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THE INFLUENCE OF ANGIOTENSIN II ON AIRWAY FUNCTION IN ASTHMA

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A thesis submitted to the University of Glasgow for the degree of Doctor of Medicine.
April 1996.

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ABSTRACT

The renin-angiotensin system (RAS) is an endocrine system principally involved in the maintenance of cardiovascular homeostasis and electrolyte balance, its actions being mediated by the octapeptide hormone angiotensin II (ANG II).

In a preliminary observation we found marked activation of the RAS in acute severe asthma. The stimulus to this activation, and the possible consequences of elevated ANG II on lung function in man were unknown, although ANG II had recently been shown to potentiate bronchoconstriction of rabbit airway smooth muscle *in vitro*. The purpose of our investigation was to examine possible stimuli to ANG II release in acute asthma and also the direct and indirect effects of this hormone on airway function *in vivo* in man. Initial studies confirmed that single and multiple high doses of nebulised and also intravenous β_2 agonists used in the treatment of asthma eg salbutamol, elevate ANG II levels in the plasma, but the levels achieved were less than those observed in acute severe asthma. Further studies revealed that hypoxia did not cause activation of the RAS in normal volunteers, nor did it potentiate the effect of nebulised β_2 agonists. We also demonstrated that the elevation of ANG II levels by nebulised β_2 agonists can be prevented by pretreatment with ACE inhibitor drugs (lisinopril), suggesting that this action of β_2 agonists on the RAS is mediated via the classical components of the RAS, including ACE, rather than involving alternative pathways of ANG II generation.

When administered by intravenous infusion to produce similar plasma levels to those observed in acute asthma, ANG II caused bronchoconstriction in mild asthmatic patients, and in lower (subthreshold) doses potentiated the effect of inhaled methacholine both *in vitro* in isolated human bronchi and also *in vivo*, in mild asthmatic patients. We therefore conclude from this series of experiments that the RAS is activated in acute severe asthma and that high dose β_2 agonists are likely to be partly responsible for this activation. The role

of other possible stimuli, perhaps inflammatory mediators such as proteinase enzymes released from mast cells, remains to be established. Our findings also suggest a role for ANG II as a mediator in asthma, and further studies to examine the effects of ANG II receptor antagonists in different forms of experimental asthma are now indicated.

ACKNOWLEDGEMENTS

I have great pleasure in thanking Professor Neil Thomson for giving me the opportunity to undertake this project in his department and for his encouragement and helpful criticism of the manuscripts arising from this work and drafts of the thesis. I would also like to thank my research colleague Robert Angus for his enthusiasm for this work and able assistance. Several of the studies were undertaken in the CIRU (Clinical Investigation and Research Unit), University Department of Medicine and Therapeutics, Western Infirmary and I am grateful to the staff of the unit for accommodating me in their ward and providing me with so many willing control subjects. I also express my thanks to Professor John Connell and Dr Gordon McInnes (University Department of Medicine and Therapeutics, Western Infirmary) for their advice, particularly regarding study design and also to Jane Nally for allowing me to collaborate with her exciting *in-vitro* work on angiotensin II.
The expertise of Dr JJ Morton and Dr Richard Spooner in performing the hormone assays, and the assistance of Miss Wendy Fallon of our pharmacy sterile department with drug preparation and blinding of the studies was greatly appreciated, and I am also grateful to all the asthmatic patients in the Western Infirmary and Gartnavel General Hospitals who participated so willingly in these studies.
Finally, I acknowledge the support of the National Asthma Campaign for funding this research and hope that our results may be of some future value, perhaps by helping to throw some light on one of the many pathophysiological processes in asthma.

DECLARATION

I am the sole author of this thesis and I have personally consulted all the references listed. This work was undertaken by myself in the Departments of Respiratory Medicine, Gartnavel General Hospital and Western Infirmary, Glasgow and also the Clinical Investigation and Research Unit, (C.I.R.U.), University Department of Medicine and Therapeutics, Western Infirmary, Glasgow. Some of the preliminary results in acute severe asthma were provided by my colleague Dr Geoffrey Hulks, and the *in-vitro* work with angiotensin II was performed in collaboration with Dr Jane Nally in the Respiratory Physiology Laboratory, Western Infirmary, Glasgow.

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

ABBREVIATIONS

ACE	angiotensin converting enzyme
ANG I	angiotensin I
ANG II	angiotensin II
ANG III	angiotensin III
ABG's	arterial blood gases
BHR	bronchial hyperresponsiveness
FEV_1	forced expiratory volume (in one second)
MCh	methacholine
PaO_2	partial pressure of arterial oxygen (units: kPa)
$PaCO_2$	partial pressure of arterial carbon dioxide (units: kPa)
PEFR	peak expiratory flow rate
SD	standard deviation
SEM	standard error of mean

CHAPTER 1

INTRODUCTION

1.1 THE PATHOGENESIS OF ASTHMA

Asthma is one of the most common medical disorders affecting over 5% of the adult population in the Western world and around 10% of children (1). There is evidence that morbidity and mortality from asthma is increasing in many countries, despite our increased understanding of its pathogenesis and advances in therapy. The American Thoracic Society defines asthma as "a clinical syndrome characterised by increased responsiveness of the tracheo-bronchial tree to a variety of stimuli. The major symptoms of asthma are paroxysms of dyspnoea, wheezing and cough, which may vary from mild and almost undetectable to severe and unremitting (status asthmaticus). The primary physiological manifestation of this hyperresponsiveness is variable airways obstruction, which can take the form of spontaneous fluctuations in the severity of obstruction following bronchodilators or corticosteroids, or increased obstruction caused by drugs or other stimuli" (2). Many attempts have been made to further classify asthma, the most commonly used being based on immunological status (atopic or non-atopic), as well as whether inhaled allergens provoke symptoms (extrinsic) or not (intrinsic). In addition, there is a category whose asthma is solely provoked by exercise (exercise-induced asthma).

One of the most characteristic features of asthma is the phenomenon of bronchial hyperresponsiveness (BHR), which is best described as an exaggerated bronchoconstrictor response to a variety of inhaled stimuli such as histamine, cold air or cholinergic agonists, eg methacholine. Over the last 5 years or so it has become apparent that asthma is a chronic inflammatory disease of the bronchi in

which many cell types play a role, and that BHR is a likely to be a consequence of this inflammation. Thus, inflammatory changes in the bronchial mucosa are believed to play a fundamental part in the pathophysiology of asthma regardless of aetiology. Histological studies of the lungs of asthmatics who have died during acute attacks show several common features with thickening of the bronchial wall due to hypertrophy of smooth muscle cells (3,4) and mucus plugging of the lumen. Inflammatory cells such as eosinophils and T lymphocytes are present, and the airway epithelium is often damaged with widespread shedding (5). By using the techniques of bronchial biopsy and bronchoalveolar lavage, similar, though less severe mucosal and submucosal abnormalities have been shown to be present in the lungs of even mild athmatic patients. Many different inflammatory mediators have been implicated in asthma although the exact role of each mediator in the pathophysiology of asthma is not yet clear, and the sequence of events which leads to asthmatic symptoms uncertain. The immediate stimulus is probably the release of mediators from a variety of inflammatory cells, which interact to cause the effects that are characteristic of asthma such as bronchoconstriction, mucus hypersecretion, bronchial oedema and plasma extravasation, as well as causing chemotaxis of other inflammatory cells.

1.1.1 Inflammatory cells

It is now clear that several different cell types may be involved in asthma, although the precise role of each is uncertain. Activation of mast cells is important in initiating immediate bronchoconstriction in response to allergen and other indirect stimuli such as exercise, cold air and hyperventilation (6). However it seems likely that in more chronic inflammatory events, including airway

hyperresponsiveness, other cells such as macrophages, eosinophils and T lymphocytes are more important. Macrophages are derived from blood monocytes and are activated in the airways in asthma by allergen combining with low affinity Ig E receptors on their surface. Dendritic cells, which are specialised macrophages in the airway epithelium, may then act as antigen presenting cells which process allergen for presentation to T-lymphocytes (7). They can also produce a large variety of proteins called cytokines which initiate an inflammatory response. Eosinophil infiltration is a characteristic feature of asthma, migrating into the airway in response to various chemotactic factors. Eosinophils were originally viewed as beneficial cells in asthma, but it is now more likely that they may play a damaging role via release of mediators which are cytotoxic for airway epithelial cells, for example basic proteins and oxygen-derived free radicals (8). The role of B lymphocytes in the synthesis of IgE is well documented, and more recently a role for T lymphocytes in asthma has also been recognised. T-lymphocytes are a feature of asthmatic airways and in particular, activated TH_2-lymphocytes may be involved in orchestrating the inflammatory response via the secretion of various cytokines such as IL-3, IL-4, IL-5 and GM-CSF. These products may in turn recruit eosinophils (9) and maintain the presence of mast cells in the airway (10). Platelets and neutrophils have also been implicated in asthma and the phenomenon of BHR but their exact role is uncertain.

1.1.2 Inflammatory mediators

For an inflammatory mediator to have a potential role in the pathogenesis of asthma, it must generally fulfill a number of criteria. When identified and administered (usually by inhalation) to asthmatic subjects it should mimic some component of the asthmatic response. The mediator, or a metabolite should be

measurable in a biological fluid during the asthmatic response, it should be antagonised either by direct antagonists or inhibitors of its synthesis and most importantly it should be determined whether the mediator antagonists or synthesis inhibitors are of value in treating asthmatic patients. Many different mediators have been implicated in asthma and can produce several of the pathological features of asthma including bronchial smooth muscle contraction, mucus hypersecretion, microvascular leakage and inflamatory cell chemotaxis. Such mediators include histamine, prostaglandins, thromboxane, leukotrienes, bradykinin, platelet activating factor, and more recently cytokines and growth factors.

Histamine is formed within the mast cell or basophil and is rapidly metabolised in the tissues after release. Elevated levels of histamine may be detected in stable asthmatic subjects (11) and small rises have been described during allergen and exercise-induced bronchoconstriction (12). H_1 receptor antagonists have been reported to partially inhibit some asthmatic responses, for example, exercise (13) but even very potent and longer acting antihistamines are not effective bronchodilators nor do they have have a useful role in the management of asthma. **Platelet activating factor (PAF)** is released into the plasma following allergen challenge in mild asthmatic patients (14) and in normal subjects can cause bronchoconstriction and airway hyperresponsiveness when inhaled (15). PAF can recruit a variety of inflammatory cells including eosinophils and neutrophils and it was initially suggested that it may be an important mediator in allergen-induced asthmatic responses and in airway inflammation in asthma. However, recent potent antagonists have been shown to be ineffective in inhibiting allergen - induced asthmatic responses (16) or airway hyperresponsiveness (17) and oral

PAF antagonists have recently been shown to have no beneficial effect in chronic asthma (18). The role of high concentrations delivered by inhalation has not yet been established.

Prostaglandins PGD_2 and $PGF_{2\alpha}$ are released from human airways challenged with allergen (19) are potent bronchoconstrictors(20,21) and subthreshold contractile concentrations have been demonstrated to increase airway responsiveness to inhaled histamine and methacholine in asthmatic subjects (22,23). Both PGD_2 and $PGF_{2\alpha}$ cause bronchoconstriction via a direct effect on airway receptors and also indirectly through cholinergic mediated bronchoconstriction (24). However specific receptor antagonists are not available to allow further characterisation of their importance in causing asthmatic responses. PGE_2 and PGI_2 are the only mediators with significant bronchodilator activity. There is evidence that manoevres that stimulate production of endogenously produced PGE_2 protect against bronchoconstriction and recently it has been postulated that impaired production of PGE_2 may contribute to the pathogenesis of asthma (25)

Leukotrienes LTC_4 and LTD_4 are the most potent bronchoconstrictor agents studied in humans, being up to 10,000 times more potent than methacholine in some normal subjects (26). Increased urinary levels of LTE_4, the metabolite of LTC_4 and LTD_4 occurs after allergen-induced early responses and in patients with acute severe asthma (27,28). Initial studies with potent and specific leukotriene antagonists and synthesis inhibitors have demonstrated clinical efficacy in asthma (29).

Cytokines More than 30 different types of protein mediators or cytokines have been described. Examples include interleukin (IL)-3, IL-5 and granulocyte macrophage-colony stimulating factor (GM-CSF). They are produced by cells such as the helper T cell in asthmatic airways (30) and have the ability to promote eosinophil and mast cell differentiation and recruitment into the airways. GM-CSF is present in increases amounts in biopsies from asthmatic airways and levels increase after allergen challenge (31). It is likely that the cytokines play a dominant role in chronic inflammation, rather than the acute inflammatory response and further studies with specific antagonists are required to establish their precise role in the pathogenesis of asthma.

Endothelin-1 is a 21 amino-acid peptide which is a potent and long lasting constrictor of airway smooth muscle and bronchial vessels (32). It is expressed in airway epithelial cells in asthma (33) and raised concentrations of endothelin-1 have been detected in bronchoalveolar lavage fluid from asthmatic patients (34). Accordingly, a role for this mediator is likely in asthma.

In summary, it is possible that a complex interaction between various inflammatory mediators might account for BHR, but at present the multiplicity of mediators makes it unlikely that antagonism of a single mediator will result in significant clinical improvement.

1.1.3 Airway smooth muscle

There is much debate about the role of abnormalities in airway smooth muscle activity in the pathogenesis of asthma and in the generation of BHR, and it is still not entirely clear whether an abnormality in airway smooth muscle itself, or in its control by nerves and mediators is responsible for the abnormalities of asthma.

The exaggerated bronchoconstrictor response in asthma suggests some intrinsic abnormality of asthmatic airway smooth muscle, but studies of human airway contraction *in vitro* have failed to demonstrate any significant association with airway hyperresponsiveness *in vivo* (35-37). A small number of studies of airway smooth muscle from asthmatic subjects suggest that the smooth muscle is hyperresponsive to agonists *in vitro* when compared to airways from non-asthmatic subjects (38,39) implying that there may be an inherent defect in asthmatic airway smooth muscle accounting for airway hyperresponsiveness.

In asthmatic airways, hypertrophy and hyperplasia of airway smooth muscle is evident, although the functional significance of this is uncertain. This may be the result of stimulation by growth factors released from inflammatory cells, or conversely, be a consequence of, rather than the cause of airway hyperresponsiveness. A recent study (40) raises the possibility that an increase in airway smooth muscle volume in asthmatics might cause airway hyperresponsiveness by thickening of the airway wall. Using modelling studies it was demonstrated that a small increase in the thickness of the airway wall, undetectable by changes in spirometry, could result in airway hyperresponsiveness.

1.1.4 Neural mechanisms

Neural control of the human airway is complex and abnormalities of all components of the airway autonomic system have been suggested in the pathogenesis of asthma. Cholinergic nerves are the dominant neural bronchoconstrictor pathway, with the human airway being under continuous parasympathetic tone, as evident by the fact that anticholinergic drugs cause bronchodilation in asthmatic and non-asthmatic subjects (41).

In man, airway sensory receptors can be stimulated by the inhalation of histamine, cold air, sulphur dioxide and mechanical irritation, resulting in vagally mediated bronchoconstriction. Other postulated mechanisms which might contribute to cholinergic bronchoconstriction in asthma include increased central vagal drive, enhanced neurotransmission in cholinergic ganglia, either from release of other neurotransmitters or facilitation of acetylcholine release from postganglionic nerve terminals (42).

Adrenergic nerves do not control airway smooth muscle directly, and it is circulating catecholamines which play a more important role in regulation of bronchomotor tone. Several possible abnormalities in adrenergic control have been proposed in asthma (43) including enhanced α-adrenergic responses or defects in ß-adrenergic responses, although presently these abnormalities are thought to be secondary to the disease rather than the primary cause. The role of non-adrenergic non-cholinergic (NANC) nerves in asthma remains uncertain, although neuropeptides which may act as neurotransmitters of NANC nerves have now been identified in human airways, eg nitric oxide and vasoactive intestinal peptide and are currently the subject of much research.

1.2 DRUG TREATMENT OF ASTHMA

Drugs used to treat asthma are conventionally divided into bronchodilator and prophylactic drugs. Examples of bronchodilator drugs include the β_2 agonists, anticholinergic agents and methylxanthines (eg theophylline), and prophylactic drugs are the corticosteroids and sodium cromoglycate.

1.2.1 Beta-2 (β_2) adrenoceptor agonists

Beta-2 agonists are the most effective and widely prescribed bronchodilators. They are particularly useful for mild episodic attacks of asthma, exercise-induced asthma and to supplement prophylactic agents in more severe cases. They are most effective when given by inhalation, as this delivers the drug directly to the site of action, maximising the bronchodilator effect and minimising generalised systemic effects. Examples include salbutamol (Ventolin®) and terbutaline (Bricanyl®), administered by aerosol. In acute attacks of asthma these drugs may be given by nebuliser, thus allowing greater drug delivery to the airways.

Mode of action

ß adrenoceptors are widely distributed in the lung and have been identified by autoradiography on airway epithelium, alveoli, submucosal glands and also on airway and vascular smooth muscle (44). The receptors on airway and vascular smooth muscle appear to be entirely of the β_2 subtype, whereas β_1 receptors account for 10 - 30% of ß- receptors on submucosal glands and alveolar walls. β_2 agonist drugs have several actions which affect airway function. They are potent bronchodilators by causing relaxation of bronchial smooth muscle (45) and therefore immediate relief of symptoms. Although their predominant effect is on

bronchial smooth muscle, other actions include effects on some of the processes of acute inflammation, including suppression of inflammatory mediator release from mast cells (46) and possibly inhibition of vascular permeability and attenuation of inflammatory cell recruitment.

ß agonists act through ß adrenoceptors, activating the enzyme adenylate cyclase via a stimulatory guanine nucleotide regulatory protein (Gs). This enzyme catalyses the conversion of adenosine triphosphate (ATP) to adenosine 3'5' monophosphate (cAMP). Intracellular cAMP is responsible for activation of specific protein kinases which, in bronchial smooth muscle, cause a reduction of phosphorylation of myosin light chain to reduce calcium-dependent coupling of actin and myosin and produce smooth muscle relaxation (47). ß agonists also open potassium channels (maxi-K channels) in airway smooth muscle. This effect is observed at low concentrations of ß agonists *in vitro*, suggesting that this may be a major mechanism of airway smooth muscle response to ß agonists (48).

Cardiovascular and metabolic effects of $ß_2$ agonists

$ß_2$ agonists cause vasodilatation, tachycardia and and increase in cardiac output (49). The latter effects have previously been attributed to reflex changes following peripheral vasodilatation, although recent work suggest that part of the response may be due to stimulation of cardiac $ß_2$ receptors. The metabolic and hormonal effects of $ß_2$ agonists include glycogenolysis, lipolysis and stimulation of renin, insulin, glucagon, parathyroid hormone and antidiuretic hormone (49). The decrease in serum potassium concentrations after $ß_2$ agonists is thought to be due to stimulation of membrane-bound Na/K ATPase activity, particularly in skeletal

muscle, rather than to insulin release. ß agonists also cause an increase in physiological tremor, although this is rarely severe.

$ß_2$ agonists are widely prescribed and have been used for three decades as first line bronchodilator therapy in asthma. In general these drugs have a very high safety profile, however there is concern in a number of recent studies that regular use of $ß_2$ agonists is associated with increased morbidity and mortality from asthma. Inhaled fenoterol has been implicated as a possible contributing factor to the rise in asthma mortality in New Zealand since the late 1970's on the basis of a series of case-control studies (50-52). In addition, Sears et al showed that regular (as opposed to required) inhaled fenoterol resulted in a significant deterioration in disease control (53). A study of non-epidemic deaths in Saskatchewan noted a relationship between fatal and near fatal episodes of asthma and the regular use of $ß_2$ agonists (54), although it was unclear whether ß agonists were directly responsible for these adverse effects or simply a marker for more severe asthma. More recently, Van Schayak noted a significant decline in lung function with regular inhaled bronchodilator drugs in general (55). The exact mechanisms whereby $ß_2$ agonists could have adverse efects on asthma mortality and morbidity are uncertain. Proposed theories have included hypokalaemia, cardiac dysrhythmias, tachyphylaxis (desensitisation) and exacerbation of airway hyper-reactivity, but no satisfactory explanation has been provided. On present evidence it seems likely that the use of high dose and possibly less $ß_2$ selective preparations (eg fenoterol) were causally associated with increased mortality in the epidemics of asthma deaths. The studies of morbidity suggest that tolerance can occur to $ß_2$ agonists in the form of a deterioration in asthma control, and it is recommended

that patients receive the minimum doses of β_2 agonist required once their inhaled steroid requirements are optimised.

1.2.2 Inhaled corticosteroids

These drugs represent a significant advance in the management of asthma. All patients who have persisting symptoms despite β_2 agonist therapy require regular treatment with inhaled corticosteroids eg; beclomethasone, fluticasone or budesonide. Their exact mode of action is unknown but probably reflects their ability to directly inhibit inflammatory cells in the airway (56) thus reducing airway hyperresponsiveness. The main local side effects are candidiasis and dysphonia. Minor adrenal suppression and some systemic corticosteroid side effects may occur with higher doses.

1.2.3 Others

Other adjuncts include anticholinergic drugs eg inhaled ipratropium bromide (Atrovent), and oral theophylline preparations. High doses of oral corticosteroids eg prednisolone are often required for patients with severe exacerbations of asthma and lower doses for asthmatics who are not optimally controlled on inhaled therapy alone.

1.3 ACUTE SEVERE ASTHMA

Most acute severe attacks of asthma occur in patients whose asthma has been poorly controlled for days or weeks, although in some individuals, symptoms of asthma can develop rapidly, transforming the patient to a state of severe disability over a few hours or even minutes. Symptoms of an acute asthma attack include severe dyspnoea, chest tightness and often inability to complete sentences in one breath. Examination of the patient typically reveals a hyperinflated chest, rapid breathing and usually widespread wheeze on auscultation.

Useful indicators of a severe attack of asthma are a tachycardia greater than 110 beats per minute and the most important respiratory signs of a severe attack are a respiratory rate of 25 per minute or more, a silent chest and central cyanosis (57). Exhaustion and hypotension are serious signs indicating impending cardio-respiratory failure. Arterial blood gas analysis is essential in the assesment of disease severity, and repeated measurements are necessary to monitor response to treatment. Most patients admitted to hospital with an exacerbation of asthma are hypoxaemic and about a third of patients admited with severe asthma have a PaO_2 of less than 8kPa on air (58,59). Ventilatory function tests such as the peak expiratory flow rate (PEFR) are of limited value in assessing the patient with acute severe asthma, but in general a PEFR of $< 33\%$ predicted normal or best should be regarded as evidence of a potentially life threatening attack of asthma (60). Prolonged severe asthma can often lead to fluid deprivation, either from the breathless patient avoiding drinking or insensible loss from the respiratory tract and rarely intravenous fluids may be necessary for rehydration if the attack has been of several hours duration. Electrolyte imbalance such as hypokalaemia can also occur in some patients as a result of β_2 agonist or corticosteroid therapy.

If not recognised or treated appropriately, acute severe asthma may be fatal. Most asthmatic subjects admitted to hospital are hypoxaemic, and oxygen therapy should be administered as early as possible in the attack by face mask in the highest concentration available with the aim of raising the paO_2 to above 8kPa in all patients and preferably to between 10kPa and 14kPa in most patients (61). High doses of inhaled $ß_2$ agonists (eg salbutamol 5mg) are administered nebulised with oxygen, or if this is not available, by multiple actuations of a metered dose inhaler. High doses of systemic steroids are also recomended, for example prednisolone 30-60mg or intravenous hydrocortisone 200mg, or both. If life-threatening features are present, ipratropium bromide and intravenous aminophylline may be added. Recent guidelines on the management of acute asthma issued by the British Thoracic Society (60) recommend that if there is no improvement after 15-30 minutes, nebulised $ß_2$ agonists should be administered more frequently (up to every 15 minutes) and the use of parenteral $ß_2$ agonists (eg intravenous salbutamol) be considered. Patients with deteriorating peak flow rate, worsening hypoxia or hypercapnia, exhaustion or respiratory arrest merit subsequent admission to an intensive care unit to be considered for assisted ventilation.

