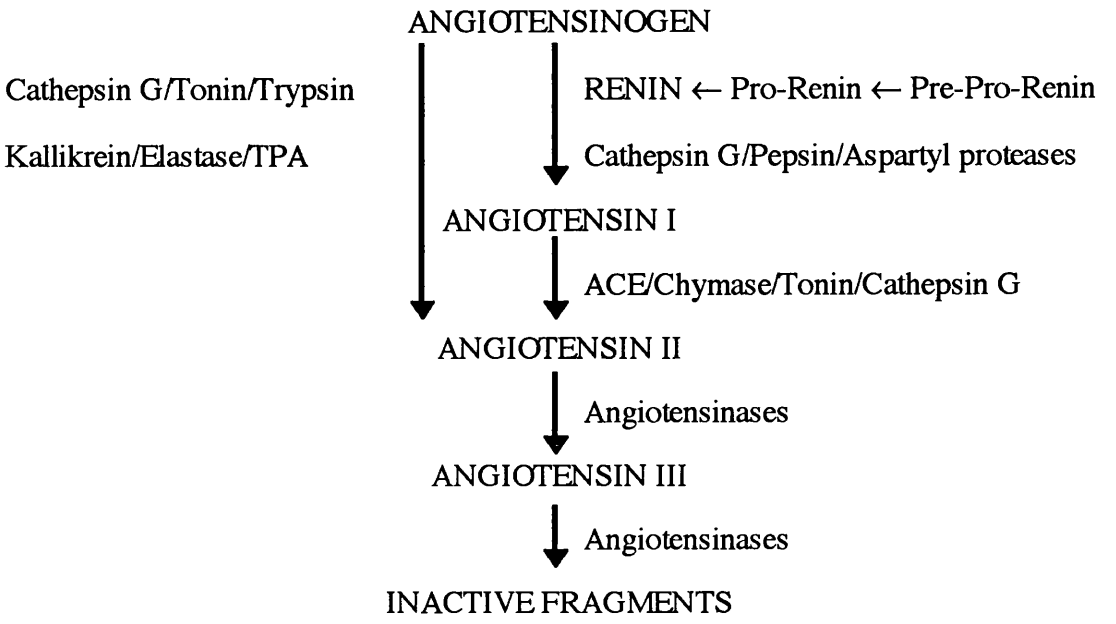
Growth inhibitors	Cell type	Experiment	Species	Reference
Isoprenaline	ASMC	*In vitro*	Human	(105)
Salbutamol	ASMC	*In vitro*	Human	(30)
Heparin	ASMC	*In vitro*	Cow	(106)
Heparan sulphate	ASMC	*In vitro*	Cow	(106)
Fragmin	ASMC	*In vitro*	Cow	(106)
Nedocromil sodium	ASMC	*In vitro*	Rat	(107)
LTD_4 antagonist (MK-571)	ASMC	*In vitro*	Rat	(38)
VIP	ASMC	*In vitro*	Human	(108)
TGF-β_1	ASMC	*In vitro*	Human	(109)

Abbreviations: ASMC - Airway smooth muscle cell; LTD - Leukotriene D; VIP - Vasoactive intestinal peptide; TGF - Transforming growth factor.

1.3 The Renin-Angiotensin System

The renin-angiotensin system is primarily involved in sodium and water homeostasis but also has an important role in cardiovascular disease. Angiotensin II, the active octapeptide hormone is the end result of a cascade shown in Figure 2 beginning with angiotensinogen.

Figure 2 *The renin-angiotensin system cascade.*



Angiotensinogen is synthesised primarily in the liver and circulates in plasma. Its production increases in response to angiotensin II (110), acute inflammation (111), glucocorticoids, haemodilution, oestrogens and hypoxia (112). Angiotensinogen is cleaved by renin to angiotensin I which is in turn converted by angiotensin-converting enzyme (ACE) to angiotensin II, which is subsequently degraded by angiotensinases to angiotensin III and inactive peptide fragments (Figure 2).

Direct cleavage of angiotensinogen to form angiotensin II can also be achieved by the action of the enzymes human neutrophil cathepsin G (113), tonin (114), trypsin (115), kallikrein, elastase and tissue plasminogen activator. In addition, cathepsin G, cathepsin D (116) pepsin (117), chymase (118, 119) and aspartyl proteases can convert angiotensinogen to angiotensin I. Conversion of angiotensin I to angiotensin II is also mediated by cathepsin G and mast cell chymase (120). An alternative ACE-independent pathway for the formation of angiotensin II occurs during exercise (121).

Renin is a peptidase, first identified 100 years ago. Its release is increased by renovascular receptors responding to alterations in renal perfusion pressure, macula densa renal tubule receptors responding to alterations in sodium, stimulation of renal α- and β-adrenoceptors, neurohypophyseal hormones, catecholamines and cardiopulmonary receptors. Renin release is suppressed by atrial natriuretic factor, adenosine, angiotensin II and vasopressin. Renin is predominantly synthesised in the juxta-glomerular cells in the kidneys then stored until required (122). Many other tissues are a source of renin including the brain (123), arteries, veins, heart (124), adrenal glands (125), submandibular glands (126), testes (127), ovaries (128) and uterus (129).

Circulating angiotensin II is formed mainly in the pulmonary capillaries where up to 80% of ACE conversion occurs due to the high concentration of endothelial cells (130). Angiotensin II acts on the adrenal gland to release aldosterone but also has many other actions: It is a potent vasopressor, stimulates growth of vascular smooth muscle and other tissues (see Section 1.5), acts on the liver to stimulate glycogenolysis, gluconeogenesis and angiotensinogen synthesis, stimulates prostaglandin formation in the rat renal medulla and acts as a neurotransmitter in the brain to increase blood pressure, stimulate drinking, increase salt appetite and to release pituitary hormones including adreno-corticotrophic hormone, oxytocin and luteinising hormone (131). Angiotensin II is also a weak bronchoconstrictor (132) and induces endothelin-1 secretion (133). The renin-angiotensin system is also stimulated by physical exercise (121, 134), especially in the presence of hypoxia (135).

Angiotensin II receptors are coupled to G proteins and are found in vascular smooth muscle, adrenal cortex and medulla, kidney, myocardium, liver, brain, pituitary, hypothalamus, platelets, monocytes, macrophages (131), uterus, gut, gonads and lung (136, 137). There are three angiotensin II receptor subtypes with the type 1 receptor (AT_1) predominating. These are further subdivided into AT_{1A} and AT_{1B} which mediate most known actions of angiotensin II. The type 2 receptor (AT_2) mediates an inhibitory effect of angiotensin II on cell proliferation in some tissues (138) and may be involved in fetal growth (139). The function of the type 3 (AT_3) receptor is not known. It has extra sites for protein kinase C phosphorylation and may be involved in the differential regulation of angiotensin II receptors in response to variations in dietary sodium (140).

1.4 The Renin-Angiotensin System In Asthma

The renin-angiotensin system is activated in acute severe asthma although not in stable asthmatics (40, 132). The reason for this and the mechanisms involved are unclear.

When infused intravenously, angiotensin II causes bronchoconstriction in mild asthmatics (132). *In vitro* it constricts human (141) and bovine bronchial rings (26). Angiotensin II potentiates the bronchoconstrictor effects of methacholine on human airway *in vivo* and *in vitro* (141) and endothelin-1 on bovine airway *in vitro* (26). It also potentiates vagally mediated contraction of rabbit airway smooth muscle via a pre-junctional effect on cholinergic nerve terminals (44).

Inhaled and intravenous salbutamol raise plasma angiotensin II levels by an ACE-dependent mechanism (142, 143). Plasma levels of salbutamol in acute severe asthma vary widely (144), however plasma levels of angiotensin II induced by salbutamol experimentally do not attain the values seen in acute severe asthma, therefore additional mechanisms seem to be involved. Catecholamine release, hypoxia, electrolyte imbalance, hypotension, vasodilatation and the influence of other circulating mediators may have a role. Angiotensin II could also be formed by tissue chymases and inflammatory proteases in acute asthma.

Recent work has shown that angiotensin II induces human airway smooth muscle cell proliferation and hypertrophy *in vitro* (101). Therefore it is possible that angiotensin II has a role in airway remodelling.

1.5 The Role Of The Renin-Angiotensin System In Remodelling

The renin-angiotensin-aldosterone system is one of the most important growth promoters. Angiotensin II stimulates growth of fibroblasts, bovine adrenocortical cells (145), cardiac myocytes, renal tubular cells (146) and can increase collagen and fibronectin synthesis by vascular smooth muscle cells *in vitro* (147). Angiotensin II is mitogenic for vascular smooth muscle *in vitro* causing hyperplasia and hypertrophy (148) mediated by the AT_1 subtype of angiotensin II receptor (149). *In vivo* angiotensin II increases vascular smooth muscle cell proliferation both in normal arteries (150) and following intimal injury in rats (151).

The vascular myointimal hyperplasia in response to angiotensin II *in vivo* may be mediated by induction of both PDGF (which has proliferative effects) and also TGF-β_1 (which has anti-proliferative effects)(109), with a resultant net hypertrophic effect.

Vascular endothelium releases prostacyclin and nitric oxide which antagonise these actions, but endothelial injury may permit angiotensin II to cause vascular smooth muscle cell proliferation (152). A similar situation could operate in the airways as airway epithelium expresses TGF-β_1 mRNA (51, 153) and epithelial shedding is a recognised feature of asthma (48).

Angiotensin II has also been implicated in cardiac remodelling following myocardial infarction, in left ventricular hypertrophy and in arterial medial thickening in hypertension. In the arterial wall the effect of angiotensin II on vascular smooth muscle cells is independent of blood pressure (154) and is increased following intimal injury. ACE inhibitors and angiotensin II receptor antagonists inhibit angiotensin II-mediated vascular smooth muscle cell proliferation *in vivo* and *in vitro* (149, 155) and can inhibit cardiac muscle hypertrophy in hypertensive rats (145).

1.6 Evidence For A Local Renin-Angiotensin System In The Lungs

Most tissues that respond to the circulating renin-angiotensin system also have the capacity to synthesise its components. Local renin-angiotensin systems are established in the kidney (124), brain (123), heart, vasculature (124), adrenal glands (125), salivary glands (126), testes (127), ovary (128) and uterus (129, 156). The actions of circulating angiotensin II in asthma causing airways constriction (132) and pulmonary vasoconstriction (157) suggest that the lungs may have a local renin-angiotensin system.

It is well known that the pulmonary capillaries are rich in ACE (158, 159). Renin has been identified in human lung vasculature around a variety of pulmonary tumours (160), in an anaplastic pulmonary adenocarcinoma (161), in an oat cell bronchial carcinoma (162) and in a pulmonary metastasis from an epithelioid sarcoma (163). Renin mRNA has also been isolated from rat lung (164).

Angiotensinogen mRNA (165) and AT_2 receptor mRNA have been isolated from rat lung (139). AT_1 angiotensin II receptor mRNA has been extracted from human lung (137), adult rat (166) and fetal rat lung (167). AT_1 receptors have been localised immunohistochemically to bronchial muscle in rat lungs (136). The AT_{1A} receptor subtype accounts for 85% of the angiotensin II receptor mRNA expressed (168). A third type of angiotensin II receptor (AT_3) has also been demonstrated in rat lung (140).

However, like other local renin-angiotensin systems, a local pulmonary system would be unlikely to make a significant contribution to circulating levels of renin and

angiotensin II; however, it could influence airway bronchoconstriction and remodelling at a tissue level.

1.7 The ACE Gene Insertion/Deletion Polymorphism

Angiotensin-converting enzyme is a zinc metallo-peptidase which cleaves the dipeptide His-Leu from angiotensin I and is responsible for the generation of the majority of angiotensin II. It also inactivates bradykinin and substance P. Most ACE is found in the lungs and is membrane-bound on endothelial, epithelial and neuro-epithelial cells (169). It is also found in T-lymphocytes (170) and free in plasma, amniotic and seminal fluids (169).

There is a wide variation in serum ACE activity within the general population and around 50% of this can be attributed to a variation in genotype (169). A polymorphism of the ACE gene involving the insertion or deletion of a 287 base-pair repeat sequence within intron 16 has been identified (171). The deletion homozygote (DD) has twice the ACE activity of the insertion homozygote (II) with the heterozygote (ID) intermediate (169). Ueda *et al.* (172) confirmed this finding demonstrating higher ACE activity *in vivo* for the DD genotype, with a greater rise in blood pressure following infusion of angiotensin I. It is also suggested that this polymorphism could affect individual responsiveness to ACE inhibitors.

There is now an extensive literature examining the association of the ACE gene polymorphism with predominantly cardiovascular disease. The ACE DD genotype is associated with idiopathic and ischaemic dilated cardiomyopathy (173), fatal myocardial infarction and sudden cardiac death (174), left ventricular hypertrophy (with the strongest association in normotensive subjects) (175), diabetes, sudden cardiac death from hypertrophic obstructive cardiomyopathy (176) and coronary arterial disease but not angiographic coronary arterial stenosis (177). Cardiac tissue ACE activity is highest in those with the DD genotype in non-cardiac deaths (178).

A meta-analysis of ACE genotype and myocardial infarction confirms that the DD homozygote confers an increased risk with an odds ratio of 1.26. However, the clinical importance of this as an independent risk factor is unclear with the authors pointing out that many of the studies are retrospective resulting in a survival bias (179), although the risk appears highest in those with few other conventional risk factors (180).

An association has been demonstrated between the DD genotype and common carotid artery intimal and medial thickness although the difference is small (12%) and unlikely to be of clinical significance as no difference was found in the internal carotid arteries or in plaque formation (181).

Although an association has been shown in the above instances this does not prove causality and the mechanisms involved remain to be clarified. There may yet be hitherto unidentified mutations of the ACE gene within the 10,000-100,000 base pair range which could be involved in the variation in ACE activity (177).

Other polymorphisms of the renin-angiotensin system have been identified including that of the AT_1 angiotensin II receptor which has a small association with hypertension (182) and the angiotensinogen gene T235 variant which is associated with an increased risk of hypertension, coronary heart disease and myocardial infarction independent of ACE genotype (183). The possibility of synergistic increased risk of myocardial infarction between ACE DD genotype and a variant of the AT_1 receptor gene has also been suggested (184).

The ACE gene polymorphism may thus partly explain the variation in levels of angiotensin II found in asthma.

1.8 Salt And Asthma

The association between salt intake and asthma is an area of controversy. A high salt intake may be associated with an increase in wheezing and increased bronchial hyper-responsiveness. This is related to the sodium rather than the chloride component and the association is stronger in men than in women. One explanation may be the reduction in circulating catecholamines which occurs with salt loading (185).

The renin-angiotensin system is involved in salt and water homeostasis and is activated by a low salt intake. This would increase angiotensin II which is known to have an effect on asthmatic airways (132), upregulate adrenal zona glomerulosa angiotensin II receptors but downregulate vascular smooth muscle cell receptors (131). Indeed rats on a low sodium diet have increased plasma renin activity and increased vascular DNA synthesis in the media of the mesenteric arteries (186).

Conversely, if a high salt intake is associated with asthmatic symptoms and causes lower angiotensin II levels due to negative feedback, it would appear at first glance that the bronchoconstrictor effects of angiotensin II may not be of primary

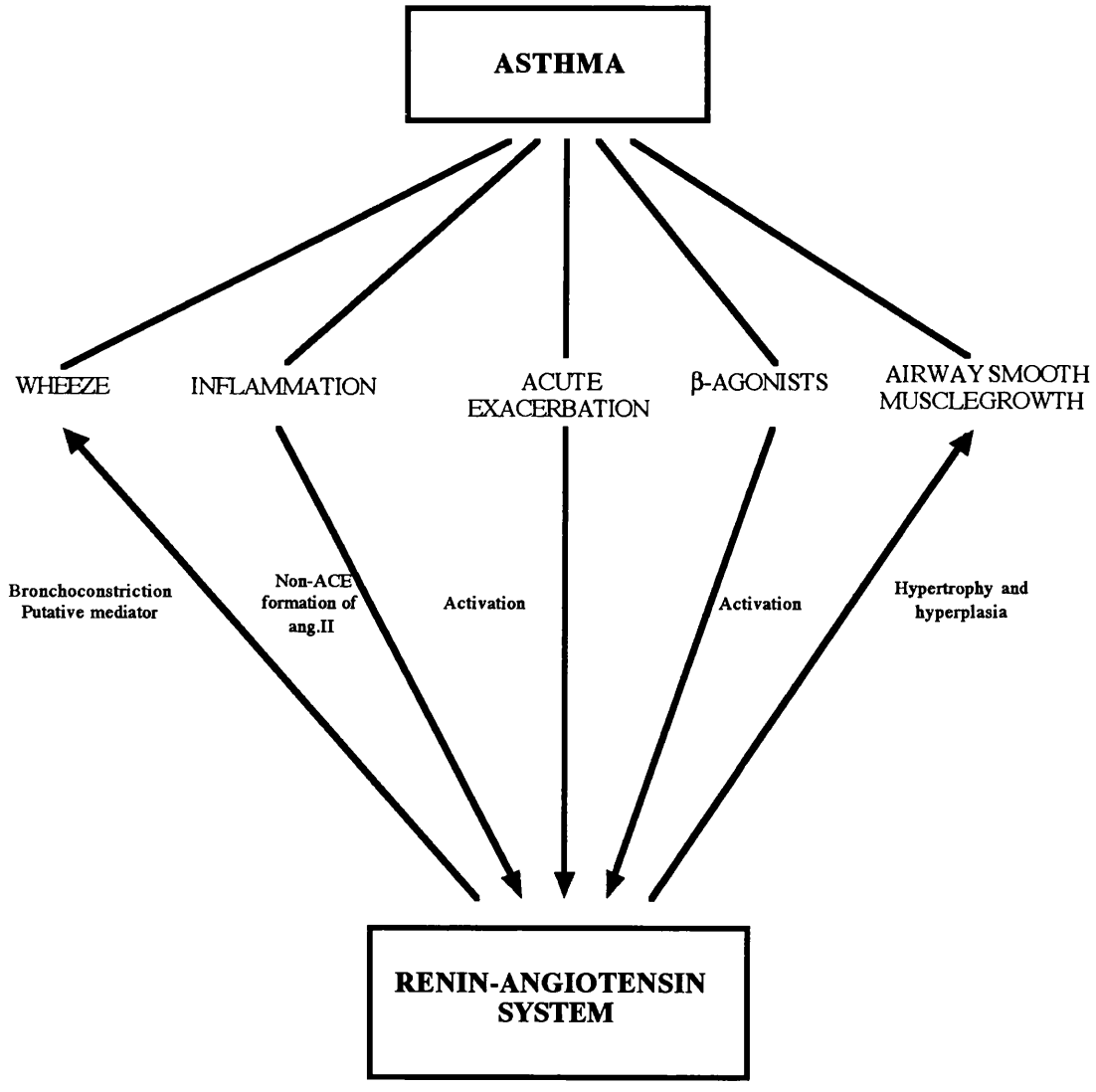
importance in the salt and asthma equation. However, since a high salt intake upregulates vascular smooth muscle cell angiotensin II receptor density it may also do the same for airway smooth muscle cells, thereby increasing the sensitivity to angiotensin II.

2. HYPOTHESES

2.1 Hypotheses

Asthma is a complex disease, the clinical manifestations of which are the net result of the interaction of many different mediators and cytokines involving multiple cell types and tissues. The renin-angiotensin system appears to play a part within this complicated *milieu* but its precise role is unclear. Figure 3 represents the schematic relationship between asthma and the renin-angiotensin system postulated by the work of this thesis.

Figure 3 *The proposed relationship between the renin-angiotensin system and asthma.*



The hypotheses to be tested in the work of this thesis are as follows:

2.1.1 Activation of the renin-angiotensin system in acute severe asthma may be due primarily to the large amounts of β_2-agonist medication administered during attacks. However, many different factors are altered during acute attacks of asthma which may also play a role in activating the renin-angiotensin system, possibly by synergistic mechanisms. These include endogenous catecholamines, hypoxia, hypotension, inflammatory mediators, proteases and cytokines, which will also be measured and correlated.

2.1.2 Angiotensin II which is known to have a putative effect on the bronchoconstrictors methacholine and endothelin-1 may also potentiate the effects of histamine, another clinically important bronchoconstrictor, in the human airway both *in vitro* and *in vivo*.

2.1.3 Given that most organs which respond to the renin-angiotensin system also appear to have local tissue systems, it would seem logical to suggest that the lungs also have a local renin-angiotensin system. This would necessitate the presence of angiotensinogen, renin, ACE and angiotensin II receptors within lung tissue.

2.1.4 Angiotensin II is a growth promoter for airway smooth muscle *in vitro* and thus may cause remodelling of the rat airway *in vivo* by subcutaneous infusion and by endogenous activation of the renin-angiotensin system by dietary sodium depletion.

2.1.5 The homozygote deletion genotype of the ACE gene insertion/deletion polymorphism is associated with higher plasma levels of angiotensin II and thus may be more prevalent in asthmatics, particularly those with activation of the renin-angiotensin system.

2.2 Aims of the Thesis

The work of this thesis aims to define the pathological or physiological purpose which activation of the renin-angiotensin system serves in asthma. If this is a clinically important pathological role either by causing bronchoconstriction directly or potentiating the effect of other bronchoconstrictors, or by increasing airway smooth muscle growth then potential intervention with ACE inhibitors or, better still, angiotensin II receptor antagonists may be beneficial. This would require further study and clarification but could be of clinical relevance in the treatment of asthma.

3. THE MECHANISM OF ACTIVATION OF THE RENIN-ANGIOTENSIN SYSTEM IN ACUTE SEVERE ASTHMA

3.1 Introduction

The renin-angiotensin system is activated in some cases of acute severe asthma (40, 132). The mechanism is unclear, but recent evidence has shown that plasma renin and angiotensin II levels rise in response to nebulised and intravenous salbutamol (142, 143). There is wide variation in plasma β_2-agonist levels in acute asthma (144) and this could account for the variation found in renin and angiotensin II levels.

However, the degree of activation of the renin-angiotensin system following administration of β_2-agonists is less than that seen in acute asthma, suggesting the involvement of additional mechanisms. The generation of angiotensin II via an "alternative" pathway through the action of inflammatory proteases (113) either circulating or within the airways could augment the "classical" activation of the renin-angiotensin system. Endogenous catecholamines released during an acute attack of asthma (39) could also stimulate β-adrenoceptors on juxta-glomerular cells in the kidney (187) resulting in renin release. These may act synergistically with systemically absorbed salbutamol. Variations in serum ACE activity could also contribute to the wide range of plasma angiotensin II levels found in acute asthma.

This study aims to clarify the role of plasma levels of circulating β_2-agonists in activation of the renin-angiotensin system in acute severe asthma and to define the contribution of other factors and explore the possibility of a synergistic effect as suggested above. Since it has been established that angiotensin II levels are elevated in acute severe asthma (132), no control group has been examined.

3.2 Methods

3.2.1 *Study Subjects*

A non-consecutive series of forty adult asthmatic patients (26 female) with mean (standard deviation) age 44.8 (17.1) years were recruited into the study. All presented non-consecutively at the accident and emergency department with acute exacerbations of asthma unresponsive to their regular medication and requiring hospital admission for treatment. Admission parameters are shown in Table III. Spirometry was not measured during the acute admission but pre-morbid values were available for 19 of the 40 study subjects. Due to the acute nature of the admissions 12 of the 40 patients were already receiving supplementary inspired oxygen at concentrations varying from 24-100% by the time arterial blood gases were measured. The values for PaO_2 therefore over-estimate the true PaO_2 on breathing air alone.

Table III *Study patient characteristics on hospital admission.*

	Mean	Standard Deviation
Age	44.8	(17.1)
Peak Expiratory Flow Rate (% Predicted)	35.2	(18.3)
FEV_1 (% Predicted) [n=19]	52.1	(28.4)
Pulse Rate	108.1	(16.4)
Oxygen Saturation (%) *	94.0	(3.7)
PaO_2 (kPa) *	11.3	(4.6)
$PaCO_2$ (kPa)	4.9	(1.4)
Systolic BP (mmHg)	139.5	(22.8)
Diastolic BP (mmHg)	86.0	(16.0)
Sex Ratio (M:F)	14 : 26	
Smokers	21	

n = 40 unless otherwise stated in the text.

* Denotes values obtained when patients receiving supplementary oxygen.

FEV_1 - Forced Expiratory Volume in One Second; BP - Blood Pressure.

All patients were treated with nebulised salbutamol. Thirty-one also received oral prednisolone (dose range 15-40 mg), thirteen received intravenous hydrocortisone, one received intravenous salbutamol and one intravenous aminophylline. Thirty-three patients were taking regular inhaled corticosteroids, eleven were previously taking oral

prednisolone, seven oral theophylline, nine inhaled salmeterol and one was on no medication. Thirty-nine used inhaled β_2-agonists as required (nine via a home nebuliser), seven inhaled ipratropium bromide, one inhaled sodium cromoglycate and one was on oral β_2-agonists. Only two were on regular diuretic treatment and none were on angiotensin-converting enzyme inhibitors.

Ethical approval for the study was obtained from the Glasgow West Ethical Committee and written, informed consent was obtained from the study volunteers prior to entering the study (Appendix 1).

3.2.2 *Sample Collection*

All patients had blood samples measured within 24 hours of admission (mean (SD) time post-admission 13.5 (9.6) hours) for estimation of plasma renin and angiotensin II, plasma salbutamol, serum angiotensin-converting enzyme activity, adrenaline, noradrenaline, histamine, eosinophilic-cationic protein, endothelin-1, IgE, sodium, potassium, urea and creatinine. Plasma salbutamol levels were measured 3.4 (1.9) hours following the previous dose of salbutamol and the cumulative dose of salbutamol administered in the previous 24 hours was estimated. All samples were immediately placed on ice until separated by centrifugation at 4°C for 15 minutes at 3,000 *g*, then plasma or serum were removed and kept frozen at -20°C until analysis.

3.2.3 *Plasma Assays*

3.2.3.1 Renin: Plasma renin concentration was measured by an antibody trapping technique (188). The intra-assay coefficient of variation is 5.5% and inter-assay variation is 11%. The normal reference range for our laboratory is 9-50 μU/ml.

3.2.3.2 Angiotensin II: The assay is a modified radioimmunoassay which uses C_{18} cartridges (Sep-Pak; Waters, Milford, MA, USA) to extract angiotensin II from plasma (189). The intra-assay coefficient of variation is 6.4% and inter-assay variation 10%. The normal reference range for our laboratory is 3-12 pg/ml.

3.2.3.3 Catecholamines: The assays for adrenaline and noradrenaline use a liquid chromatography technique with electrochemical detection (190). Both the intra- and inter-assay coefficients of variation are below 10%. The reference ranges for our laboratory are up to 0.4 nmol/L for adrenaline and up to 5.0 nmol/L for noradrenaline.

3.2.3.4 Endothelin-1: This is measured by radioimmunoassay using a commercially available kit (Nichols Institute, Saffron Walden, UK). The intra-assay coefficient of variation is 4.5% and the inter-assay variation 6.8%. The normal reference range for our laboratory is 1.6-4.9 pg/ml.

3.2.3.5 Salbutamol: This is a high performance liquid chromatography with solid phase extraction using a mobile phase of 39.4% methanol, 59.1% acetonitrile and 1.5% ammonium acetate (191). Salbutamol is extracted from plasma using Bond-Elite silica cartridges (Jones Chromatography Ltd., Mid Glamorgan, UK) then high performance liquid chromatography (10cm Scherisorb S3W-Silica, Hichrom, Berkshire, UK) is performed and the drug detected in the effluent by an LS-1 Fluorescence Detector (Perkin-Elmer, Beaconsfield, UK). The intra-assay coefficient of variation is 3.9% and the inter-assay variation is 4.1%.

3.2.3.6 Angiotensin-Converting Enzyme Activity: Serum ACE activity is determined by a continuous monitoring spectrophotometric method based on the hydrolysis of N-(3-(2-furyl)-acrylyl)-L-phenylalanyl-glycyl-glycine (192). The intra-assay coefficient of variation is 1.7-6.3% and the inter-assay variation is 3.7-9.7%. The normal range for our laboratory is below 88 Units/L.

3.2.3.7 Histamine: This is a radioimmunoassay using a commercial kit (Immunotech S.A., Marseille, France) with high-affinity monoclonal antibodies to histamine following acylation. The sensitivity of the assay is 0.2 nM. The intra-assay coefficient of variation is 3.4-8.9% and the inter-assay variation is 6.4-13.0%. The normal range is < 10 nM.

3.3.3.8 Eosinophilic Cationic Protein: This is a double antibody radioimmunoassay using a commercially available kit (Pharmacia U.K. Ltd. Milton Keynes, U.K.). The sensitivity of the assay is <2μg/l. The intra-assay coefficient of variation is 4.8-10.9%. The normal range for the assay is <20μg/l.

3.2.3.9 Immunoglobulin E: Total serum IgE was measured with a Phadebas RAST kit (Pharmacia, U.K.) according to the manufacturer's recommendations. An estimate of error was made at 20% based on the coefficient of variation of the measurements of the same internal quality control samples over 50 consecutive weeks. The normal range for our laboratory is <120 International kiloUnits/l.

3.2.3.10 Electrolytes: The sodium and potassium assays use standard ion selective electrodes. The normal range for our laboratory is 135-144 mmol/l for sodium and 3.5-5.1 mmol/l for potassium.

3.2.3.11 Renal Function: The assay for urea uses a standard urease method. The normal range for our laboratory is 2.5-7.5 mmol/l. The assay for creatinine uses the Jasse method measuring in infra-red. The normal range for our laboratory is 60-110 μmol/l.