1.4 THE RENIN-ANGIOTENSIN SYSTEM

1.4.1 Historical background

The renin-angiotensin system (RAS) is an endocrine system which exerts its main physiological actions through a circulating effector peptide, angiotensin II (ANG II) to regulate cardiovascular homeostasis and electrolyte balance. The hormone renin was first discovered in 1898 by Tigerstedt and Bergman in an elegant experiment where saline extracts of the renal cortex of rabbits were injected intravenously into anaesthetised rabbits. A rapid and significant rise in blood pressure was observed (62). This initial work failed to achieve full recognition until 1934, when Goldblatt and colleagues demonstrated that clamping of the renal arteries could produce hypertension (63), with renin later being successfully extracted and purified from the kidney. Soon afterwards, further studies revealed that in fact renin was not itself vasoconstrictor, but acted upon a substrate in the plasma to form the vasoactive material which is now known as ANG II (64,65). A new dimension to the system was added in 1960 with the finding that ANG II stimulates the secretion of aldosterone, with consequent effects on salt and water balance.

1.4.2 The components of the renin-angiotensin system.

Since the discovery of renin nearly 100 years ago there have been major advances in our understanding of the RAS. The components of the RAS have now been identified and form a biological cascade (fig 1.1). The initiating enzyme of the system is renin, an aspartyl protease enzyme which is secreted into the circulation from the juxtaglomerular apparatus (JGA) in the afferent arteriole of the kidney. The JGA is an area adjacent to the segment of the distal convoluted tubule known

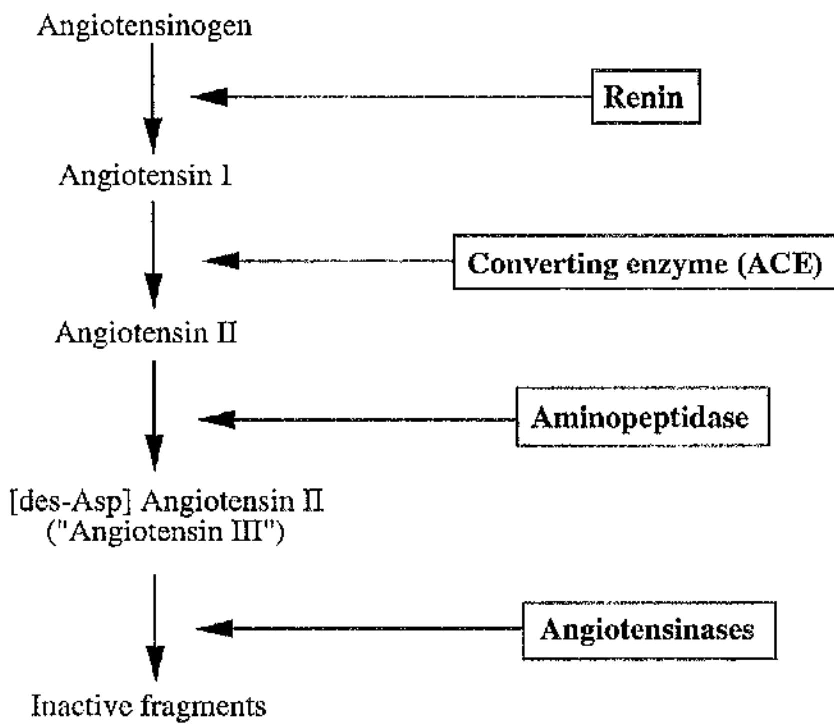

Fig 1.1 Schematic diagram of the renin-angiotensin system and its biosynthetic pathways.

as the macula densa. Release of renin is regulated by several mechanisms which will be reviewed later. Once released into the circulation, renin cleaves the large molecular weight α_2 globulin angiotensinogen to generate the inactive decapeptide angiotensin I (ANG I). The carboxy terminus of ANG I is then cleaved by angiotensin converting enzyme (ACE) to form ANG II. ACE is a membrane-bound dipeptidyl peptidase enzyme, present in vascular endothelium, with particularly high concentrations in the pulmonary circulation. Thus most activation of angiotensin occurs in the lungs. ACE has little substrate specificity also being involved in the processing of bradykinin, hence the alternative name of kininase. Although many studies suggest that ACE is the primary pathway leading to ANG II formation, alternative pathways have been proposed; ANG I has been shown to be rapidly converted to ANG II by neutrophil and mast cell proteinases (66) suggesting high local release of ANG II at inflammatory sites where both neutrophils and mast cells may be sequestered. In the failing human heart most ANG II can be formed by a pathway sensitive to a membrane-bound serine proteinase and not to an ACE inhibitor (67). Whether these alternative modes of ANG II generation are of clinical importance remains the focus of much debate. ANG II is the biologically active component through which the RAS exerts its effects. It has a short half-life (30 seconds) before undergoing further metabolism in the blood and other tissues to form the heptapeptide des-asp ANG II (or ANG III). ANG III retains partial activity, being a weak pressor agent in comparison with ANG II. It appears to be of minor physiological importance as a circulating hormone, except in the brain where it may function as a neurotransmitter (68). Other smaller degradation products of ANG II and III are most likely inactive.

1.4.3 The control of renin seceretion

It is known that the release of renin is regulated by several mechanisms (fig 1.2) :

1. A renal baroreceptor within the JGA which responds to changes
in blood pressure in the afferent renal arteriole.

2. The macula densa which detects changes in the rate of delivery of sodium and / or chloride to the distal tubule.

3. A negative feedback of circulating angiotensin on renin secretion

4. The central nervous system via either the renal sympathetic nerves or circulating catecholamines.

Role of the sympathetic nervous system in the control of renin secretion.

The juxtaglomerular apparatus is densely innervated by postganglionic sympathetic nerves and stimulation of the renal nerves has been shown to increase renin secretion (69,70). Slices of renal tissue and cortical cell suspensions are also responsive to catecholamines indicating that renin release is due to a direct effect of these hormones on the juxtaglomerular cells rather than via alterations in renal haemodynamics (71).

Renin levels in the plasma are known to be increased in several situations where sympathetic activity is increased, including exercise (72), standing or tilting (73), hypoglycaemia (74) and non-hypotensive haemorrhage (74). It is now generally accepted that the direct effect of catecholamines on renin-secretion is mediated by ß adrenoceptors as this effect can be blocked by the non-selective ß antagonist propranolol (75). Although autoradiography has demonstrated that the ß receptors on the juxtaglomerular cells in the dog, rat and guinea-pig are $ß_1$ in nature (76-78). the ß adrenoceptor subtype involved in renin release in man has been the

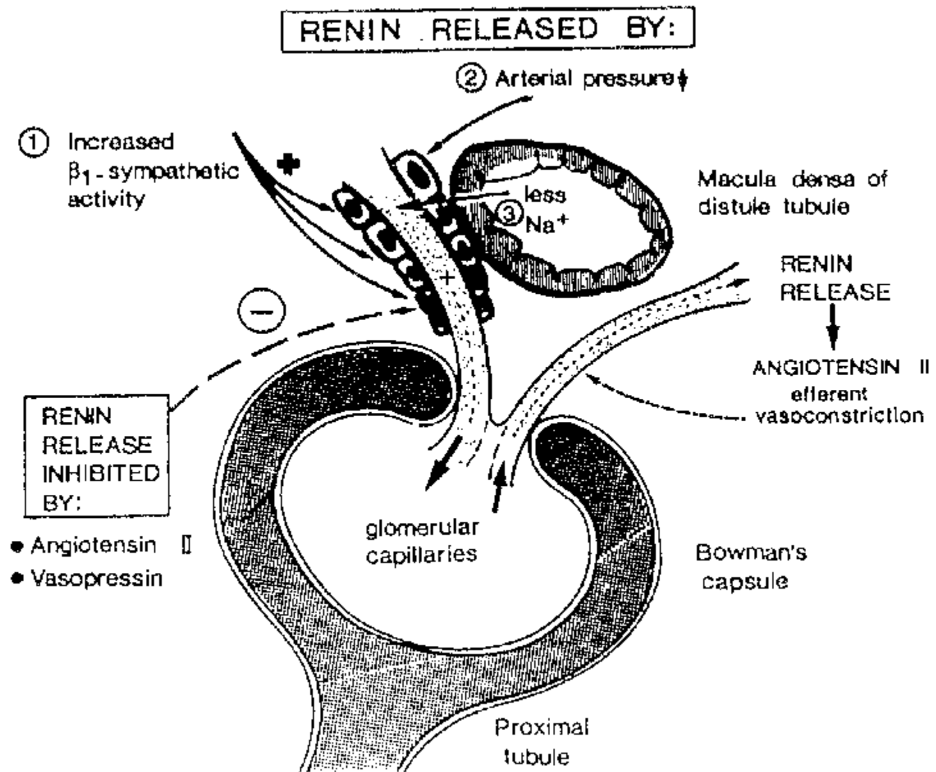

Fig 1.2 Mechanisms for the release of renin from juxtaglomerular cells of the kidney. From ref (79).

1. beta-1 sympathetic activity

2. hypotension or decreased renal blood flow

3. decreased tubular reabsorption of sodium eg; during a low sodium diet or diuretic therapy.

subject of much controversy. On the basis of pharmacological evidence it is now generally believed that the β_1 adrenergic receptor is probably the predominant subtype involved. This is supported by the observation that pretreatment of volunteers with the selective β_1 antagonists atenolol, metoprolol or acebutolol prevents the rise in renin produced by isoprenaline (a non-selective ß agonist) as effectively as propranolol (a non-selective ß antagonist)(80,81).

When selective ß agonists are studied however, the results are conflicting, and the evidence for β_1 mediated release of renin in man becomes less conclusive. Selective β_2 agonists eg salbutamol, stimulate renin release in some studies (82,83) but not in others (84,85). It remains unclear whether the effect of β_2 agonists on plasma renin is, in fact, a result of their ability to cause hypokalaemia, to increase plasma noradrenaline levels or perhaps by a partial agonist effect on β_1 receptors, rather than a direct interaction with juxtaglomerular β_2 receptors. Studies of the effect of selective β_1 agonists on renin release has also produced conflicting results (86-88). The reason for this is unclear but may be due to activation of mechanisms that actually inhibit sympathetic stimulation of renin release eg the positive inotropic, chronotropic and pressor actions of the β_1 agonists.

Other factors influencing renin secretion

Many other physiological and pharmacological stimuli can influence the activity of the RAS in man and an awareness of these is important prior to interpreting changes in circulating levels of renin and ANG II:

1. Electrolyte and water intake - a low dietary sodium (89), dehydration and to a lesser extent potassium depletion (90) stimulates the RAS.

2. Haemorrhage - bleeding is a marked stimulus to renin secretion, particularly when associated with hypotension and hypovolaemia (91).

3. Arterial pressure - in normal subjects and those with essential hypertension, there appears to be no relationship, or an inverse relationship between blood pressure and plasma renin. However, in experimental and clinical renovascular hypertension there is a clear positive correlation between ANG II and blood pressure (92,93).

4. Diurnal changes - a modest variation in plasma renin concentration occurs during a 24 hour period with a peak at 1000 and a trough at 2200 (94).

5. Age, gender and race - there is a progressive fall in renin with age, plasma renin activity is lower in women than age-matched men and Negroes have been found to have lower plasma renin activity than Caucasians (95).

6. Cigarette smoking - mean plasma renin activity has been found to be approximately 20% higher in smokers than non-smokers (95).

7. Drugs - diuretic agents such as thiazides (96) and loop diuretics (97) increase renin as a result of their natriuretic and potassium losing effects. Other drugs reported to increase renin include lithium (98) and calcium antagonists (99). Non-steroidal anti-inflammatory drugs eg indomethacin have been reported to reduce plasma renin activity after single or multiple doses (100).

1.4.4 ANGIOTENSIN II

Acute effects

Angiotensin II (ANG II) is an octapeptide hormone with several important physiological actions. The first of these to be identified was its pressor action (101), and for many years it was believed that the main role of the RAS was to regulate blood presssure. ANG II can rapidly raise the blood pressure by several mechanisms (102) : It may act directly on specific angiotensin receptor sites on the vascular wall to cause vasoconstriction. In addition ANG II has a facilitatory role on the sympathetic nervous system, enhancing the release of noradrenaline from adrenergic vasoconstrictor nerves by stimulation of presynaptic angiotensin receptors. This action is independent of its direct effect on the vascular receptor. ANG II can also cause vasoconstriction by potentiating catecholamine release from the adrenal medulla.

Besides acting as a vasoconstrictor, ANG II stimulates the secretion of the sodium-retaining hormone aldosterone from the zona glomerulosa of the adrenal cortex (102). The action of aldosterone is to increase sodium reabsorption from urine, sweat, saliva and gastointestinal secretions. By promoting sodium and water reabsorption at the distal tubule and collecting duct of the kidney, aldosterone increases potassium and hydrogen-ion secretion, thus maintaining extracellular fluid volume and acid-base balance. The RAS is the chief regulator of aldosterone in states of volume depletion.

ANG II as a growth factor

In addition to its acute effects on aldosterone secretion and vasoconstriction, ANG II exerts long term effects on cellular regulation and growth. Recent *in vitro* studies have demonstrated that ANG II has a direct hypertrophic effect on cardiac myocytes (103), and ANG II has been reported to stimulate the growth of cultured human smooth muscle cells in vitro (104). The intracellular pathways mediating this effect are the subject of current research although second messengers related to the activation of protein kinase C are known to be involved (105). Although there is little direct evidence for such an effect *in vivo*, local generation of ANG II in the heart and vascular wall may play a role in causing vascular and cardiac hypertrophy. Such changes may be important in the long term, either by causing increased vascular resistance in hypertension or left ventricular hypertrophy, a risk factor for cardiovascular mortality.

Angiotensin II receptors and intracellular messenger systems

The effects of ANG II are now known to be mediated by its interaction with specific, high affinity receptors on the plasma membrane of its target cells. Recently, the development of highly selective ANG II receptor ligands has led to the identification and characterisation of these receptors in a number of tissues, including vascular smooth muscle, heart, uterus, the adrenal zona glomerulosa and the brain (106). The receptors are coupled to cellular activation by a variety of biochemical signalling systems which include guanyl nucleotide regulatory proteins (G proteins), membrane enzymes and ion channels. Two receptor subtypes termed AT_1 and AT_2 have recently been described.

The AT_1 receptor mediates all or most of the known biochemical and physiological actions of ANG II, including contraction of smooth muscle and aldosterone production. AT_1 is a membrane linked protein which transmits ANG II activity via a G-protein linked pathway. Two G proteins are known to be associated with the AT_1 receptor, the major of which (Gq) is coupled to a phosphodiesterase enzyme known as phopholipase C, and the other (Gi) to the inhibition of adenylate cyclase. Most of the known actions of ANG II are exerted through receptor-mediated hydrolysis of inositol phospholipids and increased cytoplasmic calcium levels via the Gq protein (108,109). The primary event is the cleavage by phospholipase C of phophatidylinositol 4, 5 bisphosphate (PtdIns(4,5)P_2) to form Ins (1,4,5)P_3 and sn-1,2 diacylglycerol. These products subsequently act as intracellular second messengers to mediate hormone action (79). IP_3 liberates calcium from the intracellular sarcoplasmic reticulum whereas diacyl glycerol activates protein kinase C, which is thought to act on contractile proteins to produce a sustained response. When vascular angiotensin receptors are activated it is believed that two mechanisms lead to an increased release of calcium from the sarcoplasmic reticulum (79). Firstly the formation of IP_3 and secondly via an additional direct G-protein mediated stimulation of a calcium channel. The resultant release of calcium increases vascular contraction and tone. Similarly, the cellular mechanisms whereby ANG II stimulates aldosterone release from the adrenal zona glomerulosa are also thought to be initiated by a receptor-mediated rise in cytoplasmic calcium concentration. This effect, in conjunction with protein kinase C produces phosphorylation of key enzymes involved in the conversion of stored cholesterol and corticosterone to aldosterone in the cells of the adrenal zona glomerulosa (110).

The AT_1 receptor subtype is sensitive to the nonpeptide antagonist losartin, a drug which has been shown to have potent inhibitory effects on ANG II induced vasoconstriction in animal studies. Being orally active, drugs such as losartin are currently being introduced in the management of certain forms of hypertension.

In contrast, $\mathbf{AT_2}$ receptors do not appear to be coupled to G-proteins and as yet there is little evidence for a functional role of this subtype. However their recent identification in the foetal and immature brain and subcutaneous tissues suggests a possible role for these receptors in mediating developmental actions of ANG II (111).

1.4.5 Local renin-angiotensin systems

Although many of the actions of the RAS are typical of an endocrine system, where the effects are mediated entirely by circulating hormones, there is now evidence to suggest that the components of the RAS are also present and functional in a number of tissues including blood vessels, heart and brain. Such local or tissue RAS's are believed to have a local or paracrine role, probably being responsible for the maintenance of vascular structure and the long term control of blood pressure, in contrast to the plasma RAS which has a more acute regulatory role. The brain RAS is one of the most studied tissue RAS (112), and the components of the system have been localised in many areas of the brain thought to be involved in cardiovascular regulation such as the hypothalamus and medulla. There is currently considerable evidence that the brain RAS plays a role in the development or maintenance of hypertension, with ANG II acting as a neurotransmitter or neuromodulator. Results from other studies suggest that a local renin-angiotensin system also exists in the heart ; cardiac myocytes and isolated perfused rat hearts are known to produce renin and ANG II (113), and

ACE activity has been detected in the atria and ventricles of rat hearts (113). It has been proposed that ANG II produced locally by the myocardium may have important physiological and pathological functions eg ventricular remodelling and compensatory hypertrophy after myocardial infarction. Similarly, local generation of ANG II in blood vessels may play a role in the modulation of vascular tone, and interference with the intrinsic RAS in the blood vessels could contribute to the fall in blood pressure with ACE inhibitor treatment.

The potential functions of these and other tissue RAS eg; the reproductive tract and kidney are at present a rapidly developing and exciting area of research.

1.4.6 Inhibitors of the renin-angiotensin system

Angiotensin converting enzyme is a zinc dependent metallo-peptidase enzyme. The first ACE inhibitor agent to be discovered in 1977, captopril, incorporated a sulfhydryl group to bind to the zinc (114). Subsequent ACE inhibitors have been developed with alternative zinc ligands, better potency or longer durations of action. All the ACE inhibitor drugs block the enzyme both in the plasma and in the tissues, although the degree of inhibition varies with different inhibitors (115). Orally active ACE inhibitor drugs eg; captopril and lisinopril, are now established therapy in two of the most common cardiovascular conditions, hypertension and congestive cardiac failure. In the treatment of hypertension, several mechanisms may be involved; The main acute effect is clearly to decrease circulating ANG II levels and thus cause vasodilatation with another possible major site of action being the tissue RAS, including ACE activity in the vascular bed. Other postulated mechanisms include increased formation of bradykinins or prostaglandins with vasodilatating properties (116), modulation of adrenergic

activity (117) eg; by inhibiting noradrenaline release from adrenergic nerves, or by causing natriuresis (118). Extensive studies have also demonstrated the benefits of acute and chronic use of ACE inhibitors in congestive heart failure. They have been shown to relieve symptoms and improve exercise tolerance, and in addition, delay the progression of left ventricular dysfunction after myocardial infarction (119) and to reduce mortality in moderate to severe heart failure (120,121).

The recent development of non-peptide (and hence orally active) specific ANG II antagonists eg losartan, offers the possibility of long term blockade of the AT_1 receptor. Although preliminary observations in hypertension are encouraging, it remains to be seen how effective these drugs will be in the long term.

1.4.7 Genetic aspects of the RAS : the ACE genotype

A polymorphic marker which correlates with circulating ACE levels has recently been identified in the ACE gene. Posession of this marker (D) (deletion of a 287 base pair) has been shown to be a risk factor for coronary artery disease (122), particularly in men otherwise considered to be at low risk. DD genotype is associated with higher levels of circulating ACE in these patients, and is significantly more frequent in people who have had a myocardial infarction than in age matched controls. The DD genotype frequency has also been shown to be a risk factor for the development of end-stage heart failure due to cardiomyopathy (123), and it is proposed that molecular variants in ACE may, by altering local cardiac ANG II levels, influence ventricular structure and function, via the trophic effect of ANG II on cardiac myocytes.

1.5 THE RENIN-ANGIOTENSIN SYSTEM IN ASTHMA : BACKGROUND TO CURRENT STUDIES.

Activation of the RAS has been described in a variety of pathophysiological states in man including myocardial infarction (124), acute renal failure (125) and severe haemorrhage (91). However, the activity of the RAS in asthma has not been previously described, and the direct effect of ANG II on bronchomotor tone is unknown. In a preliminary study in which we measured hormonal influences in patients with acute severe asthma, marked activation of the RAS was noted. The stimulus to this activation, and the possible significance of the effects of elevated plasma levels of ANG II in asthma was unclear.

A subsequent search of the relevant literature revealed that ANG II could potentiate contraction of rabbit airway smooth muscle *in vitro* (126), thus raising the possibility that this hormone may possess bronchoconstrictor activity *in vivo*. Intrigued by our initial observations in acute asthma, we then decided to confirm and extend this preliminary observation in a larger number of patients.

Possible stimuli which could potentially cause activation of the RAS in acute severe asthma include hypoxia (127,128), β_2 agonists (129,82), circulating catecholamines (130) and inflammatory mediators (131,66) and the contribution of some of these stimuli was investigated in subsequent studies in this thesis. The effect of β_2 agonists were of particular interest in view of the current concerns over the safety of these agents in asthma (50-54).

Although the effects of elevations of ANG II on pulmonary function are unclear, ANG II could, in theory, adversely affect the lungs by several mechanisms, including bronchoconstriction (126). The remainder of the studies were designed

to examine the direct and indirect effects of ANG II on airway function *in vivo* in mild asthmatic patients, and also *in vitro* on human airway smooth muscle.

The results of these investigations should increase our understanding of the role of ANG II on airway function in asthma, and in particular will allow us to establish whether the elevated levels of ANG II in acute severe asthma have an adverse effect on lung function. These results will hopefully clarify some of the pathophysiological processes occurring in acute severe asthma and in addition determine whether ANG II might be a mediator in asthma.

CHAPTER 2

GENERAL METHODS AND MEASUREMENTS

2.1 SUBJECTS

Patients presenting with acute severe asthma were identified at the Accident and Emergency Unit of the Western Infirmary and respiratory wards at Gartnavel General Hospital, Glasgow. Mild asthmatic subjects were recruited from outpatient asthma and general respiratory clinics at Gartnavel General Hospital, the Western Infirmary, Glasgow and Dumbarton Health Centre. Healthy volunteers were comprised mainly of nursing, medical and secretarial staff in these hospitals. None of the asthmatic subjects or healthy volunteers were smokers. Further demographic data for each subject group is discussed in the appropriate chapter.

2.2 LUNG FUNCTION

Peak expiratory flow rate (PEFR), as measured by a mini Wright's peak flow meter, provides a simple measurement of airflow obstruction and is often used for the home monitoring of asthma. In addition, this test forms part of the assessment of acutely ill patients presenting to hospital with severe asthma.
In all cases the PEFR was recorded as the best of 3 readings (unless otherwise stated).

Spirometry is performed using a dry wedge spirometer (Vitalograph-S) or portable pneumotachograph spirometer (Vitalograph-Compact), both from Vitalograph, Buckinghamshire, UK, the machines being calibrated prior to each study. The most common measurement to determine limitation to airflow is the volume expired in the first second (FEV_1 or forced expiratory volume in the first

second of expiration), in general values below 70% predicted for age (132) indicating airflow obstruction. However mild asthmatic subjects (as in the present study) often have FEV_1 values in excess of this. For all studies the best of 3 readings was recorded.

2.3 METHACHOLINE CHALLENGE (PROVOCATION TEST)

A number of different agents have been used to demonstrate bronchial hyperreactivity in asthma, methacholine and histamine being the most common and reliable of the non-specific bronchoconstrictors. These agents can also produce airway narrowing in normal subjects, but at much higher doses than those affecting asthmatic patients. Methacholine responsiveness is affected by asthma medication and this should be taken into account prior to testing. Tests were performed at the same time of day for each subject.

Short acting β_2 agonists (eg; Salbutamol) were withheld for 8 hours, long acting β_2 agonists (eg; Salmeterol) were withheld for 24 hours and slow release theophylline preparations were withheld for 48 hours before the test. Inhaled corticosteroids continued unchanged. The method of mesurement of bronchial reactivity to methacholine used a method described by Cockroft (133) and Hargreave (134). Aerosols were generated using the same Wright nebuliser and delivered through a loose fitting facemask. Subjects wore a noseclip and inhaled the aerosol through the mouth by normal tidal breathing. The nebuliser contained a volume of 3ml of solution and was driven by a continuous airflow at 8l/min from a compressed air source of 50 lbs/in (345 kPa) giving an output of approximately 0.15ml/min. Baseline FEV_1 was measured as previously described and until reproducible within 5%. Subjects than inhaled phosphate buffered saline

for 2 minutes and FEV_1 recorded again at 30, 90 and 180 seconds. The lowest post-saline value was then used as the baseline from which subsequent falls were measured. At 5 minute intervals, subjects received doubling concentrations of methacholine (0.0625mg to 8mg/ml) and FEV_1 repeated at 30, 90 and 180 seconds. Inhalations were continued until the FEV_1 fell by 20% or more from the lowest post-saline value. Results were then expressed as the PC_{20} (provocation concentration 20%), which was calculated by linear interpolation of the dose-response curve for each individual. All mild asthmatic subjects in the present study demonstrated at least mild hyperresponsiveness to methacholine with a $PC_{20} < 8$mg/ml. The value for normal individuals is > 8mg/ml.

At the end of each methacholine challenge, subjects received nebulised salbutamol to reverse the bronchoconstriction.

2.4 HORMONE ASSAYS

Hormone assays were performed by Dr JJ Morton in the Blood Pressure Unit, Western Infirmary, Glasgow. Accuracy of ANG II measurement has improved over the last decade and sample handling bias has become appreciated. Since ANG II can be generated in the plasma after blood sampling, it is therefore neceassary to ensure that samples are collected into prechilled tubes containing inhibitor cocktails, with rapid centrifugation and quick freezing of the plasma thereafter. Other pitfalls of blood sampling for both renin and ANG II measurement are the effects of factors such as posture, diurnal variation, concurrent medication and food intake (chapter 1.4.3).

As measurement of plasma renin and ANG II levels in asthma and healthy volunteers constitutes an important aspect of the methods used in this thesis, a more extensive description of the assays for these particular hormones is included.

Angiotensin II

7mls venous blood was collected rapidly into an iced glass sample tube containing 1ml EDTA / o-phenanthroline to inhibit angiotensin converting enzyme and angiotensinase enzymes. After thorough mixing, samples were immediately centrifuged at 3000rpm for 10 minutes at 4^{0}C and plasma stored at -20^{0}C pending hormone analysis.

The assay for ANG II is a modified radioimmunoassay originally developed by Dusterdieck and McElwee (135) with modifications by Morton and Webb (136). ANG II was extracted from the plasma using Sep-Pak C18 cartidges pretreated with methanol (5mls) and water (5mls). After washing with water ANG II was eluted from the column with 80% methanol (2mls). The extracts were dried under a stream of air and redissolved in 4000μl of Tris Buffer for assay. Radiolabelled ANG II and ANG II antibodies were added to tubes containing 100μl of sample and a standard curve made up using dilutions from 100pg to 1.56pg unlabelled ANG II per tube. All the tubes were then incubated at 4^{0}C for 18 hours. 1ml of dextran-coated charcoal was then added to separate unbound ANG II and mixed thoroughly before centrifugation at 3000rpm for 7 minutes. The supernatant was removed and the residue counted for 100 seconds in a gamma counter (Nuclear Enterprises NE 1612). Calculation of ANG II levels was performed automatically by an on-line computer. Recovery from plasma is >95%. The intra-assay coefficient of variation is 6.4% and inter-assay variation 10%.

The normal reference range for our laboratory is 3-12pg/ml.

Renin

5ml venous blood was collected into potassium EDTA tubes and kept at room temperature. Plasma was separated by centrifugation at 3000rpm for 10 minutes at 4°C and samples stored at -20°C until assay. The assay for renin is based on the method developed by Millar et al (137) which measures plasma renin concentration rather than plasma renin activity, and is based on antibody trapping. This assay is capable of determining concentrations of both total and active renin. For the active renin assay 20μl of plasma was added to a standard mixture containing purified ox renin substrate, Tris buffer and antibody to ANG I and the mixture incubated at 37° C for 30 minutes. Active renin within plasma converts renin substrate to ANG I which is bound by the antibody. The reaction is stopped by the addition of cold Tris buffer. A standard curve is then derived by adding serial dilutions of ANG I standard to the mixture, and blank tubes with no ANG I and empty tubes for total counts also included. Labelled ANG I was then added to all the tubes and incubated at 4° C for 18 hours. Free and bound ligand were separated by the addition of 1 ml of dextran coated charcoal and free ligand was counted in a gamma counter (Nuclear Enterprises NE 1612) for 100 seconds and the results computed automatically.

The intra-assay range for active renin is 5.5% and inter-assay range 11%.

Our normal laboratory reference range for active renin is 9-50 μU/ml.

Catecholamines

5mls of venous blood were collected into chilled lithium heparin tubes and kept on ice until centrifugation at 4° C. Plasma noradrenaline and adrenaline were then measured by reverse phase high performance liquid chromatography and

electrochemical detection after extraction from plasma using activated alumina as described by Goldstein et al (138). The coefficient of variation is less than 10% and normal values are < 5.0 nmol/l and < 0.4 nmol / l for noradrenaline and adrenaline respectively.

ACE (Angiotensin Converting Enzyme)

Measurement of serum ACE employs a continuous spectrophotometric method based on the hydrolysis of furylacrylpenylalanineglycylglycine (FAPGG) and applied to an IL Multistat II centrifugal analyser. The method is that described by Maguire and Price (139).

The reference range for our laboratory is 20-95U/L.

2.5 ARTERIAL BLOOD GASES

Arterial blood gases (ABG's) were sampled on air from the radial artery using a pulsator blood sampling system (Concord laboratories) and analysed using a Ciba-Corning 288 blood gas system.

Normal values are PaO_2 11-13kPa and $PaCO_2$ 4.5-6kPa.

2.6 HAEMATOCRIT (or PACKED RED CELL VOLUME)

Measured by an automated Coulter Counter.

Normal values are 0.4 -0.54 l/l (males) and 0.37-0.47 l/l (females)

2.7 24 HOUR URINARY SODIUM

This was measured using diluting ion selective electrodes (Olympus 5200 analyser).

The normal reference range is 100-250mmol / 24hr

2.8 DRUGS

Angiotensin II (Hypertensin®, Sigma Chemical Company, Poole, Dorset, UK) kindly donated by Dr A. Morris, University Department of Medicine and Therapeutics, Western Infirmary, Glasgow. Angiotensin II was prepared in 5% dextrose as a solution for intravenous infusion.

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

Salbutamol (Ventolin ®) solution for nebulisation 5mg prepared in 3.5 ml normal (0.9%) saline.

Fenoterol (Berotec®) solution for nebulisation 2.5mg prepared in 3.5ml normal saline.

Lisinopril (20mg) and identical placebo capsules prepared in the hospital pharmacy sterile unit.

For the *in vitro* studies **methacholine** and **angiotensin II** were supplied from Sigma Chemicals, Poole, Dorset, and prepared in distilled water with serial dilutions in Krebs-Henseleit solution.

2.9 STATISTICAL ANALYSIS

Statistical analysis was performed on an Apple Macintosh LC II computer using the Statview® software package.

In the acute asthma study (Chapter 3) differences in plasma renin and ANG II levels between the different groups of asthmatic patients and control subjects was evaluated using the Mann-Whitney U-test, and the absolute numbers of patients with elevated plasma renin and ANG II levels within each group was compared using the X^2 test. p values below 0.05 were accepted as significant.

For the studies outlined in Chapters 4 to 8, comparison of baseline levels and the significance of changes in these at each time point was by analysis of variance, corrected for multiple comparisons. p values below 0.05 were accepted as significant.

In Chapter 9, statistical significance between data samples for the *in vitro* work was tested by two-way ANOVA and statistical difference between pD2 values was by Student's t-test. For the *in vivo* study, differences between placebo and active days was by Student's t-test with subsequent Dunnett test.

In both cases, probability levels of $p < 0.05$ were considered significant.

2.10 ETHICAL APPROVAL

All clinical study protocols had prior approval by the Glasgow West Ethical Committee. Informed written consent was also obtained from all participants, with the exception of the acute asthma study as it was thought that this would delay treatment of such patients and venepuncture was considered to be a normal part of the assessment process in the Accident and Emergency Department. Following admission however, patients were informed that venous blood samples requested on days 2 and 5 were for research purposes and were allowed to decline if they wished.

CHAPTER 3

THE RENIN-ANGIOTENSIN SYSTEM IN ACUTE SEVERE ASTHMA

3.1 INTRODUCTION

This first study was stimulated by an earlier observation made by ourselves while examining hormonal responses in acute asthma. In this preliminary study we were surprised to discover marked activation of the renin-angiotensin system (RAS) in patients admitted to hospital with an acute exacerbation of asthma. However, as far as we are aware, the activity of the RAS in asthma had not been previously described. and the effect of the hormone ANG II on lung function in man was unknown. Having consulted the relevant literature, it was discovered that ANG II had recently been shown to potentiate bronchoconstriction of rabbit airway smooth muscle in vitro (126). We therefore decided to extend and confirm our earlier observation by measuring plasma levels of renin and ANG II in a larger group of patients with acute severe asthma admitted to our hospital. Furthermore, we were also interested to compare these results with those levels obtained in stable patients with mild chronic asthma taking only inhaled bronchodilator therapy on an as-required basis. a group of severe chronic asthmatics on regular home nebulisers and a healthy non-asthmatic control group.

3.2 PATIENTS AND METHODS

Plasma renin and ANG II levels were therefore measured in 4 groups of subjects :

Group 1) 20 (6 male) patients admitted non-consecutively (2 patients admitted twice) to Accident and Emergency with acute severe asthma. [Mean (SD) age 34 (12.9) years, PEFR 125 (38) l/min, PaO_2 9.2 (3.3) kPa (on air), pulse rate 120 (15) beats/min on admission]. Venous blood was withdrawn shortly after admission for estimation of plasma renin, ANG II, catecholamines (adrenaline and noradrenaline) and haematocrit. Arterial blood gases (ABG's) were sampled on air from the radial artery and peak expiratory flow rate (PEFR) was recorded. Patients were treated as deemed appropriate by the attending physician and all measurements (with the exception of ABG's) were repeated on day 2 and day 5 after admission.
All patients had received nebulised $ß_2$ agonists (salbutamol) and four were given intravenous hydrocortisone in the Accident and Emergency Department prior to venesection. Seventeen patients were taking regular inhaled corticosteroids, 5 oral theophylline and 10 were on oral prednisolone. All used as required inhaled $ß_2$ agonists via metered dose inhaler (6 of these in addition were taking $ß_2$ agonists via home nebuliser), 4 inhaled salmeterol and 4 inhaled ipratropium bromide. No patients were receiving oral ß agonists. Only one patient was on regular oral diuretic therapy (frusemide).

Group 2) 9 (2 male) patients with mild chronic asthma, mean (SD) age 40.3 (13) years, PEFR 450 (59) l/min, mean O_2 saturation 96.8 (1.2)%. All were taking as required inhaled $ß_2$ agonists via metered dose inhaler and 6 regular inhaled corticosteroids.

Group 3) 10 (2 male) patients with severe chronic asthma taking regular nebulised ß agonists (salbutamol), mean (SD) age 39.3 (15) years, PEFR 225 (48) l/min, O_2 saturation 97 (1.8)%. In addition, all were taking regular inhaled corticosteroids, 4 nebulised ipratropium bromide, 3 oral theophylline and 3 maintenance doses of oral prednisolone.

Group 4); 16 (5 males) normal volunteers, mean age 36.6 (11.4) years, PEFR 507 (77) l/min, O_2 saturation 98 (1.3) %.

Patients in groups 2 and 3 were requested to withhold their $ß_2$ agonists for 6 hours prior to venesection.

Measurements

Renin, ANG II, catecholamines see Chapter 2.4, Hormone assays

Haematocrit - see Chapter 2.5

Arterial blood gases - see Chapter 2.6

Oxygen saturation - pulse oximeter and finger probe (Ohmeda Biox 3700E; Ohmeda, Louisville, USA).

PEFR - Wright mini-peak flow meter.

Statistical analysis

Differences in plasma renin and ANG II between the groups was evaluated using the Mann-Whitney U- test. The absolute number of patients with elevated renin and ANG II levels in each group was compared using the x^2 test.

p values below 0.05 were accepted as significant.

3.3 RESULTS

In the acute severe asthma group, plasma renin was significantly elevated ($p<0.05$) on days 1, 2 and 5 when compared to the mild chronic asthmatics, the severe chronic asthmatics and our non asthmatic control group. Plasma ANG II was only significantly elevated on day 5 when compared with the other groups (figure 3.1 and table 3.1).

When the groups were compared for the absolute number of individuals with elevated ANG II levels there were also significant differences. In the acute asthma group, 15 out of 20 patients had elevated ANG II levels compared to 3 of the 9 mild asthmatics and 5 of the 16 controls [$x^2 = 7.74$ and 4.05 respectively, $p< 0.05$].

There was no significant difference in plasma renin and ANG II levels between the mild chronic asthma group, the severe chronic asthma group and the normal volunteers.

There was no correlation between plasma ANG II levels and systolic blood pressure on admission in the acute severe asthma group.

Raw data for this chapter is included in the Appendix [tables 1A to 7A].

	Acute severe asthma			Mild chronic asthma	Severe chronic asthma	Normal volunteers
Day	1	2	5			
RENIN (μU/ml)	48.7 * (24-79)	44.2 * (15-75)	45.5 * (21-70)	19 (12.5-25.5)	22 (17-32)	20 (12-24.3)
ANG II (pg/ml)	32.5 (7.5-75)	38 (6-105)	56 * (12-109)	12.5 (6-19.5)	12.2 (7.7-18.8)	11.3 (9-17)

Table 3.1. Plasma renin and ANG II levels in acute severe asthma, mild chronic asthma, severe chronic asthma and normal volunteers (results expressed as median with interquartile range) *$p<0.05$ versus mild chronic asthma, severe chronic asthma and normal volunteers.

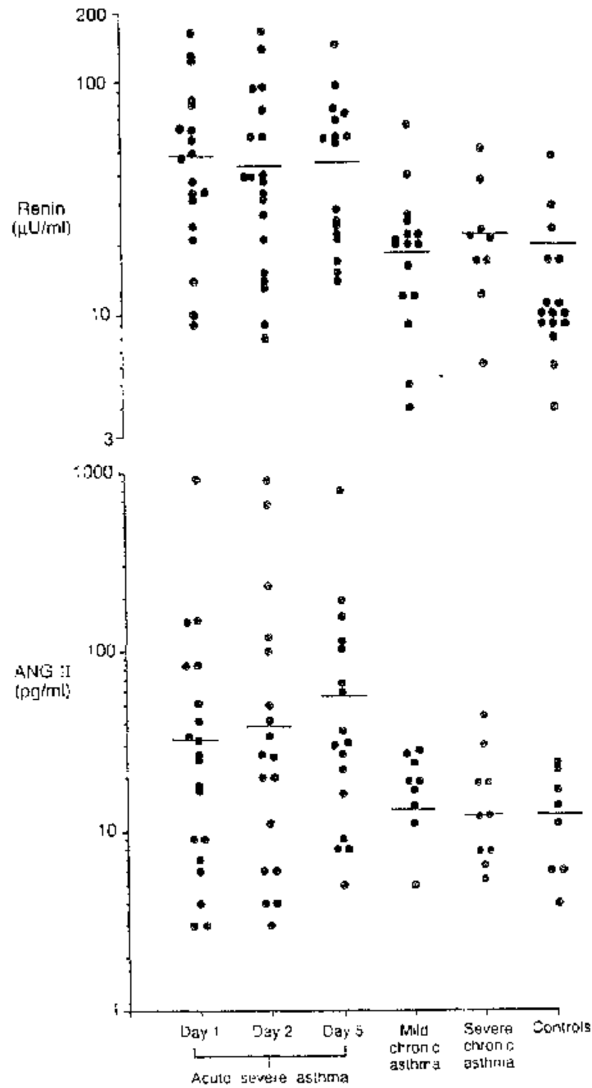

Fig 3.1 Scatter plot (log scale) illustrating individual plasma renin and angiotensin II (ANG II) levels in acute severe asthma [n=20], mild chronic asthma [n=9], severe chronic asthma [n=10], and controls [n=16]. Bars represent median values. In acute severe asthma, plasma renin was significantly elevated ($p<0.05$) on days 1, 2 and 5, and ANG II significantly elevated ($p<0.05$) on day 5 only, when compared to the other groups.

3.4 DISCUSSION

This is the first study to document activation of the RAS in acute severe asthma. In addition, we have shown that the levels of renin and ANG II in acute severe asthma are significantly elevated when compared to a non asthmatic control group and a group of mild chronic asthmatics or severe chronic asthmatics who had withheld their ß agonist therapy. Plasma renin levels were noted to be significantly elevated on days 1, 2 and 5 after admission, and although ANG II was also elevated, this only reached statistical significance on day 5. However, it is possible that the lack of significance on the first two days may be the result of the small sample size. The reason for the wide range of variations in plasma renin and ANG II levels in the patients is not certain, but may related to differences between individuals in factors influencing the RAS.

The stimulus to this activation of the RAS in asthma, and the effects of ANG II on lung function in man are unclear. Since ß adrenoceptors are known to be present on juxtaglomerular cells (74), it is possible that nebulised $ß_2$ agonist therapy used in the treatment of acute asthma could be responsible for this activation. In support of this suggestion is our observation that plasma renin and ANG II levels remained elevated on days 2 and 5 when the patients were recovering from the acute attack of asthma (as demonstrated by increased peak flow rates) but still continued to receive nebulised bronchodilator therapy. In addition, increases in plasma renin activity in normal subjects has been described following intravenous infusion of salbutamol and also after the $ß_2$ agonists fenoterol and salbutamol administered by metered dose inhaler (129,82). Hypoxia may also cause activation of the RAS, although studies which have examined this effect in animals and in man have produced conflicting results (140,141). In stable cor

pulmonale the RAS has been reported not to be activated (141) while others have observed elevated levels of ANG II in patients with airflow obstruction and arterial hypoxaemia (128). Other stimuli which could cause activation of the RAS in acute severe asthma include circulating catecholamines. Ind et al have reported elevated plasma noradrenaline (NA) levels in patients admitted with acute severe asthma (eg; mean (SEM) plasma NA of 7.7 (0.6) nmol/l), although adrenaline levels were not increased in any cases (130). We have not found convincing evidence of this in our own study, where in the majority of patients plasma catecholamines were within the normal range (Table 4A, Appendix). Other possible stimuli are hypovolaemia and drugs such as diuretics. There was a small but significant increase in the mean haematocrit value on the day of admission when compared to day 2 and 5 in our acute asthma patients, although individual values remained within the normal laboratory reference ranges on all 3 days (Table 3A, Appendix). This elevation in haematocrit on admission probably reflects the haemoconcentration resulting from insensible loss of water in the breath as a result of prolonged hyperventilation during an acute attack of asthma. However, it is unlikely that such a minor degree of haemoconcentration would explain activation of the RAS lasting until day 5 after admission. Only one acute asthma patient was receiving regular oral diuretic therapy and it is also unlikely that inhaled or oral steroid therapy is responsible for this activation of the RAS in acute asthma. Although cortisol has been reported to increase renin substrate (angiotensinogen) levels (142), prednisolone given to normal volunteers in doses of 50mg/day has no effect on plasma renin activity (143) and there was no evidence of activation of the RAS in our 3 severe chronic asthmatics who were taking oral steroids at the time of venesection. The effect of oral theophyllines on the RAS is unknown. The role of inflammatory mediators such as histamine or

prostaglandins on the activity of the RAS is also unclear, although it is of interest that proteinase enzymes secreted by mast cells have been shown to cause rapid conversion of ANG I to ANG II by an angiotensin converting enzyme (ACE) independent pathway (131).

Thus, in this initial study we conclude that the RAS is activated during acute severe asthma. Further studies should now be designed to explore possible stimuli to the release of ANG II during acute severe asthma and later to investigate whether the elevated ANG II levels found in acute asthma might adversely influence lung function.

CHAPTER 4

THE EFFECT OF NEBULISED β_2 AGONISTS ON THE RENIN-ANGIOTENSIN SYSTEM IN MILD ASTHMATIC PATIENTS AND NORMAL VOLUNTEERS

4.1 INTRODUCTION

The stimulus to the activation of the renin-angiotensin system (RAS) in acute asthma is unclear, but it is possible that high dose β_2 agonists used in the treatment of acute asthma could be responsible. In support of this is the observation that isoprenaline has been reported to stimulate the release of renin from isolated juxtaglomerular cells in vitro, this effect being mediated by ß adrenoceptors (74). In addition, an increase in plasma renin activity has been reported following intravenous infusion of salbutamol in 4 healthy male subjects (129), and a dose dependent increase in plasma renin activity was noted after fenoterol and salbutamol administered by metered dose inhaler to normal subjects (82).

In the following study, we have investigated the effect of two commonly used β_2 agonists, salbutamol and fenoterol, on the activity of the RAS in both mild asthmatic patients and normal subjects.

4.2 METHODS

Subjects

Eight stable mild asthmatic patients (2 male, 6 female), mean (SD) age 38(8.4) years, mean FEV_1 82 (15) % predicted, and 8 non asthmatic volunteers, mean age 34 (7.6) years were studied. All asthmatic patients were taking as required inhaled short acting ß2 agonists, 7 of the 8 inhaled corticosteroids, and 2 an oral theophylline. Asthmatic patients were asked to withhold ß2 agonists for at least 6 hours, and oral theophyllines for 24 hours prior to each visit.

Study design

Subjects attended the laboratory on 3 non-consecutive study days. A 24 hour urine collection was obtained prior to each visit as an indirect means of assessing their dietary sodium intake. On arrival, an indwelling catheter (Venflon ®; Viggo AB, Helsingborg, Sweden) was inserted into a forearm vein for blood sampling. After a 20 minute rest, baseline spirometry (the best of 3 readings) was recorded and 30 ml of venous blood was withdrawn for estimation of baseline plasma renin, ANG II and serum potassium.

Each subject then received either fenoterol (2.5mg in 3.5 ml normal saline), salbutamol (5mg in 3.5 ml normal saline) or placebo (3.5 ml normal saline) via a face mask and nebulizer driven by compressed air (CR 60 High Flow Compressor; Medic-Aid Ltd, Pagham, Sussex, UK.). Subsequent recordings of FEV_1 (the best of 3 readings) were made at 15, 30, 60 and 120 minutes after nebulisation, and blood withdrawn at the same time points for estimation of

plasma renin, ANG II and serum potassium. Subjects remained semi-recumbant for the duration of the test.

The study was approved by the Glasgow West Ethical Committee and informed consent was obtained from all participants. Subjects with known hypertension or taking regular diuretic therapy were excluded from participating.

All drugs were prepared in the sterile unit of our pharmacy and administered according to a randomised, double blind crossover study design.

Measurements

Spirometry (FEV_1) - see Chapter 2.2, Lung function.

Plasma renin and ANG II- see Chapter 2.4, Hormone assays.

24 hr urinary sodium - see chapter 2.7

Serum potassium - measured using standard ion selective electrodes.

Statistical analysis

Comparison of baseline values and the significance of changes in these at each time point was by analysis of variance, corrected for multiple comparisons. p values below 0.05 were accepted as significant.

4.3 RESULTS

1. Plasma renin and ANG II

There were no significant differences between baseline values of plasma renin and ANG II on either study day or between asthmatic patients and normal subjects, although we note that one individual in the asthmatic (fenoterol) group had a high baseline ANG II level which tended to skew the mean baseline value for this particular group (table 4.1).

In the asthmatic patients, renin levels were significantly elevated after both salbutamol and fenoterol at 120 minutes, and ANG II at 15 minutes after fenoterol alone, when compared to placebo $p< 0.05$ (figure 4.1).

In the normal subjects, there was a trend for renin levels to increase after fenoterol and salbutamol although the results were not statistically significant when compared to placebo. ANG II levels however were significantly higher than placebo 30 and 60 minutes after inhalation of salbutamol $p<0.05$ (figure 4.2).