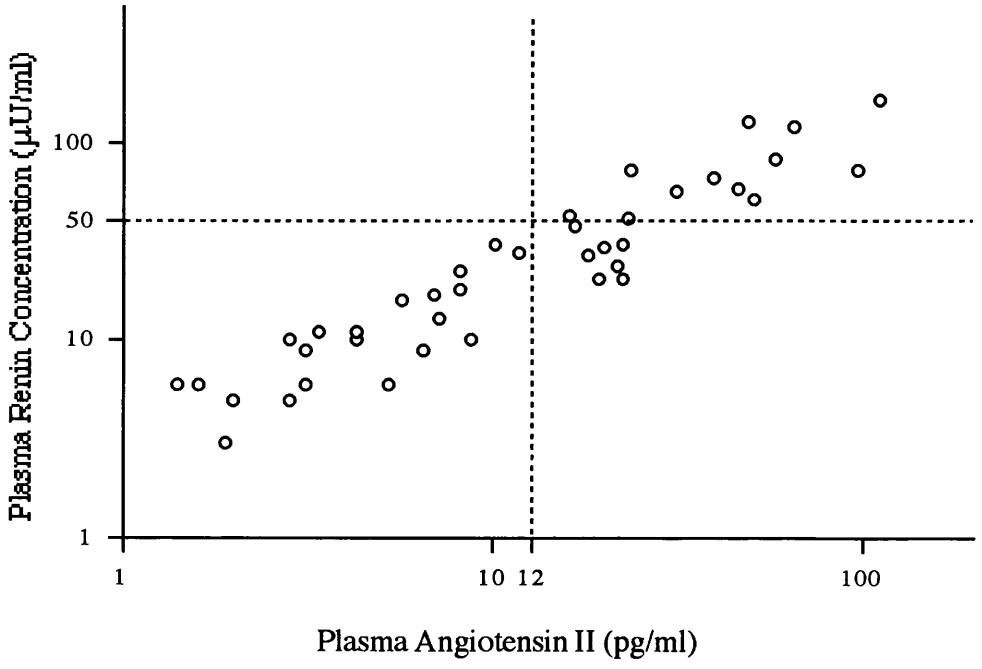
3.2.4 *Statistical Analysis*

Spearman's Rank Correlation Coefficient was used to analyse correlations between the variables and, as multiple tests were performed, $p<0.001$ was considered significant.

3.3 Results

Although the levels of renin and angiotensin II were not elevated in the study group as a whole, a sub-population of asthmatics had raised levels of renin and angiotensin II above the normal range for our laboratory, suggesting activation of the renin-angiotensin system in these patients (Figure 4). Nineteen (47.5%) of the patients had elevated angiotensin II levels and ten (25%) had elevated plasma renin. There was a strong correlation between renin and angiotensin II levels (Spearman's rank correlation coefficient 'ρ'=0.942; $p < 0.001$).

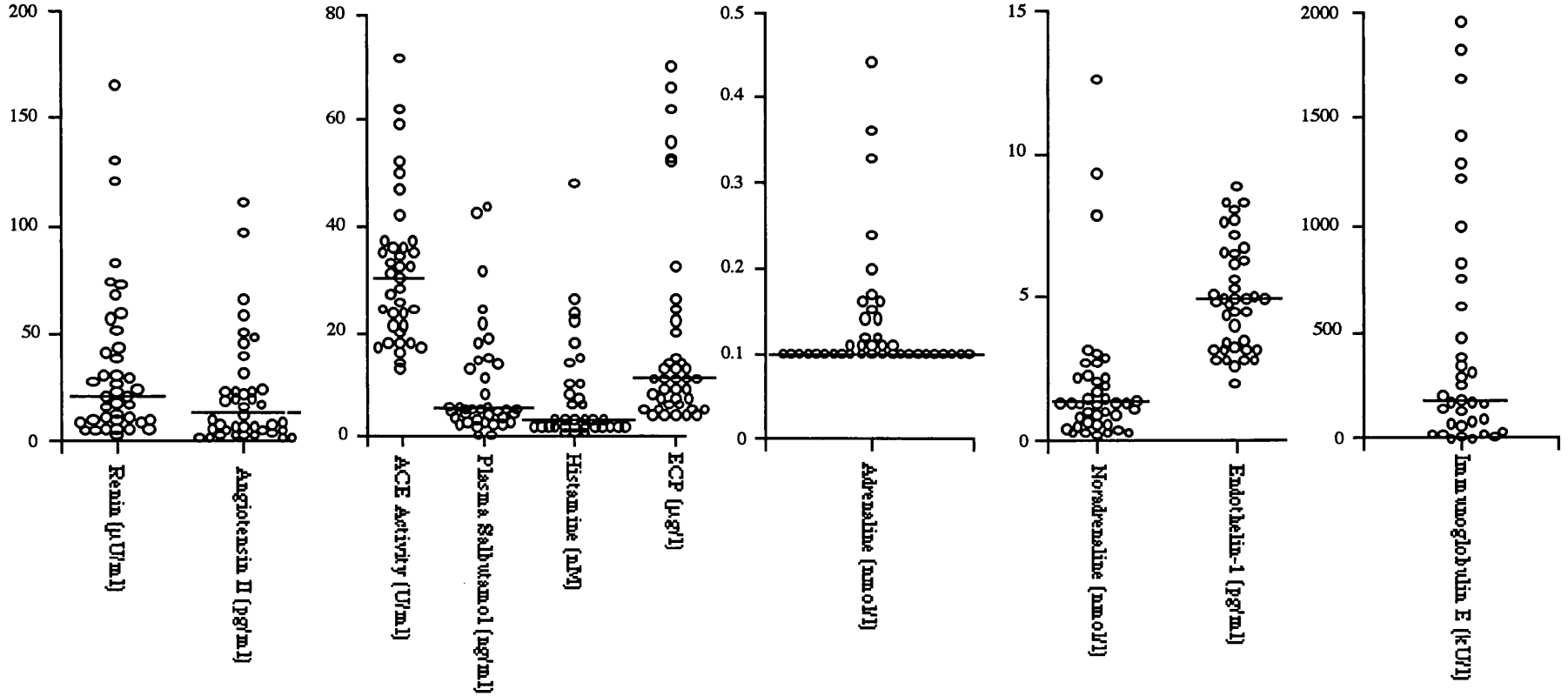
Figure 4 *Plot of plasma renin and angiotensin II levels.*



Dotted lines indicate the upper limit of the normal range for plasma renin and angiotensin II in our laboratory.

The results of the other assays measured are shown in Figure 5 and listed fully in Table IV.

Figure 5 Plot illustrating plasma levels of assays measured in acute asthmatics



Bars indicate median values. References are stated in the text.

Adrenaline and noradrenaline correlated strongly with each other (ρ =0.794; $p < 0.001$), although all except one patient had adrenaline levels within the normal range. Noradrenaline levels were elevated in only three of the forty asthmatics. Histamine was associated positively with eosinophilic cationic protein (ρ =0.412; p = 0.008) and negatively with oxygen saturation (ρ = - 0.473; p = 0.002), but these values were just outwith our limit of statistical significance. Sixteen patients (40%) had raised plasma endothelin-1 levels which correlated negatively with peak expiratory flow rate (ρ = -0.37; p = 0.02) but did not reach statistical significance.

The measured plasma salbutamol level related to the estimated dose of salbutamol administered in the previous 24 hours but did not attain statistical significance (ρ =0.327; p = 0.045).

Table IV *Measurements of serum and plasma levels made during acute severe asthma.*

	Median	**Inter-quartile range**	**Normal range**
Angiotensin II (pg/ml)	10.9	(4.3-23.5)	[3-12]
Renin (μU/ml)	22.0	(10.0-50.0)	[9-50]
Adrenaline (nmol/l)	0.1	(0.10-0.14)	[<0.4]
Noradrenaline (nmol/l)	1.3	(0.6-2.2)	[<5.0]
Endothelin-1 (pg/ml)	4.9	(3.2-6.4)	[1.6-4.9]
Salbutamol (ng/ml)	5.2	(3.0-6.4)	--------
ACE Activity (U/ml)	30.0	(20.5-36.5)	[<88]
Histamine (nM)	3.0	(2.0-7.8)	[<10]
Eosinophilic Cationic Protein (μg/l)	11.0	(6.0-21.5)	[<20]
Immunoglobulin E (kU/l)	182.0	(61.1-744.0)	[<120]
Urea (mmol/l)*	4.9(mean)	(1.6) [SD]	[2.5-7.5]
Creatinine (μmol/l)*	84.9(mean)	(25.6) [SD]	[60-110]
Sodium (mmol/l)*	138.2(mean)	(3.5) [SD]	[135-144]
Potassium (mmol/l)*	4.1(mean)	(0.4) [SD]	[3.5-5.1]

The results are expressed as median (inter-quartile range) except for those marked (*) which are expressed as mean (standard deviation).

There was no association between plasma renin or angiotensin II levels and plasma levels of salbutamol, adrenaline, noradrenaline, endothelin-1, serum total IgE, histamine, eosinophilic cationic protein, serum ACE activity, renal function (urea, creatinine) or electrolytes (sodium, potassium), any measures of the clinical severity of the asthma attack (oxygen saturation, peak expiratory flow rate) or the cumulative doses of salbutamol or corticosteroids administered (Table V).

Table V *Spearman's rank correlation coefficients between renin, angiotensin II and the parameters measured.*

	Renin	**Angiotensin II**
Renin	------	0.94 *
Angiotensin II	0.94 *	------
Plasma Salbutamol	0.26	0.11
Adrenaline	0.29	0.35
Noradrenaline	0.17	0.22
Histamine	- 0.02	- 0.07
Endothelin-1	0.13	0.09
Serum ACE Activity	- 0.10	0.03
Eosinophilic Cationic Protein	0.11	0.10
Immunoglobulin E	0.15	0.16
Urea	- 0.10	- 0.08
Creatinine	0.23	0.26
Sodium	- 0.07	- 0.04
Potassium	- 0.01	- 0.03
Peak Expiratory Flow Rate	0.11	0.20
Oxygen Saturation	0.33	0.40
Age	- 0.24	- 0.20

* Denotes significant correlation ($p < 0.001$).

Mean (SD) blood pressure was 139.5 (22.8) mmHg systolic and 86.0 (16.0) mmHg diastolic. There was no difference in blood pressure between those with elevated plasma renin (mean [SD] 135 [16.5] mmHg systolic, 81.1 [15.1] mmHg diastolic) and those with normal levels (140.9 [24.4] mmHg systolic, 87.5 [16.2] mmHg diastolic) or in those with elevated plasma angiotensin II (135.5 [15.4] mmHg

systolic, 82.6 [12.7] mmHg diastolic) and those with normal levels (143.0 [27.4] mmHg systolic, 89.0 [18.2] mmHg diastolic). There was no correlation between systolic or diastolic blood pressure measurements and either renin or angiotensin II levels.

3.4 Conclusion

Therefore, although plasma renin and angiotensin II levels are once again found to be elevated in a sub-group of asthmatics during acute attacks, no association has been found with any of the clinical or the circulating parameters measured.

4. THE ACE GENE INSERTION/DELETION POLYMORPHISM IN ASTHMA

4.1 Introduction

An insertion/deletion polymorphism of the 16th intron of the angiotensin-converting enzyme gene has been identified in the general population (171). This may be an independent risk factor in cardiovascular disease with a deletion homozygote genotype being least desirable (180). The deletion homozygote of the polymorphism is associated with higher serum ACE activity (169) and the propensity to produce higher levels of angiotensin II (172). This polymorphism accounts for half of the variation in serum ACE activity found in the general population. The results in Chapter 3 again show a wide variation in plasma renin, plasma angiotensin II and serum ACE activity in acute asthmatic subjects. This study examines the hypothesis that the ACE gene polymorphism has a role in the variation in plasma angiotensin II levels found in acute asthmatics.

4.2 Materials And Methods

Forty adult asthmatic subjects (26 female), mean (standard deviation) age 44.8 (17.1) years, with acute exacerbations of asthma requiring hospital admission were recruited for the study as previously described and characterised in chapter 3. One sample for ACE genotype was damaged leaving n=39. For comparison, serum ACE activity and ACE genotype were also measured in a control group of 78 healthy volunteers from the West of Scotland, the same geographical area as the asthmatic subjects. Of the 78 control volunteers, 47 were female with a group mean (standard deviation) age of 35.6 (11.0) years.

A further twenty non-asthmatic subjects (10 female) with mean (standard deviation) age 57.2 (17.9) years, were also recruited after hospital admission for acute medical emergencies. These included chest pain/angina (n=8), deep venous thrombosis (n=2), arrhythmia/palpitations (n=2), pulmonary thrombo-embolism (n=2), syncope (n=1), headache (n=1), diabetes mellitus (n=1), vomiting (n=1), back pain (n=1) and urinary tract infection (n=1). None were taking ACE inhibitors but six were taking beta-blockers, three were taking calcium-channel antagonists and one was taking a diuretic. Nineteen were normotensive with one having a blood pressure of 180/100 mmHg.

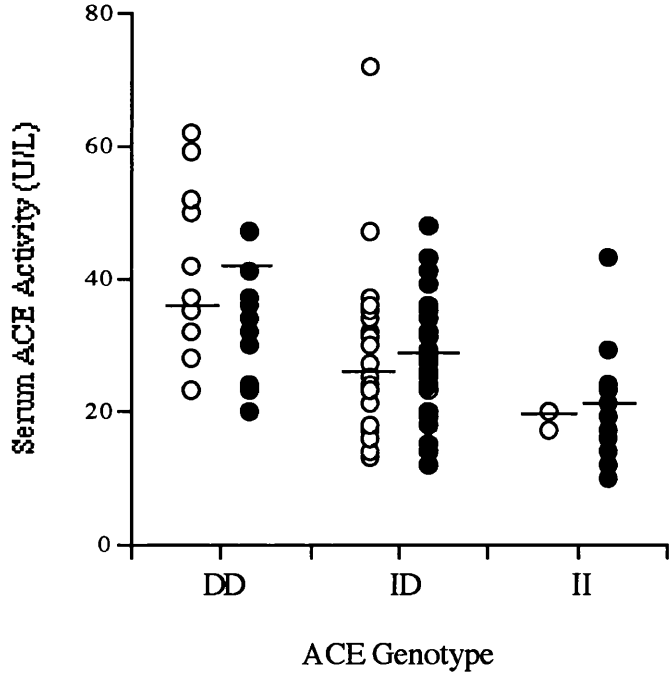
Blood samples were obtained in a plain glass tube for serum ACE activity and in an EDTA tube for ACE genotype. Serum ACE activity was measured by a continuous monitoring spectrophotometric method as previously described in Chapter 3 (192). ACE genotype was determined by both a 2-primer method of polymerase chain reaction according to Rigat *et al.* (193) and a 3-primer method according to Evans *et al.* (174) (Appendix 2). These two methods were used to ensure that no error occurred during the genotyping procedure. Assays for ACE activity and ACE genotyping were kindly performed by Dr. Richard Spooner and Dr. Caroline Jagger in the Department of Clinical Biochemistry, Gartnavel General Hospital, Glasgow, using techniques which they have established in the Department.

Statistical analysis of the results was performed using one-way analysis of variance to assess the relationship between ACE genotype and both serum ACE activity and plasma angiotensin II, and the age distribution among the ACE genotype sub-groups. A chi-squared test was used to compare the ACE gene allele frequencies. Comparison of ACE activity for each genotype between groups was by the Mann-Whitney U-test.

4.3 Results

The ACE genotypes derived for each individual were in agreement when determined by both the 2-primer and the 3-primer methods of analysis. In the asthmatic subjects, the deletion homozygote (DD) for the ACE gene was associated with a significantly higher (p=0.009) serum ACE activity (median [range] 39.5 [23-62] U/L) than the heterozygote (ID) (24.5 [13-72] U/L) and the insertion homozygote (II) (18.5 [17-20] U/L). This was also the case in the control group, with a significantly higher (p=0.007) serum ACE activity (34 [20-47] U/L) in those with the DD genotype than those with the ID genotype (27 [12-48] U/L) and the II genotype (20 [10-43] U/L) (Figure 6).

Figure 6 *Serum ACE activity from acute asthmatics and controls plotted against ACE genotype.*



Open circles represent acute asthmatics and black circles represent controls.
Bars indicate median values.

There was an increased frequency of the deletion allele in the asthmatic group (61.5%) compared to the control group (50.6%) although this did not reach statistical significance. In addition, the DD homozygote is present in greater frequency in the asthmatic group than the controls, but again this difference is not statistically significant (Table VI).

Table VI *ACE genotypes, allele distribution and serum ACE activity in asthmatic subjects and controls.*

	ASTHMATICS (n=39)		**CONTROLS** (n=78)	
	Frequency (%)	**ACE activity** (U/L)	**Frequency** (%)	**ACE activity** (U/L)
ACE GENOTYPE				
DD	28.2	39.5 (23-62)	23.1	34.0 (20-47)
ID	66.7	24.5 (13-72)	55.1	27.0 (12-48)
II	5.1	18.5 (17-20)	21.8	20.0 (10-43)
ALLELE FREQUENCY				
D	61.5		50.6	
I	38.5		49.4	

Results for ACE activity are expressed as medians (range).

In the two groups studied, the control group were younger ($p<0.05$) than the asthmatic group by just over 9 years. In addition, although the age distribution for each genotype was similar for the control group, the DD genotype sub-group of the asthmatic subjects was significantly ($p=0.003$) older (mean [SD] age 58.6 [12.7] years) than the ID genotype sub-group (38.4 [15.0] years) and the II genotype sub-group (38.0 [22.6] years). These facts must be borne in mind when interpreting the results obtained, but may simply be a consequence of the relatively small sample size of the asthmatic group. In the asthmatic group it may be that older asthmatics with an exacerbation are more likely to require hospital admission than young asthmatics, thus skewing the age distribution.

An additional analysis, not included in the original hypothesis, compared a group of acute hospital admissions with the control group. This group comprised the 40 asthmatics and a further 20 non-asthmatic acute medical admissions on whom data was collected during the course of the study. When these two groups were compared there was again no difference in allele frequency or ACE genotype (Table VII).

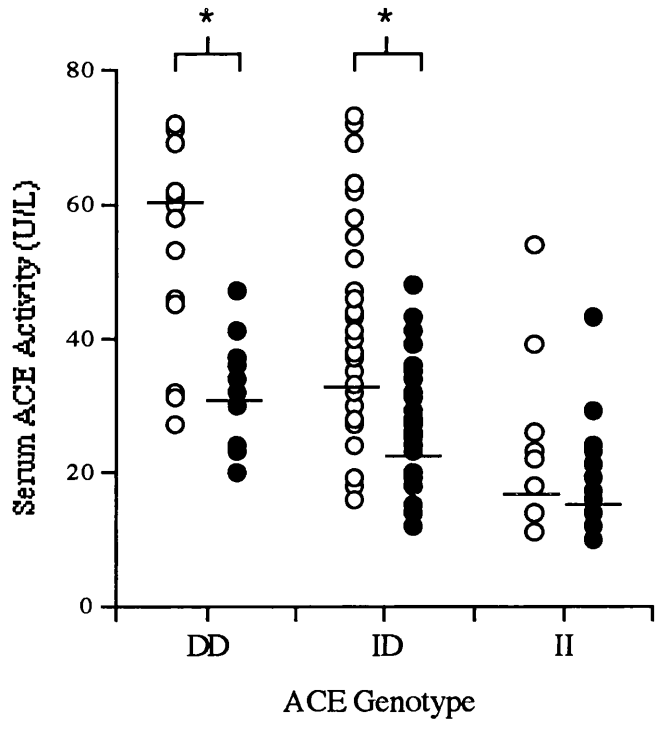
Table VII *ACE genotypes, allele distribution and serum ACE activity in hospital admissions and controls.*

	HOSPITAL ADMISSIONS (n=39)		**CONTROLS** (n=78)	
	Frequency (%)	**ACE activity** (U/L)	**Frequency** (%)	**ACE activity** (U/L)
ACE GENOTYPE				
DD	27.1	59.0 (27-72)	23.1	34.0 (20-47)
ID	57.6	36.0 (16-73)	55.1	27.0 (12-48)
II	15.3	22.0 (11-54)	21.8	20.0 (10-43)
ALLELE FREQUENCY				
D	50.6		50.6	
I	49.4		49.4	

Results for ACE activity are expressed as medians (range).

However, when ACE activity was graphed against ACE genotype it was noted that the ACE activity was significantly higher (p=0.002) for the DD and ID genotype in the acute hospital admissions group than the control group. No difference was found for the II genotype (Figure 7). Once again there was a discrepancy in the ages of the two groups with the hospital admission group being older (mean [SD] age 48.9 [18.2] years) than the control group (35.6 [11.0] years). This finding must be interpreted with caution as this analysis was not a part of the original hypothesis.

Figure 7 *Serum ACE activity for acute hospital admissions and controls plotted against ACE genotype.*



Open circles represent acute hospital admissions and black circles represent controls. Bars indicate median values. * p=0.002.

4.4 Conclusion

Thus the ACE gene insertion/deletion polymorphism has again been shown to determine serum ACE activity with the highest found for the DD homozygote and the lowest for the II homozygote with the ID heterozygote intermediate. There is a trend towards an increased frequency of the D allele in asthmatics but no association is found with activation of the renin-angiotensin system. There appears to be a higher ACE activity for the DD and ID genotypes in those individuals requiring hospital admission for an acute medical illness compared with a healthy control population.

5. THE EFFECT OF ANGIOTENSIN II ON HISTAMINE-INDUCED BRONCHOCONSTRICTION IN THE HUMAN AIRWAY *IN VITRO*

5.1 Introduction

The active octapeptide hormone of the renin-angiotensin system, angiotensin II, is a potent vasoconstrictor and also causes weak bronchoconstriction when administered intravenously to mild asthmatics (132). Angiotensin II potentiates vagally-mediated contraction of rabbit airway smooth muscle via a prejunctional effect on cholinergic nerve terminals (44) and has a post-junctional potentiating effect on sympathetic contraction of rabbit saphenous artery (194-196). It also enhances tissue responses to noradrenaline (197).

Sub-threshold concentrations of angiotensin II enhance methacholine-induced bronchoconstriction both *in vitro* and *in vivo* (141) and endothelin-1-evoked bronchoconstriction of bovine airways *in vitro,* by a type-1 angiotensin II receptor-specific effect (26). The mechanism for this remains obscure but may be due to interaction with other spasmogens in the airway. The potent bronchoconstrictor histamine is released from mast cells in asthmatic airways and any interaction with angiotensin II would be of interest. This study examines the effect of angiotensin II at subthreshold doses for bronchoconstriction on histamine-induced bronchoconstriction in human airways *in vitro.*

5.2 Materials And Methods

5.2.1 *Tissue Collection and Preparation*

Macroscopically normal human bronchi (3rd to 6th order) were obtained from eight patients undergoing thoracotomy for bronchial carcinoma. All were smokers but information about pulmonary function was unavailable; none suffered from asthma. Bronchial rings were dissected free of connective tissue and fat and stored overnight at 4°C in oxygenated Krebs-Henseleit solution of the following composition; NaCl 118.4 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 24.9 mM, KH_2PO_4 1.2 mM and glucose 11.1 mM. Published data has shown that overnight storage of this tissue does not alter its reactivity (198). Four bronchial rings were prepared from each specimen.

5.2.2 *Measurement of Contractile Responses*

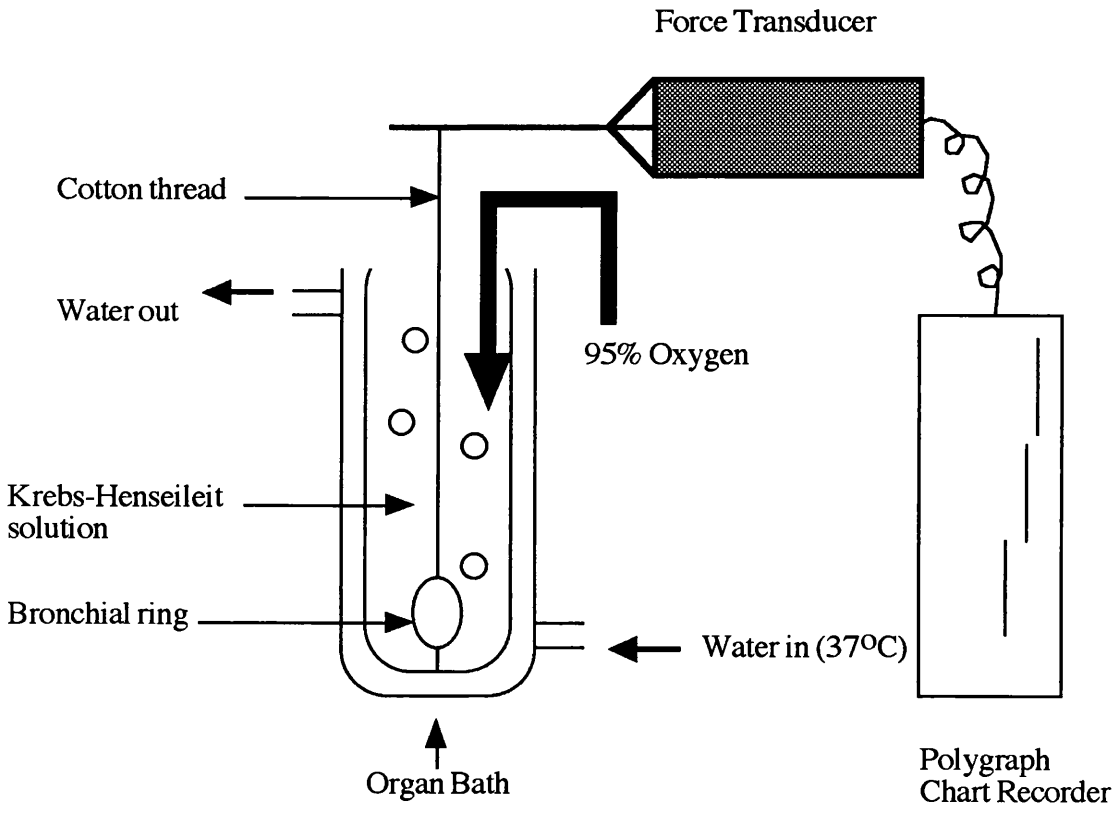
Contractile responses of bronchial rings (3-5mm) were measured in warmed vertical organ baths (10ml) at 37±0.5°C in oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution at pH 7.4. Tension was applied with an initial load of 3g via two platinum wires into the lumen. The rings were allowed to equilibrate gradually over a two hour period with adjustment of the tension to the desired load of 2g. Contractions were measured with a Grass polygraph force displacement transducer (Grass FT03T, Model 7, Quincy, Massachusetts, U.S.A.) which was calibrated with 1g weight prior to use. One wire was anchored to the base of the organ bath and the other attached by a cotton thread to the transducer (Figure 8).

A baseline contraction for each tissue was measured by adding 10^{-4}M methacholine to ensure tissue reactivity and to measure maximal baseline contraction. Any tissue which failed to contract was discarded and replaced. The organ baths were then washed three times with Krebs-Henseleit solution, the rings allowed to equilibrate once more, then tension readjusted to a base load of 2g. Drugs were added directly to the organ baths. Cumulative concentration-response curves were constructed for each bronchial ring with four rings used from each specimen. The first curve was constructed using histamine alone to provide a control, adding concentrations of 10^{-9}M, $3x10^{-9}$M, 10^{-8}M, $3x10^{-8}$M, 10^{-7}M, $3x10^{-7}$M, 10^{-6}M, $3x10^{-6}$M, 10^{-5}M, $3x10^{-5}$M, 10^{-4}M and $3x10^{-4}$M. These concentrations were equivalent to intervals of 0.5 when transformed logarithmically. Each subsequent dose of histamine was added to the bath once the previous contraction had plateaued. Cumulative concentration-response curves were also constructed in the presence of angiotensin II which was added to the three other rings in a dose of 10^{-7}M, $3x10^{-7}$M and 10^{-6}M respectively, 15 minutes

before the subsequent addition of histamine in the doses described above. All contractile responses were calculated using a Linseis chart recorder and analysed subsequently.

It was not possible to construct individual control curves to histamine alone for each bronchial ring as histamine exhibits tachyphylaxis *in vitro*. It is not removed fully from receptor sites by washing, therefore constructing serial curves would lead to reduced tissue responsiveness. To compensate for this, the tension generated by each ring in response to histamine is expressed as a percentage of the maximal baseline contraction to methacholine. This is the accepted methodology for *in vitro* studies of this nature.

Figure 8 *Apparatus used to measure the contractile responses of bronchial rings.*



5.2.3 *Drugs*

The drugs used were histamine (Sigma Chemicals, UK), methacholine chloride (Sigma Chemicals, UK) and angiotensin II (Sigma Chemicals, UK). All were prepared as a stock solution in distilled water then serial dilutions prepared in Krebs-Henseleit solution.

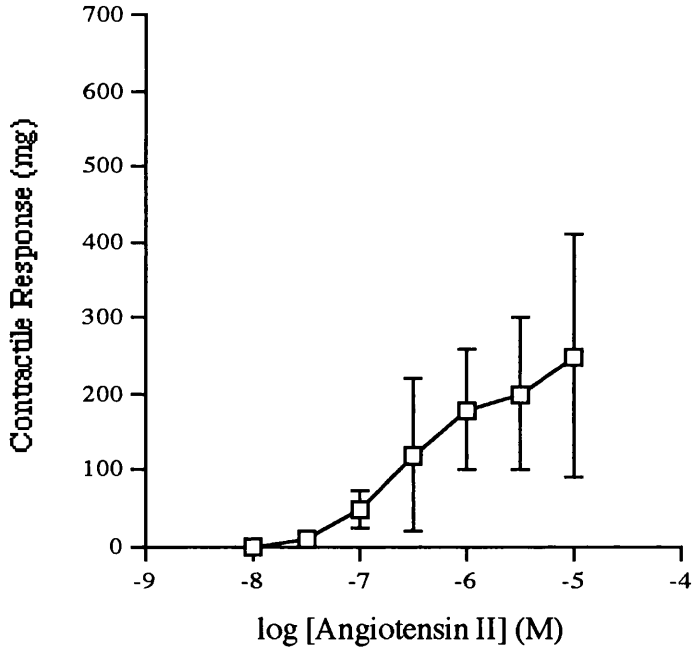
5.2.4 *Statistical analysis*

Statistical significance was determined by two-way ANOVA with a probability level of $p<0.05$ considered significant.

5.3 Results

Cumulative concentration-response curves were constructed for angiotensin II to ascertain the threshold for contraction. Angiotensin II produced small (<0.25 g maximum), concentration-dependent contractions of human bronchi, with a threshold of between 10^{-7}M and 10^{-6}M (Figure 9). This compares with the maximum contraction of mean (SEM) 1.45 (0.28) g with $3x10^{-4}$M histamine.