2. 24 hour urinary sodium

There were no significant differences between 24 hour urinary sodium concentrations on any of the study days. Mean (SEM) urinary sodium values were 145.4 (25.1), 160 (23) and 172 (23.1)mmol/ 24 hr in the asthmatic patients, and 170 (24.9), 172 (21.2) and 157 (25.8) mmol/ 24hr in the normal subjects, prior to fenoterol, salbutamol and placebo respectively

3. Serum potassium

Mean (SEM) baseline potassium levels were similar between the groups on each study day. In the asthmatic patients baseline values were 4.4 (0.2), 4.3 (0.2). and 4.5 (0.2) mmol/l, and in the normal subjects 4.8 (0.2), 4.4 (0.2) and 5.0 (0.2) mmol/l prior to inhalation of fenoterol, salbutamol and placebo respectively. Fenoterol and salbutamol both caused significant decreases in serum potassium when compared to placebo, $p<0.05$ (figure 4.3). At 60 minutes, mean (SEM) absolute fall in potassium levels were 0.8 (0.2), 0.5 (0.3) and 0.1 (0.1) mmol/l in the asthmatic patients and 0.8 (0.3), 0.5 (0.2) and 0.1 (0.1) mmol/l in the normal subjects after inhalation of fenoterol, salbutamol and placebo respectively. In addition, the effect of fenoterol was significantly greater than salbutamol at 60 minutes ($p<0.05$) in both the asthmatic patients and normal subjects. The lowest recorded potassium level in our study was 2.9 mmol/l (after fenoterol).

4. FEV_1

In the asthmatic patients, both salbutamol and fenoterol increased FEV_1 compared to placebo, the effect being maximal at 60 minutes. The degree of bronchodilatation was similar for each agent. Mean (SEM) baseline values of FEV_1 were 2.45 (0.45), 2.54 (0.31) and 2.25 (0.34) litres with a mean (SEM) absolute change in FEV_1 of 0.55 (0.04), 0.52 (0.05) and 0.04 (0.03) litres after inhalation of fenoterol, salbutamol and placebo respectively.

In the normal subjects there was no significant change in FEV_1 after either fenoterol or salbutamol (data not shown).

Raw data for this chapter is included in the Appendix [tables 8A to 13A].

	Fenoterol		Salbutamol		Placebo	
	Renin	ANG II	Renin	ANG II	Renin	ANG II
Asthmatic patients	24.2 (4.4)	19.1 (8.3)	31.5 (8.4)	8.6 (1.1)	30.4 (7.5)	8.1 (3.5)
Normal subjects	32.8 (6.1)	11.0 (2.6)	29.2 (6.3)	11.5 (3.3)	26.5 (6.7)	13.1 (3.9)

Table 4.1 Mean (SEM) baseline values of plasma renin (μU/ml), and angiotensin II (ANG II) (pg/ml) in asthmatic patients [n=8] and normal subjects [n=8].

No significant differences.

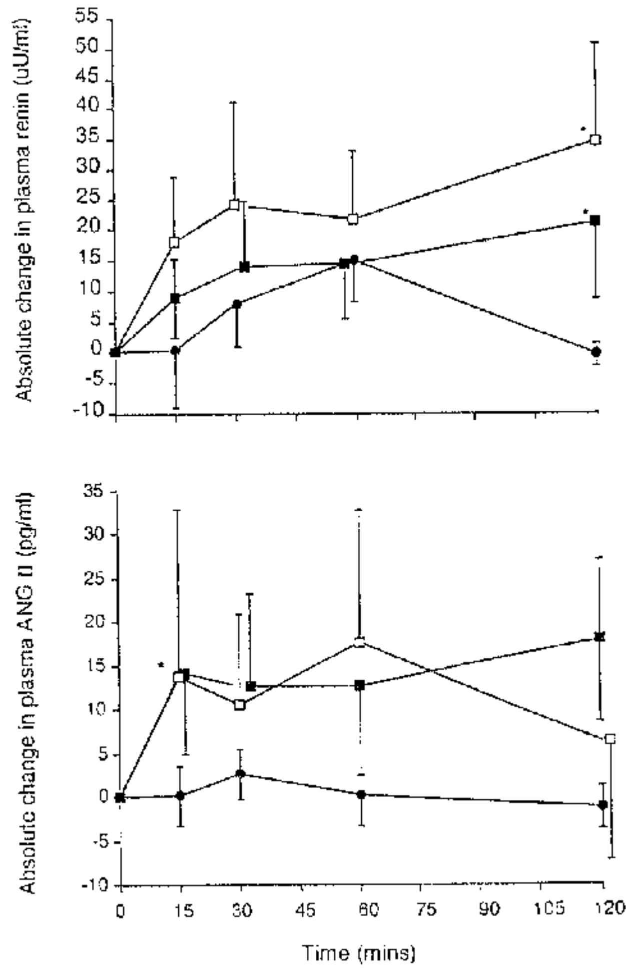

Figure 4.1 The effect of ß2 agonists on plasma renin and angiotensin II (ANG II) levels in asthmatic patients [n=8].

Results as mean (SEM) absolute change from baseline values. *$p<0.05$ vs placebo

☐ fenoterol (2.5mg), ■ salbutamol (5mg), ● placebo

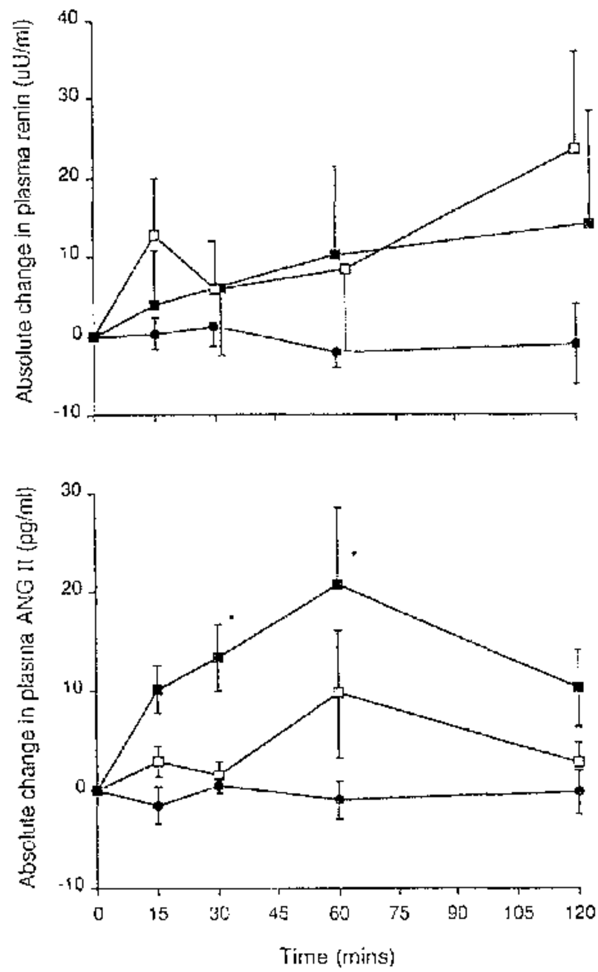

Figure 4.2 The effect of β_2 agonists on plasma renin and angiotensin II (ANG II) levels in normal subjects [n=8].

Results as mean (SEM) absolute change from baseline values. *p<0.05 vs placebo

□ fenoterol (2.5mg), ■ salbutamol (5mg), ● placebo

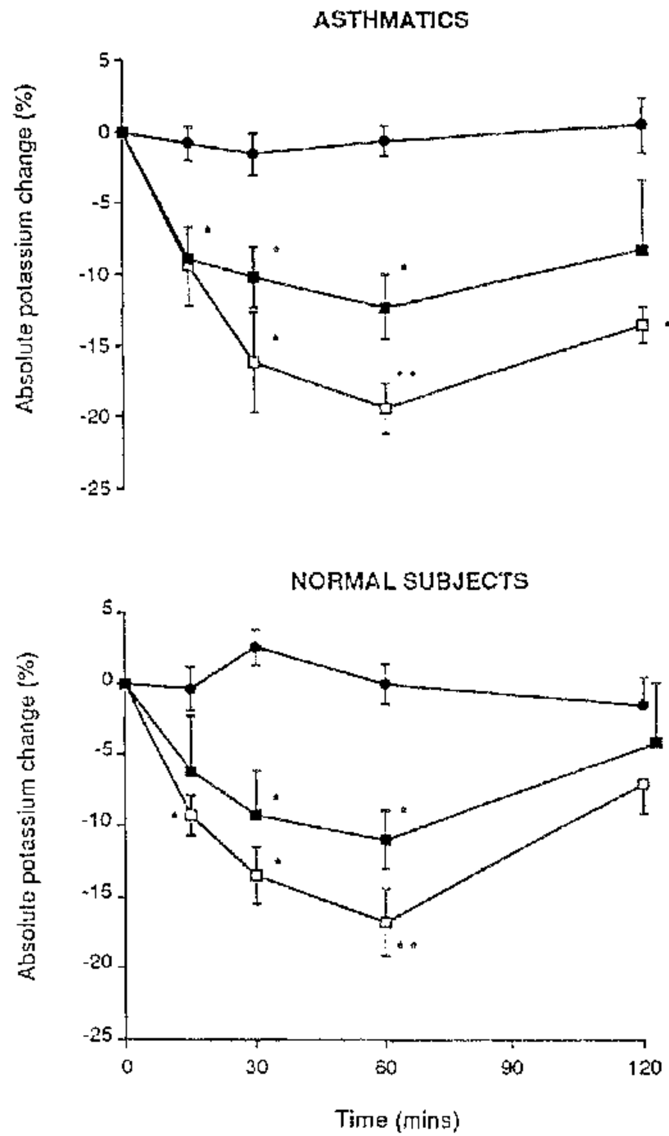

Figure 4.3 The effect of ß2 agonists on serum potassium levels in asthmatic patients and normal subjects [n=8 in each case]. Results expressed as % change from baseline values. *p<0.05 vs placebo ** p< 0.05 vs salbutamol

❒ fenoterol (2.5mg), ■ salbutamol (5mg), ● placebo

4.4 DISCUSSION

This particular study was designed to examine whether high dose β_2 agonists might be responsible for the high levels of plasma renin and ANG II noted in patients with acute exacerbations of asthma, and our results demonstrate that these agents can activate the RAS in both mild asthmatic patients and normal volunteers. We chose to study two commonly used β_2 agonists, namely fenoterol and salbutamol, administered in high doses that are recommended in the British National Formulary for the treatment of acute severe asthma, or in patients receiving domiciliary nebulised bronchodilator therapy.

Renin release from the juxtaglomerular cells of the kidney is regulated by a number of factors. These include sympathetic nervous system activity, renal perfusion pressure and sodium and potassium balance (74). The β adrenoceptor subtype involved in the renin-releasing response has been the subject of much controversy, and although it is now generally accepted that renin release is mediated primarily by β_1 receptors (144), β_2 agonists have clearly elevated plasma renin in some studies. Although Davies et al reported no effect of intravenous salbutamol on plasma renin activity in healthy subjects (145) and Rahman et al noted that plasma renin activity was unaffected by inhaled terbutaline (146), Phillips et al observed an increase in plasma renin activity following intravenous infusion of salbutamol in 4 healthy male subjects (129), and Scheinin and co-workers found a dose dependent increase in plasma renin activity after both fenoterol and salbutamol administered by metered dose inhaler to 6 normal subjects (82). However none of these studies measured changes in plasma ANG II.

The exact mechanisms whereby salbutamol and fenoterol may stimulate renin release in the present study are not entirely clear. It has been suggested that the increased renin secretion by β_2 agonists may result from a weak agonist action of these drugs on renal β_1 adrenoceptors (71), while other postulated mechanisms include increased sympathetic activity due to activation of presynaptic facilitatory β_2 adrenoceptors, local vascular effects with resultant changes in renal perfusion pressure, or that renin release is stimulated by the hypokalaemic effect of β_2 agonists (129). It is also intriguing to speculate on the possible existance of a local tissue RAS in the airway, similar to that found in other tissues (106), and whether such a mechanism may contribute to elevations in plasma renin and ANG II following the inhaled route of administration.

The hypokalaemic and bronchodilator effects of salbutamol and fenoterol are well documented, and our results are in close agreement with those of others (147-151). However, the results for renin and ANG II are more difficult to interpret. This is possibly due to our small sample size and also what appears to be a wide variability in each individual's response to the effect of β_2 agonists on the RAS. In the group of normal volunteers, there was an obvious trend for renin to increase after both salbutamol and fenoterol, although this did not quite reach statistical significance. However, plasma ANG II was elevated significantly after salbutamol but not fenoterol. In the asthmatic group renin was significantly elevated after both β_2 agonists, yet it was fenoterol and not salbutamol which sigificantly increased ANG II in the plasma. For an unknown reason, one individual in the asthmatic (fenoterol) group had a high baseline plasma ANG II which tended to skew the mean value for this group (table 4.1). However the plasma renin for this individual was normal and we think that this higher baseline ANG II is unlikely to be of any importance with respect to the results. The reason for the differences in

the ability of fenoterol and salbutamol to elevate ANG II in each group is not clear. We might hypothesise that the greater degree of hypokalaemia induced by fenoterol may stimulate the RAS more so than salbutamol. However this theory does not hold when applied to the group of normal volunteers. Although we did not measure blood pressure in the present study, increases in systolic and decreases in diastolic blood pressure have been reported following inhaled salbutamol and fenoterol (147). It is possible that the apparent differences between these agents on the activity of the RAS could be the result of differing alterations in renal haemodynamics and hence renin release. It has also been suggested that perhaps the adrenoceptor involved in the release of renin does not fit the normal classification of ß adrenoceptors (152). These theories do not however clearly explain the differing effects of fenoterol and salbutamol in the two groups of subjects.

Although the plasma levels of renin and ANG II recorded following single doses of nebulised $ß_2$ agonists are less than the range of levels we observed in acute severe asthma [e.g. renin 45.5 (21-70) μU/ml and ANG II 56 (12-109) pg/ml on day 5 after admission, results as median with interquartile range](Chapter 3), in the emergency treatment of acute asthma repeated doses of ß agonists are often administered and are recommended in recent guidelines on the management of asthma should the patient fail to improve (60). Patients admitted to hospital with acute asthma show wide variations in circulating salbutamol and terbutaline concentrations, and in some individuals the $ß_2$ agonist levels are markedly elevated (153). Thus, circulating plasma levels of renin and ANG II may be further increased as a result of multiple dosing with $ß_2$ agonists. It therefore seems likely that $ß_2$ agonists are contributing to the activation of the RAS in acute severe asthma but it would be valuable in future studies to examine whether a

dose dependent effect on renin and ANG II exists with repeated dosing, therefore reinforcing our findings.

In conclusion, these results suggest that single high doses of nebulised β_2 agonists commonly used in the treatment of asthma cause activation of the RAS both in mild asthmatic patients and normal volunteers. This appears to be an effect of β_2 agonists as a class, and further comment on our findings is not possible due to the presence of wide individual variations in response. It is possible that circulating ANG II levels might be further increased as a result of multiple dosing with β_2 agonists (as occurs in the treatment of acute severe asthma), and this hypothesis will be addressed in the next study.

CHAPTER 5

THE EFFECT OF INTRAVENOUS SALBUTAMOL AND MULTIPLE DOSES OF NEBULISED SALBUTAMOL ON THE RENIN-ANGIOTENSIN SYSTEM IN ASTHMA

5.1 INTRODUCTION

The results of the previous study demonstrated that single doses of nebulised β_2 agonists can elevate plasma renin and ANG II in mild asthmatic patients, suggesting that β_2 agonists are likely to be contributing to the activation of the RAS in acute asthma. It was noted, however, that plasma levels of renin and ANG II following a single nebulised dose of salbutamol were significantly less than those measured in acute severe asthma. We therefore hypothesised that if repeated doses of nebulised β agonist were administered, as is often the case in acute asthma or in patients receiving domiciliary nebulised bronchodilator therapy, then this could perhaps have additive effects on circulating renin and ANG II levels. In addition, we have extended our earlier observation by investigating whether similar rises in circulating plasma renin and ANG II levels can occur following salbutamol administered by intravenous infusion, employing doses that are recommended for the treatment of acute severe asthma (60).

5.2 METHODS

Patients

Eight stable asthmatic patients (3 male), mean (SD) age 34 (7.5) years, mean FEV_1 82 (14.6) % predicted, were studied. All were taking as required short acting inhaled β_2 agonists and 7 of the 8 regular inhaled corticosteroids. None were on oral β_2 agonists, theophyllines or diuretic therapy. Patients were requested to withhold β_2 agonists for at least 6 hours prior to each visit, but to continue with their inhaled corticosteroids unchanged. The study was approved by the Glasgow West Ethical Committee, and informed consent was obtained from all participants.

On arrival in the laboratory, an indwelling catheter (Venflon ®, Viggo AB, Helsingborg, Sweden) was inserted into a forearm vein for blood sampling, followed by a 30 minute rest. 30 ml of venous blood was withdrawn for estimation of baseline plasma renin, ANG II and serum potassium. A 24 hour sample of urine was collected by each patient prior to each study visit as an indirect means of assessing dietary sodium intake.

Study 1: The effect of intravenous salbutamol on the renin-angiotensin system.

Subjects attended on 2 separate study days, at least a week apart. Each then received either salbutamol (5,10 and 20μg/min in increasing increments at 30 min intervals) or placebo (0.9% saline). The drugs were prepared in our pharmacy sterile unit and administered intravenously by means of a 50 ml syringe driver (Perfusor ®, Secura E, B Braun, Melsunger AG, Germany) according to a randomised double-blind crossover study design. At the end of each 30 minute infusion period, and 30 minutes after completion of the final infusion, blood was withdrawn for estimation of plasma renin, ANG II and serum potassium.

Study 2: The effect of multiple doses of nebulised salbutamol on the renin-angiotensin system.

On 3 separate study days each subject received (at time 0 and 30 mins) either salbutamol (5mg) followed 30 mins later by a further dose of salbutamol (5mg), salbutamol 5mg followed by placebo (3.5ml normal saline), or 2 placebo inhalations. The drugs were administered by nebuliser (CR 60 High flow compressor, Medic-Aid Ltd, Pagham, Sussex, UK) and face mask in a randomised, double-blind crossover manner. At baseline, and at 15, 30, 45, 60,90, 120 and 150 mins, blood was withdrawn for estimation of plasma renin, ANG II and serum potassium.

Measurements

Plasma renin and ANG II - see Chapter 2.4 , Hormone assays.

24 hr urinary sodium - see chapter 2.7

Serum potassium was measured using standard ion-selective electrodes.

Statistical analysis

Comparison of baseline values and the significance of changes in these at each time point was by analysis of variance, corrected for multiple comparisons. p values below 0.05 were accepted as significant.

5.3 RESULTS

Study 1: The effect of intravenous salbutamol on the activity of the renin-angiotensin system in asthma.

1. Plasma renin and ANG II

There were no significant differences between baseline values of renin or ANG II on either study day [Mean (SEM) renin 21.7 (4.5)μU/ml and ANG II 6.7 (2.3) pg/ml prior to salbutamol, and renin 19.0 (6.4)μU/ml and ANG II 7.4 (2.3)pg/ml prior to placebo].

After the 10 and 20 μg/min infusions of salbutamol, plasma renin levels were significantly greater than placebo ($p < 0.05$). [Mean (SEM) absolute change in renin of 6.0 (3.5) and 5.1(4.1) μU/ml respectively after salbutamol and 0.43 (3.1) and -2.7 (2.7) μU/ml after placebo] (fig 5.1). ANG II levels were significantly greater than placebo ($p<0.05$) 30 mins after completion of the final infusion [Mean (SEM) absolute change in ANG II of 5.1(4.3)pg/ml and -0.4 (0.69) pg/ml respectively] (fig 5.1).

2. Serum potassium

There were no significant differences between baseline values of potassium on either study day. [Mean (SEM) potassium of 4.4 (0.1) mmol/l and 4.3(0.13)mmol/l prior to salbutamol and placebo respectively]. Serum potassium levels decreased after salbutamol and were significantly less than placebo after the 10 and 20μg/min infusions and 30 mins after completion of the final infusion. [Mean (SEM) absolute change in serum potassium of -0.32 (0.1), -0.62 (0.2) and -0.58 (0.28)mmol/l respectively, $p<0.05$ vs placebo] (fig 5.1).

3. 24 hr urinary sodium.

There were no significant differences between 24 hr urinary sodium values on each study visit. Mean (SEM) urinary sodium values were 234 (43) mmol/ 24 hr and 185 (17.2) mmol/ 24hr prior to salbutamol and placebo respectively.

Study 2: The effect of multiple doses of nebulised salbutamol on the activity of the renin-angiotensin system in asthma.

1. Plasma renin and ANG II.

There were no significant differences between baseline values of renin and ANG II on either study day. (renin 15.3 (3.2), 15.6 (2.6) and 14.7 (4.5) μU/ml and ANG II 8.6 (2.5), 6.1 (0.7) and 6.8 (1.3) pg/ml prior to prior to inhalation of placebo, single and double doses of salbutamol respectively).

Plasma renin levels increased after both the single[S] and double[D] doses of salbutamol and were significantly greater than placebo at 30 mins after S, and 45 and 120 mins after D. (fig 5.2) [Mean (SEM) absolute change in plasma renin from baseline of 10.6 (3.9)μU/ml after S, and 17.4 (3.2) and 14 (3.2) μU/ml after D respectively $p<0.05$ vs placebo]. However there was no significant difference between the levels of plasma renin obtained after the single or double dose of salbutamol.

Plasma ANG II also increased after S and D and was significantly greater than placebo at 30 and 45 mins after S, and at 30 mins and all time points thereafter following D. [Mean(SEM) absolute change in ANG II from baseline values of 3.0 (2.6), and 2.6 (2.5)pg/ml after S, and 3.7 (1.5), 9.9(5), 4.7(2.9), 7.0(2.1),

5.1(2.0) and 4.3 (1.6)pg/ml after D respectively, $p<0.05$ vs placebo]. In addition, the effect of D was significantly greater than S at 45 mins, $p<0.05$. (fig 5.2).

2. Serum potassium.

There were no significant differences between baseline values of potassium on either study day. [Mean (SEM) potassium of 4.4 (0.2), 4.3 (0.1) and 4.4 (0.1) mmol/l prior to placebo, single and double doses of salbutamol respectively). Potassium levels decreased after the single and double doses of salbutamol and were significantly less than placebo, $p<0.05$, at 30 mins and all time points thereafter, following both S and D. [Mean (SEM) maximum fall in serum potassium of 0.85 (0.1)mmol/l after S at 45 mins, and 1.16 (0.1)mmol/l after D at 150 mins]. In addition the effect of D on serum potassium was also significantly greater than S at 150 mins (fig 5.2).

3. 24 hr urinary sodium.

There were no significant differences between 24 hr urinary sodium values on each study visit. Mean (SEM) urinary sodium values were 140.4 (25.1), 160.9 (24.9) and 112 (45.8) mmol/ 24hr in the normal subjects, prior to S, D and placebo respectively.

Raw data for this chapter is included in the Appendix [tables 14A-15A].