Figure 9 *Cumulative concentration-response curve to angiotensin II at 10^{-8}M to 10^{-5}M showing the contractile response as mean milligram tension generated.*

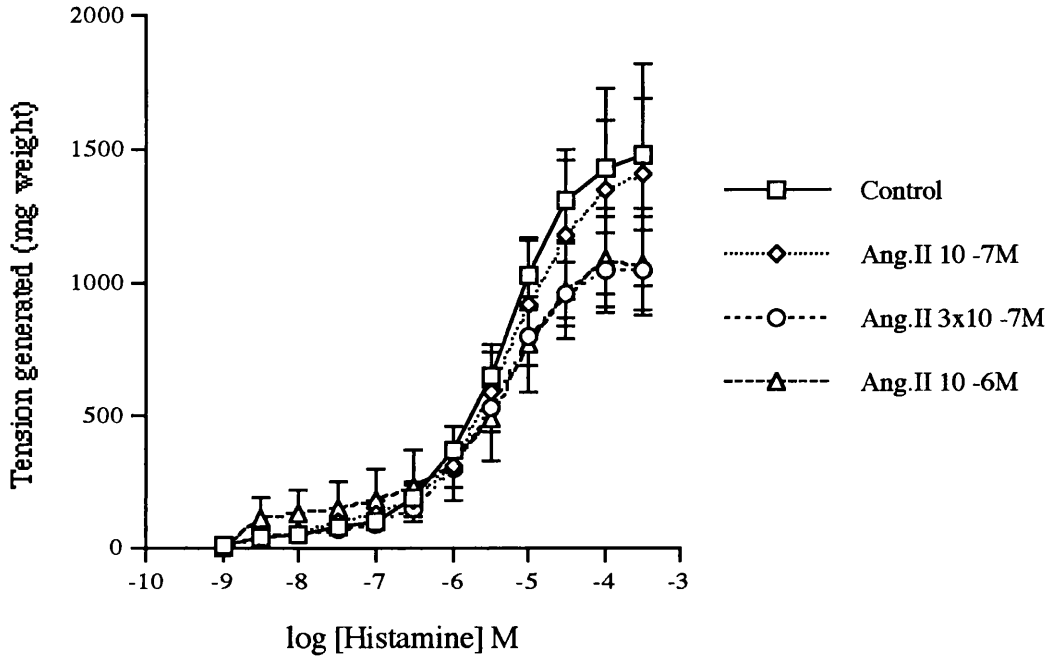


Number of observations (n) = 4. Bars represent SEM.

Pre-incubation with angiotensin II (10^{-7}M) evoked contraction in only three of the eight tissues and in all cases the contraction was less than 0.3 g compared with the control maximum contraction of mean 1.48 (0.21) g with histamine ($3x10^{-4}$M).

The results are shown in Tables VIII - X and Figures 10-12. Contractile responses are expressed as absolute values for gram weight tension generated (Figure 10), as percentage of maximum contraction in response to histamine (Figure 11) and as percentage of baseline methacholine contraction (Figure 12).

Figure 10 *Cumulative concentration-response curves to histamine alone and with angiotensin II 10^{-7}M, $3x10^{-7}$M and 10^{-6}M as milligram weight tension generated.*



Number of observations, n=8.

Responses are shown as means with SEM error bars.

Figure 11 *Responses expressed as percentage of histamine control maximum contraction.*

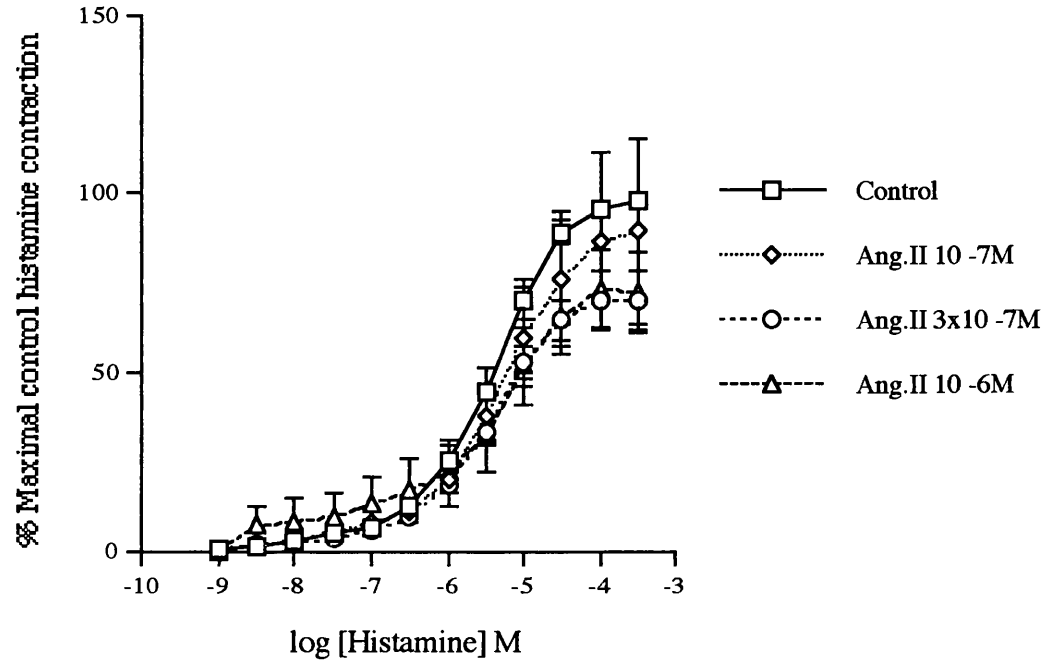
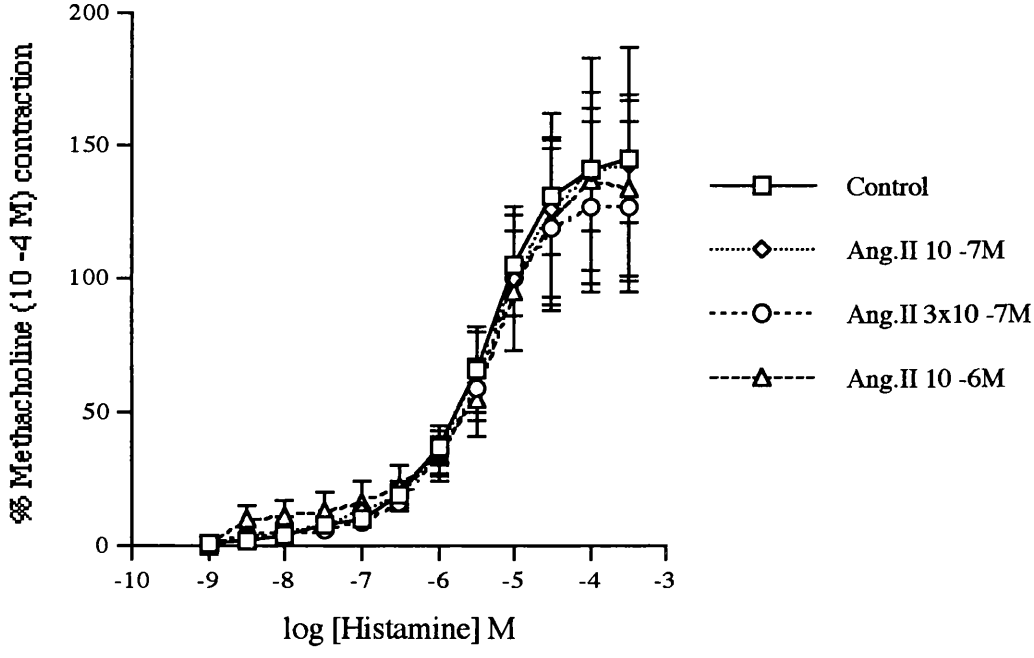


Figure 12 *Responses expressed as percentage of methacholine (10^{-4}M) evoked contraction.*



These results clearly show no enhancement of histamine-evoked contractions (10^{-9}M - 3 x 10^{-4}M) in any of the tissues following pre-incubation with angiotensin II at 10^{-7}M, 3 x 10^{-7}M and 10^{-6}M.

Table VIII *Results from the cumulative concentration response curves as milligram weight tension generated.*

[Histamine]	Control	Ang.II 10^{-7}M	Ang.II $3x10^{-7}$M	Ang.II 10^{-6}M
10^{-9}M	6.2 (6.2)	0 (0)	2.5 (2.5)	0 (0)
$3x10^{-9}$M	32.5 (25.0)	33.8 (31.0)	41.0 (29.1)	108.1 (78.4)
10^{-8}M	40.6 (24.7)	57.5 (36.7)	46.0 (29.5)	126.2 (92.8)
$3x10^{-8}$M	72.5 (29.0)	91.2 (38.2)	61.0 (29.2)	145.0 (102.0)
10^{-7}M	96.2 (38.5)	125.0 (46.7)	88.9 (32.6)	175.6 (115.1)
$3x10^{-7}$M	186.2 (44.8)	180.0 (65.4)	146.1 (43.0)	228.8 (131.3)
10^{-6}M	368.8 (81.8)	310.0 (88.2)	292.8 (64.9)	318.1 (138.8)
$3x10^{-6}$M	643.1 (117.0)	582.5 (147.6)	520.1 (89.0)	483.8 (160.4)
10^{-5}M	1026.9 (135.8)	918.8 (236.9)	793.8 (109.3)	767.5 (178.5)
$3x10^{-5}$M	1303.1 (149.3)	1177.5 (317.0)	954.1 (123.9)	963.1 (181.3)
10^{-4}M	1423.1 (177.9)	1343.8 (386.0)	1045.4 (144.6)	1081.2 (193.2)
$3x10^{-4}$M	1476.2 (205.5)	1400.6 (417.1)	1045.4 (148.1)	1060.6 (189.3)

Results are expressed as mean (SEM).

Table IX *Results from the cumulative concentration response curves as percentage of histamine control maximum contraction.*

[Histamine]	Control	Ang.II 10^{-7}M	Ang.II $3x10^{-7}$M	Ang.II 10^{-6}M
10^{-9}M	0.2 (0.2)	0 (0)	0.2 (0.2)	0 (0)
$3x10^{-9}$M	1.5 (1.0)	1.4 (1.2)	1.9 (1.1)	6.8 (5.4)
10^{-8}M	2.3 (1.0)	2.8 (1.5)	2.2 (1.2)	8.0 (6.4)
$3x10^{-8}$M	4.8 (1.5)	5.2 (1.9)	3.2 (1.3)	9.5 (7.0)
10^{-7}M	6.0 (1.6)	7.6 (2.0)	5.6 (1.4)	12.9 (8.0)
$3x10^{-7}$M	12.2 (2.4)	11.1 (3.0)	9.4 (1.9)	16.6 (9.0)
10^{-6}M	24.8 (4.7)	19.9 (3.9)	18.7 (2.5)	22.1 (9.3)
$3x10^{-6}$M	44.5 (6.7)	37.6 (6.6)	33.7 (3.8)	32.3 (10.2)
10^{-5}M	70.4 (5.5)	59.9 (14.0)	52.8 (4.7)	51.9 (10.8)
$3x10^{-5}$M	89.2 (3.5)	75.9 (18.9)	64.6 (5.9)	65.4 (10.4)
10^{-4}M	95.8 (1.3)	86.6 (24.5)	70.4 (7.6)	73.2 (11.2)
$3x10^{-4}$M	98.1 (1.3)	89.3 (26.2)	70.1 (8.0)	72.0 (11.2)

Results are expressed as mean (SEM).

Table X *Results from the cumulative concentration response curves as percentage baseline methacholine (10^{-4}M) contraction.*

[Histamine]	Control	Ang.II 10^{-7}M	Ang.II $3x10^{-7}$M	Ang.II 10^{-6}M
10^{-9}M	0.3 (0.3)	0 (0)	0.2 (0.2)	0 (0)
$3x10^{-9}$M	1.9 (1.4)	2.7 (2.0)	3.9 (2.7)	9.2 (5.3)
10^{-8}M	3.4 (1.5)	4.7 (2.6)	4.2 (2.7)	10.6 (6.2)
$3x10^{-8}$M	7.4 (2.8)	7.4 (2.7)	5.2 (2.6)	12.4 (6.7)
10^{-7}M	9.2 (2.8)	12.6 (3.8)	8.4 (2.9)	15.8 (7.5)
$3x10^{-7}$M	18.8 (4.0)	18.3 (5.3)	15.2 (3.4)	21.2 (8.7)
10^{-6}M	36.7 (7.4)	34.1 (8.7)	33.2 (7.1)	33.9 (10.1)
$3x10^{-6}$M	65.8 (13.9)	65.7 (16.1)	58.5 (11.5)	54.9 (14.0)
10^{-5}M	104.6 (19.3)	99.6 (27.3)	99.5 (27.4)	95.0 (22.8)
$3x10^{-5}$M	130.8 (22.1)	125.3 (36.3)	118.1 (30.2)	121.9 (29.7)
10^{-4}M	140.4 (22.9)	140.0 (42.7)	126.9 (32.0)	136.4 (33.4)
$3x10^{-4}$M	144.6 (24.3)	142.4 (43.9)	126.4 (32.2)	133.3 (32.8)

Results are expressed as mean (SEM).

5.4 Conclusion

These results show angiotensin II has no effect on histamine-induced contraction of human bronchial rings *in vitro*.

6. THE EFFECT OF ANGIOTENSIN II ON HISTAMINE-INDUCED BRONCHOCONSTRICTION IN MILD ASTHMATIC SUBJECTS

6.1 Introduction

In the asthmatic airway, bronchoconstriction is the end result of a complex series of interactions of the effects of neural stimuli and both circulating and locally released bronchoconstrictor agents. These include inflammatory mediators, peptides, catecholamines and cytokines. In addition to the direct bronchoconstrictor effects of these agents, some also act in synergy with other agents thus increasing the final degree of bronchoconstriction. Humoral modulation of neurally mediated bronchoconstriction involves a number of agents including prostaglandins, acetylcholine, noradrenaline, adrenaline, nitric oxide, histamine, serotonin and bradykinin (22).

Angiotensin II has synergistic effects pre-junctionally on cholinergic nerves (44), post-junctionally on sympathetic nerves (194-196) and enhances the effects of noradrenaline (197). Previous work has shown that sub-threshold concentrations of angiotensin II enhance the effects of methacholine on human airways *in vitro* and *in vivo* (141) and endothelin-1 on bovine airways *in vitro* (26). Since circulating angiotensin II is elevated in acute severe asthma this potentiating effect may be clinically relevant.

Angiotensin II may interact with other mediators of bronchoconstriction such as histamine. Chapter 5 has examined the effect of angiotensin II on histamine-induced bronchoconstriction in human bronchial rings *in vitro* and this study investigates the effect of subthreshold doses of angiotensin II on histamine-induced bronchoconstriction *in vivo* in subjects with mild asthma.

6.2 Materials And Methods

6.2.1 *Patients*

Eight volunteers (5 male) with mild asthma were recruited. Their mean (range) age was 41 (27-69) years and their baseline FEV_1 was 88 (78-106) % of predicted. Bronchial hyper-responsiveness to inhaled histamine was demonstrated by challenge testing according to the method of Cockcroft *et al.* (199). Individuals were excluded if found to be hypertensive, pregnant or taking antihistamines, diuretics or angiotensin-converting enzyme inhibitors. Two subjects were smokers and one was a former smoker. Seven subjects were taking inhaled salbutamol as required, four were taking additional low dose (<1,000 μg daily) inhaled beclomethasone dipropionate, one was taking additional salmeterol and one was on no treatment (Table XI).

Table XI *Study subject characteristics.*

	Sex	**Age** (yrs)	**FEV_1** Litres [% predicted]	**Histamine** PC_{20}(mg/ml)	**Treatment**	**BP** (mmHg)
1	M	27	4.80 [106%]	1.84	S	128/74
2	M	49	3.56 [88%]	4.21	nil	157/89
3	M	32	2.75 [82%]	3.24	S/BDP	134/68
4	F	35	2.83 [98%]	1.26	S/BDP	134/79
5	F	56	2.14 [79%]	2.61	S/BDP	148/88
6	M	30	4.37 [97%]	7.75	S	132/76
7	F	30	2.46 [80%]	5.32	S	132/78
8	M	68	2.46 [78%]	6.33	S/BDP/Sm	153/83
Mean		**40.9**	**3.18** [88.5%]	**3.48***		
(SD)		(15.0)	(0.97) (10.6)			

Abbreviations: S = salbutamol (prn), BDP = beclomethasone dipropionate (<1,000 μg/day), Sm = salmeterol, * = geometric mean.

Prior to each study day subjects were instructed to withhold short-acting beta-agonists for 8 hours and long-acting beta-agonists for 12 hours but inhaled corticosteroids were continued unchanged.

Ethical approval for the study was obtained from the Glasgow West Ethical Committee and written informed consent was obtained from the study volunteers prior to commencing the study (Appendix 3).

6.2.2 *Study Protocol*

The study was carried out in a randomised, double-blind and placebo-controlled manner. The subjects attended the laboratory on four separate occasions; initially a screening visit comprised a physical examination followed by a histamine bronchial provocation test. This was performed using a Wright's nebuliser, calibrated to give an output of 0.12 ml/min. at a flow rate of 8 l/min. air, to define the concentration required to cause a 20% fall in FEV_1 from baseline (the PC_{20} histamine). On the three subsequent visits an intravenous cannula (Venflon, Viggio AB, Helsinborg, Sweden) was inserted into a vein on each forearm then, after 30 minutes rest, a blood sample was extracted to measure baseline plasma renin and angiotensin II levels. Baseline spirometry, pulse, blood pressure and pulse oxymetry were measured then an intravenous infusion commenced via a 50ml syringe driver (Perfusor Secura E, B.B. Braun, Melsunger AG, Germany). Subjects received either placebo (5% dextrose) or subthreshold doses of angiotensin II at 1 or 2 ng/kg/min over one hour. After 30 minutes, steady state was deemed to have been reached and a further blood sample was taken from the arm opposite to the infusion and the FEV_1 repeated. A histamine bronchial provocation test was then performed and the PC_{20} noted. At the completion of the test a further blood sample was taken and the bronchoconstriction reversed with nebulised salbutamol (Ventolin, Allen and Hanburys, UK). Throughout the study pulse, blood pressure and oxygen saturation were monitored at 15 minute intervals.

6.2.3 *Measurements*

6.2.3.1 FEV_1

Spirometry was measured using a dry wedge spirometer (Vitalograph Model S, Buckinghamshire, U.K.), the highest of three measurements being taken at each time point.

6.2.3.2 Pulse and Blood Pressure

A semi-automatic sphygmomanometer (Dinamap, 1846SX Vital Signs Monitor, Critikon, Florida, USA) was used to measure pulse and blood pressure from the opposite arm to the infusion at each time point.

6.2.3.3 Oxygen Saturation

Oxygen saturation was measured by transcutaneous pulse oxymetry (Oscaroxy Pulse Oxymeter, Datex, Helsinki, Finland) at each time point.

6.2.3.4 Histamine Bronchial Provocation Test

Baseline spirometry was carried out then repeated after a two minute inhalation of 0.9% saline, administered via a Wright's nebuliser driven by compressed air calibrated to give an output of 0.12 ml/min at a flow rate of 8 l/min. Doubling doses of histamine were subsequently administered starting with a dose of 0.0625 mg/ml. The FEV_1 was measured at 30, 90 and 180 seconds following each inhalation until a 20% fall was achieved. The histamine concentration causing a 20% fall in FEV_1 was calculated by linear regression and taken as the PC_{20}.

6.2.3.5 Angiotensin II Assay

This was performed as described in Chapter 3 (Section 3.2.3.2) (189). The intra-assay coefficient of variation is 6.4% and inter-assay variation 10%. The reference range for our laboratory is 3-12 pg/ml (2.9-11.5 x 10^{-9}M).

6.2.3.6 Renin Assay

This was performed as described in Chapter 3 (Section 3.2.3.1) (188). The intra-assay coefficient of variation is 5.5% and the inter-assay variation 11%. The reference range for our laboratory is 9-50 μU/ml.

6.2.4 *Drugs*

The drugs used were histamine (Fluka Chemicals, UK) and angiotensin II (Sigma Chemicals, UK). Serial dilutions of histamine were prepared in phosphate buffered saline ranging from 0.0625-8.0 mg/ml. Angiotensin II was prepared in 5% dextrose under sterile conditions and blinded by Ms. W. Fallon in our Pharmacy Department.

6.2.5 *Statistical analysis*

Statistical significance was determined by one-way ANOVA with a probability level of $p<0.05$ considered significant. Histamine PC_{20} values were transformed logarithmically prior to statistical analysis.

6.3 Results

On each of the three study days there was no significant difference between baseline pulse, blood pressure, oxygen saturation, FEV_1, plasma renin and plasma angiotensin II measurements (Table XII).

Table XII *Baseline measurements.*

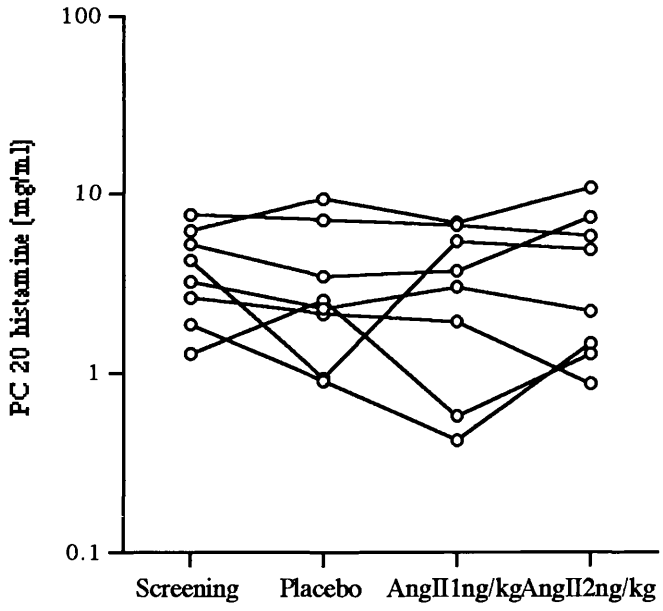
Study Day :	Screening	Placebo	Angiotensin II 1ng/kg/min.	Angiotensin II 2ng/kg/min.
FEV_1 (litres)	3.18 (0.34)	3.07 (0.28)	3.12 (0.28)	3.01 (0.30)
Pulse (bpm)	62.2 (3.1)	58.5 (2.2)	60.2 (3.1)	62.0 (3.4)
Systolic BP (mmHg)	139.8 (3.9)	132.2 (2.6)	135.2 (4.0)	130.1 (5.5)
Diastolic BP (mmHg)	79.4 (2.5)	73.8 (2.9)	77.1 (2.2)	73.2 (2.9)
O_2 Saturation (%)	96.9 (0.4)	97.1 (0.3)	96.8 (0.2)	96.2 (0.2)
Renin (μU/ml)		16.9 (7.0)	9.6 (1.9)	9.4 (2.2)
Angiotensin II (pg/ml) [Molar concentration]		7.6 (1.6) [7.3 x10^{-9}M]	7.3 (1.5) [7.0 x10^{-9}M]	8.2 (1.9) [7.9 x10^{-9}M]

Results are expressed as mean (SEM)

Prior to histamine challenge and during the infusion there was no significant change in FEV_1 noted from baseline values on each study day; mean [SEM] change in FEV_1 (litres) from baseline: placebo -0.08 [0.05], angiotensin II 1ng/kg/min. -0.12 [0.04], angiotensin II 2ng/kg/min. -0.08 [0.04].

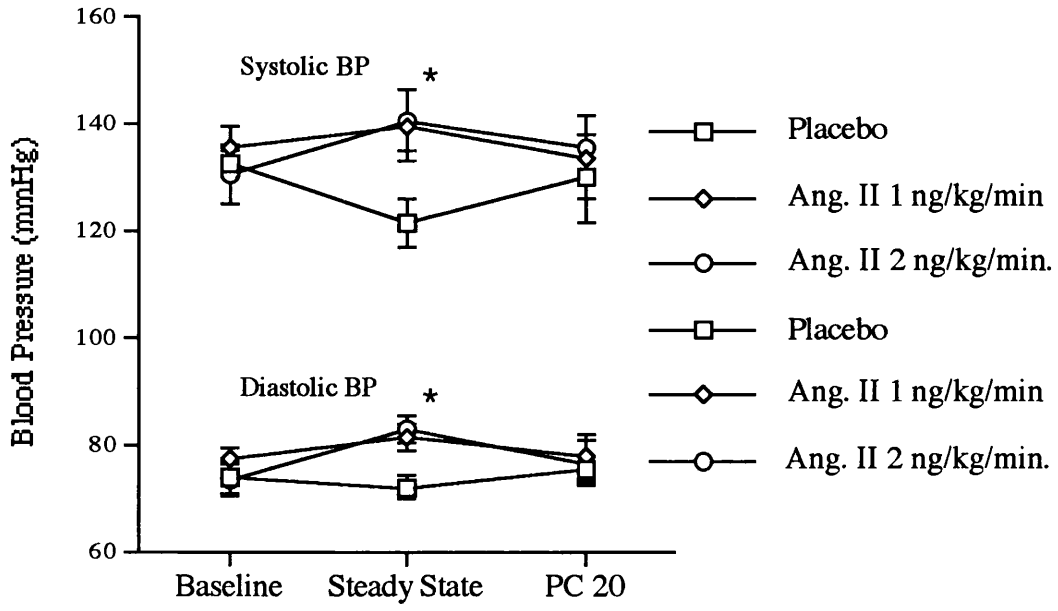
Following histamine bronchial provocation testing on each study day there was no significant difference in histamine PC_{20} (geometric mean [range]) between screening (3.48 [1.26-7.75] mg/ml), placebo (2.67 [0.89-9.57] mg/ml), angiotensin II 1 ng/kg/min. (2.45 [0.42-6.97] mg/ml) and angiotensin II 2 ng/kg/min. (3.09 [0.88-10.78] mg/ml) study days (Figure 13).

Figure 13 *Histamine PC_{20} value on each study day.*



A significant rise ($p<0.05$) in both systolic and diastolic blood pressure occurred following angiotensin II infusion on the angiotensin II 1 ng/kg/min and 2 ng/kg/min compared to placebo (systolic BP mean [SEM] mmHg 121.4 [4.57], 139.4 [6.72] and 140.5 [5.84], and diastolic BP mean [SEM] mmHg 72.0 [2.40], 81.4 [2.54] and 82.88 [2.43] on placebo, angiotensin II 1ng/kg/min and angiotensin II 2ng/kg/min study days respectively). However this difference in blood pressure was no longer evident following histamine challenge testing on each study day (Figure 14). This may reflect the vasodilating effect of histamine with a rise in blood pressure on the placebo days due to the stress associated with bronchoconstriction and the perception of dyspnoea. No changes were noted in pulse rate or oxygen saturation throughout each study day.

Figure 14 *Changes in blood pressure during the period of infusion by study day.*

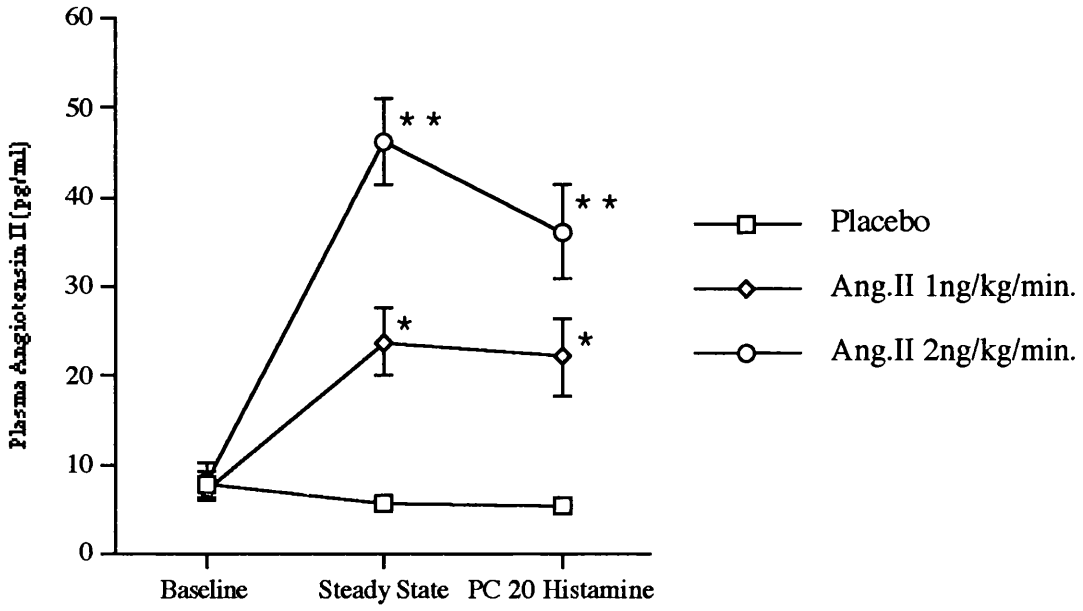


*Denotes a significant difference between placebo and both angiotensin II infusion days ($p < 0.05$).

Plasma angiotensin II was measured at baseline, steady state (30 minutes into the infusion) and following completion of the infusion on each study day. On the placebo day angiotensin II levels remained unchanged; mean (SEM) [mean molar concentration] baseline 7.6 (1.6) pg/ml [7.3 x 10^{-9}M], steady state 5.7 (1.1) pg/ml [5.5 x 10^{-9}M] and post-histamine challenge 5.4 (0.6) pg/ml [5.2 x 10^{-9}M]. On both angiotensin II study days plasma levels increased significantly ($p<0.05$) after 30 minutes compared to baseline and placebo, moreso on the 2ng/kg/min. day and remained elevated until the end of the infusion; 7.3 (1.5) pg/ml [7.0 x 10^{-9}M], 23.8 (3.7) pg/ml [2.3 x 10^{-8}M] and 22.0 (4.3) pg/ml [2.1 x 10^{-8}M] after angiotensin II 1ng/kg/min. and 8.2 (1.9) pg/ml [7.9 x 10^{-9}M], 46.2 (4.7) pg/ml [4.4 x 10^{-8}M] and 36.0 (5.2) pg/ml [3.5 x 10^{-8}M] after angiotensin II 2 ng/kg/min (Figure 15).

No adverse effects were reported by the subjects on any of the study days.

Figure 15 *Plasma angiotensin II levels measured before, during infusion and following histamine challenge testing on each study day.*



* Denotes a significant difference from placebo and angiotensin II 2ng/kg/min ($p<0.05$).

**Denotes a significant difference from placebo and angiotensin II 1ng/kg/min ($p<0.05$).

6.4 Conclusion

In summary, an infusion of angiotensin II at sub-threshold doses for bronchoconstriction causes a rise in blood pressure and plasma angiotensin II levels but has no effect on histamine-induced bronchoconstriction in mild asthmatic volunteers.

7. THE EFFECT OF ANGIOTENSIN II ON REMODELLING OF THE RAT AIRWAYS AND MESENTERIC VASCULATURE

7.1 Introduction

In chronic asthma, airway remodelling contributes to airflow obstruction by causing hypertrophy and hyperplasia of airway smooth muscle (200), increased volume of mucous glands, epithelial loss (48) and subepithelial fibrosis (49, 50). The mechanism of these structural changes is incompletely understood but recent evidence implicates growth promoters in airway smooth muscle cell proliferation (201).

Cardiovascular growth and remodelling occurs in a variety of cardiac and arterial diseases. One of the most important growth promoters is the renin-angiotensin-aldosterone system (145). Angiotensin II stimulates vascular smooth muscle cell growth both *in vitro* (148) and *in vivo* (151, 154), and promotes growth of fibroblasts and cardiac myocytes (202). Recent *in vitro* work has shown that angiotensin II induces human airway smooth muscle cell proliferation and hypertrophy (101).

Since plasma angiotensin II levels are raised in acute severe asthma (132) and in response to β_2-agonists (142, 143), and angiotensin II infusion is known to cause bronchoconstriction (132) it could be an important growth promoter in airway remodelling. However, the effects of angiotensin II on the growth of airway smooth muscle cells and other components of the airway wall have not yet been explored *in vivo*.

This study examines the effect of infused angiotensin II on the growth of smooth muscle, epithelium and connective tissue in the rat airway *in vivo*. The response of the airways is compared to the mesenteric vasculature, where angiotensin II is a known growth promoter (186).

7.2 Materials And Methods

Fourteen young male Wistar rats (weight 250-320g) were randomly allocated to an experimental (n=7) and a control group (n=7). Over fourteen days, all animals received a continuous subcutaneous infusion of the thymidine analogue, bromo-2′-deoxyuridine (BrdU) [Sigma Chemicals, U.K.] at a dose of 210 μg/kg/hr. BrdU is taken up by cells undergoing DNA synthesis, either during replication or repair of damaged DNA, therefore both hypertrophy and hyperplasia of cells will result in increased uptake of BrdU. The experimental group also received angiotensin II (Hypertensin, Ciba Geigy, Basle, Switzerland) at a dose of 200 ng/kg/min., while control animals received only normal saline. All solutions were administered via osmotic minipumps (ALZET Model No. 2002, ALZET Corporation Ltd., California, U.S.A.) implanted subcutaneously in the neck under halothane anaesthesia by Dr. C.J. Kenyon.

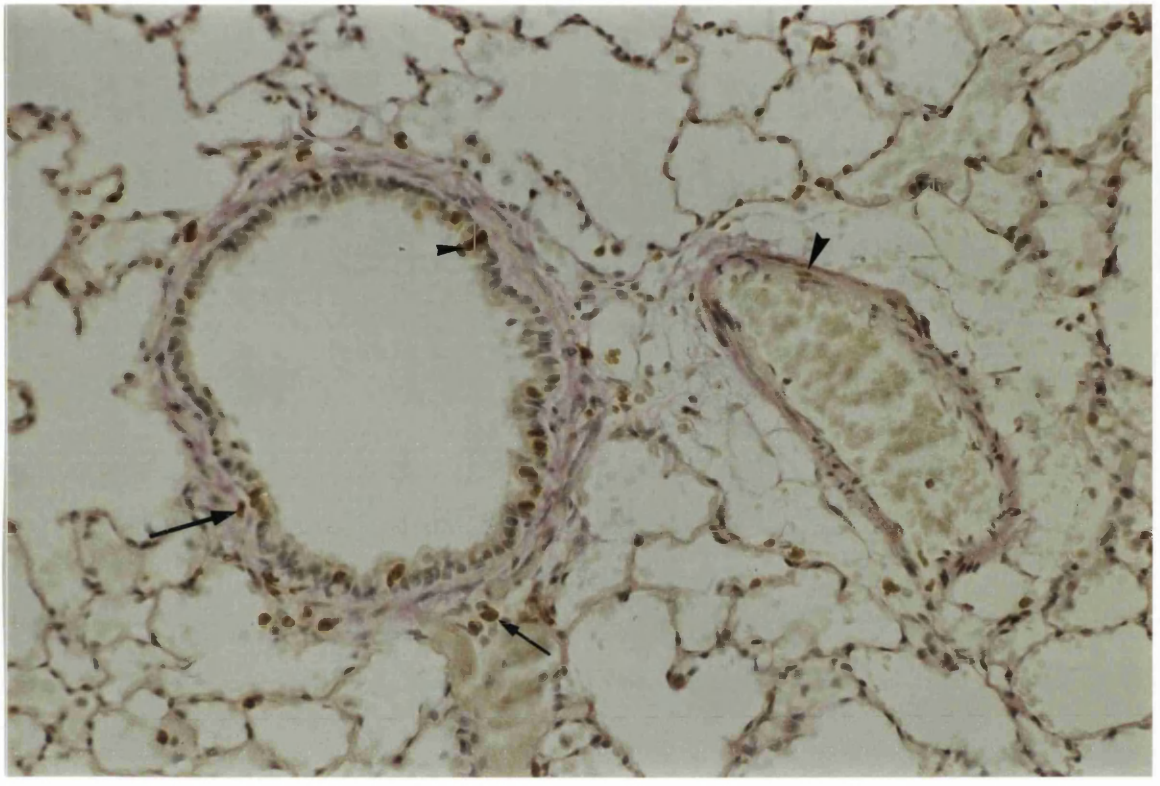
After two weeks, the animals were sacrificed by stunning then decapitation to avoid activating the renin-angiotensin system. Trunk blood was collected for plasma renin and angiotensin II analysis. Systolic blood pressure, measured by an automated tail cuff method (203), weight and pulse rate were documented prior to and on completion of the experiment.

The left lung was inflated *in situ* via the trachea with 10% neutral buffered formalin at a pressure of 25 cm of water then fixed overnight. Transverse, coronal slices were obtained from the perihilar and basal regions of the lung. The kidneys and mesentery were also removed, fixed in formalin, then all tissues were processed for histological examination.

7.2.1 *Immunostaining for BrdU*

Lung and mesentery sections, 3μm thick, were prepared then underwent microwave antigen retrieval followed by immunocytochemistry with a mouse monoclonal anti-BrdU antibody (Bionuclear Services Ltd., Bude, Cornwall, U.K.) following a modification of a standard alkaline phosphatase anti-alkaline phosphatase (APAAP) technique described fully in Appendices 4 & 5 (Figure 16). Control sections had mouse IgG substituted for the primary antibody. To define the anatomy, lung sections were counter-stained with periodic acid-Schiff reagent (PAS) and the mesentery sections with alcian blue-PAS.

Figure 16 *Rat lung demonstrating airway and artery stained with anti-BrdU monoclonal antibody.*

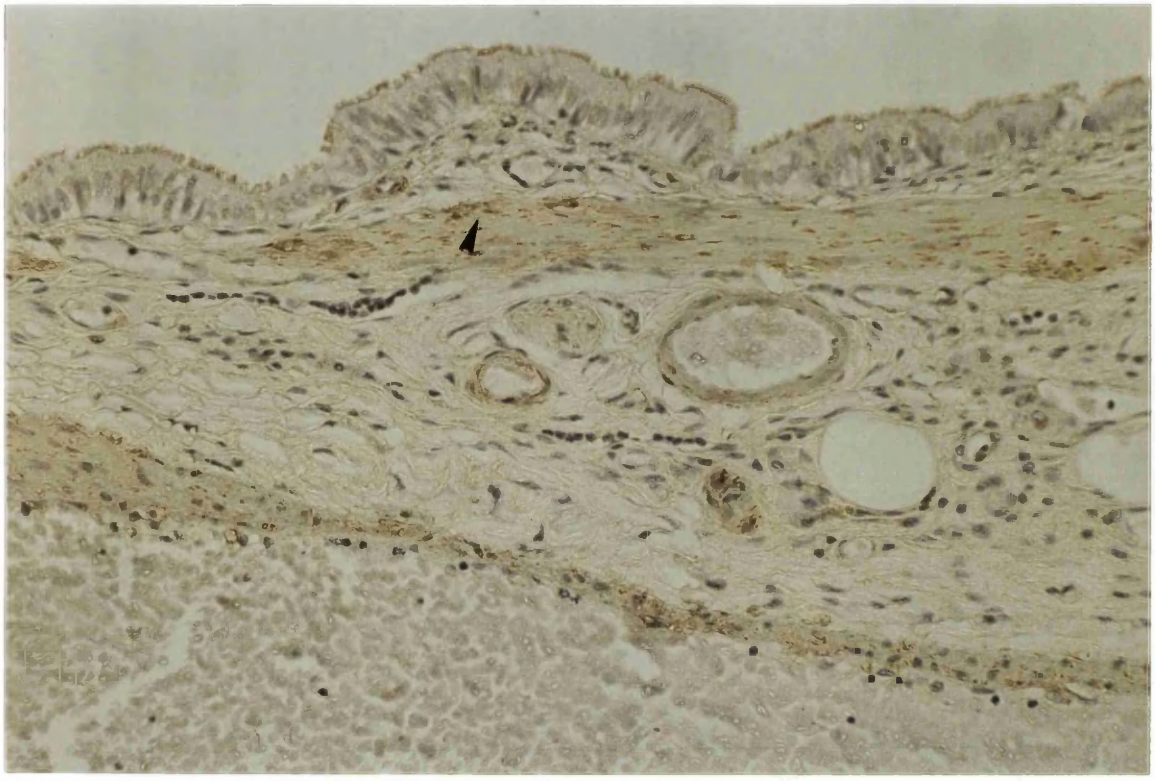


BrdU uptake is shown in the epithelium (small arrowhead), airway smooth muscle (long arrow), adventitia (short arrow) and arterial media (large arrowhead).

7.2.2 *In Situ Hybridisation for Renin mRNA*

Digoxygenin-labelled riboprobes (1200 bp) were prepared from a cDNA complementary to most of the coding sequence of rat renin mRNA. Kidney sections, 3μm thick, were cut onto 3-amino-propyl-triethoxy-silane coated glass slides and a digoxygenin-labelled rat renin riboprobe (1 in 50 dilution) was applied and left to hybridise overnight at 42 °C. Hybridisation was revealed by applying mouse anti-digoxin monoclonal antibody (Sigma, U.K.) as described fully in Appendix 6. Negative control sections were hybridised with "sense" riboprobes and buffer substituted for riboprobes.

Figure 17 *Rat airway stained with α-smooth muscle actin.*



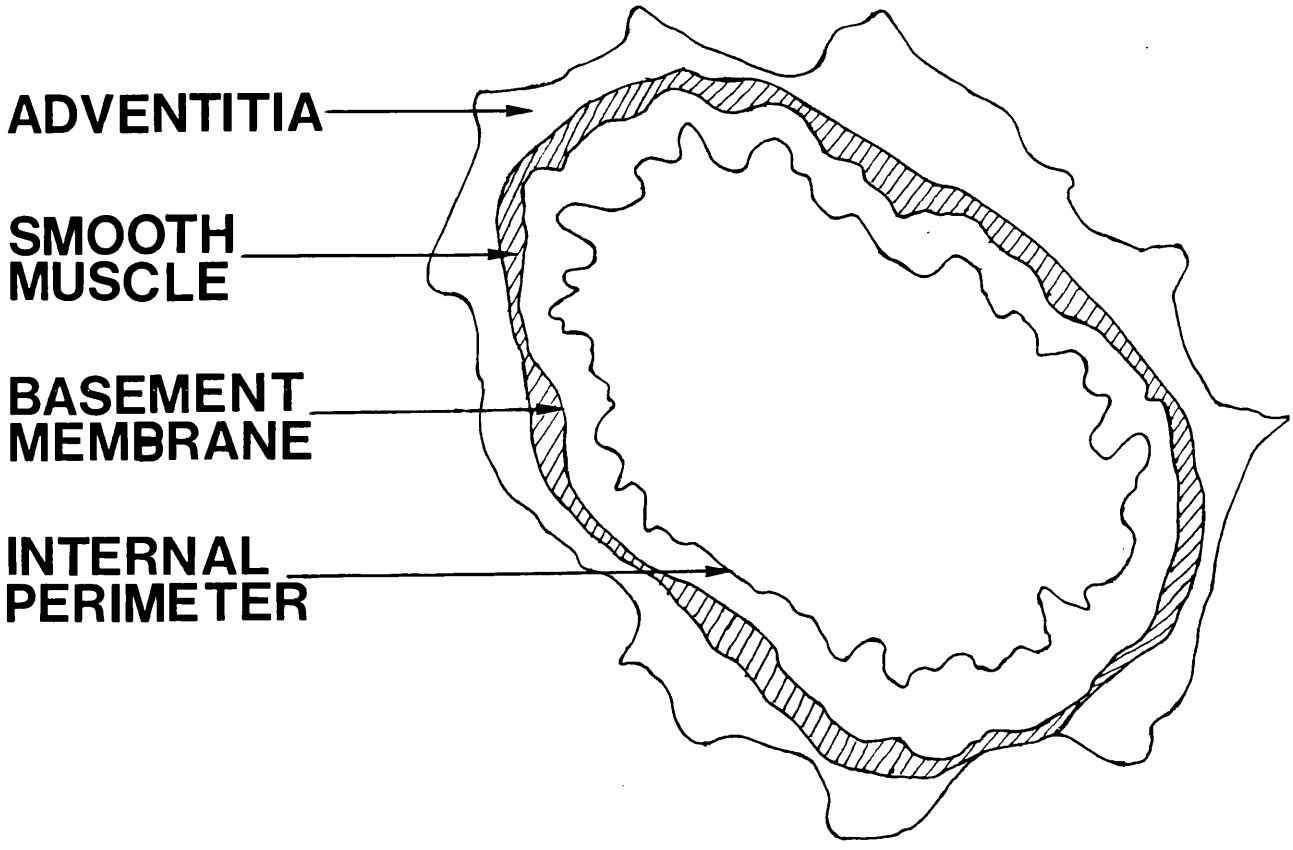
Airway smooth muscle cells are labelled brown (Arrowhead).

7.2.3 *Morphometry*

Two lung sections per animal were stained with Masson's trichrome and smooth muscle identified by immunocytochemistry using α-smooth muscle actin antibodies (Sigma, U.K.) [Figure 17] according to the method in Appendix 4. All transversely cut airways (i.e. with length:breadth ratio < 2.0) were measured in a blinded fashion as follows: outlines of the internal perimeter, epithelial basement membrane, bronchial smooth muscle internal and external boundaries, and the outer adventitial perimeter (Figure 18) were traced using a camera lucida (Wild, Heerbrug, Switzerland). The tracings were digitised using a camera (JVC TK-870E) linked to an automated computerised image analysis system (Optomax V, IBM PS/2 8570, Analytical Instruments, Cambridge, U.K.). The area occupied by the epithelium, bronchial smooth muscle and adventitia, and the length of the internal perimeter were calculated using Vids-V software (Analytical Instruments, U.K.). To correct for shrinkage artifact during processing the calculated areas were transformed by square root and plotted against the length of the internal perimeter for that airway. The internal

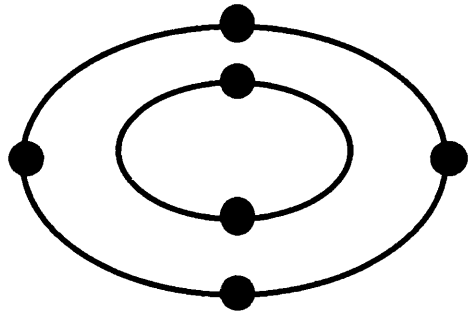
perimeter has been shown previously to remain constant despite any shrinkage which occurs in the tissues (204).

Figure 18 *Tracing of a rat airway section showing the tissue boundaries measured by morphometry.*



Morphometry of the mesenteric arteries and veins was performed on sections stained with elastica-Martius' scarlet blue which defines the internal and external elastic laminae and distinguishes arteries from veins. These were examined by Mr. Niall Whyte. For each animal all intact arteries and veins within one section of the entire mesentery were measured by a technique that is a modification (205) of the method of Short (206). In brief, six Cartesian co-ordinates were recorded for each vessel: two delineating the longest diameter, two the shortest internal diameter and two the shortest external diameter of the media (Figure 19). These co-ordinates enable the wall thickness and wall-lumen ratio to be calculated, even in obliquely sectioned vessels.

Figure 19 *Diagram illustrating the six points measured for morphometry of the mesenteric vasculature.*



7.2.4 *DNA Synthesis*

In each airway the total number of nuclei in the epithelium, smooth muscle and connective tissue were counted. In small airways all nuclei were counted. In larger airways a maximum of 300 nuclei were counted for each cell type. The number of BrdU-positive nuclei in each tissue was expressed as a percentage of the total number of nuclei creating a BrdU index. In the mesenteric blood vessels, the BrdU index was similarly assessed in medial smooth muscle cells.

7.2.5 *Blood Samples*

Plasma renin activity was measured in trunk blood collected into heparinised containers then separated by centrifugation at 3,000 *g* for 15 minutes and frozen at -20 °C until analysis. This was performed by a radioimmunoassay of angiotensin I generated from a known amount of renin substrate and expressed as ng/ml/hour (207). Blood samples for angiotensin II were assayed as described in Chapter 3 (Section 3.2.3.2) (189).

7.2.6 *Observer Variation*

Intra-observer variation was tested by counting the epithelial, smooth muscle and adventitial cells in 32 randomly selected airways in a blinded manner on two separate occasions giving a Pearson product moment correlation coefficient of 0.98, and for morphometric measurements a correlation coefficient of 1.0. Inter-observer variation was tested by two observers counting cells in 10 randomly selected airways giving a correlation coefficient of 0.92.

7.2.7 *Statistical Analysis*

Group values for blood pressure, heart rate and weight were compared using Student's t-test. Plasma renin and angiotensin II levels were compared using the Mann-Whitney U-test. Comparison of the BrdU indices between the two groups was performed by calculating the mean values for each animal then comparing the two groups of rats by Student's t-test.

The square root of the area of each component of the airway wall was plotted against the length of the internal perimeter and a regression analysis performed for each animal (208). The values of the slope and intercept with the y-axis were grouped for the control and the experimental animals then compared by Student's t-test.

Changes in the geometry of the mesenteric vessels were assessed by plotting cumulative distribution curves of the wall-lumen ratio for the arteries and veins.

7.3 Results

There was no difference in any parameter between the two groups of animals prior to commencing the study (Table XIII). Blood pressure rose in the group infused with angiotensin II to a mean (SD) level of 197.1 (22.5) mmHg [p = 0.0001] but no change in blood pressure occurred in the control group. The angiotensin II group were heavier following the study, probably reflecting fluid retention secondary to aldosterone release.

There was a trend towards a lower plasma renin activity in the angiotensin II infused animals although this did not attain statistical significance. The plasma levels of angiotensin II were not significantly different in the control and angiotensin II infused animals (Table XIII).

Table XIII *Physiological measurements in the control and angiotensin II animals before and after the two week infusion.*

	Control Group		**Angiotensin II Group**	
	Pre-infusion	Post-infusion	Pre-infusion	Post-infusion
Blood Pressure (mmHg)	126.3 (14.7)	139.4 (12.4)	134.9 (14.7)	197.1* (22.5)
Renin Activity (ng/ml/hr)		0.88 (0.7-2.7)		0.33 (0.1-0.9)
Angiotensin II (pg/ml)		65.0 (34-125)		78.0 (48-110)
Heart Rate (bpm)	406.1 (36.5)	427.0 (37.8)	422.9 (31.7)	404.0 (38.9)
Weight (g)	288.0 (22.5)	316.4 (28.5)	290.3 (10.9)	321.9† (11.6)

Results are given as mean (standard deviation) except for plasma renin activity and angiotensin II which are expressed as median (interquartile range).

* Denotes $p < 0.01$ compared to the pre-infusion value and to the control group.

† Denotes $p < 0.01$ compared to pre-infusion value.

7.3.1 *In Situ Hybridisation*

In situ hybridisation revealed almost complete suppression of renin mRNA expression in the juxta-glomerular apparatus in the animals receiving angiotensin II (Figure 20) as shown by the median [inter-quartile range] glomerular labelling index (0.79 [0-1.2]) compared to the control animals (50.2 [47-60.2]) [p=0.002] (Figure 21).

Figure 20 *In situ hybridisation of rat kidney showing suppression of renin mRNA expression in juxtaglomerular cells following angiotensin II infusion.*

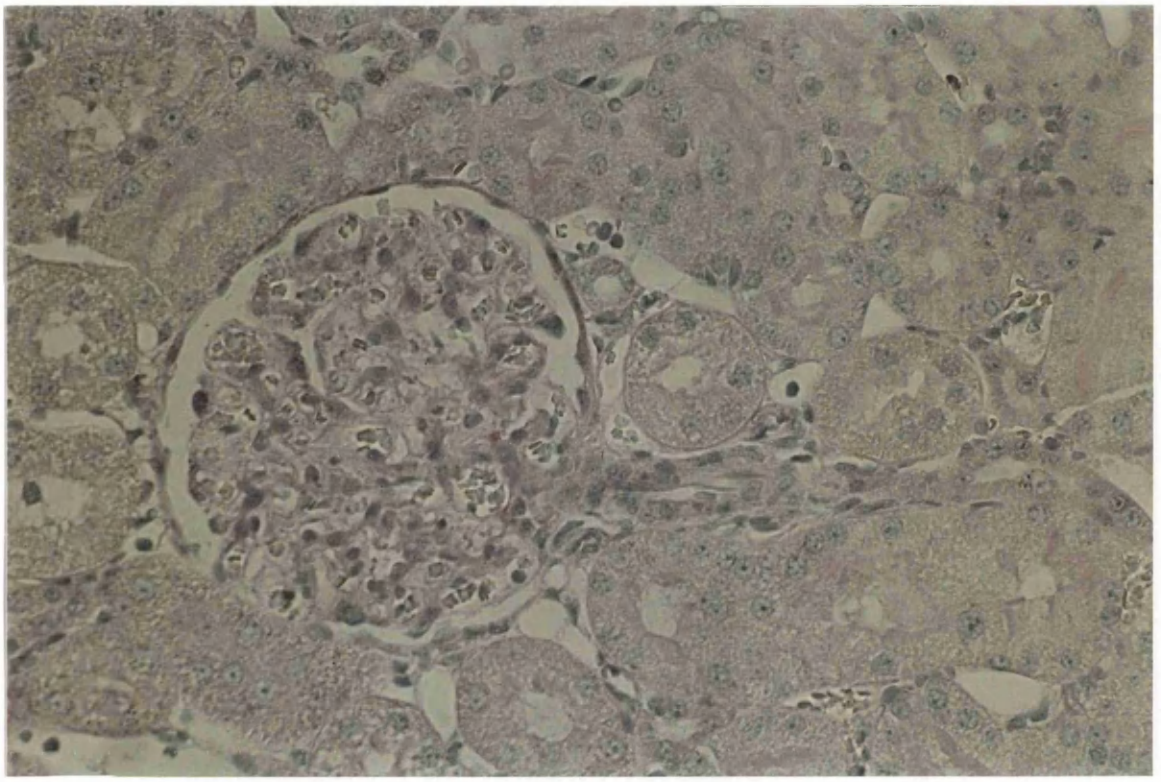
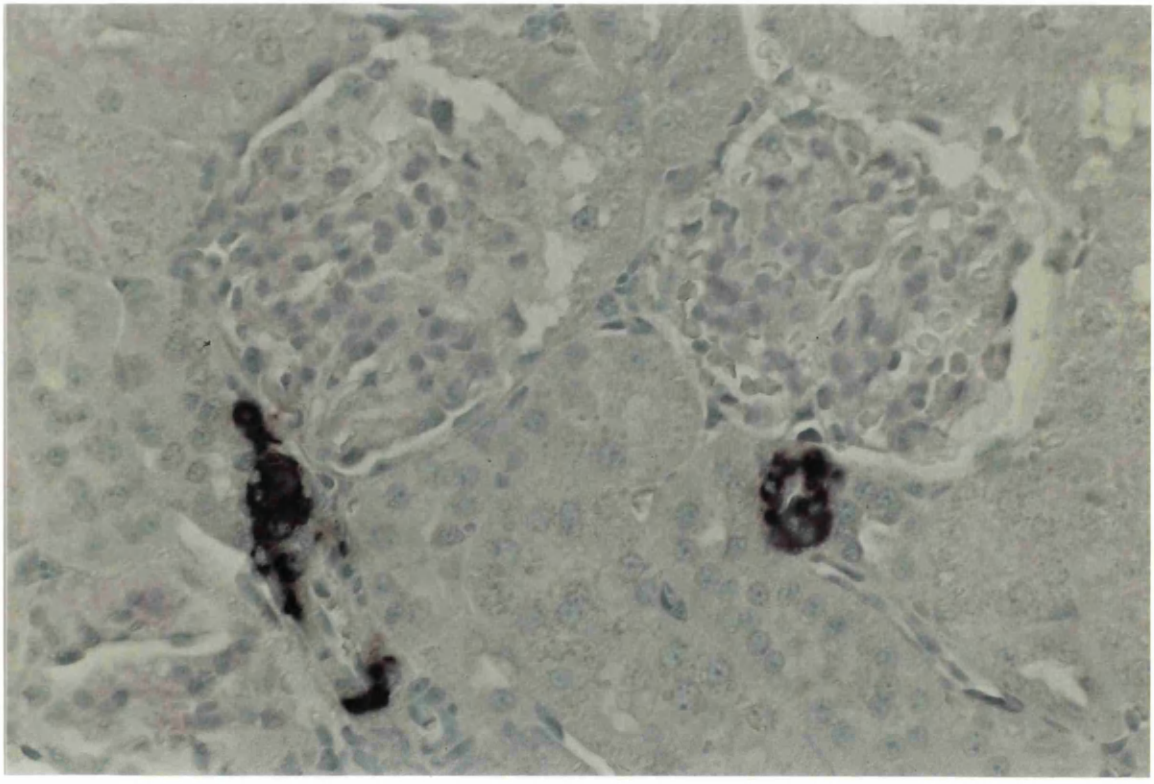


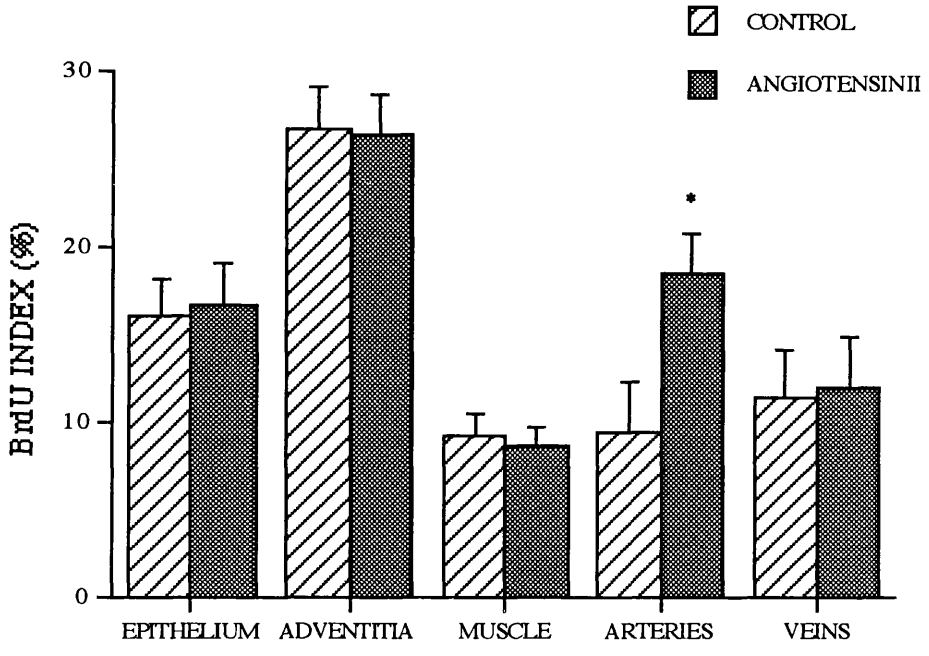
Figure 21 *In situ hybridisation of rat kidney showing normal renin mRNA expression in control animals.*



7.3.2 *DNA Synthesis*

In the airways, there was no difference in the BrdU indices of any of the components of the airway wall between the two groups of animals: epithelium - control group mean [SEM] 16.7 [2.3]%, angiotensin II group 16.0 [2.2]%; airway smooth muscle - control group 8.6 [1.1]%, angiotensin II group 9.3 [1.1]%; adventitia - control group 26.4 [2.2]%, angiotensin II group 26.6 [2.4]% (Figure 22).

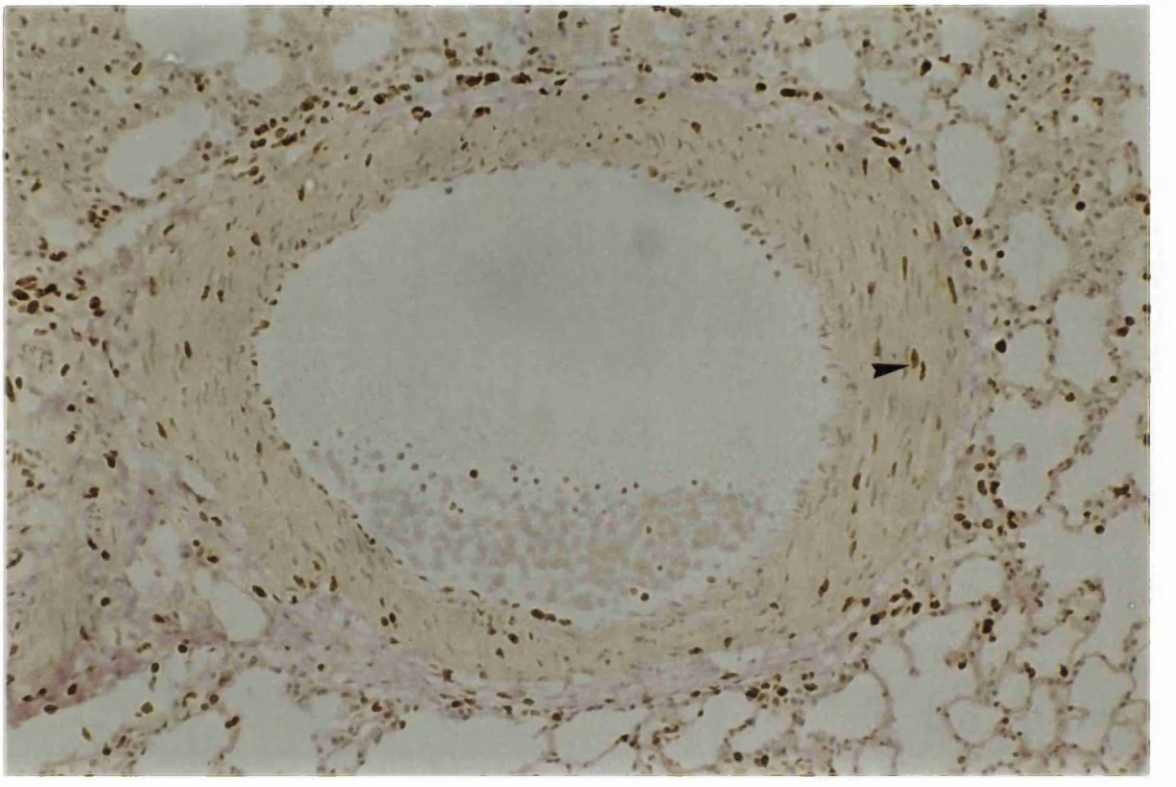
Figure 22 *Comparison of DNA synthesis between the control and angiotensin II-infused animals.*



Values are expressed as mean ± SEM. * p=0.03 compared to control animals.