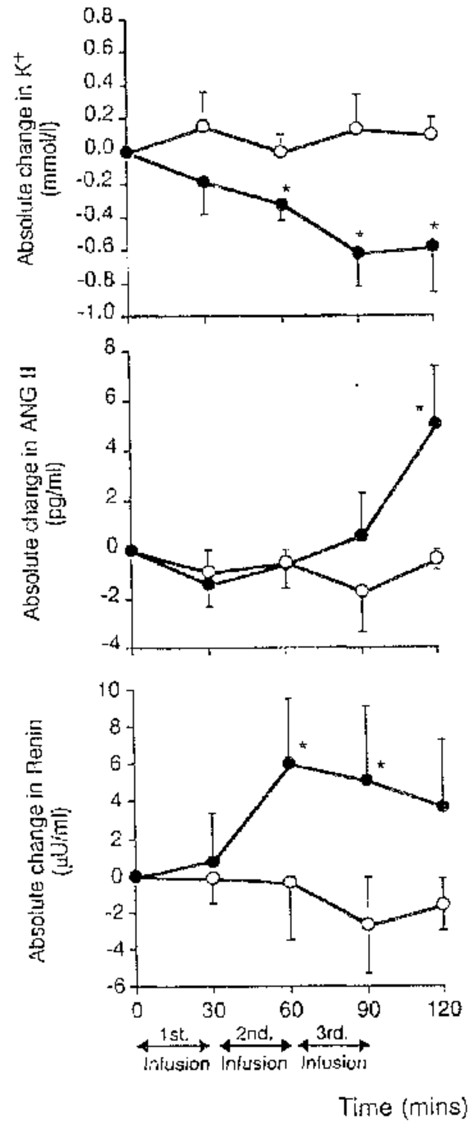

Fig 5.1 The effect of intravenous salbutamol (●) [after the first (5µg/min), second (10µg/min) and third (20 µg/min)infusions], and placebo [5% dextrose] (O) on plasma renin, ANG II and serum potassium (K^+) in mild asthmatic patients. [n=8]

Results as mean (SEM) absolute change from baseline values. * $p<0.05$ vs placebo

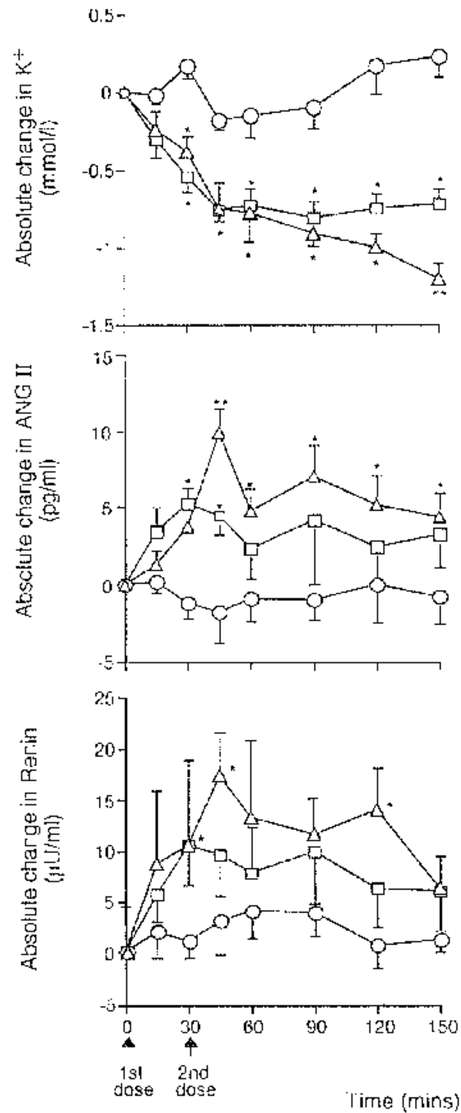

Fig 5.2 The effect of single (❒) and double (Δ) doses of nebulised salbutamol [5mg], and placebo (O) on plasma renin, ANG II and serum potassium (K^+) in mild asthmatic subjects [n=8]

Results as mean (SEM) absolute change from baseline values.

* $p<0.05$ vs placebo **$p<0.05$ vs single dose of salbutamol.

5.4 DISCUSSION

The β_2 agonist salbutamol administered either by intravenous infusion or by aerosol causes activation of the RAS in asthma. Administration of a second dose of nebulised salbutamol has further additive effects on plasma ANG II levels. This finding therefore confirms and extends our earlier observations in asthmatic subjects that a single nebulised dose of the β_2 agonist fenoterol can activate the RAS (Chapter 4). Although there was also a trend for plasma renin to increase after the second dose of ß agonist in this study, the results did not reach statistical significance. This may, however, be partly explained by the small sample size and an apparent wide variation in individual responses to the effect of β_2 agonists on the RAS. In addition, there is activation of the RAS by intravenous salbutamol when administered in doses recommended for the management of acute severe asthma (60). These results are in agreement with those of Phillips et al who described increases in plasma renin activity and decreases in serum potassium following intravenous infusions of the β_2 agonists salbutamol (0.1, 0.4 and 0.7 µg/kg/min) and rimiterol (0.5,1.0 and 2.0 nmol/kg/min) in 4 healthy male subjects (129).

It is unclear why the rises in plasma renin and ANG II following intravenous salbutamol are smaller than those observed in the nebulised study. This may simply be a reflection of lower plasma drug concentrations following the intravenous route of administration. Presumably renin release follows systemic absorption of the drug to stimulate ß adrenoceptors on the juxtaglomerular cells of the kidney directly, or alternatively, it is interesting to speculate that the more marked elevations in renin and ANG II following the inhaled route may be due to

an additional activation of a local RAS in the airway, similar to those found in other tissues (106).

We previously hypothesised that ß agonist treatment may contribute to the high levels of plasma renin and ANG II noted in patients with severe asthma (Chapter 3). However, even following repeated dosing with nebulised $ß_2$ agonists it is apparent that the plasma levels of renin and ANG II attained are still less than those measured in acute severe asthma [eg results as median with interquartile range; renin 45.5(21-70)μU/ml and ANG II 56(12-109)pg/ml on day 5 after admission]. This raises the possibility that there must be other additional stimuli to the activation of the RAS in acute asthma such as hypoxia or inflammatory mediators, thus leading to marked elevations in circulating plasma renin and ANG II.

CHAPTER 6

AN INVESTIGATION OF THE MECHANISM OF β_2-AGONIST-INDUCED ACTIVATION OF THE RENIN-ANGIOTENSIN SYSTEM

6.1 INTRODUCTION

In the renin-angiotensin system (RAS), the octapeptide hormone ANG II is generated in a biological cascade in which the enzyme renin cleaves ANG I from the large molecular weight precursor angiotensinogen. ANG I is subsequently activated to ANG II by angiotensin converting enzyme (ACE), mainly in the pulmonary circulation, but also in other vascular beds and other tissues including the myocardium and coronary arteries. In addition, there is currently interest in the existance of alternative pathways for ANG II synthesis, for example Reilly et al have reported that ANG I can be rapidly converted to ANG II by human neutrophil cathepsin G and also by skin mast cell chymase enzymes (66).

We have reported that high dose nebulised β_2 agonists used in the treatment of asthma can elevate plasma renin and ANG II in mild asthmatic patients and healthy volunteers, suggesting that β_2 agonists are likely to contribute to the activation of the RAS in acute asthma. In this study we sought to determine the mechanism responsible for this activation. We examined the influence of ACE inhibition with the drug lisinopril in a group of healthy subjects, thereby testing our hypothesis that high dose β_2 agonists cause elevation in plasma ANG II via the classical components of the RAS including ACE.

6.2 METHODS

Subjects

Eight healthy volunters (3 male), mean (SD) age 28 (4.6) years were recruited to the study. All were non-smokers, with no history of hypertension or heart disease. None was taking any regular medication. Informed, written consent was obtained from each subject and the study was approved by the Glagow West Ethical Commitee.

Study design

Subjects attended the laboratory on 4 separate study days at least a week apart, and all at approximately the same time of day (12.00-14.00 hrs). Three hours prior to attending, subjects took either lisinopril (20mg) or an identical placebo capsule. On arrival, an intravenous cannula (Venflon®, Viggo AB, Helsingborg, Sweden) was inserted into a forearm vein following which subjects rested for 30 mins in the supine position. Baseline blood pressure (mean of 3 readings) was measured and blood was withdrawn for estimation of plasma renin and ANG II, serum ACE and potassium. Each subject then received either salbutamol or placebo inhalation. Thereafter subjects remained supine for the duration of the visit, and blood was withdrawn at 15, 30, 60, 90 and 120 minutes for measurement of plasma renin, ANG II and serum potasium. Serum ACE was measured 120 mins after nebulisation. Blood pressure was monitored at 15 minute intervals throughout the study.

Salbutamol (5mg) or placebo inhalation (3.5ml normal saline) were given by nebuliser (CR60 High Flow Compressor, Medic-Aid Ltd, Pagham, Sussex, UK) and face mask. All drugs were prepared in the sterile unit of the pharmacy, and

administered according to a randomised, double-blind, crossover study design, so that after all four vists each subject had received all possible combinatons of capsules and nebuliser solution.

Measurements

Plasma renin, ANG II and serum ACE see Chapter 2.4 , Hormone Assays.

Serum potassium - measured using standard ion-selective electrodes.

Blood pressure recorded by semi-automatic sphygmomanometer (Dinamap®, 1846 FX vital signs monitor, Critikon, Berkshire, UK) and the mean of 3 readings at each time point recorded.

Statistical analysis

Comparison of baseline values and the significance of changes in these at each time point was by one way analysis of variance corrected for multiple comparisons.

p values below 0.05 were accepted as significant.

6.3 RESULTS

1. Plasma renin and ANG II

Baseline plasma renin levels were significantly higher after lisinopril but there were no significant differences between baseline renin levels on the non-lisinopril days nor between the lisinopril days (table 6.1)

Following salbutamol alone, plasma renin levels increased, being significantly higher than placebo at 15, 30 and 45 mins [Mean (SEM) absolute change in plasma renin of 26.1(7.9), 16.6(3.9) and 14.1(4.9) μU/ml respectively, $p<0.05$ vs placebo].

When lisinopril was administered prior to salbutamol, although initial baseline levels of renin were high, there were also further significant rises in plasma renin. [mean (SEM) absolute increase of 38.2 (15.6) and 26.7(9.3) μU/ml at 15 and 30 mins, $p< 0.05$ vs placebo inhalation] (fig 6.1).

Baseline ANG II levels were similar on the non-lisinopril days, and were significantly lower after lisinopril (table 6.1). Following salbutamol alone, plasma ANG II increased, being significantly higher than placebo at 15, 30, 45 and 90 mins after nebulisation (Mean (SEM) absolute change in ANG II of 9.5 (3.4), 5.7(1.9), 4.5(2.6) and 2.5(1.7) pg/ml, $p< 0.05$ vs placebo). However, when salbutamol was preceded by lisinopril, this rise in plasma ANG II was completely inhibited (fig 6.1).

2. Serum ACE

Mean (SEM) serum ACE levels were 38.7(5.6) and 31.5 (9.6)U/L on the non-ACE inhibitor days prior to placebo and salbutamol inhalations respectively, and 35.7 (6.5) and 32.5 (8.4) U/L at the end of the study.

Following lisinopril, serum ACE was <5 U/L at baseline in all except two subjects, in whom ACE levels were 5 and 8 U/L. Since our laboratory level of detection of ACE was 5U/L with a coefficient of variation of 30%, these values would lie within 2 standard deviations of this level and we can therefore assume that in all cases ACE activity was effectively suppressed. In addition, ACE levels remained suppressed at the end of the study.

3. Serum potassium

Baseline serum potassium levels were higher, although not significantly so, after lisinopril when compared to the non-lisinopril days (table 6.1). Salbutamol caused significant hypokalaemia, maximal at 45 mins after salbutamol alone (Mean (SEM) potassium of 3.08 (0.2) mmol/l), and at 30 mins when preceded by lisinopril (3.4(0.1)mmol/l). The hypokalaemic response to salbutamol alone was greater than when salbutamol was preceded by lisinopril [Mean(SEM) decrease in serum potassium of 1.2 (0.2) mmol/ compared to 0.8 (0.2) mmol/l] (fig 6.1).

4. Blood pressure and side effects

Mean blood pressure was not influenced by any of the treatments. One subject suffered dizziness and headache after lisinopril on each exposure. These symptoms were associated with marked falls in systolic blood pressure.

Raw data for this chapter is included in the Appendix [tables 16A-19A].

	renin	ANG II	ACE	K^+
Plac / Plac	27.5(5.1)	9.5(2.2)	38.7(5.1)	4.24(0.1)
Plac / Sal	35.6(8.3)	8.2(2.2)	31.5(9.1)	4.26(0.16)
Lis / Plac	150.6(30.4)*	1.3(0.4)*	<5*	4.31(0.07)
Lis / Sal	160.1(20.6)*	1.4(0.1)*	<5*	4.36(0.12)

Table 6.1

Mean (SEM) baseline values [ie; prior to administration of nebulised drug] of plasma renin (μU/ml), angiotensin II (ANG II) (pg/ml), serum ACE(U/L), and potassium(K^+)(mmol/l) for the following treatment combinations ; plac/plac=placebo capsules and placebo inhalation, plac/sal=placebo capsules and salbutamol, lis/plac = lisinopril and placebo inhalation, lis/sal= lisinopril and salbutamol. *$p<0.05$ vs plac/plac and plac/sal

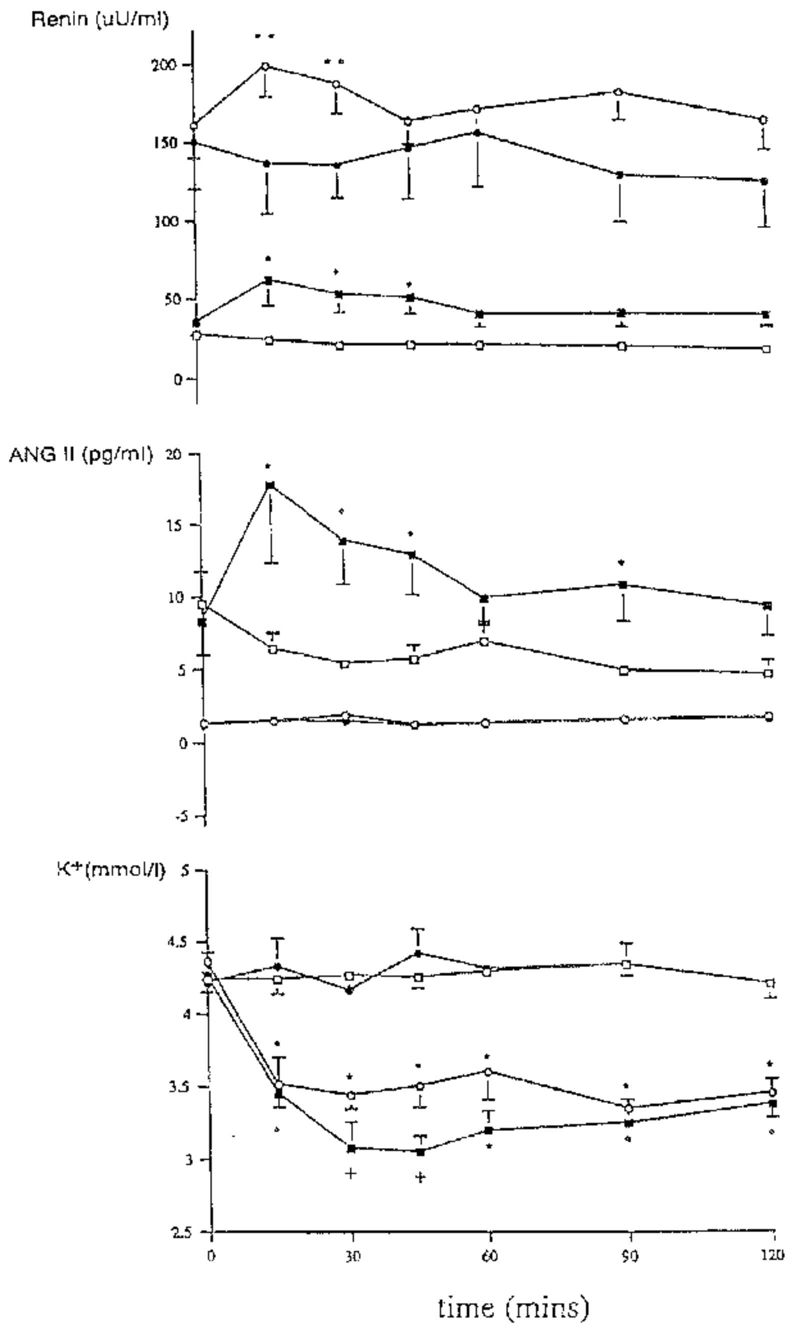

Fig 6.1 The effect of lisinopril pretreatment on salbutamol induced changes in plasma renin, ANG II and serum potassium (K^+) in healthy volunteers [n=8]. Results expressed as mean (SEM).

□ placebo / placebo inhalation ■ placebo/salbutamol [5mg]

● lisinopril [20mg] /placebo inhalation ○ lisinopril / salbutamol [5mg]

* p<0.05 vs plac /plac ** p<0.05 vs lis /plac + p<0.05 vs lis / sal

6.4 DISCUSSION

These results confirm our earlier findings that ß2 agonists cause activation of the RAS. Pretreatment with lisinopril resulted in elevated plasma renin concentrations and reciprocal low plasma ANG II concentrations as a result of inhibition of ACE and consequent withdrawal of the inhibitory effect of ANG II on renin release (154). Although peak plasma concentrations of lisinopril occur approximately 6 hours after administration (155), it was evident that serum ACE activity was effectively inhibited 3 hours following a single 20 mg oral dose in all our subjects and complete inhibition persisted throughout this observation period.

During ACE inhibition, the increase in plasma renin concentrations induced by nebulised salbutamol persisted but the rise in plasma ANG II concentration was completely suppressed. These findings strongly support the hypothesis that activation of the RAS by nebulised ß2 agonists involves the classical components of the RAS, with conversion of ANG I to ANG II being mediated by ACE. The exact mechanisms whereby ß2 agonists actually initiate renin release are not entirely clear. Alternative pathways for the conversion of ANG I to ANG II might also be involved. ANG I is rapidly converted to ANG II by neutrophil and mast cell proteinase enzymes (66); neither enzyme is inhibited by the ACE inhibitor captopril. The findings from this study suggest that elevation of plasma ANG II by ß2 agonists is mediated by ACE. In inflammatory processes such as acute severe asthma, where very high levels of plasma ANG II were observed however (Chapter 3), it is conceivable that such alternative pathways of ANG II generation may contribute to the development of these higher levels of plasma ANG II.

Our results also confirm previous observations concerning the hypokalaemic effect of β_2 agonists (148,156). ACE inhibition tended to increase serum potassium and although potassium decreased in response to salbutamol even in the presence of lisinopril, this fall in serum potassium was less than that when salbutamol was administered alone. These findings differ from those of Rahman et al (146), who showed that although enalapril for one week protected against the hypokalaemic effect of inhaled terbutaline in healthy volunteers, there was no alteration in in the magnitude of fall in potassium following terbutaline. One explanation for these disparate findings may be more complete and prolonged inhibition of ACE with lisinopril.

In conclusion, the results of this study suggest that the effect of β_2 agonists is mediated primarily by ACE and not by alternative pathways of ANG II generation. However, it seems unlikely that β_2 agonists are solely responsible for the the very high levels of plasma ANG II observed in acute severe asthma, raising the possibility that perhaps hypoxia or inflammatory mediators such as mast cell proteinase enzymes may be contributing to this activation.

CHAPTER 7

THE EFFECT OF HYPOXIA AND β_2-AGONISTS ON THE ACTIVITY OF THE RENIN-ANGIOTENSIN SYSTEM IN NORMAL VOLUNTEERS

7.1 INTRODUCTION

Activation of the RAS in asthma is likely to be due partly to β_2 agonist therapy but the role of other putative stimuli such as hypoxia or inflammatory mediators is unclear. Patients admitted to hospital with acute exacerbations of asthma are invariably and often profoundly hypoxaemic (57,58) and Rees et al (157) have observed that hypoxia often persists for many days and sometimes weeks despite intensive therapy and apparent symptomatic relief. Moreover, airways obstruction as measured by FEV_1 (and therefore also PEFR) was not found to be a reliable guide to the degree of hypoxaemia (157). Thus, it would seem reasonable to hypothesise that hypoxia may be stimulating the RAS in our acute asthmatic patients.

Previous studies which have examined the effect of hypoxia on the activity of the RAS in man have produced conflicting results (128,141), and therefore we were interested to investigate the effect of hypoxia on circulating renin, ANG II and ACE levels in a group of healthy volunteers. We chose to study normal subjects in the first instance, rather than asthmatic patients and, in addition, this study was extended to examine whether the effect of β_2 agonists on the activity of the RAS might be influenced by the presence of hypoxic conditions.

7.2 METHODS

Subjects

Eight healthy non-smoking volunteers (4 male) between ages 22 and 40 (mean (SD) age 29.7(4.3) years) were recruited. None of the volunteers were taking any regular medication and none had any relevant past medical history. The study was approved by the Glasgow West Ethical Commitee and informed written consent was obtained from each subject prior to beginning the study.

Study design

Subjects attended the laboratory on 4 separate occasions at least a week apart. On arrival an indwelling cannula (Venflon ®, Viggo AB, Helsingborg, Sweden) was inserted into a forearm vein for blood sampling, followed by a 30 minute rest. 30 ml of venous blood was withdrawn for estimation of baseline plasma renin, ANG II and serum ACE. In addition, we also measured plasma catecholamines (noradrenaline and adrenaline). Pulse rate and oxygen saturation were recorded at baseline with the subject breathing room air and thereafter continously throughout the study by means of a pulse oximeter and finger probe (Ohmeda Biox 3700E; Ohmeda, Louisville, USA). Using a double-blind, placebo controlled crossover study design, each subject was randomised to breathe (via a mouthpiece connected to a non-rebreathing valve while wearing a noseclip to occlude nasal airflow) either a normoxic or hypoxic ($F_I O_2$ =12%) gas mixture for a period of 30 minutes. The source of inspired gas was a reservoir bag connected to a constant flow of a mixture of oxygen and nitrogen. Oxygen and nitrogen were obtained from K and G sized cylinders respectively (BOC, Medishield, Harlow, Essex, UK), the oxygen and nitrogen being mixed in an oxygen blender prior to

entering the reservoir. The fractional inspired oxygen content of the mixture was determined by means of an oxygen analyser (5120 oxygen monitor, Ohmeda, Louisville, USA) and was controlled by changing the relative mixture of air and nitrogen to produce an inspired gas composed of either 12% or 24% oxygen. After the first 10 minutes, either salbutamol (5mg) or placebo (3.5 ml 0.9% saline) was introduced into the circuit via a nebuliser [CR 60 High Flow Compressor, Medic-Aid Ltd, Pagham, Sussex, UK] and nebulisation continued for 10 minutes or dryness, whichever occurred first. The nebuliser was then disconnected from the circuit and the gas mixture continued for the remaining 10 minutes.

Blood samples for hormone estimation as above were withdrawn at 10, 20, 30, 45, 60, 90 and 120 mins. Subjects remained semi-recumbant for the duration of the study.

Measurements

Plasma renin, ANG II, catecholamines and serum ACE - see chapter 2.4, Hormone assays.

Statistical analysis

Comparison of baseline values and the significance of changes in these at each time point was by one way analysis of variance corrected for multiple comparisons.

p values below 0.05 were accepted as significant.

7.3 RESULTS

1. Oxygen saturation.

During the first 30 minutes peripheral oxygen saturations [mean (SEM)] were 96 (1.6), 82 (1.5), 85 (3.9) and 84 (3.0)% on the combined hypoxia/salbutamol day and 97 (0.7), 83 (2.0), 84 (2.3), and 85 (1.9)% on the hypoxia/placebo day, at 0, 10, 20 and 30 minutes respectively, returning to normal values within seconds of discontinuing the hypoxic mixture. There were no significant differences between oxygen saturations on the two hypoxia days.

2. Plasma renin and ANG II

There was no significant difference between baseline values of plasma renin and ANG II on either study day (table 7.1). Following the period of hypoxia alone, there were no significant changes in plasma renin and ANG II (fig 7.1). When salbutamol was included in the hypoxic mixture, there were significant increases in plasma renin and ANG II levels. [mean (SEM) increase in ANG II of 7.2(4.9),4.5(3.4), 5.4(2.9) and 3.6(3.1) pg/ml at 30, 45, 60 and 90 mins, and renin of 12.8(4.8), 15.5(6.3), 4.0(3.2) and 4.0(3.9) μU/ml at 45, 60, 90 and 120 mins respectively, compared to 1.1(0.8), 1.1(1.3), -0.5(0.9), 1.5 (2.7) and -4.4(2.6), -2.1(2.6),-4.0(3.3) and -6.0(2.7)μU/ml after normoxia and placebo inhalation at the same time points, $p < 0.05$] (fig 7.1).

However, although nebulised salbutamol in the normoxic mixture also led to significant rises in plasma renin and ANG II when compared to nebulised placebo in normoxic mixture, [mean(SEM)increase in renin of 17.4 (6.4), 12.7(3.1), 12.9(3.8), 9.7(3.8) and 11.4(3.5) μU/ml at 30,45, 60, 90 and 120 mins, and ANG II of 5.5(2.4), 8.6(4.1) and 8.3(4.1)pg/ml at 30, 45 and 60 mins respectively,

$p<0.05$], there was no significant difference between the effect of salbutamol on plasma renin and ANG II levels when administered in either the normoxic or hypoxic mixture (fig 7.1).

3. Serum ACE

Baseline serum ACE levels were similar (table 7.1) and did not change significantly at any time point on each study day (data not shown).