In the mesenteric arteries, the BrdU index was significantly higher in the animals receiving angiotensin II (mean [SEM] 18.4 [2.3] %) compared to the control animals (9.4 [2.8]%) [p = 0.028]. There was no difference between the BrdU index in the veins: angiotensin II group (12.0 [2.9]%), control group (11.4 [2.6]%) (Figures 22 & 23).

Figure 23 *Transverse section of rat bronchial artery.*



BrdU uptake is seen in medial smooth muscle cell nuclei (Arrowhead).

7.3.3 *Morphometry*

Morphometry showed no difference in the volume of the airway wall occupied by epithelium, smooth muscle and adventitia between the two groups of animals (Table XIV).

Table XIV *Slopes and intercepts from linear regressions of the square root of areas of airway wall tissues against internal perimeter.*

	Control Group (n=7)	**Angiotensin II Group (n=7)**	
Slope			
Epithelium	0.08 (0.006)	0.07 (0.006)	p=0.09
Smooth Muscle	0.09 (0.005)	0.08 (0.009)	p=0.29
Adventitia	0.16 (0.01)	0.14 (0.02)	p=0.53
Intercept			
Epithelium	31.4 (4.0)	38.0 (3.9)	p=0.27
Smooth Muscle	10.9 (2.9)	16.8 (7.7)	p=0.50
Adventitia	8.7 (12.0)	5.9 (20.0)	p=0.91

Results are expressed as mean (SEM) with 'p' values are given for the test of equality of the two groups.

Cumulative distribution curves of wall thickness showed that the arteries in the angiotensin II infused animals were thicker than those in the controls but this difference was not statistically significant. There were no changes in the veins.

7.4 Conclusion

Therefore, a two week infusion of angiotensin II in Wistar rats causes a rise in blood pressure and weight gain with suppression of renin mRNA expression in renal juxta-glomerular cells. There is increased DNA synthesis in the media of the mesenteric arteries but no evidence of DNA synthesis or remodelling in the rat airway.

8. THE EFFECT OF SODIUM DEPLETION ON REMODELLING OF THE RAT AIRWAY

8.1 Introduction

In Chapter 7 I have shown that a two week infusion of angiotensin II has no effect on rat airway remodelling. However, systemic administration of exogenous angiotensin II may not be as physiologically relevant as elevation of endogenous levels; homeostatic regulatory mechanisms may compensate, at least in part, for the infusion of angiotensin II or local airway levels may not be raised. This study examines the effect of endogenous activation of the renin-angiotensin system by sodium depletion on airway remodelling in a rat model previously shown to induce remodelling of the mesenteric arteries (186).

8.2 Methods

Twelve young male Sprague-Dawley rats were randomised to two groups (each n=6). All animals received 2 week infusions of the thymidine analogue bromo-2'-deoxyuridine (BrdU) [Sigma Chemicals, U.K.] 1.25 mg/day via subcutaneous osmotic minipumps (ALZET Model No. 2002, ALZET Corporation Ltd., California, U.S.A.). The osmotic minipumps were implanted subcutaneously in the neck of the animal under halothane anaesthesia by Dr. C.J. Kenyon.

The experimental group were fed a low salt diet (10 mmol Na^+) [Special Diets Services] and the control group were fed normal rat chow. After two weeks, the animals were sacrificed by stunning then decapitation. Trunk blood was collected for analysis of plasma renin activity and aldosterone.

The lungs, kidneys and mesenteric vascular bed were dissected out and immersion-fixed in formalin. The mesenteric vessels have been examined previously and the results reported in abstract form (186). The lungs and kidneys were processed for histological examination and embedded in wax. Immunocytochemistry was performed as outlined in Appendix 5 with an anti-BrdU monoclonal antibody (Boehringer Mannheim). The BrdU index was calculated for each tissue by expressing the number of BrdU-positive nuclei as a percentage of the total number of nuclei counted.

In situ hybridisation was performed according to the method described in Appendix 6 with a digoxygenin-labelled renin riboprobe to demonstrate renin mRNA expression in kidney sections. The glomerular labelling index was calculated by expressing the number of renin-positive cells as a proportion of the total number of glomeruli.

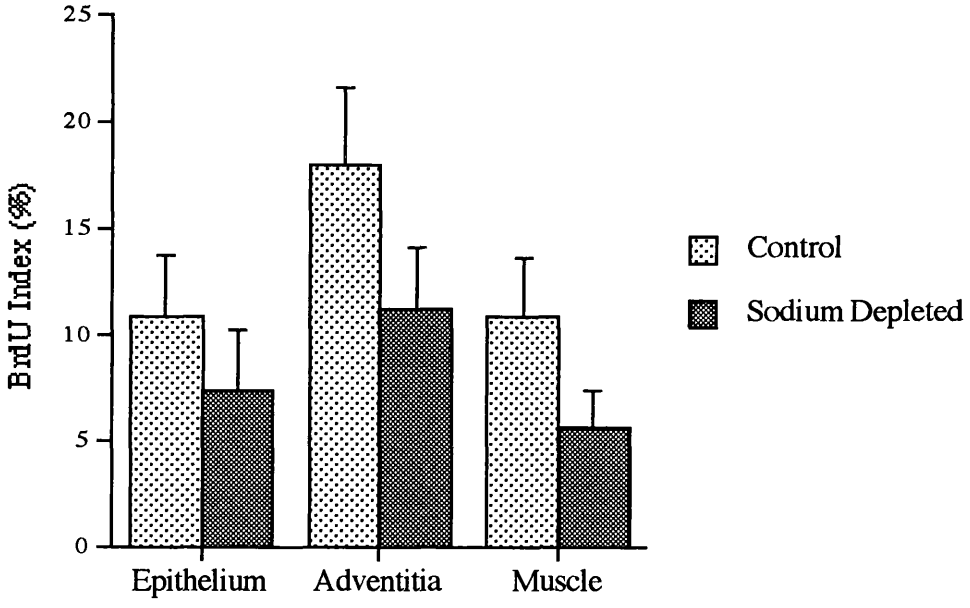
Morphometry of the airways was carried out on all transversely cut airways as described in Chapter 7 (Section 7.2.3).

Inter- and intra-observer variation were as stated in Chapter 7 (Section 7.2.6). All analyses were performed in a blinded fashion in every case with each animal having been assigned a code number which was not decoded until all data had been collected.

8.3 Results

Minor insignificant variations in blood pressure (5-10 mmHg) were seen between the two groups. The differences in the BrdU indices of the three tissues in the control and sodium depleted animals were not statistically significant. Control animals: mean [SEM] BrdU index: epithelium 10.8 [3.0] %, smooth muscle 10.9 [2.8] %, adventitia 18.0 [3.6]. Sodium depleted animals: epithelium 7.4 [2.8] %, smooth muscle 5.6 [1.8], adventitia 11.2 [2.8] (Figure 24).

Figure 24 *Comparison of DNA synthesis between sodium depleted and control animals.*



Morphometry showed no difference in the volume of airway smooth muscle, epithelium or adventitia between the 2 groups (Table XV).

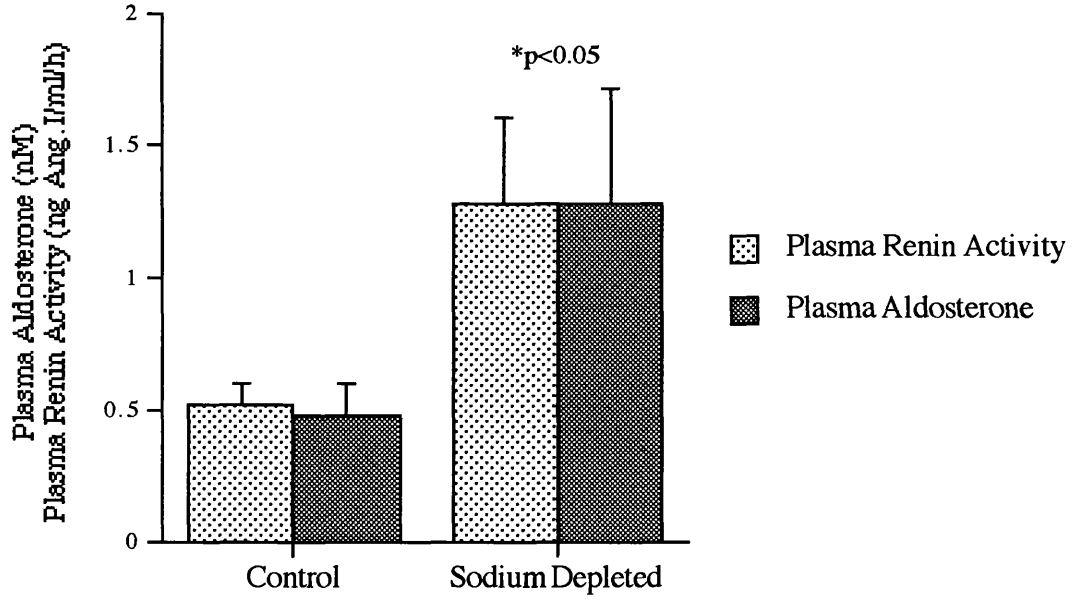
Table XV Slopes and intercepts from linear regressions of the square root of area of airway wall tissues against internal perimeter.

	Control Group	Sodium Depleted Group	
Slope			
Epithelium	0.09 (0.005)	0.11 (0.02)	p=0.22
Smooth Muscle	0.08 (0.009)	0.08 (0.02)	p=0.92
Adventitia	0.08 (0.02)	0.08 (0.01)	p=0.90
Intercept			
Epithelium	41.25 (3.7)	30.1 (7.3)	p=0.21
Smooth Muscle	33.15 (4.7)	33.37 (7.3)	p=0.98
Adventitia	57.35 (7.2)	52.25 (9.7)	p=0.68

Results are expressed as mean (SEM) with 'p' values given for test of equality of the two groups.

Plasma renin activity & aldosterone were significantly higher ($p < 0.05$) in the sodium depleted rats (mean [SEM] plasma renin activity 1.28 [0.32] ng/ml/hr, plasma aldosterone 1.28 [0.44] nM) than in the control animals (plasma renin activity 0.52 [0.08] ng/ml/hr, plasma aldosterone 0.48 [0.12] nM) (Figure 25).

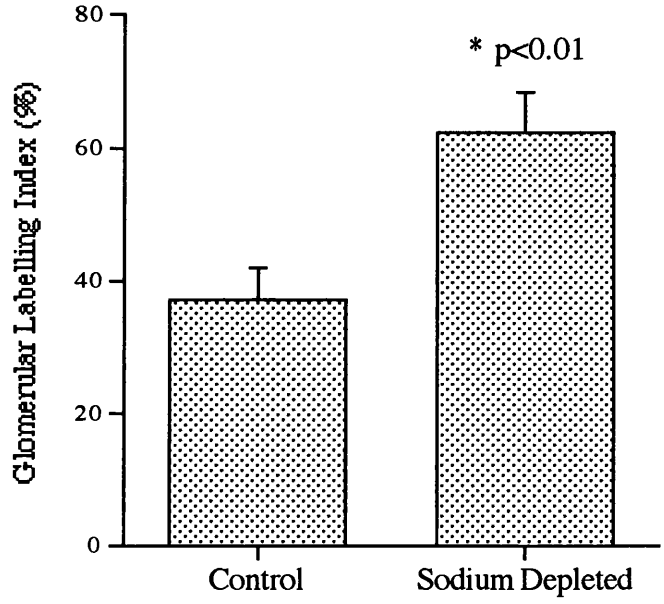
Figure 25 *Plasma renin activity and aldosterone levels in sodium depleted and control animals.*



* Denotes significantly ($p < 0.05$) higher levels of plasma renin activity and aldosterone in sodium depleted animals compared to controls.

In the kidneys, renin mRNA expression was significantly greater ($p < 0.01$) in the sodium depleted group than in the controls as calculated from the glomerular labelling index: Mean (SEM) glomerular labelling index 62.5 (5.8) % in sodium depleted animals and 37.4 (4.7) % in controls (Figure 26).

Figure 26 Glomerular labelling index in the renal juxta-glomerular cells of the sodium depleted and control animals.



8.4 Conclusion

Therefore, a two week dietary sodium restriction in Sprague-Dawley rats causes endogenous activation of the circulating renin-angiotensin system, increased plasma renin activity and plasma aldosterone, with an associated increase in renin mRNA expression in renal juxta-glomerular cells but no significant DNA synthesis or remodelling in the rat airway.

9. EVIDENCE FOR THE EXISTENCE OF A LOCAL RENIN-ANGIOTENSIN SYSTEM IN THE HUMAN LUNG

9.1 Introduction

The renin-angiotensin system plays a role in acute asthma (132), bronchoconstriction (26, 132, 141), pulmonary vasoconstriction (157) and is a growth promoter for human airway smooth muscle cells *in vitro* (101). Most tissues that respond to the circulating renin-angiotensin system also have the capacity to synthesise its components: local systems are present in the kidney, heart, brain, vasculature, adrenal glands, gonads(156) and salivary glands (126).

Components of the renin-angiotensin system have been identified in lung tissue in various species, specifically ACE (159), renin mRNA (164), angiotensinogen mRNA (165), type 1 (137) and type 2 (139) angiotensin II receptor mRNA as discussed in Chapter 1 (Section 1.6). These findings suggest that a local renin-angiotensin system may exist in the human lung and this study looks for evidence of the component parts of the renin-angiotensin system.

9.2 Methods

Fresh human lung was obtained from 12 patients undergoing thoracotomy for bronchial carcinoma. Patient characteristics are shown in Table XVI. All were either current or ex-smokers and none were hypertensive. Normal lung tissue distant from the tumour margins was obtained, processed in formalin and embedded in wax for histological examination. A portion of the tissue was snap frozen in liquid nitrogen, after wrapping in silver foil to minimise trauma to the tissue, for receptor autoradiographic localisation and RNA extraction. This tissue was stored at -70°C until required for sectioning.

Table XVI *Characteristics of thoracotomy patients.*

	Mean (SD)
Age	66.4 (5.02) years
Sex	7 male: 5 female
FEV_1	78.5 (17.7) % predicted
Smoker	6 current: 6 former
Systolic BP	133.7 (18.7) mmHg
Diastolic BP	76.1 (11.3) mmHg

9.2.1 *Immunocytochemistry*

Immunocytochemistry was performed on wax-embedded lung sections from each of twelve tissues with polyclonal antibodies to ACE and renin. Antibody binding was revealed using a standard alkaline phosphatase anti-alkaline phosphatase technique as described in Appendix 4.

9.2.2 *In Situ Hybridisation*

In situ hybridisation was performed to identify messenger RNA expression in lung tissue using digoxygenin-labelled riboprobes. Riboprobes were obtained for renin, angiotensinogen, ACE and AT_1 angiotensin II receptor. *In situ* hybridisation was performed following a standard technique with tissue binding revealed using an anti-digoxygenin monoclonal antibody as outlined in Appendix 6.

The renin cDNA was kindly gifted by Dr. Kevin Lynch, University of Virginia, U.S.A. and the AT_1 angiotensin II receptor cDNA by Dr. Kathleen Curnow, INSERM, Paris, France. The angiotensinogen cDNA was obtained commercially from the

American Type Culture Collection, Maryland, U.S.A. The angiotensin-converting enzyme cDNA was gifted by Dr. Alhenc-Gelas, INSERM, Paris, France.

Riboprobes were prepared by standard techniques. The bacteria containing the plasmids were reconstituted and grown in broth. The DNA was extracted, the plasmid linearised and the desired fragment cut out by restriction digests as appropriate (Appendix 7).

9.2.3 *Northern Blot Analysis*

Frozen lung tissue from each of the twelve specimens (weight 86-350mg) and frozen control specimens of human and rat kidney, and human liver were homogenised for RNA extraction following the protocol outlined in Appendix 8. The samples were then run on a denaturing gel and transferred to a nylon filter. The extracted RNA was then probed with digoxygenin-labelled probes for ACE, renin, angiotensinogen and AT_1 angiotensin II receptor as described above. The procedures followed are set out in Appendix 8.

9.2.4 *Angiotensin II Receptor Localisation and Autoradiography*

Angiotensin II receptor localisation was performed using radiolabelled (sarcosine^1)-angiotensin II (saralasin). This technique of *in situ* receptor localisation has been developed by Dr.J.McQueen and the work described here was carried out jointly with him following his protocols.

Saralasin (Peninsula Laboratories) is an angiotensin II agonist which is known to have a higher affinity for binding to angiotensin II receptors, especially the AT_1 subtype. It is not available commercially in a radio-iodinated form and therefore iodination with ^{125}I- was performed (Appendix 9).

Frozen lung tissue from each patient was then sectioned using a microtome at -20°C, with the sections thaw-mounted onto APES-coated slides. Sections were then wrapped in aluminium foil and stored at -70°C until required. Due to the technical difficulty in sectioning frozen lung tissue, the quality of histology obtained was sub-optimal. Different methods of freezing the tissue were tried but despite attempts at prior immersion in 5% dextrose and 10% glycerol, no improvement in histological definition could be obtained. Therefore straightforward snap freezing in liquid nitrogen was used.

The frozen sections were warmed to room temperature while wrapped in foil to prevent further disruption to the tissue. The slides were rehydrated then the radio-ligand applied as described in Appendix 10.

Four sections per specimen were analysed to assess total receptor binding, non-specific tissue binding, AT_1 receptor binding and AT_2 receptor binding. For non-specific binding, an excess of unlabelled angiotensin II was also added to the sections to competitively saturate the angiotensin II receptors, therefore any radioligand which is detected must represent non-specific binding outwith receptors. To demonstrate AT_1 receptor binding, the AT_2 receptor antagonist PD123319 (Parke-Davis, USA) was added and to demonstrate AT_2 receptor binding, the AT_1 receptor antagonist DuP 753 (Losartan, DuPont) was added.

For film autoradiography slides were placed directly onto the film (Hyperfilm-^{3}H, Amersham, UK). The film and slides were placed in a cassette and left at -70°C for an exposure time of 48 hours with the use of an enhancing screen (HyperScreen, Amersham, UK) giving an effective length of exposure of 96 hours. The films were then developed by immersion in developer (Kodak D19 developer, Sigma, UK) for 6 minutes then in fixer (Kodak GBX fixer, Sigma, UK) for 12 minutes and finally rinsing in water.

The autoradiographs were photographed and digitised into PC format images by Dr.M.McJury, Clinical Physicist, using a flatbed scanner and the Photoshop application on an Apple Macintosh computer. Using Metamorph image analysis software on a PC each individual image produced by a single tissue section was cut from the overall image. Manual thresholding of each image removed background pixel noise, thus including only pixels with silver grain deposition in the analysis. Since there were four images for each lung specimen, the same threshold value was used for all four images.

For each tissue section, the mean and standard deviation pixel intensity for the whole image was calculated, along with the thresholded area for the image and the percentage of the whole image represented by the thresholded region. Luminosity of each tissue section was calculated by multiplying the average pixel intensity (subtracted from 256) by the thresholded area. The results were compared statistically by ANOVA using Minitab.

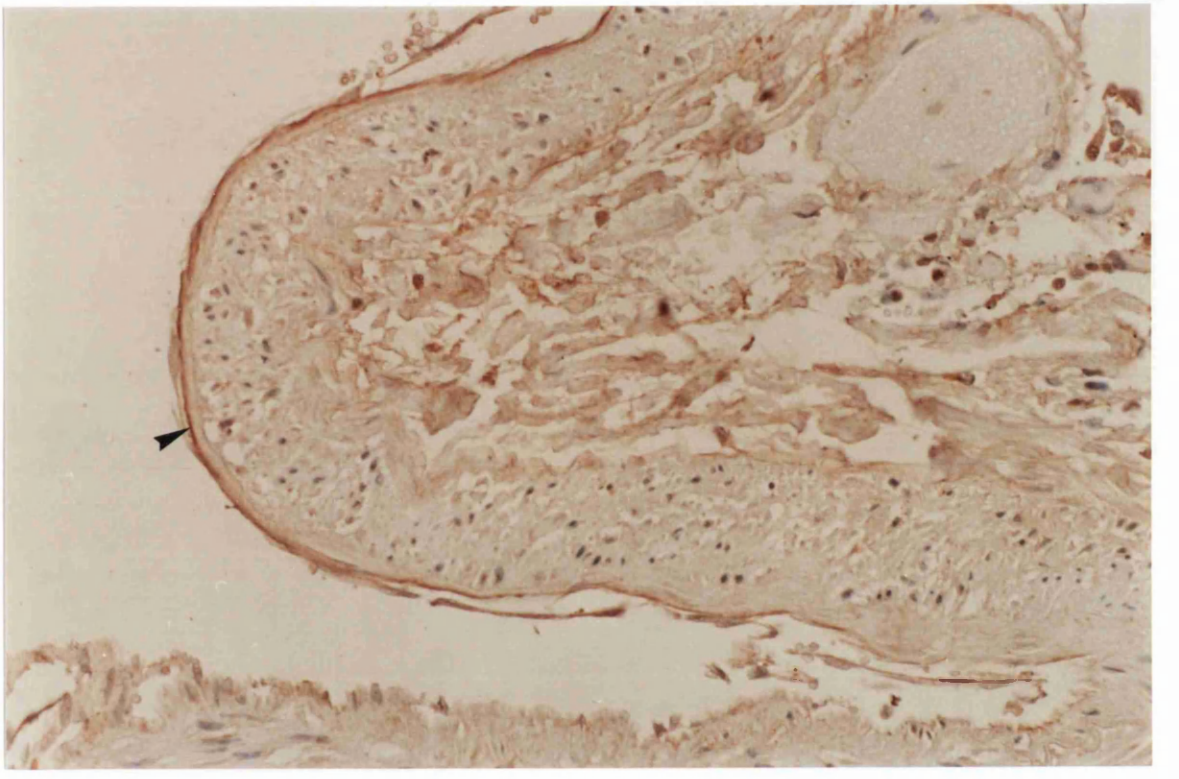
9.2.5 *In Situ Receptor Localisation*

The procedure for *in situ* emulsion receptor localisation is described in Appendix 10. Silver grains deposited over areas of receptor binding on the sections can be viewed by light microscopy. As well as the human lung sections, known positive control tissues were also examined, including rat brain, adrenal, placenta, kidney and rat lung.

9.3 Results

Immunocytochemistry demonstrated the presence of ACE in all 12 cases. Positive staining was localised to the vascular endothelium & alveolar capillaries (Figure 27). However, no evidence of immunoreactive renin could be found.

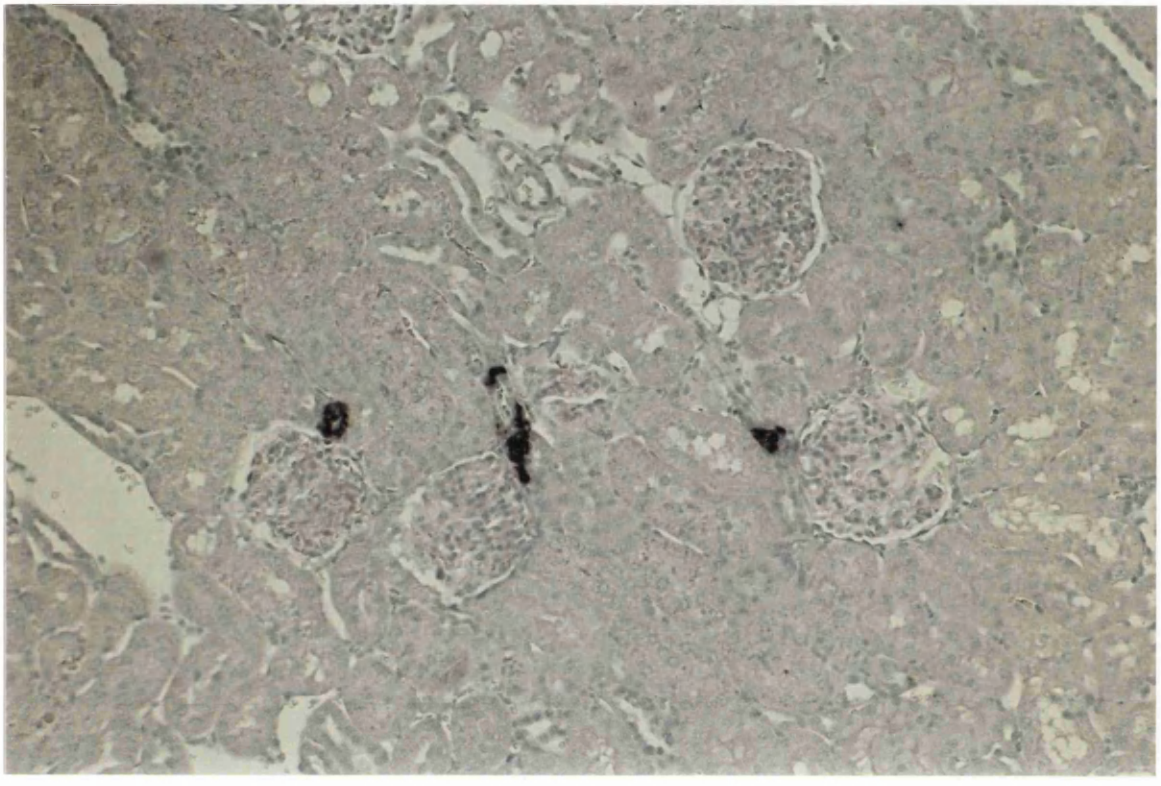
Figure 27 *Immunocytochemistry of human lung showing ACE in vascular endothelium.*



Red staining demonstrates positivity (Arrowhead).

In situ hybridisation failed to demonstrate renin mRNA, ACE mRNA, angiotensinogen mRNA or AT_1 angiotensin II receptor mRNA in human lung tissue. Our laboratory technique appeared to be satisfactory as control kidney tissue showed renin mRNA expression in juxtaglomerular cells (Figure 28). However, due to technical difficulties in the preparation of the riboprobes for angiotensinogen, ACE and AT_1 receptor mRNA, it was not possible to demonstrate their efficacy on known positive control tissues, therefore the results for these components of the renin-angiotensin system are inconclusive.

Figure 28 *In situ hybridisation of kidney demonstrating renin mRNA in juxtaglomerular cells.*

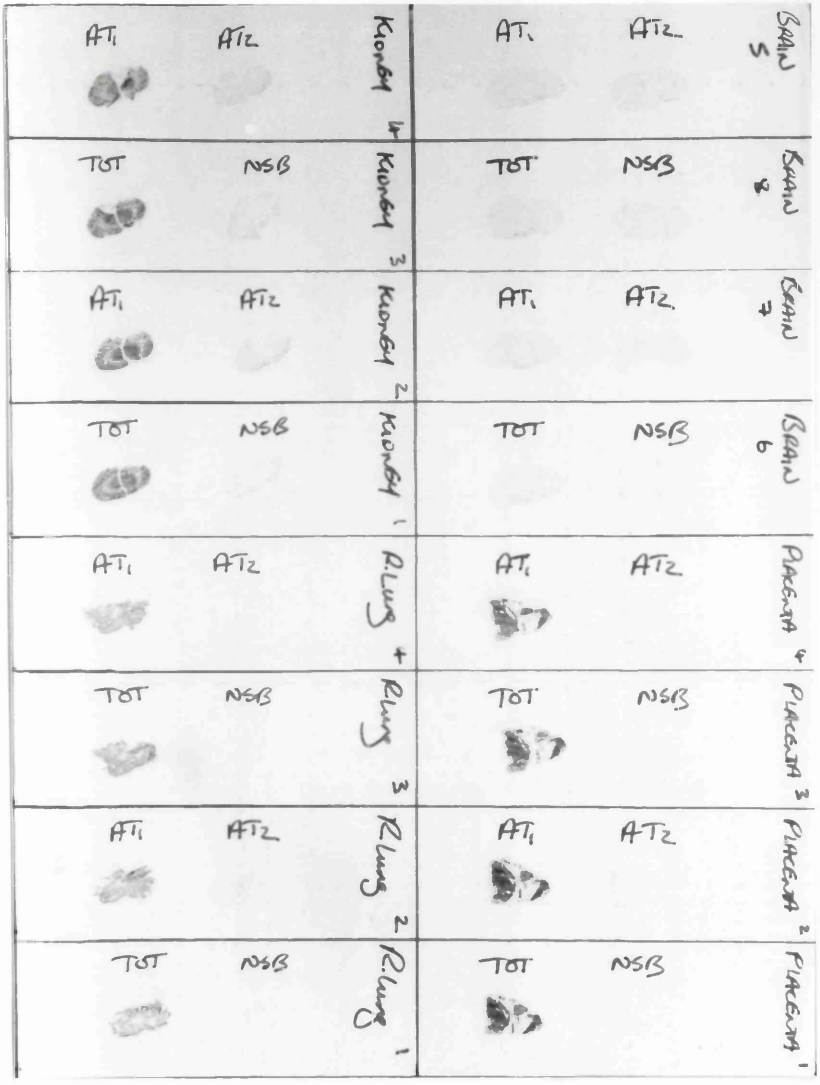


Counterstained with periodic acid-Schiff. Positive staining is black.

Northern blotting of homogenised frozen human lung tissue failed to demonstrate any evidence of renin mRNA, ACE mRNA, angiotensinogen mRNA or AT_1 angiotensin II receptor mRNA. Once again, this appears to be a true negative for renin mRNA, but the technical difficulties with the other riboprobes preclude any firm conclusions being drawn.

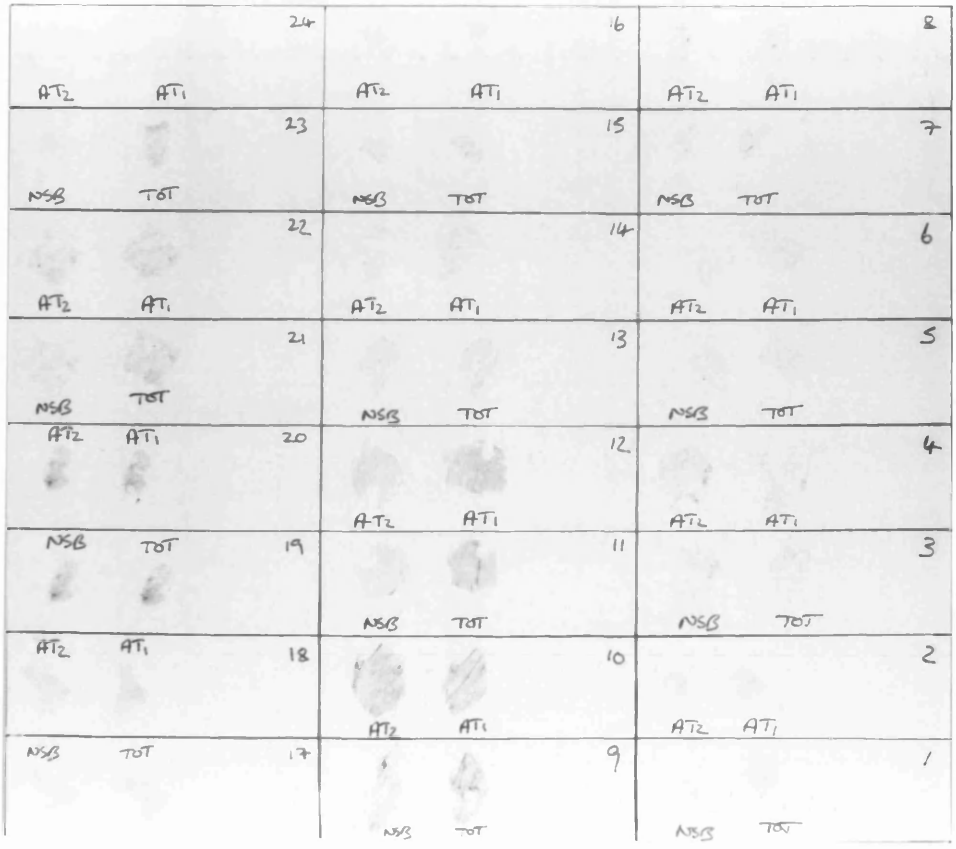
Angiotensin II receptor autoradiography shows angiotensin II receptors in rat kidney, rat brain, rat lung & human placenta (Figure 29) but no evidence of receptors in human lung (Figures 30). This is confirmed by luminosity measurements (Table XVII) in the human lung where there was no significant difference between the values for total, non-specific and AT_1 or AT_2 receptor-specific binding. The small numbers of control rat tissues did not permit statistical analysis.

Figure 29 *Autoradiographs showing angiotensin II receptor binding in rat lung, rat kidney, rat brain & human placenta.*



AT_1: type 1 AII receptor; AT_2: type 2 AII receptor; TOT: total binding; NSB: non-specific binding.

Figure 30 *Autoradiograph showing angiotensin II receptor binding in human lung.*



There are four sections per specimen.

Table XVII *Luminosity values from densitometry measurement of autoradiographs from human lung.*

Specimen	AT_2 Receptor	AT_1 Receptor	Non-specific Binding	Total Binding
1	5544	12832	21518	165371
2	183779	239389	109061	317376
3	249211	264639	246690	270040
4	141355	202831	123132	174535
5	33353	49566	40860	52680
6	58993	76579	80753	79269
7	46503	33255	31105	38213
8	93441	106820	115023	58804
9	330855	552104	173055	399152
10	650999	415154	382424	460876
11	364792	206070	145548	97058
12	143051	138232	153060	153434
Mean (SD)	**191823** (185038)	**191456** (161910)	**135186** (101026)	**188931** (142034)

In situ emulsion autoradiography fails to demonstrate angiotensin II receptors in significant numbers in human lung (Figure 31). However, angiotensin II receptors can be clearly identified over glomeruli in rat kidney control sections (Figures 32 & 33) confirming the efficacy of the technique.

Figure 31 *In situ emulsion autoradiography of human lung.*

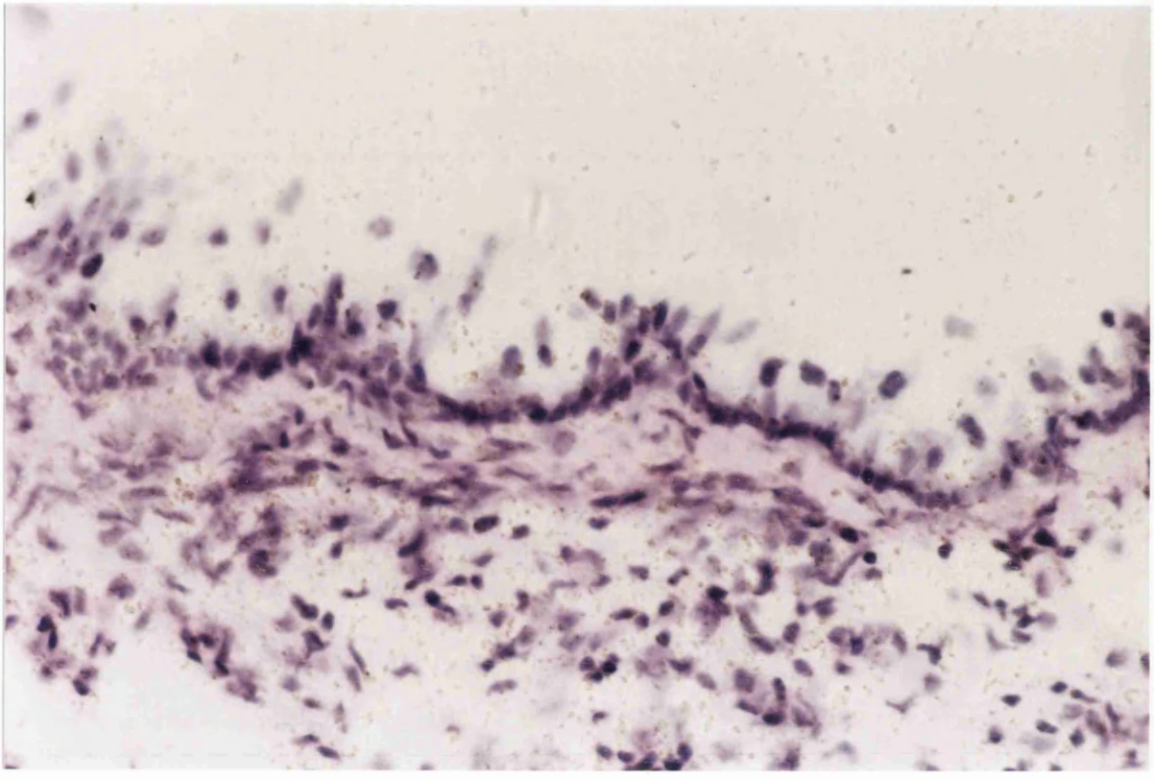


Figure 32 *In situ emulsion autoradiography of kidney - low power view.*

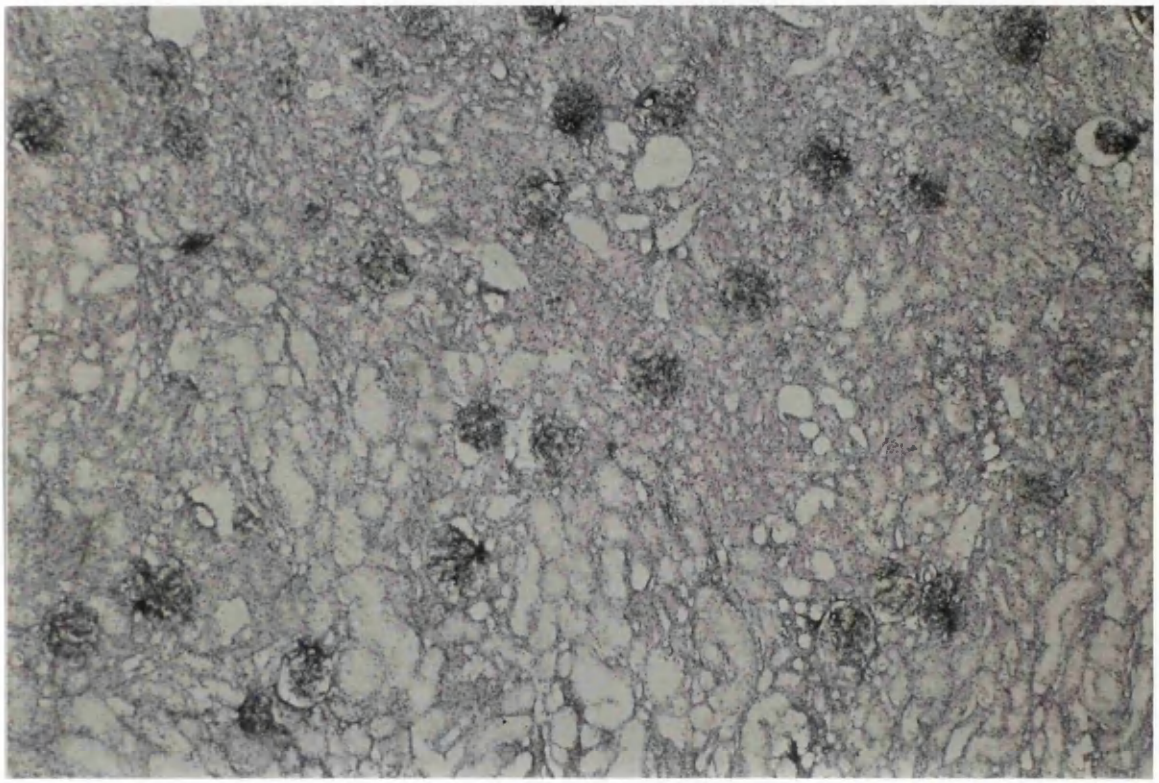
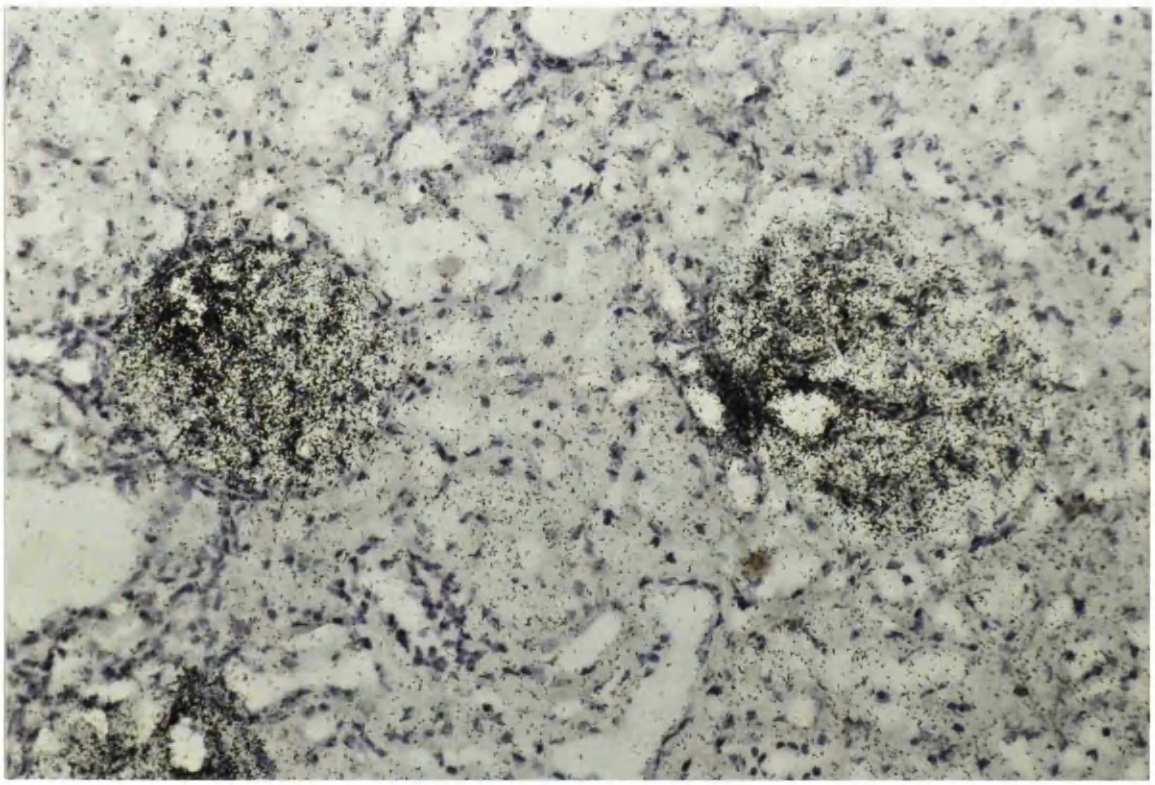


Figure 33 *In situ emulsion autoradiography of kidney - high power view.*



Silver grains can be seen deposited *in situ* over glomeruli indicating angiotensin II receptor binding.

9.4 Conclusion

This study confirms the presence of ACE in human lungs and possibly angiotensin II AT_1 receptors in rat lung, but has found no evidence of renin, angiotensinogen nor angiotensin II receptors in human lung. Thus there does not appear to be a local renin-angiotensin system in the human lung.

10. DISCUSSION

10.1 Activation of the Renin-Angiotensin System in Acute Severe Asthma

This thesis has examined several facets of the renin-angiotensin system with regard to its role in asthma. Further investigations on the mechanism of activation of the renin-angiotensin system in acute severe asthma confirms elevation of plasma renin and angiotensin II levels in some, but not all asthmatics. The strong correlation of plasma renin and angiotensin II levels suggests that the formation of angiotensin II is renin-dependent, presumably via the action of angiotensin-converting enzyme, but the trigger stimulus for activation of the renin-angiotensin system remains unknown.

10.1.1 *Inflammatory Mediators*

Certain inflammatory mediators were found to be elevated to varying extents in the group of subjects with acute severe asthma. Endothelin-1, a potent vasoconstrictor and bronchoconstrictor, is found in human respiratory epithelial cells (23) and contributes to bronchoconstriction in asthma (209). Circulating levels of endothelin-1 are raised in some patients with acute asthma (25) but not in others (81, 210). In my study plasma endothelin-1 levels were raised in 16 patients (40%). The levels showed a trend towards a negative correlation with peak expiratory flow rate suggesting a possible role in bronchoconstriction.

Histamine, derived from mast cells, is involved in both the early and late allergic asthmatic responses (211). In this study, plasma histamine was elevated in 9 subjects (22.5%) and was associated with lower oxygen saturations, which may reflect either histamine-induced bronchospasm or an increase in histamine release due to hypoxia.

Eosinophil numbers are increased in the blood and broncho-alveolar lavage fluid of asthmatics and correlate with the severity of the disease (212). Eosinophilic cationic protein is released from activated eosinophils, is a marker of eosinophil activity and is elevated in serum from asthmatics providing an indicator of inflammatory airflow obstruction (213, 214). In this study, serum levels of ECP were elevated in 10 subjects (25%).

Although the levels of the above inflammatory mediators showed varying degrees of correlation with the severity of the asthmatic attack, there was no relationship with the plasma levels of renin and angiotensin II. Therefore these mediators do not appear to have a major role either directly or synergistically in activation of the renin-angiotensin system.

The findings of this and previous work pose the question as to what purpose activation of the renin-angiotensin system serves in acute asthma. It is unclear whether this represents a pathological phenomenon, a physiological response or merely an epiphenomenon. In any case several potential mechanisms of activation exist:

10.1.2 *Pathological Activation*

The renin-angiotensin system may be activated by direct stimulation of renin secretion via the renal juxta-glomerular α- and β_2-adrenoceptors (187) either by β_2-agonists, adrenaline or noradrenaline. Recently the β_2-agonist salbutamol, administered both intravenously and by nebuliser, has been shown to cause activation of the renin-angiotensin system by a renin-driven, ACE-dependent mechanism (142, 143). In acute asthma there is a marked variation in the plasma levels of salbutamol (144) and also variation in the systemic absorption of nebulised salbutamol (215). In acute asthmatic attacks, the large doses of β_2-agonists administered may reasonably be expected to stimulate renin release since they are in excess of doses that cause elevation of renin and angiotensin II (142, 143). My work confirms a wide range of plasma salbutamol levels which correlate with the calculated dose administered in the preceding 24 hours. However, there was no correlation between plasma levels of salbutamol and renin nor angiotensin II.

Similarly, noradrenaline, which is raised in acute asthma (39) and following histamine-induced bronchoconstriction (216), induces renin release *in vitro*, a mechanism that is potentiated by theophylline (217). In my study noradrenaline was elevated in only three cases, in contrast to previous work (39, 218) and there was no correlation with renin nor angiotensin II. Adrenaline was elevated in only one subject. Thus catecholamines having an additive or synergistic effect with salbutamol on the juxtaglomerular adrenoceptors to stimulate renin release is unlikely. As adrenaline is not elevated, the acute asthma attack and admission to hospital *per se* do not appear to cause sufficient stress to contribute to activation of the renin-angiotensin system.

The role of hypoxia in activation of the renin-angiotensin system is controversial: whilst it potentiates exercise-induced activation of the system (135), it does not directly increase renin and angiotensin II at rest (219). In acute asthma no correlation has been identified between either oxygen saturation or PaO_2 and renin or angiotensin II levels.

The strong correlation of plasma renin with plasma angiotensin II levels indicates that the formation of angiotensin II is renin-dependent, presumably via the action of angiotensin-converting enzyme. Thus formation of angiotensin II by an alternative pathway such as the action of non-specific proteolytic enzymes (113) is unlikely. Although such alternative pathways do not appear to contribute to circulating levels in acute asthma, this does not preclude a role at a local tissue level in the airway.

Angiotensinogen is a member of the serpin family, a group of serine protease inhibitors (220) and may have a role in inhibiting enzymatic action in acute inflammation. Angiotensinogen synthesis has been shown to increase by around 70% in inflammatory states such as pneumonia (111). Angiotensin II may counteract the vasodilation that accompanies fever and sepsis (111). Plasma levels of angiotensinogen are also increased by oral oestrogens, hypophyseal adreno-corticotrophic hormone and glucocorticoids (221), the latter effect probably due to mineralocorticoid activity (222).

Asthma is now treated routinely with inhaled corticosteroids and both oral and intravenous corticosteroids are administered in acute exacerbations. This could result in further elevation of angiotensinogen levels. Unfortunately in this study it was not possible to measure plasma angiotensinogen, but there was no correlation between the administered corticosteroid dose and plasma renin or angiotensin II levels. It would be interesting to look further at plasma angiotensinogen and angiotensin II levels in asthma. In addition, measurement of oestrogen and angiotensinogen levels in female asthmatics who have pre-menstrual worsening of their symptoms would be of interest, particularly in the light of concerns being raised over the apparent increased risk of developing late-onset asthma through the administration of oestrogens as hormone replacement therapy (223).

10.1.3 *Physiological Activation*

Elevation of mean systolic blood pressure occurs in acute asthma (39). The absence of raised blood pressure in those asthmatics with elevation of renin and angiotensin II levels is therefore surprising. The release of renin and subsequent angiotensin II formation in acute asthma may be part of a physiologic homeostatic regulatory mechanism to maintain an adequate blood pressure in the face of arterial vasodilatation. Indeed this role for the renin-angiotensin system has been postulated in acute inflammatory conditions (111). Histamine has vasodilator properties (21) but blood levels did not correlate with renin or angiotensin II levels. Bradykinin is also a potent bronchoconstrictor and also causes arterial vasodilation and capillary leakage (224). Plasma kinin is elevated in acute asthma and has been found in bronchoalveolar

lavage fluid from asthmatics following allergen challenge (225). Therefore bradykinin-induced vasodilation could trigger activation of the renin-angiotensin system, however bradykinin levels have not been estimated in this study.

The renin-angiotensin system is also activated by a drop in renal perfusion pressure, either a local reduction in renal artery flow or a reduction in systemic blood pressure. In acute severe asthma, raised intra-thoracic pressure produces mechanical impairment of cardiac diastolic ventricular filling (*pulsus paradoxus*) leading to an intermittent drop in systemic blood pressure which could stimulate the renin-angiotensin system. The levels of urea and creatinine measured in the asthmatic subjects were normal making a chronic disturbance of renal function unlikely but no inferences can be made about acute changes in renal function.

Electrolyte imbalance, in particular hyperkalaemia, can stimulate renin release. Administration of salbutamol is known to cause a shift of potassium ions intracellularly resulting in hypokalaemia (226) and thus would antagonise renin release. However, sodium and potassium levels were normal in the study subjects.

It still remains unclear what role the renin-angiotensin system is serving in acute asthma although the administration of salbutamol may in part stimulate renin release. The absence of a rise in blood pressure suggests that angiotensin II may be playing a compensatory role in maintaining blood pressure in the face of vasodilation induced by the inflammation in acute asthma.

10.2 The ACE Gene Insertion/Deletion Polymorphism in Asthma

There is variation in serum ACE activity as the result of an insertion/deletion polymorphism of the ACE gene (169). Given the elevation of plasma angiotensin II and renin in some acute asthmatics but the lack of any association with serum ACE activity the role of the ACE genotype in asthma was studied. Individuals who are homozygous for the deletion polymorphism have a higher serum ACE activity (169) and subsequently the ability to generate greater amounts of angiotensin II from angiotensin I (172). This could potentially explain the elevation of angiotensin II in some asthmatics but would not account for elevation of renin levels.

In studies of ACE genotyping using the two-primer method of Rigat *et al.* (193), polymerase chain reaction has on occasion failed to amplify the insertion allele in heterozygotes. This can lead to overestimation of the DD homozygote. However, the three-primer method of Evans *et al.* (174) increases the accuracy of detection of the

insertion. In this study both methods have been employed and the ACE genotype was confirmed in all cases with both methods.

My results confirm that the highest ACE activity in both the asthmatic and control groups was associated with the deletion homozygote, the lowest ACE activity with the insertion homozygote and the heterozygote intermediate. In the asthmatic subjects there was an excess of the deletion allele when compared to the control population however this did not reach statistical significance. This finding is supported by a recent study by Benessiano *et al.* (227) which found a higher frequency of the deletion homozygote genotype in a group of asthmatics compared to non-asthmatic controls.

Although there may be an association between the ACE deletion allele and asthma it is difficult to implicate this in the aetiology of asthma. Benessiano was unable to demonstrate any association between ACE genotype and pulmonary function (227). Costerousse (170) showed that ACE is expressed in human T lymphocytes and monocytes and, although making little contribution to circulating ACE, it contribute to local tissue levels. T lymphocytes accumulate at sites of inflammation and angiotensin II is chemotactic for T cells. Bradykinin and substance P are also involved in lymphocyte proliferation, neutrophil chemotaxis, phagocytosis and mediator release, therefore T lymphocyte ACE may act as a regulatory mechanism to metabolise bradykinin produced locally during tissue inflammation. As a consequence of this, increased amounts of angiotensin II could be produced locally.

Lung tissue ACE activity has not been studied in asthmatics although it is well known that the pulmonary capillaries are the richest source of ACE as a consequence of the size of the capillary bed. It would be interesting to compare asthmatic lung specimens with those of non-asthmatic controls to investigate whether the inflammatory response in asthma has any effect on lung tissue ACE activity.

Serum ACE activity is elevated in other pulmonary diseases, in particular sarcoidosis. This is due to activation of the monocyte-macrophage system forming granulomas (228). *In vitro*, monocytes increase ACE expression during differentiation to macrophages (170). Serum ACE is also elevated to varying degrees in many other diseases including tuberculosis, thyrotoxicosis, diabetes mellitus, silicosis, leprosy and alcoholic liver disease (228). Although not sufficiently specific to be used as a diagnostic tool in sarcoidosis, serum ACE activity is a useful index for monitoring disease activity.

It is known that ACE activity is suppressed by corticosteroids in both healthy individuals and those with lung disease including asthmatics (229). However, no association was found between ACE activity and steroid dose in this study. Combined oestrogen and progestogen hormone replacement therapy also reduces serum ACE activity in post-menopausal women by 20% over 6 months and ACE activity also falls during pregnancy (230). Not surprisingly, ACE activity also falls during treatment with ACE inhibitors (228).

A troublesome side-effect of ACE inhibitors is cough, which is attributed to increased bradykinin, but cough does not occur in all those treated. Furuya *et al.* (231) demonstrated that those with ACE inhibitor-induced cough had an increased frequency of the insertion allele and the II homozygote genotype with consequently the lowest baseline ACE activity. This appears to increase susceptibility to ACE inhibitor-induced cough. Given the effect of angiotensin II on bronchial reactivity it would be reasonable to suggest that ACE inhibitors may also have an effect on the airways, however this area remains controversial. In one series of patients with ACE inhibitor-induced cough enalapril increased bronchial reactivity to histamine (232). Another series of subjects with ACE inhibitor-induced cough found a lower histamine PC_{20} than controls on ACE inhibitors with no cough, suggesting this may be due to bradykinin (233). However, a further study of hypertensive asthmatics demonstrated that treatment with captopril had no effect on FEV_1 or bronchial reactivity to methacholine (234). Therefore if ACE inhibitors have any effect on the airways it appears to be relatively minor and of doubtful clinical significance.

Polymorphisms of the other components of the renin-angiotensin system, in particular the angiotensinogen gene (183), which is the rate-limiting element in the system, could affect angiotensin II levels. Therefore, if a sub-group of asthmatics had the propensity to produce higher amounts of angiotensinogen, that, in combination with other stimuli such as exogenous β_2-agonists could theoretically lead to the production of higher levels of angiotensin II for a given increase in renin release.

A further unexpected finding was the variation in ACE activity for a given genotype between acute medical hospital admissions and normal controls. When the data from the asthmatic subjects and a group of non-asthmatic acute medical hospital admissions were combined, the serum ACE activities for the DD and ID genotypes were higher than for the equivalent genotypes in the control population. Despite this, there was no difference in the allele frequency or genotype distribution between the two groups. This difference was not evident when comparing the asthmatic subjects alone

and may therefore simply be a consequence of the relatively small study numbers. In addition, the control population was younger than the hospital in-patient group and thus the difference seen may be an age effect. The stress of hospital admission may increase ACE activity but no association was found with circulating catecholamine levels. Indeed, the majority of the asthmatics received treatment with corticosteroids which are known to suppress ACE activity (228). Thus there appear to be other factors involved in determining ACE activity than simply ACE genotype. It is also possible that individuals with a given genotype who have the capacity to produce a higher ACE activity may be more susceptible to medical illness.

10.3 The Effect of Angiotensin II on Histamine-Induced Bronchoconstriction

Angiotensin II has weak bronchoconstrictor properties (132) but also has the capacity to potentiate the bronchoconstrictor effects of the mediators methacholine (*in vivo* and *in vitro*) (141) and endothelin-1 (*in vitro*) (26). These effects appear to be mediated by the AT_1 angiotensin II receptor subtype (26). The effect of angiotensin II on other mediators of bronchoconstriction is hitherto unknown and thus histamine-induced bronchoconstriction has been examined. However, the results show that angiotensin II does not affect histamine-induced bronchoconstriction in human bronchi either *in vitro* or *in vivo*.

In vivo, intravenous infusion of angiotensin II results in an elevation of plasma angiotensin II to a maximum of median (inter-quartile range) 43.9 (36.9-52.4) pg/ml compared to maximum levels measured by Millar *et al.* (132) in asthmatic patients of 56 (12-109) pg/ml and levels of 10.9 (4.3-23.5) pg/ml in the current study during acute severe asthma. Therefore, the levels achieved by the infusion of angiotensin II are physiologically relevant.

The rise in systolic and diastolic blood pressure during the 1 and 2 ng/kg/min. angiotensin II infusions confirms its physiological effect. The plasma levels of angiotensin II measured during this study were similar to those found in the previous study by Millar *et al.* (141) where angiotensin II potentiated methacholine-induced bronchoconstriction.

As there was no significant effect on spirometry following 30 minutes of the infusion on each study day it can be concluded that the dose of angiotensin II administered did not cause bronchoconstriction.

The reason for angiotensin II potentiating methacholine- (141) but not histamine-induced bronchoconstriction is undoubtedly complex and may be multi-factorial. The two studies have heterogeneous groups of subjects although four subjects took part in both studies. The current study group were older [mean (SD) age 41 (5.3) years compared to 27 (8) years in the study by Millar *et al.* (141)], but both groups were mild asthmatics with mean (SD) FEV_1 88 (11)% predicted in the current study and 82 (9)% predicted in the study by Millar *et al.*(141). Bronchial reactivity during placebo infusion was similar in both groups with geometric mean (range) PC_{20} histamine 2.67 (0.89-9.57) mg/ml in the current study and PC_{20} methacholine 3.09 (1.15-6.0) mg/ml in the study by Millar *et al.* (141). Therefore it is unlikely that variation in patient selection between these two studies accounts for the different effect of angiotensin II on histamine- and methacholine-induced bronchoconstriction *in vivo*.

The severity of asthma may be important: In asthmatics with greater bronchial reactivity the effect of angiotensin II on histamine-induced bronchoconstriction may be different due to greater local inflammation, the presence of higher levels of mediators or an increased volume of airway smooth muscle. However, it is established that in mild asthma angiotensin II can potentiate the effect of methacholine and thus there appears to be a fundamental difference between the interaction of angiotensin II with methacholine and histamine.

10.3.1 *Receptors*

In the human airway the lack of interaction between angiotensin II and histamine could be explained by the absence of functional angiotensin II receptors. Although there is no direct evidence of tissue-specific angiotensin II receptors in the lungs, Curnow *et al.* (137) have extracted type-1 angiotensin II (AT_1) receptor mRNA from human lung tissue. Both AT_1 (167) and AT_2 (139) angiotensin II receptor mRNA have also been identified in fetal rat lung.

In vitro, studies of reactivity in bovine bronchial rings have shown an AT_1 receptor-mediated potentiating effect of angiotensin II on endothelin-1 evoked bronchoconstriction (26). Although the mild bronchoconstrictor effect of angiotensin II in asthmatic airways *in vivo* (132) and its potentiating effect on methacholine-induced bronchoconstriction (141) are not proven to be receptor-specific phenomena, it is likely that functional angiotensin II receptors exist in the human airway.

A further potential site of interaction is at the receptor itself. Angiotensin II uncovers previously silent α_2-adrenoceptors in the rabbit distal saphenous artery (196) and if a similar interaction occurred with receptors for spasmogens in airway smooth muscle this could lead to potentiation.

10.3.2 *Second Messengers*

The lack of potentiation with angiotensin II and histamine may be due to different intracellular second messenger pathways and varied cross-talk between pathways. In vascular tissue, the type 1 angiotensin II receptor is coupled to a G-protein activating phospholipase-C, liberating diacyl-glycerol (DAG) and inositol-triphosphate (IP_3) thereby causing a rise in intracellular calcium (235). In some tissues, the angiotensin II receptor is also coupled to an inhibitory G-protein which inhibits adenylate cyclase (236). Studies also suggest that angiotensin II may be able to act directly within cell nuclei, possibly reaching the cell nucleus by receptor-mediated endocytosis (131, 237). This could circumvent second messenger pathways. However, the effect of angiotensin II on intracellular pathways in airway smooth muscle remains to be characterised.