4. Plasma adrenaline and noradrenaline.

There was no significant difference between baseline noradrenaline (NA) and adrenaline (Adr) levels on either study day (table 7.1). Small but significant increases in plasma NA were noted after hypoxia, salbutamol, and hypoxia combined with salbutamol, this effect being significantly greater than normoxia and placebo inhalation at 20, 30 and 45 mins, $p<0.05$ [Mean (SEM) absolute increase of 1.69(0.5), 1.78(0.6), 1.66(0.5)nmol/l after salbutamol alone, 0.7(0.2), 0.85(0.3) and 1.34(0.3)nmol/l after hypoxia alone and 0.6(0.3), 0.8(0.4) and 0.7(0.3)nmol/l after hypoxia combined with salbutamol] (fig 7.2). There was a transient peak in plasma Adr levels at 30 mins after hypoxia and salbutamol [mean(SEM) absolute increase of 0.83(0.6) nmol/l, $p< 0.05$ vs normoxia and placebo] (fig 7.2).

5. Heart rate

Significant increases in heart rate occurred in response to hypoxia, salbutamol and also hypoxia combined with salbutamol eg; mean (SEM) maximum increase in heart rate from baseline values (table 7.1) of 12 (1.4), 18 (2.5) and 32 (3.1) beats/min at 30 mins after hypoxia/placebo, normoxia/salbutamol and hypoxia / salbutamol respectively (fig 7.3).

Raw data for this chapter is included in the Appendix [tables 20A - 25A].

	renin	ANG II	NA	Adr	ACE	HR
norm/plac	23.0(5.0)	7.4(2.6)	2.6(0.2)	0.15(0.02)	40.7(5.0)	85(4)
norm/sal	23.3(4.8)	6.6(1.2)	2.6(0.2)	0.15 (0.02)	31.5(6.4)	82(3)
hyp/plac	30.7(6.9)	9.2(2.7)	2.6(0.4)	0.14(0.03)	39.3(5.9)	83(5)
hyp/sal	29.0(7.0)	7.5(1.6)	2.5(0.3)	0.14(0.02)	33.1(8.8	82(5)

Table 7. 1

Baseline values of plasma renin (μU/ml), angiotensin II (ANG II) (pg/ml), ACE(U/L), noradrenaline (NA)(nmol/l), adrenaline (Adr)(nmol/l) and heart rate(HR) (beats/min). Results expressed as mean (SEM) [n=8].

No significant differences.

norm/plac – normoxia and placebo inhalation, norm/sal = normoxia and salbutamol 5mg, hyp/plac = hypoxia and placebo inhalation, hyp/sal= hypoxia and salbutamol 5mg.

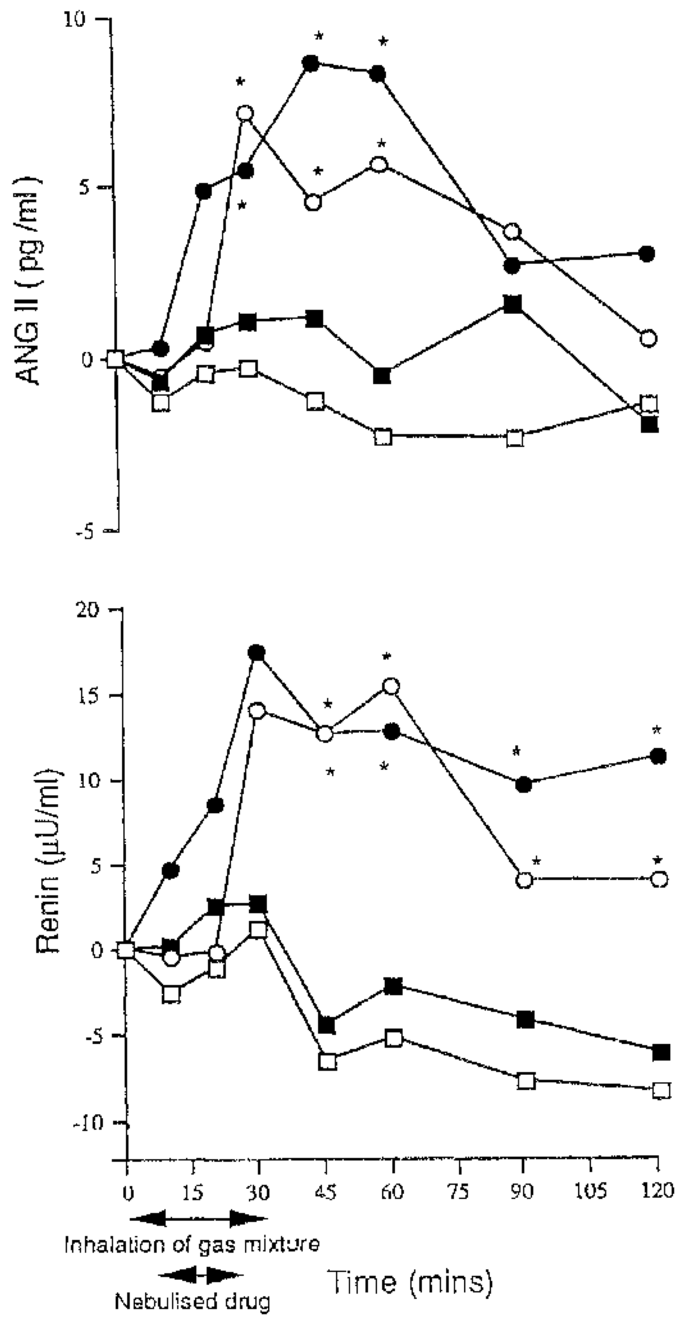

Fig 7.1 The effect of hypoxia and nebulised salbutamol [5mg] on plasma renin and angiotensin II (ANG II) levels in normal subjects. [n=8]

Results as mean absolute change from baseline values.

* $p<0.05$ vs normoxia /placebo

□ = hypoxia and placebo inhalation, ○ = hypoxia and salbutamol 5mg,

■ = normoxia and placebo inhalation, ● = normoxia and salbutamol 5mg

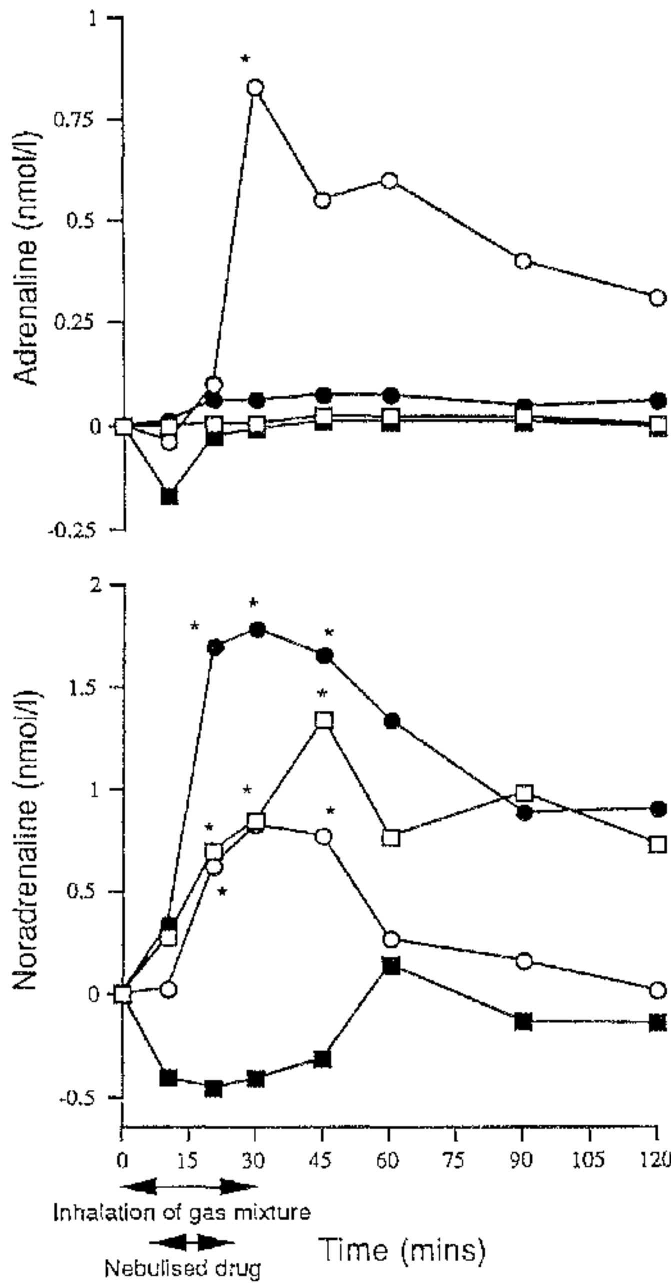

Fig 7.2 The effect of hypoxia and nebulised salbutamol [5mg] on plasma noradrenaline and adrenaline levels in normal subjects. [n=8]

Results as mean absolute change from baseline values.

* $p<0.05$ vs normoxia /placebo

□ = hypoxia and placebo inhalation, ○ = hypoxia and salbutamol 5mg,

■ = normoxia and placebo inhalation, ● = normoxia and salbutamol 5mg

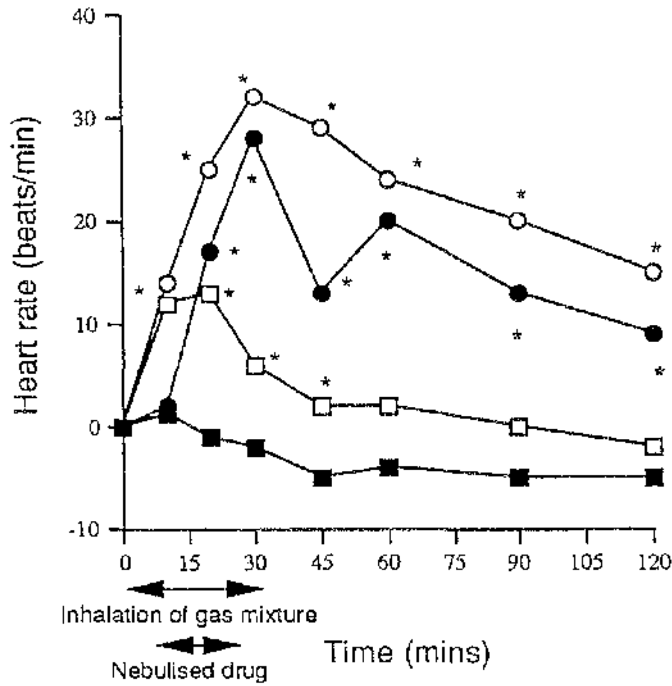

Fig 7.3 The effect of hypoxia and nebulised salbutamol [5mg] on heart rate in normal subjects. [n=8]

Results as mean absolute change from baseline values.

* $p<0.05$ vs normoxia /placebo

□ = hypoxia and placebo inhalation, ○ = hypoxia and salbutamol 5mg,

■ = normoxia and placebo inhalation, ● = normoxia and salbutamol 5mg

7.4 DISCUSSION

This study was designed to examine whether hypoxia might be contributing to the activation of the RAS in acute severe asthma and, in addition, to investigate whether the stimulatory effect of β_2 agonists on the RAS is influenced by the presence of hypoxic conditions. Our subjects received a 30 minute period of hypoxia where the FiO_2 was maintained at 12%, resulting in a mean O_2 saturation of 83.4%. During and after this period of hypoxia alone there was no change in the activity of the RAS in the group of eight healthy volunteers. Our findings also confirm our earlier observation that there is activation of the RAS by high dose nebulised β_2 agonists (Chapters 4 and 5) but that this effect is not enhanced by the presence of hypoxia.

Previous studies which have examined the effect of hypoxia on the activity of the RAS in animals and man have produced a variety of results: Rats exposed to chronic hypoxia develop a rise in renin and renin substrate (127), and hypoxaemia has been reported to cause moderate activation of the RAS in conscious dogs (158). In stable cor pulmonale the RAS has been reported not to be activated (141), while others have observed elevated levels of ANG II in patients with airflow obstruction and arterial hypoxaemia (128). Although the availability of serum ACE has been shown to be reduced by alveolar hypoxia in dogs (140), the effect of hypoxia on ACE activity in man is less clear. Some authors claim that the activity of ACE is reduced by hypoxia eg in normal subjects at high altitude (159), whereas our findings are in agreement with those of Colice and Ramirez, who found no change in ACE activity or plasma renin after a 60 minute period of

hypoxaemia in 5 normal subjects during which the oxygen saturation was maintained at 80% (160), and Lawrence et al who reported no change in plasma renin activity or ANG II in a group of 7 male subjects after a similar 60 minute period of hypoxia with an FiO_2 of 12% (161). Thus, there may be species differences and in addition the degree and duration of hypoxia may be important.
The increase in heart rate in our subjects, coupled with the small rise in plasma NA levels in response to the stress of hypoxia, suggests sympathetic nervous system activation. In previous studies, mild hypoxia (FiO_2 15%) has been reported to be without effect on circulating plasma catecholamines or heart rate (162) although higher levels of hypoxia (FiO_2 7-10%) have increased sympathetic output and caused a tachycardia (163). A rise in plasma noradrenaline by salbutamol has been described previously (82,164), and is thought to result from a direct stimulatory effect of salbutamol on prejunctional β_2 adrenoceptors, thereby facilitating neurotransmitter release from sympathetic nerve endings.

In conclusion, there is no evidence from the present study in healthy volunteers to support our hypothesis that hypoxia stimulates the RAS. Although there are characteristic pathophysiological differences between the airways of asthmatic subjects and healthy volunteers, hypoxia in a range similar to that obtained in our experiments typically occurs during acute exacerbations of asthma, thus it would seem very unlikely that hypoxia is activating the RAS in acute severe asthma. However, we cannot exclude the possibility that the asthmatic subject may respond differently or perhaps that more severe or prolonged degrees of hypoxia could cause activation of the RAS in asthma
We have also confirmed our earlier findings that high dose β_2 agonists used in the treatment of asthma can cause elevations in circulating levels of plasma renin and

ANG II, but this effect is not enhanced by the presence of hypoxia. The levels of renin and ANG II after salbutamol are less than those observed in patients with acute severe asthma (eg, plasma renin of 45.5[21-70] μU/ml and ANG II of 56[12-109] pg/ml on day 5 after admission, results as median with interquartile range) and it is possible that other factors may contribute to the activation of the RAS in acute asthma, perhaps inflammatory mediators such as histamine, prostaglandins or proteinase enzymes released from mast cells (130). The precise role of these other stimuli remains to be established in a future study.

CHAPTER 8

THE EFFECT OF INFUSED ANGIOTENSIN II ON AIRWAY FUNCTION IN ASTHMA

8.1 INTRODUCTION

Although ANG II is known to be a potent constrictor of vascular smooth muscle (102), its effects on bronchial smooth muscle remain unknown. Recent work has suggested that ANG II can potentiate contraction of rabbit airway smooth muscle *in vitro* (126) raising the possibility that this hormone may possess bronchoconstrictor activity *in vivo*. Having previously demonstrated elevated plasma levels of ANG II in patients with acute severe asthma and also after high dose β_2 agonist therapy, we have now examined the effect of ANG II on airway function in a group of mild asthmatic patients.

We chose to administer ANG II as an intravenous infusion, employing doses recommended by our colleagues in the MRC Blood Pressue Unit, Western Infirmary. Such dose ranges should be expected to increase plasma concentrations of ANG II to similar levels that occur in acute severe asthma (see Chapter 3) with only minimal increases in blood pressure.

8.2 METHODS

Patients

Eight (4 male) mild asthmatic patients (Mean (SD) age 34 (6.4) years, mean FEV_1 85 (12.5) % predicted) were studied. All were taking inhaled short acting β_2 agonists and 7 of the 8 regular inhaled corticosteroids. Inhaled β_2 agonists were withheld for 6 hours prior to each visit, while corticosteroid therapy continued unchanged.

Patients attended the laboratory on 2 separate study days. A 24 hour collection of urine was obtained prior to each visit as an indirect means of assessing dietary sodium intake.

On arrival, 2 indwelling cannulae (Venflon®, Viggo, Helsingborg, Sweden) were inserted into forearm veins for the purposes of blood sampling and administration of the intravenous infusion. Patients were asked to rest for a period of 20 minutes, following which blood pressure and baseline spirometry were recorded. Thirty mls of venous blood was withdrawn for estimation of baseline plasma ANG II levels. Each patient then received either ANG II (2, 4 and 8 ng/kg/min in increasing increments at 30 minute intervals) or placebo (5% dextrose). This was delivered intravenously by means of a 50 ml syringe driver (Perfusor® Secura E, B Braun, Melsunger AG). At the end of each 30 minute infusion period spirometry was measured (best of 3 readings) and blood withdrawn at the same time points for estimation of plasma ANG II. Patients remained semi-recumbant for the duration of the study. Oxygen saturation was monitored continuously throughout the study period.

The study was approved by the Glasgow West Ethical Committee and informed consent was obtained from all participants. Subjects with known hypertension or taking regular diuretic therapy were excluded.

Drugs were prepared in the sterile unit of our pharmacy and administered according to a randomised, double blind crossover study design.

Measurements

Blood pressure - measured using a semi-automatic sphygmomanometer (Dinamap®, 1846 FX vital signs monitor; Critikon, Berkshire, UK), and the mean of 3 readings at each time point recorded.

Plasma ANG II - see Chapter 2.4, Hormone assays

FEV_1 - see Chapter 2, Lung function

Oxygen saturation - pulse oximeter and finger probe (Ohmeda Biox 3700E, Ohmeda, Louisville, USA)

24 hour urinary sodium - see Chapter 2.7

Statistical analysis

Comparison of baseline values and the significance of changes in these at each time point was by one way analysis of variance, corrected for multiple comparisons.

We accepted p values below 0.05 as significant.

8.3 RESULTS

1. Plasma ANG II

At 0, 30, 60 and 90 mins mean (SD) plasma ANG II levels were 12.4(5.3), 25.1(4.0), 55.9 (10.2) and 121.3(20.2)pg/ml respectively after infusion of ANG II (2,4 and 8ng/kg/min), and 12.8 (7.9), 12.2 (8.0), 11.1(6.9) and 11.5 (6.3)pg/ml after infusion of placebo (fig 8.1).

2. Blood pressure and heart rate

Blood pressure did not change significantly during infusion of placebo. However following infusion of ANG II there were significant rises ($p<0.05$) in both systolic (fig 8.1) and diastolic levels. There were no significant changes in heart rate during infusion of placebo or ANG II (data not shown).

3. FEV1

There was no significant difference between baseline FEV_1 on either study day. Mean (SEM) baseline values of FEV_1 were 2.55 (0.64) and 2.63 (0.34) litres prior to infusion of ANG II and placebo respectively. A significant bronchoconstrictor response to ANG II was observed at 60 and 90 minutes, with a mean (SEM) fall in FEV_1 from baseline values of 0.18 (0.03) and 0.34 (0.13) litres or 7.8 (2.02) and 12.4 (3.3) % respectively, compared to 0.02 (0.09) and 0.01 (0.09) litres or 0.3(1.3) and 0.6 (1.2)% after placebo (fig 8.1). In addition, 5 out of 8 patients reported cough or chest tightness during the final infusion period.

4. Oxygen saturation.

Mean (SEM) baseline oxygen saturation was normal on each study day [97.5 (0.3)% prior to infusion of ANG II and 97.5 (0.2)% before placebo]. Oxygen saturation did not alter significantly during either infusion of placebo or ANG II (data not shown).

5. 24 hr urinary sodium

There were no significant differences between 24 hour urinary sodium concentrations on either of the study days. Mean (SEM) 24 hr urinary sodium values were 178 (25.2) and 145 (21.2) mmol/24hr prior to infusion of ANG II and placebo respectively.

Raw data for this chapter is included in the Appendix [tables 26A-28A].

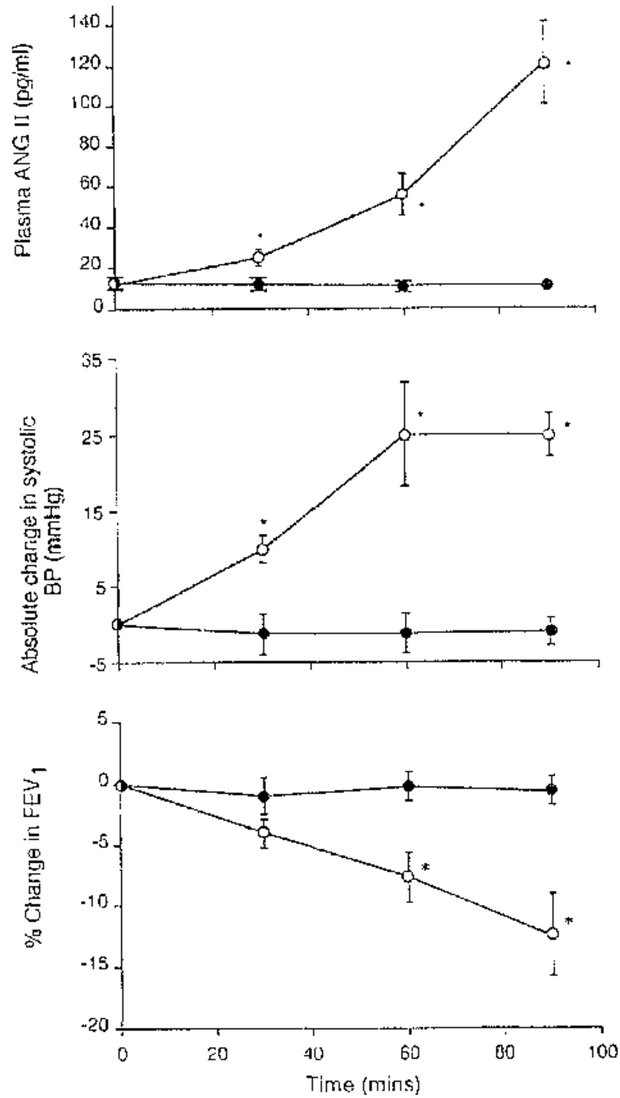

Fig 8.1 The effect of intravenous ANG II on systolic blood pressure (BP) and FEV_1 in asthmatic subjects [n=8]. Results as mean (SEM) absolute change (ANG II and BP) and % change (FEV_1) from baseline values. *p<0.05 vs placebo

○ ANG II ● placebo

8.4 DISCUSSION

Recently, ANG II has been shown to potentiate vagally mediated contraction of rabbit airway smooth muscle through the activation of prejunctional receptors (126) thereby suggesting that it may have bronchoconstrictor activity in the human airway. In this study we have demonstrated that ANG II causes bronchoconstriction when administered intravenously to mild asthmatic patients. This effect occurs at pharmacological and pathological plasma levels of ANG II, as the range of plasma levels obtained during the infusion occur after mutiple high doses of nebulised β_2 agonists (Chapter 4 and 5) and during acute attacks of asthma (Chapter 3).

The exact mechanism of bronchoconstriction by ANG II is uncertain, but probably involves a direct effect of ANG II on airway smooth muscle similar to its vasoconstrictor action on vascular smooth muscle (102). These actions of ANG II are known to be mediated by the interaction of the hormone with specific, high affinity receptors in the plasma membrane of its target cells (165,166). Recently the development of specific ANG II receptor ligands has led to the identification of at least two different ANG II receptor subtypes, which mediate their action through different signal transduction mechanisms. Most of the actions of ANG II, including vascular smooth muscle contraction, are mediated via the AT_1 receptor (167), and it is likely that bronchial smooth muscle constriction also involves the same receptor subtype. This is a classical membrane protein which transmits ANG II actions via a G-protein linked pathway (Chapter 1.4.4), stimulating the enzyme phosphodiesterase which leads to the formation of the second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG). When vascular smooth muscle AT_1 receptors are activated, it has been proposed that increased release of

calcium from the sarcoplasmic reticulum occurs via the formation of IP_3 and also directly from G-protein-gated calcium channels (109). Such increased release of calcium results in increased vascular smooth muscle contraction and tone. As the effect of ANG II on bronchial smooth muscle has not been previously described, we can only speculate at this stage that the likely mechanisms will resemble those occuring in vascular smooth muscle.

The mechanism of bronchoconstriction by ANG II could also involve the release or potentiation of other mediators of bronchoconstriction. For example, ANG II has been shown to increase prostaglandin production under a variety of circumstances (168) and can release platelet activating factor from cultured human endothelial cells (169). In addition, ANG II has also been reported to potentiate the pressor effects of endothelin-1 (itself also a potent bronchoconstrictor) (170). Another possible mechanism of airway smooth muscle contraction is by a reflex increase in vagal tone secondary to the rise in blood pressure that occurs during infusion of ANG II. However, against this theory is our finding that heart rate did not increase in our study.