Histamine acts on airway smooth muscle via the H_1 receptor which is also coupled to a G-protein activating phospholipase C and liberating DAG and IP_3 (20). The increase in IP_3 is inhibited by cyclic adenosine-monophosphate (cAMP), which attenuates the histamine-induced increase in intracellular calcium in canine airway smooth muscle (20). Histamine also exerts some of its effects by reflex bronchoconstriction through activation of cough receptors in the larynx and trachea (22).

Cholinergic muscarinic receptors in the airways are also coupled to membrane phospholipid hydrolysis to form IP_3 but this occurs by a different pathway which is insensitive to cAMP (20).

Therefore the second messenger pathways for histamine and acetylcholine /methacholine are subject to different modulatory influences and cross-talk with angiotensin II second messenger pathways intracellularly could have different end results.

10.3.3 *Innervation*

The most likely explanation for the different effect of angiotensin II on the actions of histamine and methacholine is the role of parasympathetic ganglion cells. Angiotensin II acts pre-synaptically on cholinergic neurones inducing the release of acetylcholine (44); when combined with the administration of nebulised methacholine, this would result in a greater degree of bronchoconstriction. Angiotensin II also enhances the responses of tissues to sympathetic nerve stimulation and exogenous adrenoceptor agonists by several mechanisms: i) activating pre-junctional angiotensin II receptors which facilitate noradrenaline release, ii) preventing noradrenaline reuptake by the nerve terminal, iii) increasing the synthesis of noradrenaline and iv) enhancing the response of effector cells to noradrenaline (197).

Mast cells are found around parasympathetic ganglion cells in the guinea pig airway and degranulate following antigen exposure releasing histamine and other preformed mediators locally. Following both antigen challenge and the direct administration of histamine, enhanced excitability occurs in guinea pig bronchial ganglion neurones (43). Therefore, if histamine is already having a direct effect on presynaptic cholinergic ganglion cells then it is unlikely that angiotensin II could enhance this significantly via the release of further acetyl choline.

This is undoubtedly an over-simplification of the complex process occurring within these ganglia in the airway wall. These neurones are subject to many different modulations of their activity by both facilitatory and inhibitory influences (22). Many pre-ganglionic axons converge on parasympathetic ganglion neurones which may require the input of many sub-threshold stimuli to elicit a post-ganglionic effect (238).

Thus there are several ways to explain the difference between the effects of angiotensin II on methacholine and histamine in human airways. It will be interesting to assess the effect of angiotensin II on endothelin-1-induced bronchoconstriction in the human airway in future studies.

10.4 Angiotensin II as a Growth Promoter in Airway Remodelling

In chronic asthma, airway remodelling causes changes in the epithelium, basement membrane, mucous glands, smooth muscle and connective tissue (48). The two most important functional changes are first, increased airway smooth muscle which amplifies the effects of bronchoconstrictor stimuli (54) and secondly, thickening of the adventitia which uncouples the airway from the elastic recoil forces of the surrounding lung parenchyma (68).

Many growth promoters contribute to airway remodelling (73, 201) and these are listed in Table I. In some cases, these agents have a synergistic effect on airway smooth muscle cells (32). Recent *in vitro* work shows that angiotensin II to be a growth promoter for human airway smooth muscle cells (101).

Angiotensin II is an important growth promoter in cardiovascular remodelling. It increases DNA synthesis in arteries both by raising blood pressure and by a direct effect on the arterial wall independent of blood pressure (154). There are many parallels between the structural changes in the vasculature and in the airways although the physiology of the two systems is quite different.

The work in Chapters 7 and 8 examining the structural changes in the rat airway and the systemic vasculature has manipulated the renin-angiotensin system by different means. In the first study, a two week infusion of angiotensin II increased circulating angiotensin II. In the second study, dietary sodium restriction was used to cause endogenous stimulation of the renin-angiotensin system through systemic sodium depletion.

In the first study a two week infusion of angiotensin II causes a significant rise in blood pressure and suppresses renin mRNA expression and presumably renin synthesis in the juxtaglomerular apparatus of the kidney. This confirms the findings of previous studies in rats where infused angiotensin II caused a 45% reduction in renin mRNA expression in the kidneys (110). The animals infused with angiotensin II had significantly higher BrdU indices in arteries and although the arteries tended to be thicker in the angiotensin II group, there was no significant difference in geometry of the mesenteric arteries and veins between the two groups. These findings are in keeping with previous angiotensin II infusion studies (150, 186). The absence of a rise in plasma angiotensin II at the end of the infusion may reflect homeostatic compensatory reduction in endogenous angiotensin II formation.

In the airway, there was no difference in either the BrdU indices or the volume of the epithelium, airway smooth muscle and adventitia in the control and angiotensin II infused animals.

In the second study sodium depletion caused minor, insignificant variations in blood pressure, with elevation of plasma renin activity and aldosterone confirming activation of the renin-angiotensin system. Kidney renin mRNA expression increased, confirming the findings of previous sodium depletion studies where renal

angiotensinogen mRNA was also increased (239). A separate study by McEwen *et al.* (186) has examined the mesenteric vasculature from these animals and found a significant increase in DNA synthesis in the arterial media of the sodium-depleted group.

By contrast, in the airways sodium depletion had no effect on DNA synthesis in the epithelium, airway smooth muscle or adventitia and no morphometric changes were found in the volume of these three tissues.

The lack of increased DNA synthesis in the airways makes hyperplasia and hypertrophy unlikely. This is supported by the lack of morphometric changes. Thus there is no evidence of remodelling in the rat airway in response to systemically infused angiotensin II or to endogenous activation of the renin-angiotensin system by dietary sodium restriction despite changes in the vasculature.

There are several possible reasons for the discrepancy between the effects of angiotensin II on arteries and airways. First, it is well established that angiotensin II is a growth promoter in the vasculature (152), the heart (145) and the kidneys (146). This effect is mediated primarily by AT_1 angiotensin II receptors (240) and although these receptors are present in arteries (131) their existence has yet to be confirmed in the airways. AT_1 receptor mRNA has been isolated from human (137) and rat (136) lung, however, my histological studies of human lung tissue (Chapter 9) have failed to demonstrate angiotensin II receptors *in situ* although they may be present in rat lung. Wharton *et al.* (241) have presented contrasting evidence which demonstrates AT_2 receptors in human fetal lung and both AT_1 and AT_2 receptors in human adult lung using similar techniques.

In vitro, angiotensin II potentiates endothelin-1-evoked bovine bronchoconstriction by an AT_1 receptor-mediated effect (26). This provides indirect evidence for the existence of functional angiotensin II receptors in bovine airways, but there may be species variation in the number and distribution of angiotensin II receptors.

Angiotensin II receptor expression is regulated both by changes in sodium homeostasis and by the renin-angiotensin system. In sodium deficiency, angiotensin II receptors increase in the adrenal zona glomerulosa and decrease in the vasculature, renal glomeruli, platelets, uterine and bladder smooth muscle. These changes appear to be related to raised circulating angiotensin II levels and can be reproduced by angiotensin II infusion and reversed by administration of an ACE inhibitor. Uterine angiotensin II

receptors are also modulated by oestrogen levels. Brain angiotensin II receptors are not affected by changes in either circulating or local angiotensin II levels. Angiotensin II receptors in the anterior pituitary are reduced by chronic oestradiol administration but are not affected by angiotensin II infusion or sodium balance (131). Glucorticoids potentiate the vasoconstrictor action of angiotensin II by increasing angiotensin II receptor numbers (242).

Thus, in both of the studies manipulating the renin-angiotensin system there will be down-regulation of angiotensin II receptors in the vasculature. The regulation of angiotensin II receptors in the lungs is not known, but is likely to follow that of receptors in other tissues and therefore may be down-regulated. Despite down-regulation of angiotensin II receptors in the vasculature, increased DNA synthesis still occurs, so down-regulation *per se* is not sufficient to prevent the mitogenic effect of angiotensin II. However, if the initial receptor density were lower in the lungs than in the vasculature, a further reduction in receptor numbers may be sufficient to fall beneath a threshold level whereby angiotensin II may not exert proliferative effects.

Secondly, systemic infusion of angiotensin II causes changes in the vasculature, but concentrations of angiotensin II reached in other tissues are not known. Local angiotensin II levels in the airway may not reach threshold concentrations for induction of cell growth. However, certain inflammatory proteases can cleave angiotensinogen to form angiotensin II (118) and could feasibly produce higher tissue levels in the presence of inflammation.

Thirdly, although angiotensin II promotes growth, other mediators have anti-proliferative properties and antagonise its effects. In the arterial wall TGF-β_1 secreted by endothelium antagonises the effect of angiotensin II on medial smooth muscle (152). This inhibition is removed by endothelial damage. Bronchial epithelium also synthesises TGF-β_1 (153) thus epithelial damage and shedding that occur in asthmatic airways could facilitate growth promotion by angiotensin II through removing an important inhibitor. Conversely, the absence of structural changes in the rat airway in these studies could reflect the presence of compensatory anti-proliferative stimuli in an intact epithelium. The AT_2 angiotensin II receptor subtype has an anti-proliferative effects in some tissues (138) and if predominant in rat airways, would result in a net negative effect of angiotensin II on cell growth.

Angiotensin II has synergistic properties potentiating some bronchoconstrictors (26, 141) but not others (Chapters 5 and 6). In asthma, inflammation with capillary

leakage results in the presence of many different inflammatory mediators which could act synergistically to cause airway cell growth and bronchoconstriction. As airway remodelling is probably the end result of a combination of many growth promoters and inhibitors, any effect of angiotensin II on other modulators of cell growth could alter the final outcome even if angiotensin II itself has no proliferative effect. Thus, the absence of airway inflammation in both studies may be masking a potential growth promoter effect of angiotensin II if co-mitogens were present. A suitable model of airway inflammation would be required to test this point further.

Finally, although angiotensin II promotes growth of human airway smooth muscle *in vitro,* my results may simply indicate that angiotensin II infusion has no effect on cell growth in the normal rat airway *in vivo.*

In summary, neither a two week infusion of angiotensin II sufficient to cause an elevation of blood pressure nor a two week period of dietary sodium restriction, both of which are sufficient to increase DNA synthesis in the mesenteric arteries, cause significant airway remodelling in the rat. Further *in vivo* manipulations of the renin-angiotensin system in a suitable model of airway inflammation are required to firmly establish or refute the role of angiotensin II in airway remodelling in asthma.

10.5 Is There a Local Renin-Angiotensin System in the Lungs?

The renin-angiotensin system involves several organs including the liver, kidneys, lungs, vasculature and adrenal glands. However, local renin-angiotensin systems have been identified in many organs and tissues as reviewed in the Introduction (Section 1.6). Circumstantial evidence from many existing studies would support the existence of a local renin-angiotensin system within the lungs but hitherto no studies have examined this directly.

To prove the existence of a local renin-angiotensin system it is necessary to demonstrate the presence of the components, namely angiotensinogen, renin, ACE and functional angiotensin II receptors in lung tissue. Histological techniques can be used to identify peptides and enzymes directly by applying specific, labelled antibodies by immunocytochemistry. Using this method my results confirm the presence of ACE distributed in pulmonary capillaries and on vascular endothelium. Antibodies for renin failed to demonstrate any evidence of this.

More sensitive molecular biological techniques can be used to identify the presence of messenger RNA which gives the tissues the ability to transcribe the

peptides and enzymes being studied. *In situ* hybridisation allows this to be performed on tissue sections permitting histological identification of the positive cell or tissue types. However, using labelled riboprobes for angiotensinogen, ACE, renin and the AT_1 angiotensin II receptor, no evidence of these was found in human lung tissue.

Finally, homogenised lung tissue was examined by Northern Blot analysis to look for the presence of mRNA fragments but again no evidence of the components of the renin-angiotensin system was found. Given these negative results it was decided not to proceed to polymerase chain reaction, the most sensitive investigation available.

The search for angiotensin II receptors with autoradiographic film and *in situ* techniques failed to demonstrate receptors in human lung, although films suggested the presence of AT_1 receptors in rat lung. This may be due to species differences in receptor numbers and expression. However, this may also be a consequence of infiltration by inflammatory cells as many of the rat lung specimens showed histological evidence of pulmonary infection, a common problem in animal houses.

I found no evidence of any of the components of the renin-angiotensin system in human lung apart from ACE which is well known to be present in pulmonary capillaries. The renin riboprobe was technically satisfactory as shown by the strongly positive control thus supporting the negative findings in human lung as a true negative for renin mRNA. These negative findings for the other components may also be a consequence of inadequacies in our molecular techniques as difficulties with the riboprobes led to failure on positive control tissues with the probes for angiotensinogen, ACE and the AT_1 receptor. In addition, low levels of tissue expression of these components may be undetectable by the sensitivity of the techniques employed.

The existing evidence for the presence of the components of the renin-angiotensin system in the lungs is mainly circumstantial from various studies in both rat and human tissue. ACE is undeniably present (158, 159). Although renin has been demonstrated immunocytochemically in human lung this has only been in pathological conditions such as bronchial carcinoma of different histological types (161, 162). It is possible that renin is taken up from the circulation by cells in these pulmonary lesions and stored in lysosomes rather than being synthesised within the lungs (243). Renin mRNA has been found in normal rat lung (164) but there are no reports in normal human lung. A case-report describes the presence of renin mRNA localised to tumour cells in a pulmonary metastasis from an epithelioid soft tissue sarcoma but there was no evidence of positive cells in the unaffected human lung tissue (163). Angiotensinogen

mRNA has been identified in rat lung (165) but there are no reports in human lung. The results of my work are in accord with these findings.

Angiotensin II receptor mRNA, both AT_1 (136, 166), predominantly the AT_{1A} subtype (167, 168), and AT_2 (139) has been identified in normal rat lung. Curnow *et al.* (137) have identified AT_1 mRNA in normal human lung where it is expressed in similar amounts to the kidneys and testes. Since the completion of the work of this thesis another group using a similar technique with radio-labelled angiotensin II have demonstrated the presence of angiotensin II receptors in normal fetal and adult human lung (241). In fetal lung the AT_2 subtype was predominant and localised to the bronchi while the AT_1 subtype was present in lower density in the mesenchyme. In adult lung receptors were found in the vasculature (mainly AT_1) and parenchyma (both AT_1 and AT_2). Alveolar cells expressed AT_2 receptors but no comment was made about bronchial smooth muscle.

The failure to demonstrate angiotensin II receptors in my human lung specimens does not appear to be a technical problem as both subtypes of receptors are clearly demonstrated in several control tissues. Collection and processing procedures were identical for both lung and control tissues however, frozen lung proved more difficult to section. All the human lung specimens were obtained from cigarette smokers with bronchial carcinoma and it is possible that exposure to cigarette smoke or factors released from the tumour cells may cause down-regulation of receptor expression.

In summary, although there is some doubt whether the lungs possess all components of a local renin-angiotensin system, they play an important role in the systemic renin-angiotensin system through the actions of pulmonary ACE. The lungs appear to be another target organ for the renin-angiotensin system. AT_2 receptors may play a role in fetal development (139) and thus may be involved in the regulation of fetal lung development. In addition, functional angiotensin II receptors respond to circulating angiotensin II by bronchoconstriction (132). However the physiological relevance of this remains uncertain. It is possible that some components of the renin-angiotensin system may be present in small amounts below our limits of detection or may interact with the circulating renin-angiotensin system. Active inflammation within the lungs may be required to "switch on" the production of components locally, for example it is known that inflammatory proteases can produce angiotensin II from angiotensinogen (113) and angiotensin I (120).

11. CONCLUSION

11.1 Conclusion

The work described in this thesis has examined the role of the renin-angiotensin system in asthma. Elevation of plasma renin and angiotensin II levels was again demonstrated in some asthmatics during episodes of acute severe asthma. Despite an extensive evaluation of clinical features, circulating peptides, inflammatory mediators and catecholamines no correlation was found. In particular, plasma salbutamol levels, despite administration in high doses, showed no relation with either plasma renin or angiotensin II levels. Various mechanisms of activation have been discussed but my results do not implicate any single factor. The many inflammatory mediators and cytokines released during acute attacks of asthma, as well as exogenously administered medications could interact synergistically to activate the system, but no evidence has been found to support this.

Although serum ACE activity did not correlate with plasma renin or angiotensin II levels, a higher ACE activity was associated with the deletion homozygote than the heterozygote and insertion homozygote. In addition, there was a trend for the deletion allele to be present in a higher frequency than expected in the group of acute asthmatics. This finding needs further evaluation to determine its clinical relevance.

Although angiotensin II can potentiate the effects of other bronchoconstrictors such as methacholine and endothelin-1, it has no effect on histamine-induced bronchoconstriction either *in vitro* or *in vivo*. The possible reasons for this have been discussed already (Section 10.3). It will be interesting to see the results from future studies examining the interaction of angiotensin II with other mediators such as endothelin-1 and leukotrienes in human airways.

Despite angiotensin II being a likely candidate as a growth promoter for airway smooth muscle and airway remodelling, infusion of angiotensin II into rats over a two-week period and endogenous activation of the renin-angiotensin system by dietary sodium restriction had no effect on DNA synthesis or airway morphometry. However, DNA synthesis increased in the mesenteric arteries in both these cases. This may represent a species-specific effect as angiotensin II has mitogenic properties on human airway smooth muscle cells *in vitro* (101).

Immunohistochemical examination of human lung tissue has confirmed the presence of ACE in pulmonary capillary vascular endothelium but neither renin, angiotensinogen nor type 1 or type 2 angiotensin II receptors were found. Angiotensin II receptor labelling did however appear to demonstrate angiotensin II receptors in rat lung, but further evaluation is required to confirm this and to localise the receptors to

particular cell types. My work has failed to confirm the findings of Wharton *et al.* (241) who demonstrated angiotensin II receptors in both fetal and adult human lung using similar techniques. Circumstantial evidence as discussed previously makes the presence of functional angiotensin II receptors in human lungs likely.

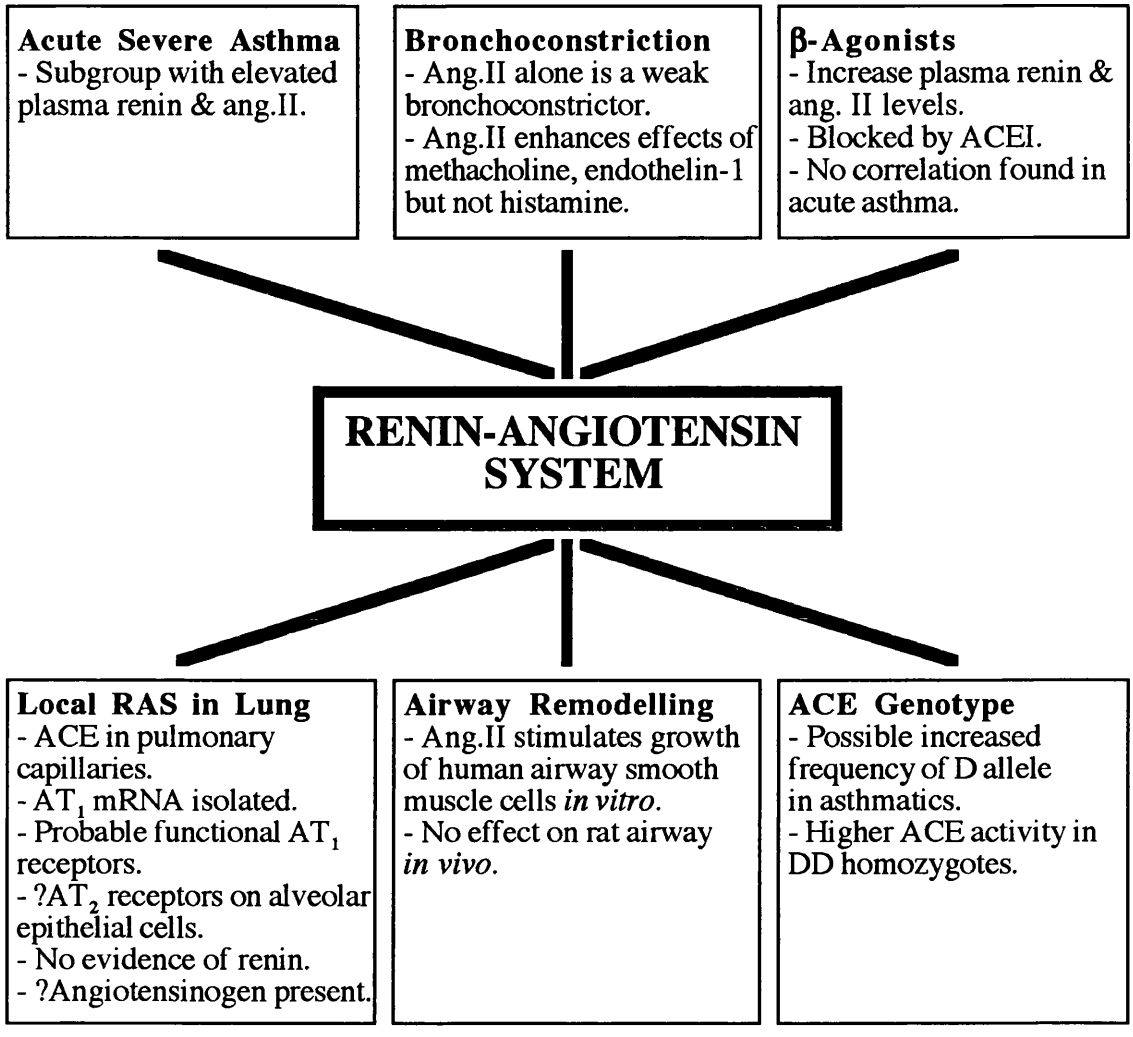
The clinical relevance of the renin-angiotensin system's involvement in asthma is of questionable significance. Although the lungs are organs which are intimately part of and respond to the renin-angiotensin system they do not appear to possess the components necessary to comprise a local system.

The fall in FEV_1 in response to infused angiotensin II is only 12% (132) which is a marginal change in airway calibre in normal lungs but may be more significant if occurring in airways which are already narrowed. This fall is also much less than other bronchoconstrictors of known clinical relevance such as histamine and acetylcholine. Therefore angiotensin II has at best only a minor role in bronchoconstriction occurring in clinical situations in the presence of other more potent spasmogens. The synergistic effects of angiotensin II are of more interest and may be of more clinical relevance particularly in acute severe asthma. However, these synergistic effects vary among different bronchoconstrictors.

If angiotensin II is shown to have mitogenic effects on airway smooth muscle in human lung it will join the growing list of substances identified as growth promoters in airway remodelling (73). This is a relatively recent concept which explains the irreversible component to airflow obstruction found in some chronic asthmatics. These growth promoters remain to be ranked by their relative potency to allow targeting of specific agents for therapeutic intervention, however it is currently believed that inhaled corticosteroids commenced early in the course of the disease may help to prevent structural changes.

The current state of knowledge regarding the renin-angiotensin system and asthma is summarised in Figure 34.

Figure 34 *The current role of the renin-angiotensin system in asthma.*



Key: Ang.II: angiotensin II; ACE: angiotensin-converting enzyme; ACEI: ACE inhibitor; RAS: renin-angiotensin system; AT_1: type 1 angiotensin II receptor; AT_2: type 2 angiotensin II receptor; D: deletion allele.

The renin-angiotensin system does appear to have a minor involvement in asthma with a borderline clinical relevance in some asthmatics. It is unlikely to have a major role in chronic or stable asthma and the evidence currently available for its effects, if any, in acute severe asthma is not sufficient to warrant trials of therapeutic intervention with drugs such as ACE inhibitors and angiotensin II receptor antagonists. However, the elevation of plasma renin and angiotensin II should be borne in mind in the future as the results of further studies on the pathogenesis of asthma become available.

12. LIMITATIONS OF THE WORK OF THE THESIS

12.1 Clinical Studies

The numbers of patients recruited into the acute asthma study were relatively small given that only a sub-group had activation of the renin-angiotensin system and although the results obtained were unequivocally negative it may be possible that a minor association could have been missed.

The study of ACE genotype in asthmatics has demonstrated a trend towards a higher than expected frequency of the deletion allele however, this did not reach statistical significance. It is possible that if a larger number of asthmatics were studied this may reveal a significant difference.

12.2 *In Vitro* Studies

The studies investigating the effect of angiotensin II and sodium depletion on remodelling of the rat airway are limited by the fact that although angiotensin II is raised in asthma, in the rat airways studied there was no underlying inflammation as would be found in asthmatics. Furthermore, it is not clear if rat airway smooth muscle is responsive to angiotensin II and *in vitro* cell culture studies may be useful to clarify this.

The human lung specimens obtained for the study were unfortunately not from normal, healthy volunteers but from patients undergoing thoracotomy and lung resections for bronchial carcinomas, all of whom were smokers. Therefore the lung tissue used was "normal" lung from the resected tissue taken as far from the tumour margins as possible. However it is not possible to exclude an effect caused by a field change induced by exposure to tobacco smoke or the effects of tumour factors released locally.

In addition, time limitations and technical problems with riboprobe preparation prevented the histological section of the thesis from being more comprehensive. Despite this valuable experience was gained in the molecular techniques outlined in Chapter 9.

Although the autoradiographs obtained from the rat lung tissues appear to show evidence of AT_1 receptors, it was not possible to localise these histologically. A problem encountered with the laboratory animals used was that of pulmonary infections which are common in animal houses. It is possible that the receptor binding demonstrated may be a consequence of an inflammatory cell infiltrate in the lung.

13. SUGGESTIONS FOR FURTHER WORK

13.1 Acute Severe Asthma

Recruiting greater numbers of subjects may reveal a significant difference in ACE genotype distribution between asthmatics and controls. As plasma angiotensinogen may be the rate-limiting factor in the formation of angiotensin II it would be worth measuring this in acute asthma as it is elevated in acute inflammation and in response to corticosteroid administration. Angiotensinogen is also increased in response to oestrogens and it would be interesting to measure angiotensinogen levels in female asthmatics who have pre-menstrual deterioration of their asthma control.

Another possible reason for activation of the renin-angiotensin system in asthma may be to counteract the vasodilator effects of other mediators such as bradykinin. It would be interesting to measure bradykinin in acute asthma and to compare this with renin and angiotensin II.

Although it is known that circulating angiotensin II is raised in acute asthma, it is not clear whether this reflects the local *milieu* in the airway wall. It would be interesting to obtain induced sputum from asthmatic subjects and to examine the supernatant for angiotensin II to find out if this is reaching the epithelial surface of the airway or is generated locally.

13.2 Challenge Studies

Angiotensin II can enhance the bronchoconstrictor effects of methacholine but not histamine in the human asthmatic airway. Endothelin-1 is another potent bronchoconstrictor and angiotensin II can potentiate its effects *in vitro* in bovine bronchial rings. It would be valuable to investigate the effect of infused angiotensin II on endothelin-1-induced bronchoconstriction in human asthmatics.

Exercise is known to stimulate the renin-angiotensin system and also to be a trigger for asthma. Comparison of plasma renin and angiotensin II levels in response to exercise challenge in asthmatics and non-asthmatic controls would be of interest. The response of the renin-angiotensin system to allergen challenge in sensitive individuals is not known and would be worth exploring further.

If any clinically relevant elevation of renin and angiotensin II is found then attempts to attenuate these changes by pre-treatment with ACE inhibitors or angiotensin II receptor antagonists may prove to be of therapeutic benefit.

Although intravenous angiotensin II is known to have bronchoconstrictor properties, the effect of inhaled angiotensin II on both the normal and asthmatic airway is not known. It would be interesting to compare this with known bronchoconstrictors and to elucidate whether any effect on the airway was irritant or receptor-mediated by using angiotensin II receptor antagonists.

Previous studies have demonstrated elevation of renin and angiotensin II in asthmatics in response to inhaled and intravenous salbutamol, however the effects of this in healthy non-asthmatic volunteers has not been studied.

13.3 *In Vitro* Bronchial Reactivity Studies

In the light of my findings regarding the discrepancy between the effects of angiotensin II on methacholine and histamine it would be useful to re-examine the mechanism of potentiation *in vitro* in human bronchial rings; in particular, clarifying if this is a receptor-mediated phenomenon using type-1 and type-2 angiotensin II receptor antagonists. Further teasing out of the second messenger pathways involved could be done using phorbol esters, charybdotoxin, cholera toxin and specific inhibitors of the arachidonic acid pathway such as indomethacin and newer cox-2 selective inhibitors such as meloxicam.

Angiotensin II is known to enhance the effect of endothelin-1 on bovine bronchial rings *in vitro*, but this has not been studied on human bronchi as yet.

Another angle to the *in vitro* work would be to add angiotensinogen and angiotensin I to the organ baths and look for any signs of bronchoconstriction and measure angiotensin II levels in the bath fluid, which would indicate local formation of angiotensin II by ACE or protease enzymes.

13.4 Airway Remodelling

It would be of value to confirm the effects of angiotensin II on human airway smooth muscle cell growth *in vitro* and to compare this with its effects, if any, on rat airway smooth muscle *in vitro*. The use of specific angiotensin II receptor antagonists would elucidate whether this was a type-1 or type-2 effect. In view of the potentiating effects of angiotensin II on bronchoconstriction it would be interesting to study its mitogenic effects in combination with other known growth promoters such as histamine, endothelin-1, PDGF and leukotrienes.

Although my studies of airway remodelling in rats were negative it would be worthwhile repeating these experiments in a model of airway inflammation such as sensitising rats or guinea pigs to ovalbumin then infusing angiotensin II. If epithelial-derived mediators are antagonising the effects of angiotensin II then stripping of the epithelium to mimic the pathological changes of asthma may be helpful. If a successful model were developed other growth promoters could be studied either alone or in combinations.

Finally, obtaining bronchial biopsies from asthmatic subjects would give some measure of cell turnover in the airway wall by using immunocytochemistry to detect proteins expressed by replicating cells such as proliferating cell nuclear antigen, Ki67 and histone gene expression. It would also be interesting to measure tissue ACE activity in bronchial biopsies from asthmatics and to compare this with normal controls.

14. PRESENTATIONS TO LEARNED SOCIETIES

14.1 British Thoracic Society Winter Meeting, London, December 1995:
Effect of Angiotensin II on Histamine Induced Bronchoconstriction in the Human Airway Both *In Vitro* and *In Vivo*.