In summary, this study is the first report of a bronchoconstrictor action of ANG II in asthma. Since the range of plasma levels of ANG II achieved during the infusions are comparable to those found in acute severe asthma, it is possible that in some individuals this hormone may be contributing to a proportion of the bronchoconstriction in acute severe asthma.

CHAPTER 9

THE EFFECT OF ANGIOTENSIN II ON METHACHOLINE-INDUCED BRONCHOCONSTRICTION *IN VITRO* AND *IN VIVO*

9.1 INTRODUCTION

In a number of tissues, pre-treatment with ANG II can uncover or enhance contractions evoked by other agonists, for example, in rabbit saphenous artery. α2-adrenoceptor-mediated contractions are enhanced in the presence of low concentrations of ANG II (171). In addition ANG II has been shown to potentiate vagal-mediated contractions of rabbit trachea, probably by pre-junctional stimulation of acetylcholine release (126).

We have recently observed elevated plasma ANG II levels in patients with acute severe asthma (Chapter 3) and in the previous chapter have also shown that when mild asthmatic patients receive this hormone intravenously at doses which evoke similar plasma levels of ANG II to that observed in acute asthma, it causes bronchoconstriction. The mechanism of action of ANG II in causing this contraction is as yet unknown and may involve either a direct action on airway smooth muscle, or perhaps modulation of the effects of other mediators of bronchoconstriction.

In this present study, we firstly examined the direct effects of ANG II *in vitro* on human bronchial smooth muscle. We also investigated the ability of ANG II to interact with the bronchoconstrictor, methacholine. This study was then extended to examine *in vivo* the ability of ANG II to modulate bronchoconstriction evoked by methacholine in patients with mild asthma, where these patients were known to exhibit hyperresponsiveness to methacholine.

9.2 MATERIALS AND METHODS

In vitro

Tissue Collection and Preparation.

Macroscopically normal human bronchial tissues (3rd to 6th order) were obtained from patients undergoing thoracic surgery for bronchial carcinoma. Tissues were dissected free of connective tissue and fat and stored overnight at 4°C in oxygenated Krebs-Henseleit solution of the following composition (mM). NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2 and glucose 11.1. Published data has shown that overnight storage of this tissue does not alter its reactivity (172).

Measurement of Contractile Responses

Contractile responses were measured from rings of human bronchi (3-5mm) in vertical organ baths (10ml) at 37 ± 0.5°C in oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution. Tension (2g) was applied via two platinum wires into the lumen. One wire was anchored and the other attached to a force displacement transducer (Grass FT03T). Tissues were allowed to equilibrate for 2h, during which time applied tension was readjusted to the initial level. Concentration-response curves were constructed to ANG II to ascertain the threshold for contraction to this hormone. In a subsequent set of experiments, concentration-response curves were constructed to methacholine in the presence and absence of ANG II (10^{-7}M). Drugs were added directly to the organ bath and ANG II was added 15min before methacholine concentration-response curves.

In vivo

Patients

7 mild asthmatic patients (3 female) were recruited from the outpatient clinic, mean (SD) age 26.8(8) years, mean (SEM) FEV1 82.1 (9.4)% predicted. All patients demonstrated at least mild bronchial hyperreactivity to inhaled methacholine, according to the method of Cockroft et al (133). All patients were taking inhaled short acting β_2 agonists as required in addition to regular inhaled corticosteroids. None were on long acting inhaled β_2 agonists or oral theophylline. All patients were otherwise in good health, in particular, none had a history of hypertension, nor were on diuretic therapy.
For the purposes of each study visit, inhaled β_2 agonists were discontinued for at least 8 hours prior to attendance, but inhaled corticosteroids continued unchanged.
The study had the approval of the Glasgow West Ethical Committee and informed, written consent was obtained from each patient.

Study design

A randomised, double-blind study design was employed. At an initial screening visit the individual dose of methacholine required to cause a 20% fall in their FEV_1 was determined (ie the PC_{20}). Bronchial reactivity to methacholine was determined using methods previously described (Chapter 2.3). Thereafter, patients attended the laboratory on 3 separate study days. At each visit, baseline FEV_1 (the best of 3 readings) was measured by dry wedge spirometer (Vitalograph, Buckinghamshire, UK), and blood pressure recorded.

An intravenous cannula (Venflon ®, Viggo, Helsingborg, Sweden) was inserted into a forearm vein followed by a 20 min rest. A baseline blood sample was obtained for estimation of plasma ANG II. Patients then received an intravenous infusion of placebo (5% dextrose) or ANG II (1 or 2 ng/kg/min) for a period of 40 min. These doses were chosen as being below threshold for bronchoconstriction in a previous study (Chapter 8). The drugs were administered by 50ml syringe driver (Perfusor Secura E, B B Braun, Melsunger AG, Germany). A second blood sample was obtained (25 min from commencement of infusion) from the contralateral arm for estimation of plasma ANG II and the FEV_1 was recorded. While the intravenous infusion still continued, patients then received a methacholine challenge using the methods previously described (131,132), to determine the PC_{20}. Following completion of the methacholine challenge, a final blood sample was withdrawn from the contralateral arm for plasma ANG II measurement and the intravenous infusion was discontinued. The effect of methacholine was then rapidly reversed with inhalation of nebulised salbutamol.

Blood pressure was monitored at 15 minute intervals and patients remained semi-recumbent for the duration of the study.

Measurements

Blood pressure - measured by semi-automatic sphygmomanometer (Dynamap, 1846 FX Vital Signs Monitor, Critikon, Berkshire, UK) and the mean of 3 readings recorded at each time point.

Plasma ANG II - see Chapter 2.4 , Hormone assays.

Drugs - see Chapter 2.8

Statistical analysis

Statistical significance between data samples in the *in vitro* studies was tested by two-way ANOVA. Statistical difference between pD_2 values was by Student's t-test. In the *in vivo* studies, differences between placebo and active days was by Student's t-test with subsequent Dunnett test. A probability level of $p<0.05$ was considered significant. Number of observations (**n**) refers to the number of patients tested or from which tissue was obtained.

9.3 RESULTS

In vitro studies

ANG II produced small (<0.25 g wt maximum), concentration-dependent contractions of human bronchi, with the threshold for contraction occurring between $3x10^{-8}$ and $3x10^{-7}$M. Pre-incubation with ANG II (10^{-7}M) evoked contraction in only two tissues and in those cases the level of contraction was less than 0.1 g wt (compared with control maximum contraction of 2.5-4.5 g wt with methacholine ($3x10^{-4}$M)). This concentration of ANG II however significantly ($p<0.001$) enhanced contractions to methacholine. This did not manifest itself as a shift in pD_2 values (pD_2 represents -log EC_{50}, the concentration of drug producing 50% maximal response) but rather as an increase in the magnitude of contractions evoked at concentrations of methacholine above 10^{-6}M. The maximum response was increased by 39.1% in human tissues [n=6] (Fig 9.1).

In vivo studies

There was no significant difference between baseline FEV_1 (mean baseline FEV_1 (SEM) were 2.98(0.4)l, 2.8(0.4) and 2.9(0.3) prior to placebo, ANG II 1ng/kg/min and ANG II 2ng/kg/min respectively).

After infusion of ANG II (1 or 2 ng/kg/min) and prior to methacholine challenge, there was no significant change in baseline FEV_1 values (Student's t-test). (mean baseline FEV_1 (SEM) were 2.8(0.4)l, 2.74(0.4) and 2.96(0.3) after placebo, ANG II 1ng/kg/min and ANG II 2ng/kg/min respectively). The PC_{20} for methacholine

(expressed as geometric mean with range) after placebo infusion was 3.09 (1.15-6.0) mg/ml. After infusion with ANG II 1ng/kg/min, this decreased to 2.14(0.85-3.8) mg/ml, although this did not reach statistical significance. In 6 out of 7 patients, ANG II 2ng/kg/min potentiated the effect of inhaled methacholine. There was a significant ($p=0.006$) decrease in PC_{20} compared to placebo (geometric mean 1.2 (0.45-2.08) mg/ml) (Fig 9.2). Plasma ANG II concentrations were measured at baseline, prior to and on completion of each infusion. Results [mean (SEM)] were; 9.2(1.8), 9.5(2.0) and 9.9(1.8)pg/ml after placebo, 5.1(1.2), 16.2(0.9) and 27.3(5.2) pg/ml after ANG II 1ng/kg/min and 6.0(1.5), 32.9(5.1) and 37.7(9.5) pg/ml after ANG II 2ng/kg/min.

Blood pressure did not alter significantly during ANG II infusion (data not shown), and patients did not report any adverse symptoms.

Raw data for this chapter is included in the Appendix [tables 29A-31A].

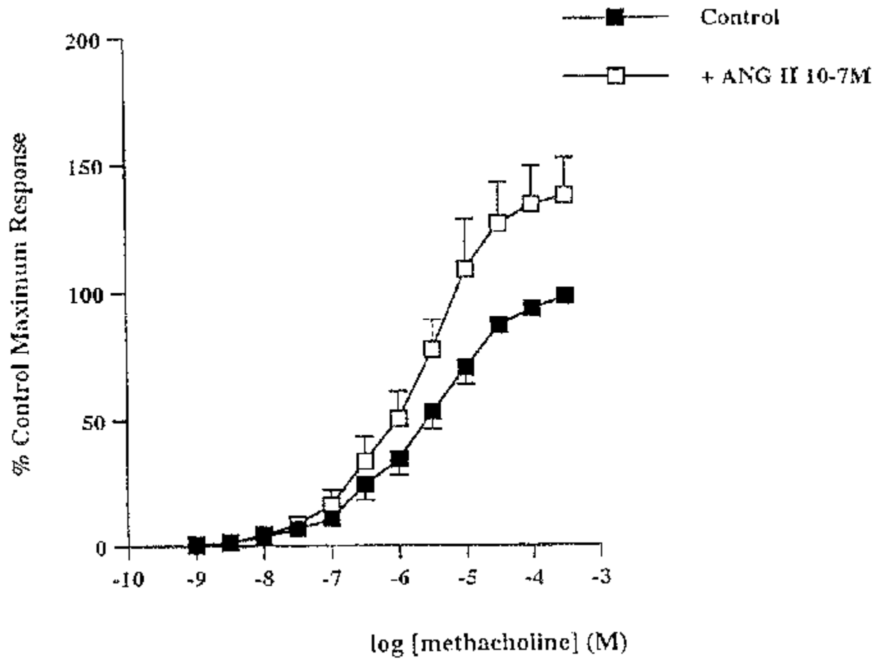

Fig 9.1 Concentration-response curves evoked by methacholine (10^{-9} - $3x10^{-4}$M) alone (■) or in the presence of ANG II 10^{-7} M (□). ANG II produced a small contraction in only two tissues, however a significant ($p<0.001$ by two-way ANOVA) potentiation of the concentration response curves evoked by methacholine was observed. Number of observations (n)=6 in each case.

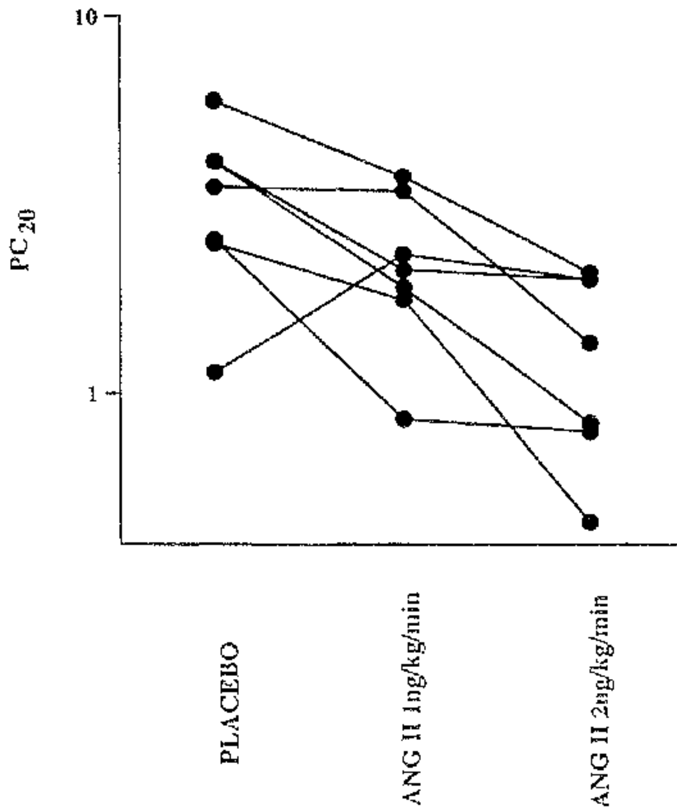

Fig 9.2 Individual PC_{20} values for methacholine (mg/ml) after placebo infusion (5% dextrose) and after IV infusion with ANG II 1ng/kg/min or ANG II 2ng/kg/min in mild asthmatic patients. ANG II 1ng/kg/min produced an insignificant fall in PC_{20}.

However, ANG II 2ng/kg/min potentiated the effect of inhaled methacholine in 6 out of 7 patients resulting in a significant decrease of PC_{20} compared to placebo, $p=0.006$.

9.4 DISCUSSION

The presence of ANG II in subthreshold concentrations markedly enhanced contractions evoked by the cholinergic agonist methacholine both *in vitro* in human bronchial smooth muscle and also *in vivo* in mild asthmatic patients. Furthermore, the potentiating effect of ANG II occured at doses which did not themselves cause bronchoconstriction. The levels of ANG II used *in vitro* were higher than occurs in the plasma of normal subjects (2.3×10^{-11})(141). However local levels in the airway may not match the general plasma level, and in addition, in conditions such as acute severe asthma (Chapter 3) or severe congestive cardiac failure (173) ANG II levels can be markedly elevated. *In vivo*, the circulating levels of ANG II produced were similar to those seen during exercise (174). Thus the potentiating action observed with ANG II on airway responsiveness is likely to have important effects on airway function at both physiological and pathological circulating concentrations of this hormone.

The means by which ANG II may potentiate bronchoconstriction is unclear, although a number of possibilities exist. Yamawaki and colleagues demonstrated in rabbit trachea that ANG II potentiated contractions evoked by electrical field stimulation, by increasing acetylcholine release (126). This occurred in the absence of any change in baseline tone evoked by ANG II alone. It is unlikely that reflex changes in vagally-mediated tone account for this action of ANG II in the present study, as blood pressure and heart rate were unaltered in the subjects during ANG II infusion. In addition, in the present study, *in vitro*, we have demonstrated a post-junctional effect of ANG II in potentiating methacholine-

evoked contractions. The possibility therefore exists that ANG II may act both pre- and post-junctionally to facilitate bronchoconstriction.
Other means by which ANG II may enhance bronchoconstriction include the release of potential spasmogens such as endothelins (175), platelet activating factor (169), or arachidonic acid metabolites (168).

It has previously been described that ANG II evokes bronchoconstriction when present in higher concentrations, such as occurs in acute severe asthma (Chapter 8). It now seems likely that the presence of relatively low concentrations of this hormone may result in paradoxically large constrictions by potentiating other mediators of bronchoconstriction. This may be of clinical importance not only during acute attacks of asthma, but also in patients with exercise-induced asthma where ANG II levels are also elevated (own unpublished observations). In adddition our findings may be of relevance to patients receiving high dose nebulised β_2 agonists since plasma ANG II levels comparable to those in the present study have been observed in mild asthmatics given nebulised salbutamol (Chapters 4 and 5).

In conclusion, ANG II potentiates methacholine-evoked bronchoconstrictions, both in isolated human bronchi and in mild asthmatic patients. This may represent an important mechanism by which levels of ANG II which have little direct activity may produce substantial changes in airway tone. The mechanism for this interaction has yet to be elucidated.

CHAPTER 10

CONCLUSIONS

This series of studies is the first to document activation of the renin-angiotensin system (RAS) in acute severe asthma and to report that the hormone ANG II not only has direct bronchoconstrictor effects on the human airway, but can also potentiate the bronchoconstrictor response to other mediators such as methacholine. These new observations are exciting and although this area of research is still in its preliminary stages, our findings raise the possibility that ANG II may play a role in the pathogenesis of asthma.

The initial studies were designed to identify possible stimuli to the RAS in acute severe asthma. Our results suggest that elevation of plasma renin and ANG II in acute asthma is likely to be a multifactorial phenomenon, although further work on this aspect is required. Hypoxia is unlikely to be activating the RAS in acute asthma, and the small elevation of haematocrit on admission reflecting a degree of haemoconcentration does not explain the prolonged elevations of renin and ANG II that persisted until day 5 after admission. There may be a minor effect of increased sympathetic drive in some patients with acute severe asthma, although [unlike the results of Ind et al (130)] there was no consistent increase in plasma adrenaline and noradrenaline in most of our cases. There is no doubt, however, that inhaled high dose β_2 agonist therapy causes activation of the RAS , and we have confirmed this finding in several studies (Chapters 4, 5 and 6). A wide range of plasma renin and ANG II levels was noted in patients with acute asthma (Chapter 3) and although the reason for this variation is unclear, we suggested it could be the result of individual differences in factors influencing the RAS.

Several studies could be devised to extend this observation. Since it is known that patients admitted to hospital with acute asthma show wide variations in circulating salbutamol and terbutaline concentrations, with some having markedly

elevated plasma levels of ß agonist (153) it would be useful to measure individual plasma salbutamol levels on admission in acute severe asthma and determine whether any correlation exists between ß agonist levels and circulating renin and ANG II concentrations. The role of allergen exposure, an event which is associated with mediator release from mast cells, is also unclear. Inflammatory mediators such as mast cell chymotryptases and neutrophil proteases released during an acute attack of asthma could cause a rapid formation of ANG II by an ACE independent pathway (66,131). This could be further investigated by measuring circulating inflammatory mediators such as plasma histamine or tryptase in venous blood in patients admitted to hospital with acute severe asthma, and determining whether these levels also correlate with those of plasma renin and ANG II. It might also be useful to examine whether allergen-induced early asthmatic responses which are associated with mast cell mediator release cause elevations in circulating ANG II levels.

Currently there is interest in the existance of polymorphism in the human ACE gene, with the DD genotype correlating with increased risk of myocardial infarction, perhaps as the result of increased local generation of ANG II in the coronary circulation (122). It is intriguing to speculate that if this were also the case in our asthmatic patients, then those who exhibit such polymorphism could have increased circulating levels of ACE, and resultant greater elevations in circulating ANG II levels in acute severe asthma and after high dose $ß_2$ agonists. Determination of the ACE genotype in each of these groups of asthmatic patients would be an interesting future exercise.

The potential role of ANG II in exercise-induced asthma also warrants further studies. The RAS is known to be activated by exercise. Hespel et al have described an increase in plasma renin in normal volunteers following exercise on a bicyle ergometer (72) and Kosunen et al reported marked increases in plasma renin and ANG II in male athletes after running exercise (174). We have also noted that asthmatic individuals have elevated plasma ANG II levels in response to graded exercise testing [mean (SEM) increase in ANG II of 22.6 (4.7)μU/ml from baseline, n=9] (own unpublished observations). As similar plasma levels of ANG II can potentiate bronchoconstriction (Chapter 9), then the possibility exists that ANG II may contribute to bronchoconstriction in exercise-induced asthma. The exact mechanism of exercise-induced asthma is currently an area of much research. One hypothesis is that the airway narrowing in this condition is the result of thermal effects of water loss, with increased osmolarity of airway surface fluid (176). This effect is known to increase mediator release from human mast cells which cause bronchial smooth muscle contraction (177). The finding that sodium cromoglycate, a drug known to inhibit the release of mast cell mediators (178), can reduce exercise induced asthma, has added support to the view that mast cell mediator release plays a contributory role. Future studies should examine whether there is an exaggerated plasma ANG II response to exercise in asthma compared to normal individuals and also whether pre-treatment of asthmatic patients with an ANG II antagonist (such as losartan) can attenuate exercise-induced asthma.

The finding that ANG II is elevated in the plasma in patients with acute asthma may be of clinical importance for several reasons. Firstly, at similar plasma levels we have demonstrated that this hormone causes bronchoconstriction *in vivo* and at

subthreshold doses it can also potentiate bronchoconstriction, both *in vitro* and *in vivo*. This suggests that ANG II is likely to be contributing to bronchoconstriction during an acute attack of asthma, and perhaps more importantly, may be enhancing the effects of other mediators of bronchoconstriction, even when present in very low concentrations itself. Secondly, ANG II could have several other potentially adverse effects on the airway in acute asthma. It is known to cause pulmonary vasoconstriction and hypoxaemia (179) which could contribute to ventilation-perfusion imbalance during an acute attack of asthma, or perhaps cause reduced clearance of inflammatory mediators. Other reported actions include increased vascular permeability in experimental animals (180), raising the possibility that in asthma, elevated levels of this hormone could contribute to the inflammatory response within the airway. ANG II can also influence inflammatory cell function by several mechanisms: It is known from *in vitro* studies to have suppressive effects on lymphocyte proliferation (181), chemotactic properties for mononuclear cells (182) and regulatory effects on macrophage Fc and C3b receptor function (183,184). The presence of angiotensins has also been documented in alveolar macrophages and peripheral blood monocytes (185), raising the possibility that they may have some functional importance. Thus, it is conceivable that many of these actions of ANG II could assume clinical significance during the inflammatory processes of acute asthma.

Circulating ANG II levels are also elevated in cardiac failure (173). It has been suggested that high levels of ANG II in this condition may exert a range of adverse effects on the cardiovascular system, including cardiac myocyte necrosis (186), coronary vasoconstriction and left ventricular hypertrophy (113). It seems unlikely, however, that transient elevations of ANG II represent any serious risk

to asthmatic patients with normal coronary circulation and left ventricular function. However, in the situation of severe hypoxaemia during acute severe asthma, more prolonged elevations of plasma ANG II, perhaps allied with electrolyte imbalance and ß adrenergic stimulation, may be of greater significance. Finally, by acting directly on the adrenal cortex, ANG II increases the secretion of aldosterone (102), resulting in sodium retention and potassium loss. High levels of ANG II may therefore exacerbate hypokalaemia, particularly during an acute attack of asthma when high doses of $ß_2$ agonist are used (see below). Thus, there are several mechanisms whereby the hormone ANG II could potentially cause adverse metabolic, pulmonary and cardiac effects in acute asthma. However, the exact clinical significance of these in patients with acute asthma is not certain.

Several investigations have questioned the safety of inhaled $ß_2$ agonist therapy in asthma (Chapter 1.2.1). In the 1960's it was suggested that the introduction of inhaled isoprenaline might have contributed to an increase in deaths from asthma (187) and more recently a particular cluster of asthma deaths in New Zealand has been linked to the introduction of the $ß_2$ agonist fenoterol in that country (50-52). The question of whether the apparent adverse effects of prolonged use of ß agonists is a class-specific effect of all ß agonists, or is simply confined to fenoterol has not been satisfactorily resolved. It is currently thought that fenoterol is used at a particularly high dosage level compared to other ß agonists such as salbutamol and its $ß_2$ adrenoceptor selectivity is only marginal (151). It may therefore have its adverse effects mediated via cardiac $ß_1$ adrenoceptors thus contributing to its association with increased mortality. Another unwanted effect may be the hypokalaemia that occurs when $ß_2$ agonists reach the systemic

circulation. This may in turn increase susceptibility to cardiac arrythmias, although there is little evidence to suggest that arrythmias occur in near-fatal asthma attacks in patients admitted to hospital (188). ß agonists may also be associated with increased morbidity from asthma. Single doses of inhaled $ß_2$ agonists are known to exhibit a protective effect against bronchial hyperreactivity, which appears to be functionally separate from their bronchodilator actions (189,190). However, it is widely believed that tolerance (tachyphylaxis) develops to protection against hyperreactivity during repeated dosing, thereby resulting in increased frequency of use and consequently an exacerbation of adverse effects (191). There is also evidence that prolonged treatment with regular ß agonists may cause a deterioration in disease control (55), although the exact mechanism remains unclear. Van Schayck et al recently showed that inhaled salbutamol given continuously versus on demand led to a small but significant decline in FEV_1 over a duration of 12 months although there was no difference in airway reactivity or symptom scores (55).