14.2 West of Scotland Biochemists Society, Glasgow, January 1996:
The Renin-Angiotensin System and Asthma.

14.3 American Thoracic Society International Conference, New Orleans, U.S.A., May 1996:

14.3.1 Effect of Angiotensin II on Histamine Induced Bronchoconstriction in the Human Airway both *In Vitro* and *In Vivo*.

14.3.2 The Mechanism of Activation of the Renin-Angiotensin System in Asthma.

14.3.3 The Role of Angiotensin II in Rat Airway Remodelling.

14.4 Pathological Society of Great Britain and Ireland Winter Meeting, London, January 1997:
The Role of Angiotensin II in Airway Remodelling.

14.5 British Thoracic Society Winter Meeting, London, December 1997:

14.5.1 Effect of Endogenous Activation of the Renin-Angiotensin System by a Low Salt Diet on Rat Airway Remodelling.

14.5.2 Is There a Local Renin-Angiotensin System in the Lungs?

15. PUBLICATIONS ARISING FROM THE THESIS

15.1 Editorial: Humoral Control of Airway Tone.
Thomson, N.C., Dagg, K.D., Ramsay, S.G.
Thorax 1996; **51:** 461-464.

15.2 Papers: Effect of Angiotensin II on Histamine Induced Bronchoconstriction in the Human Airway both *In Vitro* and *In Vivo*.
Ramsay, S.G., Clayton, R.A., Dagg, K.D., Thomson, L.J., Nally, J.E., Thomson, N.C.
Respiratory Medicine 1997; **91:** 609-615.

Investigations on the Renin-Angiotensin System in Acute Severe Asthma.
Ramsay, S.G., Dagg, K.D., McKay, I.C., Lipworth, B.J., Thomson, N.C.
European Respiratory Journal 1997; **10:** 2766-2771.

15.3 Submitted: Effect of Angiotensin II on Remodelling of the Airway and the Vasculature in the Rat.
Ramsay, S.G., Kenyon, C.J., Whyte, N., McKay, I.C., Thomson, N.C., Lindop, G.B.M.
Clinical Science

15.4 Abstracts: Effect of Angiotensin II on Histamine Induced Bronchoconstriction in the Human Airway both *In Vitro* and *In Vivo*.
Ramsay, S.G., Clayton, R.A., Dagg, K.D., Thomson, L.J., Nally, J.E., Thomson, N.C.
Thorax 1995; **50(suppl.2):** A61

Effect of Angiotensin II on Histamine Induced Bronchoconstriction in the Human Airway both *In Vitro* and *In Vivo*.
Ramsay, S.G., Clayton, R.A., Dagg, K.D., Thomson, L.J., Nally, J.E., Thomson, N.C.
Am J Respir Crit Care Med 1996; **153 (4):** A622

The Mechanism of Activation of the Renin-Angiotensin System in Asthma.
Ramsay, S.G., Dagg, K.D., McKay, I.C., Lipworth, B.J., Thomson, N.C.
Am J Respir Crit Care Med 1996; **153 (4):** A516

The Role of Angiotensin II in Rat Airway Remodelling.
Ramsay, S.G., Kenyon, C.J., Dagg, K.D., Lindop, G.B., Thomson, N.C.
Am J Respir Crit Care Med 1996; **153 (4):** A842

The Role of Angiotensin II in Airway Remodelling.
Ramsay, S.G., Kenyon, C.J., Whyte, N., Thomson, N.C., Lindop, G.B.M.
Journal of Pathology 1997; **181 (suppl.1):** 29A.

Effect of Endogenous Activation of the Renin-Angiotensin System by Low Salt Diet on Rat Airway Remodelling.
Ramsay, S.G., Kenyon, C.J., Thomson, N.C., Lindop, G.B.M.
Thorax 1997; **52 (suppl.6):** A53.

Is There a Local Renin-Angiotensin System in the Lungs ?
Ramsay, S.G., McQueen, J., Ferrier, R.K., Thomson, N.C., Lindop, G.B.M
Thorax 1997; **52 (suppl.6):** A53.

16. APPENDICES

APPENDIX 1

GREATER GLASGOW HEALTH BOARD
THE WEST ETHICAL COMMITTEE

Form Of Consent For Patients/Volunteers In Clinical Research Project

Brief Title of Project
The effect of acute severe asthma on the activation of the renin-angiotensin system and its relation to plasma salbutamol concentration, atopic status and circulating inflammatory mediators.

Patient's Summary (Purpose of study, nature of procedure, discomfort and possible risks in terms which the patient or volunteer can understand.)

We have found that a hormone called angiotensin II is elevated in the blood of patients during acute attacks of asthma. We have also shown that salbutamol (Ventolin) can cause increased levels of angiotensin II when given by a nebuliser or intravenously. In addition, we know that angiotensin II can cause narrowing of the airways when given intravenously and that other substances released by cells in response to inflammation during acute asthmatic attacks can also increase the levels of this hormone.

We are now interested in measuring the levels of angiotensin II, as well as the plasma levels of salbutamol and also several chemicals in the blood, which are raised in the inflammation occurring during an acute attack of asthma. We also wish to make similar measurements in patients who do not suffer from asthma for comparison.

We would be most grateful if you would permit a blood sample to be taken in addition to those routinely checked on admission to the Accident & Emergency Department. This would involve the removal of around 100mls. of venous blood and 3mls. of arterial blood to measure the blood oxygen level, in asthmatic patients only. You would also have your blood pressure measured and undergo a simple breathing test. A finger probe will be attached to one of your fingers to measure the oxygen level in the blood indirectly.

If you do wish to take part, your General Practitioner will be advised of your participation and the clinical management you will undergo. It should be noted that your participation in this study may not be of direct benefit to you, but could help develop treatment for the benefit of future patients. If you do not wish to participate in this trial or wish to withdraw at any time after commencing the trial, you are free to do so and your normal care will not be affected. If you are pregnant or likely to become so, you should not take part in the trial.

Consent

I, ... of .....
give my consent to the research procedures described above, the nature, purpose and possible consequences of which have been described to me by .....

Signed ..... Date .....

Witness .....

APPENDIX 2

ACE Genotyping Method

1. 5ml EDTA samples of blood were obtained from subjects and frozen until analysis.
2. The buffy coat was resuspended in nuclei lysis buffer (10mM Tris-HCl, 400mM NaCl, 2mM Na_2EDTA, pH 8.2) (244).
3. Cell lysates were digested overnight at 37°C with 0.2ml 10% SDS & 0.5ml protease K solution (1mg protease K in 1% SDS & 2mM Na_2EDTA).
4. 1ml saturated NaCl (6M) was added then shake for 15 secs. and centrifuge at 2,500 rpm for 15 mins.
5. Supernatant containing the DNA was removed and precipitated by adding 2 volumes of absolute ethanol.
6. Precipitated strands of DNA were removed and dissolved at 37°C for 2 hours in 100-200μL TE buffer (10mM Tris-HCl, 0.2mM Na_2EDTA, pH 7.5)
7. DNA was then diluted 10-fold to ensure approximately 50ng of template DNA per 20μL reaction volume.
8. Polymerase chain reaction was then performed according to the method of Rigat *et al.* (193). The reaction mixture contains 10 pmol of each of 2 primers:
   i) Sense oligo 5' CTGGAGACCACTCCCATCCTTTCT 3'
   ii) Anti-sense oligo 5' GATGTGGCCATCACATTCGTCAGAT 3'
   in a final reaction volume of 50μL containing 3mM $MgCl_2$, 50mM KCl, 10mM Tris-HCl pH 8.4, 0.1mg/ml gelatin, 0.5mM of each dNTP (Pharmacia), 1 unit of *Taq* polymerase (Cetus). 10% Dimethyl-sulphoxide is also added to the reaction mixture to prevent the under-expression of I allele amplification in heterozygotes which can lead to mis-classification of the DD genotype.
9. DNA was amplified for 30 cycles with denaturation at 94°C for 1 min., annealing at 58°C for 1 min. and extension at 72°C for 2 min. using a PTC-100 thermal cycler.
10. The PCR products were analysed by submarine agarose gel electrophoresis, then gels were stained with ethidium bromide and examined under ultraviolet light. The amplified product is 190bp for the deletion allele and 490bp in the presence of the insertion.
11. Samples identified as DD homozygotes were confirmed by re-analysis using the 3-primer method according to Evans *et al.* (174), using the following primers:
    i) ACE 1 - 5' CATCCTTTCTCCCATTTCTC 3'
    ii) ACE 2 - 5' TGGGATTACAGGCGTGATACAG 3'
    iii) ACE 3 - 5' ATTTCAGAGCTGGAATAAAATT 3'

The reaction products were analysed by vertical polyacrylamide gel electrophoresis and stained with ethidium bromide. The amplified products are 84bp for the deletion allele and 65bp for the insertion allele.

APPENDIX 3

GREATER GLASGOW HEALTH BOARD
THE WEST ETHICAL COMMITTEE

Form Of Consent For Patients/Volunteers In Clinical Research Project

Brief Title of Project
The effect of angiotensin II on histamine-induced bronchospasm in mild asthmatics.

Patient's Summary (Purpose of study, nature of procedure, discomfort and possible risks in terms which the patient or volunteer can understand.)

We have found that a hormone called angiotensin II (AII) is raised in the blood of patients during acute attacks of asthma and more recently have shown that when administered intravenously to mild asthmatics, AII can cause narrowing of the airways. In this study we wish to determine whether low concentrations of AII given intravenously can increase the effect of a substance called histamine. When inhaled as a mist in high enough doses, histamine causes bronchoconstriction rather like a mild asthma attack..

You are invited to visit the lab on 4 study days. On the first visit you will have a standard histamine challenge test. This will involve inhaling increasing concentrations of histamine to determine the dose that produces a mild wheeze. We shall measure this by breathing tests like those performed at clinics. This wheeze can easily be reversed by Ventolin. Each of the remaining 3 visits will last about 90 mins. On arrival we shall measure your breathing by asking you to blow into a tube. Then a needle will be inserted into a vein on each forearm for blood sampling & administration of AII. After a short rest you will then receive a drip into the arm of either low dose AII, higher AII or placebo. At the time of the study neither your nor the doctor will know which one, to avoid bias. The drip will last for the duration of the study (approx. 60mins.) After 30 mins. we will ask you to inhale the dose of histamine required to cause mild wheeze. Breathing tests will be performed every 5 mins. for 20 mins.

AII is a hormone which will cause a rise in blood pressure. This effect is likely to be mild at the concentrations used in this study. We shall however take the precaution of monitoring your blood pressure during the study & stop the drip if this rise is thought to be unacceptable. Histamine may cause mild wheeze, cough or a tight feeling in the chest; this will be reversed by the Ventolin. Blood samples (30ml) will be taken at 0, 30 & 60 minutes, the total amount of blood taken being 120mls. per visit.

If you do wish to take part, your General Practitioner will be advised of your participation and the clinical management you will undergo. It should be noted that your participation in this study may not be of direct benefit to you, but could help develop treatment for the benefit of future patients. If you do not wish to participate in this trial or wish to withdraw at any time after commencing the trial, you are free to do so and your normal care will not be affected. If you are pregnant or likely to become so, you should not take part in the trial.

Consent

I, ... of .....
give my consent to the research procedures described above, the nature, purpose and possible consequences of which have been described to me by .....

Signed ..... Date .....

Witness .....

APPENDIX 4

Alkaline Phosphatase-Anti-Alkaline Phosphatase Immunocytochemistry

1. Tissue sections were de-waxed in xylene for 10 minutes, then hydrated to methanol.
2. Endogenous peroxidase was removed by treating in 0.5% hydrogen peroxide in methanol for 30 minutes.
3. Rinse in water for 15 minutes.
4. Wash in 0.05M Tris buffered saline (TBS).
5. Dry slides then delineate sections with a P.A.P. pen to prevent loss of applied antibody and lay slides on a tray.
6. Sites for non-specific binding were blocked by applying 20% normal swine serum in TBS for 15-20 minutes for polyclonal antibodies (use 2% bovine serum albumin for monoclonal antibodies).
7. Drain and wipe sections.
8. Apply the primary antibody 1:1,000 dilution and incubate overnight at 4°C.
9. Wash sections with TBS three times over 10 minutes.
10. Apply secondary antibody: Biotinylated anti-rabbit immunoglobulin from ExtrAvidin kit (DAKO) 1:250 dilution in TBS and pooled normal human serum (Sigma Chemicals, U.K.) 1:250 in TBS to prevent non-specific binding. Leave for 30 minutes at room temperature.
11. Wash several times in TBS.
12. A third layer is applied of Streptavidin-antibody-alkaline phosphatase (biotinylated) [DAKO], diluted 1:110 in TBS, for 30 minutes at room temperature.
13. Wash in TBS as before.
14. Visualise using 3,3-Diaminobenzidine Tetrahydrochloride in Tris buffer for 5-10 minutes.
15. Wash in water and check control sections.
16. Counterstain with a light haematoxylin nuclear stain.
17. Dehydrate, cover and mount.

APPENDIX 5

Modification of Immunocytochemistry for Bromodeoxyuridine Staining

1. Dewax sections and treat with hydrogen peroxide, then rinse in water as above in Appendix 1.
2. Microwave on high power in citrate buffer for 10 mins. then stand at room temperature for 20 mins. This is to facilitate antigen retrieval from the tissue sections (245).
3. Wash well with tap water.
4. Digest in 0.1% trypsin and 0.1% calcium chloride in TBS at pH 7.6 for 2 minutes.
5. Wash well with tap water.
6. Incubate with 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS) pH 7.6 for 10 mins. at room temperature then drain and wipe slides.
7. Treat with mouse monoclonal anti-BrdU antibody (Eurodiagnostics, UK) diluted 1:20 in 2% PBS/2% BSA at 4°C overnight.
8. Wash with PBS several times over 15 mins., drain and wipe.
9. Treat with rabbit anti-mouse antibody-peroxidase conjugate diluted 1:20 in 2% PBS/ 2% BSA for 30 mins. at room temperature (100 µL per section).
10. Wash with PBS several times over 15 mins.
11. Visualise in diamino-benzidine (DAB) (450 mL PBS, 50 mL imidazole, 250 µL hydrogen peroxide, 2 mL DAB solution) for around 8 mins.
12. Lightly counterstain with haematoxylin and mount.

APPENDIX 6

In Situ Hybridisation

1. Hydrate sections to phosphate buffered saline (PBS).
2. Remove surface proteins in 0.2N hydrochloric acid for 15 minutes then rinse in PBS
3. Remove lipids in 0.3% Triton X100 in PBS for 15 minutes then rinse in PBS.
4. Proteinase K (1 in 10 dilution in PBS) digestion (≈200µL per slide) at 37°C for 15-45 minutes then rinse in PBS.
5. Fix in 4% Paraformaldehyde in PBS for 5 minutes then rinse in PBS
6. Prehybridise for 1 hour at 37°C in 2x standard saline citrate (SSC) / 50% formamide
7. Hybridise overnight at 42°C with digoxygenin-labelled riboprobe diluted 1 in 50 in hybridisation buffer (1mM Tris pH 7.0, 1x Denhardt's solution, 2x SSC, 50% formamide, 0.5% sodium dodecyl sulphate, 0.5% dextran sulphate, 0.25mg/ml denatured and sonicated salmon sperm DNA, and water for dilution) allowing 15 µL solution per coverslip. Remove coverslips in 4x SSC.
8. Wash in 2x SSC twice for 30 minutes at room temperature, then 0.1% SSC for 10 mins. at room temperature, then 0.1% SSC for 30 mins. at 37-45°C, then 0.1% SSC for 20 mins. at room temperature.
9. Apply Digoxygenin buffer 1 (100mM Tris HCl, 150mM sodium chloride at pH 7.5, 20°C) for 5 minutes.
10. Apply Digoxygenin antiserum for 2 hours (diluted 1 in 2,000 in Digoxygenin buffer 1 and 10% normal rat serum).
11. Wash in Digoxygenin buffer 1 twice for 15 minutes.
12. Wash in Digoxygenin buffer 3 (100mM Tris, 100mM sodium chloride, 50mM magnesium chloride at pH 9.5, 25°C) for 5 minutes.
13. Apply 22µL nitro blue tetrazolium, 16.7µL 5-bromo, 4-chloro, 3-indolyl phosphate and 1.15mg levamisole in 5ml of digoxygenin buffer 3 and incubate at room temperature in a humid atmosphere away from direct sunlight.
14. Counterstain with haematoxylin, dehydrate and mount.

APPENDIX 7

DNA Extraction and Riboprobe Preparation

1. Freeze-dried bacteria containing the plasmids with the DNA fragment of interest are reconstituted in medium containing an appropriate antibiotic.
2. 2ml of medium is inoculated with bacteria from a glycerol stock solution then grown for 8 hours at 37°C in a shaking incubator, then 0.5ml of this solution added to 10ml of medium and incubated as above, then this is added to 400ml of medium and incubated overnight.
3. The bacterial growth is centrifuged in 500ml polycarbonate tubes at 6000 rpm for 30 minutes at 4°C in an Ultra centrifuge using the JA10 rotor.
4. Supernatant is discarded and bacteria resuspended in Qiagen buffer P1 (50mM Tris/HCl, 10mM EDTA pH 8.0, 100µg/ml RNAse A)for 5 minutes at room temperature.
5. Add 10ml of Qiagen P2 buffer (200mM sodium hydroxide, 1% sodium dodecyl sulphate), mix gently and stand for 5 minutes at room temperature.
6. Add 10ml of chilled Qiagen P3 buffer (3M potassium acetate, pH 5.5), mix and stand on ice for 15 minutes.
7. Centrifuge at 4°C for 30 minutes at 17,000 rpm, remove supernatant, spin again and retain supernatant.
8. Equilibrate Qiagen Tip-500 with 10ml of Qiagen QBT buffer (750mM sodium chloride, 50mM 4-MorpholinePropaneSulfonic acid[MOPS], 15% ethanol, 0.15% Triton X-100 at pH 7.0) by allowing tip to empty by gravity.
9. Pour supernatant into tip and allow to empty.
10. Wash Tip-500 twice with 30ml of Qiagen QC buffer (1.0M sodium chloride, 50mM MOPS, 15% ethanol at pH 7.0).
11. Elute plasmid DNA with 15ml Qiagen QF buffer (1.25M sodium chloride, 50mM Tris, 15% ethanol at pH 8.5) and collect.
12. Precipitate DNA with 0.7 volumes of isopropanol for 5 minutes at room temperature
13. Centrifuge at 4°C for 30 minutes at 12,000 rpm to produce a pellet of DNA.
14. Pour off supernatant, add 2ml of 70% ethanol, centrifuge at 4°C for 30 minutes at 13,000 rpm then pour off supernatant.
15. Air dry for 5 minutes, then resuspend in 200-400µl of distilled water.
16. If necessary the DNA fragment can be cut from the plasmid and subcloned into a more suitable plasmid with more appropriate restriction sites.
17. Linearise plasmid using an appropriate restriction enzyme to cut the plasmid at the desired site. Set up reaction in an autoclaved ICC tube containing 2µl of reaction

buffer, 1µg plasmid, 2µl restriction enzyme and DEPC-treated water to a total volume of 20µl. Allow to digest for 2 hours at 37°C in a water bath.

18. Add 280µl water and 300µl phenol / chloroform, mix by vortexing for 30 seconds, then centrifuge for 30 seconds at 13,000 rpm.
19. Remove and retain the upper aqueous phase, add 300µl chloroform, mix and spin as above, then repeat once more.
20. Remove and retain the aqueous phase, add 0.5ml ice cold 100% ethanol and 1µl of glycogen / 2.5M sodium acetate, allow to precipitate DNA for at least 1 hour to overnight.
21. Centrifuge at 4°C for 15 minutes at 13,000 rpm, remove supernatant, replace with 70% ethanol and repeat.
22. Pour off excess alcohol, air dry and reconstitute in 20µl water.
23. Nature and purity of the plasmid can be checked on an agarose gel.
24. Riboprobes (single-stranded RNA, either sense or anti-sense) are produced by setting up the reaction solution in an autoclaved ICC tube as follows: 4µl of transcription buffer, 2µl 1-4-Dithiothreithol, 2µl nucleotide tri-phosphate mix (2µl ATP, 2µl CTP, 2µl GTP, 1µl UTP [all 100mmol], 5.41µl Digoxygenin-11-UTP [10mmol], 7.59µl water), 2µl linearised plasmid, 0.5µl RNAse inhibitor (500 units/ml), 7.5µl water and 2µl RNA polymerase (40 units) as appropriate for the restriction sites. All reagents must be added in this order. Incubate at 37°C for 4 hours in a water bath.
25. Stop the reaction and precipitate with ethanol and glycogen as above, wash with 70% ethanol, pour off supernatant, air dry and reconstitute in 20µl water. Riboprobe can be frozen until required.

ALL water, solutions, glassware, tubes, etc. to be used should be pre-treated with 0.2% Diethyl Pyrocarbonate (DEPC) [Sigma D-5758].

APPENDIX 8

RNA Extraction From Tissues and Northern Blot Analysis

1. Frozen tissue samples, 100-350 mg weight, are homogenised by adding 1mL of TRIzol reagent (Life Technologies) per 100 mg tissue, then mixing in a glass mortar and pestle and incubate at room temperature for 5 mins.
2. Add 0.2 mL chloroform per 2 mL of homogenate, cap tube, shake vigorously, incubate at room temperature for 2-3 mins. then centrifuge at 12,000 *g* for 15 mins. at 4°C.
3. Transfer the upper colourless aqueous phase to a fresh tube, add 0.5 mL of isopropyl alcohol per 1 mL TRIzol reagent and incubate at room temperature for 10 mins.
4. Centrifuge at 12,000 *g* for 10 mins. at 4°C. The RNA precipitate forms a white-yellow pellet at the bottom or side of the tube.
5. Remove the supernatant and wash once with 75% ethanol (1 mL per mL of TRIzol reagent used initially), mix by vortexing then centrifuge at 7,500 *g* for 5 mins. at 4°C.
6. Allow pellet to air dry, but not completely then reconstitute the RNA pellet in 100 μL of DEPC-treated water, vortex briefly then incubate for 10 mins. at 60°C to ensure dissolution.
7. Prepare agarose gel by microwaving 0.6 g of agarose in 43.75 mL of DEPC-treated water for 2 mins. on full power. Cool slightly then add 2.5 mL 20x MOPS buffer (see appendix 4) and 3.75 mL formaldehyde. Mix gently and cool to about 60°C. Pour 20 mL into minigel apparatus and allow to set.
8. Prepare samples by heating 10 μL of RNA solution with 10 μL of sample buffer (5x MOPS, formamide, 10% formalin, and loading buffer [ethidium bromide and bromophenol blue dye]) at 70°C for 15 mins. then cool on ice for 15 mins.
9. Cover the agarose gel with 1x MOPS and load 10-20 μL of prepared sample into each well on the gel. Set electrophoresis at 0.12 kV / 42 mAmps for 2 hours until the front reaches the middle of the gel.
10. Wash the gel in 20x SSC for 10-15 mins.
11. Pre-soak the nylon membrane (Hybond-N+, Amersham, UK) in 20x SSC
12. Place 1 litre of 20x SSC in a large rectangular dish, put a support in the base of the dish and cover with 2 pieces of 3MM filter paper (Whatman, UK). Place the gel on top of this with the lower surface uppermost.
13. Place a piece of the nylon membrane on top of the gel and roll a pipette over the surface to expel any bubbles and ensure a good contact.

14. Cut 3 pieces of 3MM filter paper to size, wet two and cover the membrane then place the third piece on top and then put several paper towels (3cm thick) on top of this.
15. Seal with cling film to prevent drying and weight the whole assembly with a glass plate and a 500g weight to compress and leave overnight.
16. The membrane is removed, allowed to air-dry, then placed between two sheets of filter paper and baked at 60°C for 1 hour.
17. Rehydrate blot in 20x SSC.
18. Make up a small plastic bag around blot and add pre-hybridisation buffer (5x SSC, 0.5% sodium dodecyl sulphate (SDS) & 5x Denhardt's solution) and incubate for 1.5 hours at 50°C.
19. Dilute labelled riboprobe to be used in pre-hybridisation buffer to give a concentration of 1nM.
20. Remove old buffer from sealed bag and add fresh buffer containing labelled probe, reseal bag and incubate overnight at 50°C.
21. Cut open bag and wash filter twice for 10 mins. at 50°C in 1x SSC/0.5% SDS, then twice for 10 mins. at 50°C in 0.25x SSC/0.5% SDS, then for 5 mins. in 1x SSC at room temperature.
22. Wash blot in digoxygenin buffer 3 (see appendix 3) for 5 mins. then transfer blot to nitro blue tetrazolium/5-bromo,4-chloro,3-indolyl phosphate dye solution, cover with aluminium foil and incubate until bands are visible (1-2 hours). A digoxygenin-labelled RNA ladder is run with the samples to allow sizes to be compared.

APPENDIX 9

Iodination of Sarcosine^1-Angiotensin II (Saralasin)

1. Sarcosine^1-angiotensin II (saralasin) [Peninsula Labs., USA], Iodogen (Pierce, cat. no.28600X) and $^{125}I^-$ radio-iodine are mixed in the ratio of 2: 0.75: 1 moles in a total volume of 120 μL of 20 mM phosphate buffer at pH 7.5. The iodogen must be prepared shortly prior to use due to its instability and will convert iodide to iodine which will substitute into a tyrosine ring on the saralasin peptide.
2. Incubate the vial for 30 mins. at room temperature, mixing every 5 mins.
3. Add 120 μL of 0.2M triethanolamine buffer pH 8.5 to terminate the reaction.
4. Radiolabelled peptide is extracted by high performance liquid chromatography (HPLC) using a reverse-phase silica column 250 x 4.6mm, 5 μm C_{18} (Sigma-Aldrich). Flush column with acetonitrile.
5. Equilibrate column for 30-60 mins. with 0.1M triethanolamine pH 8.5 at 1.0 mL/min.
6. Inject the initial incubation sample containing labelled peptide.
7. Collect first 15 mL of effluent into a flask, then collect and retain 60 aliquots of 333 μL fractions into tubes containing 166 μL of methanol (i.e. one fraction every 20 seconds). Finally collect about 100 mL into another flask. The elution volume of the labelled peptide is about 20 mL (around fraction 30).
8. The fractions containing the highest radioactivity are identified by scanning the tubes of effluent with a hand-held gamma detector. Plot the radioactive content against fraction number to identify the fractions with the labelled probe.
9. The five fractions with the peak radioactivity after the peak fraction are pooled in a 10 mL glass tube and 10 μmol of benzamidine hydrochloride is added.
10. The volume is reduced to 0.1-0.2 mL by evaporation under a stream of dry, filtered air (or nitrogen) at room temperature.
11. Add 0.8 mL of methanol, seal and vortex then store at -20°C until use.

APPENDIX 10

Angiotensin II Receptor *In Situ* Autoradiography

1. Warm frozen tissue sections to room temperature.
2. Rehydrate tissue sections in Tris/NaCl buffer (see below) for 10 minutes at 4°C. Tris/NaCl buffer: Tris 50mM, NaCl 120mM, $MgCl_2$ 12.5mM, benzamidine HCl 12.5mM and NaN_3 0.025% (w/v) pH 7.4.
3. Shake off excess liquid from slides.
4. Apply radiolabelled saralasin, 120µL per cm^2, in assay buffer (see below) at a concentration of 10 nM which produces virtually maximal receptor binding. Assay buffer: Tris 50mM, NaCl 120mM, $MgCl_2$ 12.5mM (Mg^{2+} increases angiotensin binding in some tissues), benzamidine HCl 12.5mM (an endopeptidase inhibitor; Sigma), acitonin 0.1mM (an aminopeptidase inhibitor; Peninsula), bestatin 0.1mM (an aminopeptidase inhibitor; Peninsula), bacitracin 0.3mg/mL (a peptide antibiotic which inhibits various enzymes; Sigma) and BSA 0.5% (w/v) pH 7.4 (prevents non-specific adsorption of the radioligand; Sigma).
5. Add 400 µM unlabelled angiotensin II to a final concentration of 10 µM to block receptors in the tissue section for non-specific binding.
6. Add Dup753 (Losartan, DuPont) in a concentrated solution to a final concentration of 10-20 µM to block AT_1 receptors on tissue sections to show AT_2 receptor binding.
7. Add PD123319 (Parke Davis) in a concentrated solution to a final concentration of 10-20 µM to block AT_2 receptors on tissue sections to show AT_1 receptor binding.
8. Incubate all slides for 60-75 minutes at 22°C in a humidified chamber.
9. Rinse off incubation solution with Tris/NaCl buffer at 0-4°C to remove unbound ligand.
10. Wash three times for 5, 5 & 10 minutes in Tris/NaCl containing 0.1% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Tween 20 at 0-4°C.
11. Wash once for 5 minutes in Tris/NaCl buffer at 0-4°C.
12. Sections for film autoradiography should be dipped in cold (0-4°C) distilled water to remove soluble material then dried in a stream of air. The sections are now ready for film exposure as described in chapter 4.
13. Sections for emulsion coating are dipped in cold water, then incubated in cold (0-4°C) phosphate buffered saline (PBS) pH 5.0 containing 12.5 mM $MgCl_2$ for 10 minutes to rinse off Tris buffer.
14. Incubate sections overnight in a humidified container at 2°C with PBS pH 5.0 containing 12.5 mM of $MgCl_2$ and 0.5M EDAC cross-linking agent (EDAC = 1-

ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma, cat. no. E-7750).

15. Incubate sections in PBS pH 3.0 containing 0.1% (w/v) BSA and 0.1% (v/v) Tween 20 for 90 minutes at room temperature.
16. Rinse in water to remove any non-covalently bound radioligand.
17. Fix sections in formalin or glutaraldehyde if required then rinse in water.
18. Rinse slides in alcohol and dry in a stream of air.
19. In a dark-room, warm the emulsion (Amersham LM-1) to 43°C and stir slowly to obtain a uniform consistency then pour into a dipping vessel.
20. Slowly dip each slide into the emulsion for 4 seconds then remove slowly.
21. Wipe off excess emulsion from the back of the slide and leave to stand vertically on paper tissue for at least 20 minutes at room temperature to allow excess emulsion to run off.
22. Pack slides into boxes maintaining the vertical orientation for about one hour to ensure a uniform film of emulsion over the sections then transfer to a refrigerator (~4°C) to expose for 96 hours (Time varies with amount of activity per section).
23. Equilibrate slides to room temperature then develop in Kodak D19 developer (Sigma) for 6 minutes, fix in GBX fixer (Sigma) for 12 minutes, then dip in water to remove processing chemicals.
24. Lightly counterstain if required in haematoxylin and mount.

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