Could our own observations that high dose $ß_2$ agonists elevate plasma ANG II levels be of any relevance to the ß agonist debate? Apart from the multitude of potentially adverse acute effects of ANG II described above, of more interest and perhaps significance are the mitogenic properties of ANG II. Angiotensin II causes vascular smooth muscle cell growth both *in vitro* and *in vivo* by increasing the expression of growth-promoting oncogenes *c-fos*, *c-jun* and *c-myc*, by mitosis, by an increase in cell size and by increased synthesis of extracellular matrix proteins (192). If such effects occured on airway smooth muscle as a result of generation of excess ANG II in the lungs by regular administration of high doses of $ß_2$ agonists, then this could be of importance in the long term by

contributing to the development of airway smooth muscle hypertrophy (as occurs in chronic asthma) and hence a decline in lung function. Thus, it would be relevant to pursue this theory by examining whether ANG II could be a growth factor for airway smooth muscle. Lindop et al have recently infused the thymidine analogue bromodeoxyuridine subcutaneously into rats via osmotic minipumps and confirmed an increase in DNA synthesis (as measured by uptake of bromodeoxyuridine into cell nuclei) in rat mesenteric resistance arteries, but not veins, in response to low dose subcutaneous infusions of ANG II (Dr G Lindop, personal communication). It would be relevant in a future study to employ similar techniques to examine the effect of ANG II on bronchial smooth muscle. If we could confirm that ANG II is also a growth factor for airway smooth muscle, then this would clarify the role of ANG II in the pathogenesis of asthma and would also strengthen our hypothesis that high dose ß agonists could adversely affect asthma control as a result of elevating circulating ANG II levels.

Local renin-angiotensin systems exist in many tissues and all the components of the system have been shown to be present and functional in a large number of sites such as the heart, kidney and brain (106). However it is not yet known whether such systems exist in human airway. Renin secreting tumours of the lung have recently been described, strengthening the possibility that renin producing cells exist in the lung (193) and renin mRNA has been detected in rodent but not human lung (194). In view of the results of our clinical studies and biological properties of ANG II, the presence of a local RAS in human airways could have important implications for the pathogenesis of asthma, and clearly this possibility needs to be further explored. It is possible to measure angiotensin I and II in human lung by specific radioimmunoassay techniques, and ANG II receptors can

be identified using autoradiography on airway tissue sections (Dr J McQueen, personal communication). In addition, sensitive methods are also available to detect renin mRNA (194) and immuno-reactive renin (195) in cells from human lung. It is interesting to speculate that if a tissue RAS does exist in the lungs as occurs in other tissues, then this may lead to high local levels of ANG II being generated, eg in response to inhaled high dose $ß_2$ agonists, with the prospect of local modulation of bronchial smooth muscle tone or, in the longer term, airway smooth muscle hypertrophy. In Chapter 5, where we examined the effect of nebulised and intravenous ß agonists on the activity of the RAS, we hypothesised that if a local RAS existed in the airways then perhaps the more marked elevations in plasma renin and ANG II noted after the inhaled route of administration could be the result of stimulation of a local RAS, with spill-over into the systemic circulation.

The effects of ANG II on airway tone also warrant further investigation. It would now be relevant to begin to explore possible pathways whereby ANG II could cause bronchoconstriction in asthma. From previously known effects of ANG II on vascular smooth muscle, the most obvious mode of action is a direct effect on airway smooth muscle, but the possibility of other mechanisms, such an indirect vagally mediated action or perhaps even via the release of other mediators of bronchoconstriction such as endothelins, prostaglandins or leukotrienes, also exist. It is known that ANG II can influence the autonomic nervous system both centrally and peripherally. Enhancement of peripheral adrenergic neurotransmission by ANG II has been demonstrated in man. For example, Moulds et al showed that physiological concentrations of ANG II *in vitro* could enhance noradrenaline-induced contraction of human palmer digital artery (196),

and Struthers et al demonstrated an interaction at the postsynaptic level between noradrenaline and ANG II, with subpressor doses of intravenous ANG II augmenting sympathetically mediated vasoconstriction in normal volunteers (197). In addition to the interaction between ANG II and the sympathetic nervous system, there is also evidence to suggest a peripheral interaction of ANG II with the parasympathetic nervous system. Potter has described attenuation by ANG II of the bradycardic response to vagal nerve stimulation in the dog (198), an effect unaffected by ß blockade. *In vitro* work has also shown that ANG II has no effect upon the response of isolated atria to acetylcholine (199) suggesting a presynaptic vagal influence of ANG II.

Recently, Yamawaki et al studied the effect of ANG II on cholinergic neurotransmission in rabbit tracheal segments *in vitro* (126). Cumulative administration of ANG II in concentrations of 10^{-11} to 10^{-6} M did not alter the resting tension, but did augment the contractile responses of the airway smooth muscle to electrical field stimulation in a concentration-dependent manner. This effect was attenuated by the ANG II receptor antagonist CK-2961. In contrast, muscle contraction induced by exogenous acetylcholine was not altered by ANG II (10^{-6} M), thereby suggesting that the ANG II-induced augmentation of bronchoconstriction is pre- rather than post-junctional, probably by stimulation of acetylcholine release from cholinergic nerve terminals. Although it was widely known from previous studies that ANG II could enhance adrenergic neurotransmission, this was the first study to report facilitation of cholinergic neurotransmission by ANG II. Our own results in human airway (Chapter 9) are in agreement with those of Yamawaki, in that ANG II produced little or no direct effect on bronchial smooth muscle tone. However, we have also demonstrated a

post-junctional action of ANG II in potentiating bronchoconstriction, thus raising the possibility that ANG II can act pre and post junctionally to facilitate bronchconstriction. Previous studies have shown that ANG II can also stimulate the release of arachidonic acid metabolites (168), amongst which the prostaglandins D_2 (200) and $F_{2\alpha}$ (201) and thromboxane A_2 (202) are known to augment parasympathetic bronchoconstriction by enhancing the release of acetylcholine. In addition, Nally et al have recently demonstrated that ANG II can enhance contractions of bovine bronchi by endothelin-1 (203). This potentiating action of ANG II appears to involve the AT_1 receptor subtype, as demonstrated by the inhibition of this effect of ANG II by the AT_1 antagonist losartan.

Several studies could be developed to further examine the mechanism of ANG II-induced bronchoconstriction in asthma. The effect of pharmacological blockers on ANG II induced bronchoconstriction should be investigated by pretreatment of the asthmatic patient with an ANG II antagonist such as losartan, inhaled ipratropium bromide (cholinergic mechanisms) or oral indomethacin (cyclo-oxygenase products) and administering ANG II in doses previously known to cause bronchoconstriction. Having previously demonstrated a potentiating effect of ANG II on methacholine-induced bronchoconstriction (chapter 9), further examination of the effect of ANG II on bronchoconstriction induced by other mediators such as histamine, is also warranted in order to attempt to disentangle the pathways involved in the interaction of ANG II with other agonists in asthma. Future studies should also be directed at investigating the intracellular mechanisms involved in the actions of ANG II on human airway smooth muscle. The effect of ANG II on second messengers such as cGMP, cAMP and I-(1,4,5)P_3 during receptor-mediated activation could be measured in the organ

bath after modulating airway tone with ANG II alone and in the presence of agonists or electrical field stimulation.

A putative inflammatory mediator in asthma should fulfil certain criteria as recently outlined by O'Byrne (204). These criteria are, in effect, modifications of the original Koch's postulates (205), a set of guidelines which were used to identify a microbe as the cause of a particular disease. The development and application of these steps in 1890 by Koch to the demonstration of the tubercle bacillus as the cause of tuberculosis is considered to be one of the milestones of medical history (206). Such postulates have been altered on several occasions and are still being applied to the identification of new agents of infectious disease.
For an agent to be considered as an inflammatory mediator in asthma it is said that the mediator and its structure must firstly be identified and when administered (usually by inhalation) to asthmatic patients it should mimic the asthmatic response. If we apply these criteria to ANG II, our results so far have confirmed this to be the case using intravenous infusions of ANG II, but not yet by inhalation, and so a future study should examine the effect of inhaled ANG II on airway tone in asthma. Secondly, with assay measurements being available for the potential mediator, it should be possible to measure it during asthmatic responses. Elevation of ANG II in the plasma has been demonstrated in acute severe asthma (Chapter 3), but we should now aim to measure this hormone in the plasma during asthmatic responses induced, for example, by stimuli such as cold air or allergen. Thirdly, when antagonists of the mediator or its synthesis are available, they should be studied in clinical models of asthma. The use of ACE inhibitor drugs in asthma is complicated by the fact that ACE appears to play a role in the genesis and metabolism of mediators of bronchoconstriction eg bradykinin and

prostaglandins, and captopril-related asthma has been previously reported (207). However, the recent availability of specific ANG II antagonists such as losartan should now allow us to begin to examine these agents in different types of experimental asthma. Only once these questions have been answered will it then be possible to address the most difficult question, namely whether the mediator antagonists or synthesis inhibitors are actually of any value in treating asthmatic patients.

In summary, the results of this thesis have begun to provide evidence for a putative role of ANG II as a mediator in the pathogenesis of asthma. We believe that our results are promising and warrant further studies in order to increase our understanding of the effects of ANG II on airway function in asthma. It is interesting to speculate that, in the future, specific pharmacological targetting of the ANG II receptor may form the basis of a novel approach to the treatment of certain types of asthma.

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APPENDIX

Subject	Sex	Age (yrs)	pO_2 (kPa)	SBP (mmHg)	DBP (mmHg)	Pulse (bpm)	Peak Expiratory Flow Rate (litres/min)		
							Admission	Day 2	Day 5
1	M	50	8.8	160	108	116	120	290	340
2	F	32	8.0	120	70	140	150	380	460
3	M	30	8.5	150	80	128	100	350	450
4	F	29	8.0	140	85	122	90	300	300
5	M	23	8.7	130	90	118	160	300	430
6	F	39	7.6	160	90	104	170	290	350
7	F	19	6.4	110	70	120	60	280	240
8	F	42	9.2	170	110	120	110	200	250
9	F	29	9.3	130	90	134	100	220	300
10	F	28	12.5	150	90	118	100	200	490
11	F	59	7.8	110	70	110	110	350	320
12	F	17	17.8	110	76	100	140	300	360
13	M	39	8.2	150	90	120	110	220	460
14	F	62	9.2	150	82	114	180	290	450
15	F	21	8.8	130	80	120	200	300	400
16	M	29	8.4	150	75	120	170	250	330
17	F	28	9.0	125	70	120	80	200	200
18	F	44	9.2	110	66	110	100	110	330
19	M	30	8.8	130	80	140	120	280	380
20	F	29	9.0	120	80	120	120	230	350
MEAN		**34**	**9.2**	**136**	**78**	**120**	**125**	**267**	**359**
(SEM)		**(2.8)**	**(0.5)**	**(4)**	**(4)**	**(2)**	**(8)**	**(14)**	**(18)**

Table 1A: Data of patients admitted with acute severe asthma, Chapter 3. (SBP/DBP, systolic / diastolic blood pressure)

Subject	RENIN (μU/l)			ANG II (pg/ml)		
	Admission	Day 2	Day 5	Admission	Day 2	Day 5
1	10	31	17	3	20	8
2	31	15	54	9	4	22
3	33	14	14	18	6	5
4	79	8	146	27	4	66
5	49	40	73	32	27	30
6	84	166	58	34	120	31
7	130	140	-	25	-	-
8	47	13	-	83	-	156
9	163	-	97	85	26	-
10	62	58	68	920	100	192
11	-	95	28	52	910	115
12	63	75	77	150	660	790
13	37	27	21	146	230	104
14	34	58	57	41	50	59
15	56	39	24	17	34	27
16	24	21	15	6	6	9
17	9	33	25	4	11	8
18	124	94	58	7	41	36
19	14	37	22	3	20	16
20	21	9	-	9	3	-

Table 2A: Individual plasma renin and angiotensin II (ANG II) levels in acute severe asthma, Chapter 3

		HAEMATOCRIT (%)		
Subject	**Sex**	**Admission**	**Day 2**	**Day 5**
1	M	0.45	0.46	0.45
2	F	0.44	0.42	0.42
3	M	0.46	0.43	0.42
4	F	0.45	0.43	0.41
5	M	0.47	0.43	0.43
6	F	0.46	0.41	0.37
7	F	0.44	0.39	0.39
8	F	0.46	0.46	0.46
9	F	0.42	0.38	0.42
10	F	0.44	0.43	0.44
11	F	0.40	0.37	0.38
12	F	0.43	0.43	0.44
13	M	0.44	0.45	0.38
14	F	0.42	0.41	-
15	F	0.43	0.43	0.44
16	M	0.44	0.44	0.44
17	F	0.45	0.46	0.44
18	F	0.42	0.42	0.40
19	M	0.44	0.40	0.40
20	F	0.39	0.37	0.39
		0.437*	**0.42**	**0.42**
		(0.01)	**(0.01)**	**(0.01)**

Table 3A: Individual haematocrit values in acute asthma patients, Chapter 3
(*p<0.05 vs Day 2 and Day 5)

Subject	Admission		Day 2		Day 5	
	NA	Adr	NA	Adr	NA	Adr
1	3.2	0.5	1.8	0.3	2.0	0.2
2	-	-	0.9	0.4	1.4	0.2
3	-	-	2.8	-	1.0	0.5
4	3.0	0.8	-	0.4	2.2	0.3
5	-	-	1.8	-	2.6	0.4
6	-	-	-	0.3	-	-
7	5.1	0.6	4.7	0.0	3.3	0.4
8	4.2	0.4	2.7	0.1	1.5	0.3
9	0.5	0.4	2.9	0.0	-	-
10	-	-	0.8	-	1.6	0.1
11	2.8	0.2	-	0.2	2.0	0.5
12	-	-	2.4	0.0	2.5	0.1
13	2.7	0.3	1.1	0.2	1.1	0.3
14	4.1	1.1	4.0	0.2	2.4	0.4
15	1.1	0.2	1.0	0.2	1.5	0.1
16	-	-	4.2	0.4	-	0.3
17	2.4	0.6	3.1	0.4	2.6	0.2
18	0.4	0.4	3.1	0.4	2.7	0.3
19	3.2	1.1	2.1	0.5	2.1	-
20	-	0.2	-	0.2	2.1	0.2
MEAN	**2.7**	**0.5**	**2.5**	**0.3**	**2.1**	**0.3**
(SEM)	**(0.5)**	**(0.1)**	**(0.4)**	**(0.2)**	**(0.4)**	**(0.2)**

Table 4A: Individual plasma catecholamine levels [Noradrenaline (NA) (nmol/l) and Adrenaline (Adr) (nmol/l)] in acute severe asthma, Chapter 3.

(No significant differences between Admission, Day 2 and Day 5)

Subject	Sex	Age (yrs)	SBP (mmHg)	DBP (mmHg)	PEFR (l/min)	0_2 Sat[n] (%)	Renin (µU/ml)	ANG II (pg/ml)
1	M	48	130	90	500	98	5	4
2	F	65	124	78	325	97	19	17
3	F	32	108	80	440	97	19	14
4	F	25	120	74	460	97	17	22
5	F	46	120	60	500	97	14	6
6	F	37	120	76	420	94	11	11
7	F	50	120	80	500	96	24	24
8	F	34	110	82	410	97	27	9
9	M	26	130	80	500	97	28	6
MEAN		**40.3**	**120**	**78**	**450**	**96.7**	**18.2**	**12.5**
(SEM)		**(4.3)**	**(3)**	**(3)**	**(20)**	**(0.4)**	**(2.5)**	**(2.4)**

Table 5A: Data of patients with mild chronic asthma, Chapter 3
(SBP/DBP, systolic/diastolic blood pressure. PEFR, peak expiratory flow rate, ANG II, Angiotensin II)

Subject	Sex	Age (yrs)	SBP (mmHg)	DBP (mmHg)	PEFR (l/min)	0_2 Sat^n (%)	Renin (μU/ml)	ANG II (pg/ml)
1	F	21	110	70	300	97	39	18.6
2	F	51	146	80	260	96	12	5.4
3	F	28	112	80	150	97	38	3.0
4	F	38	156	80	180	96	17	18.8
5	F	42	130	94	210	97	17	12.2
6	F	42	150	80	200	97	6	7.8
7	F	50	110	90	200	97	51	4.4
8	M	42	120	70	200	97	22	7.7
9	F	52	120	74	350	98	21	6.5
10	M	27	120	76	200	97	23	12
MEAN		**39.3**	**128**	**80**	**225**	**26.9**	**21.6**	**9.6**
(SEM)		**(4.2)**	**(5)**	**(2)**	**(19)**	**(0.2)**	**(4.4)**	**(1.8)**

Table 6A: Data of patients with severe chronic asthma, Chapter 3.
(SPB/DBP, systolic/diastolic blood pressure. PEFR, peak expiratory flow rate. ANG II, Angiotensin II)

Subject	Sex	Age (yrs)	SBP (mmHg)	DBP (mmHg)	PEFR (l/min)	0_2 Sat^n (%)	Renin (μU/ml)	ANG II (pg/ml)
1	F	28	115	75	510	98	9	11
2	F	36	120	70	510	96	20	29
3	F	39	110	70	440	98	22	17
4	M	43	120	80	490	98	12	10
5	F	38	110	75	530	98	21	48
6	F	22	120	70	480	96	12	8
7	M	59	118	80	520	98	5	9
8	F	29	120	80	560	96	25	10
9	F	22	144	68	440	98	16	6
10	F	24	150	74	480	99	4	9
11	F	45	110	70	480	99	22	4
12	M	52	120	78	620	99	20	11
13	F	40	118	80	480	99	20	10
14	F	42	120	80	520	97	40	17
15	F	24	140	66	520	99	65	23
16	M	44	130	70	520	98	27	9
MEAN		**36.6**	**122**	**74**	**506**	**98**	**21.2**	**14.4**
(SEM)		**(2.8)**	**(3)**	**(11)**	**(11)**	**(0.2)**	**(3.7)**	**(2.8)**

Table 7A: Data of control (non-asthmatic) subjects, Chapter 3
(SBP/DBP, systolic/diastolic blood pressure, PEFR, peak expiratory flow rate, ANG II, angiotensin II)

PLASMA RENIN (μU/ml)

Time (mins)	0	5	10	15	30	60	120	180
Placebo	31.5 (8.4)	41.1 (9.5)	31.6 (7.1)	32.1 (9.1)	36.1 (9.9)	43.0 (13.0)	29.5 (8.0)	32.7 (7.6)
Fenoterol	24.3 (4.4)	31.1 (9.3)	36.6 (11.5)	42.4 (13.8)	48.5 (19.7)	45.8 (15.0)	58.8 (19.4)	42.0 (13.3)
Salbutamol	30.4 (7.5)	35.0 (10.8)	47.3 (17.7)	40.7 (12.2)	44.6 (15.3)	45 (14.0)	51.7 (16.1)	44.7 (14.7)

Table 8A: Mean (SEM) plasma renin levels in asthmatic subjects (n=8) after inhalation of placebo, fenoterol (2.5 mg) or salbutamol (5 mg), Chapter 4.

	PLASMA RENIN (μU/ml)							
Time (mins)	**0**	**5**	**10**	**15**	**30**	**60**	**120**	**180**
Placebo	26.5 (6.7)	25.0 (7.3)	30.5 (6.8)	26.9 (5.5)	27.5 (7.1)	26.8 (5.6)	27.0 (4.7)	38.6 (16.9)
Fenoterol	32.8 (6.1)	48.8 (9.8)	47.0 (9.2)	45.6 (10.3)	38.9 (8.1)	41.7 (9.8)	55.1 (14.3)	38.5 (6.8)
Salbutamol	29.2 (6.3)	37.3 (6.8)	34.0 (34.5)	34.5 (7.3)	33.3 (8.4)	39.4 (11.2)	43.3 (14.8)	48.8 (13.5)

Table 9A: Mean (SEM) plasma renin levels in normal volunteers (n=8) after inhalation of placebo, fenoterol (2.5 mg) or salbutamol (5 mg) Chapter 4.

	PLASMA ANG II (pg/ml)							
Time (mins)	**0**	**5**	**10**	**15**	**30**	**60**	**120**	**180**
Placebo	8.6 (1.2)	6.3 (1.8)	6.1 (1.9)	10.1 (3.7)	11.1 (3.8)	8.6 (4.2)	7.3 (2.8)	7.0 (0.6)
Fenoterol	19.1 (8.4)	25.6 (8.1)	20.4 (7.2)	32.7 (17.1)	29.6 (12.3)	36.6 (16.5)	25.4 (10.7)	18.9 (7.8)
Salbutamol	8.1 (3.5)	22.4 (13.3)	21.1 (11.1)	22.1 (10.5)	20.6 (11.7)	20.7 (11.5)	26.1 (11.0)	23.0 (11.5)

Table 10A: Mean (SEM) plasma angiotensin II (ANG II) levels in asthmatic subjects (n=8) after inhalation of placebo, fenoterol (2.5 mg) or salbutamol (5 mg), Chapter 4.

	PLASMA ANG II (pg/ml)							
Time (mins)	**0**	**5**	**10**	**15**	**30**	**60**	**120**	**180**
Placebo	13.1 (3.9)	15.4 (4.7)	15.7 (4.4)	11.5 (3.7)	13.6 (3.5)	11.8 (2.9)	12.8 (3.8)	15.3 (6.8)
Fenoterol	11.0 (2.6)	17.1 (5.0)	19.4 (6.1)	13.8 (3.7)	12.6 (2.9)	20.7 (6.3)	15.7 (5.6)	14.3 (4.3)
Salbutamol	11.5 (3.2)	15.8 (5.6)	16.8 (5.7)	21.8 (5.3)	24.8 (6.5)	32.2 (10.9)	21.8 (6.2)	17.9 (4.8)

Table 11A: Mean (SEM) plasma angiotensin II (ANG II) levels in normal volunteers (n=8) after inhalation of placebo, fenoterol (2.5 mg) or salbutamol (5 mg), Chapter 4.

	SERUM POTASSIUM (mmol/l)							
Time (mins)	**0**	**5**	**10**	**15**	**30**	**60**	**120**	**180**
Placebo	4.5 (0.2)	4.5 (0.1)	4.5 (0.1)	4.4 (0.1)	4.5 (0.2)	4.4 (0.4)	4.4 (0.3)	4.6 (0.3)
Fenoterol	4.4 (0.2)	4.4 (0.3)	4.2 (0.4)	3.9 (0.2)	3.7 (0.2)	3.6 (0.2)	3.7 (0.3)	3.8 (0.4)
Salbutamol	4.3 (0.2)	4.1 (0.3)	4.1 (0.3)	4.0 (0.4)	3.9 (0.4)	3.8 (0.4)	4.0 (0.2)	4.1 (0.3)

Table 12A: Mean (SEM) serum potassium levels in asthmatic subjects (n=8) after inhalation of placebo, fenoterol (2.5 mg) or salbutamol (5 mg), Chapter 4.

	SERUM POTASSIUM (mmol/l)							
Time (mins)	**0**	**5**	**10**	**15**	**30**	**60**	**120**	**180**
Placebo	5.0 (0.1)	4.9 (0.1)	4.9 (0.1)	4.9 (0.2)	5.2 (0.3)	5.0 (0.3)	4.9 (0.2)	4.8 (0.3)
Fenoterol	4.8 (0.2)	4.6 (0.3)	4.5 (0.3)	4.4 (0.3)	4.2 (0.3)	4.0 (0.3)	4.0 (0.3)	4.3 (0.4)
Salbutamol	4.4 (0.2)	4.4 (0.2)	4.3 (0.3)	4.2 (0.3)	4.1 (0.2)	4.0 (0.3)	3.9 (0.4)	4.2 (0.3)

Table 13A: Mean (SEM) serum potassium levels in normal volunteers (n=8) after inhalation of placebo, fenoterol (2.5 mg) or salbutamol (5 mg), Chapter 4.

Time (mins)		P/P			S/P			S/S	
	Renin	ANG II	[K+]	Renin	ANG II	[K+]	Renin	ANG II	[K+]
0	15.3	10.6	4.3	15.6	6.1	4.2	14.7	6.9	4.2
	(3.2)	(2.5)	(0.1)	(2.6)	(0.7)	(0.1)	(4.5)	(1.3)	(0.1)
15	17.3	10.4	4.2	21.4	9.5	3.8	23.4	8.0	4
	(5.2)	(2.6)	(0.1)	(2.6)	(2.0)	(0.2)	(7.3)	(1.8)	(0.1)
30	16.4	9.4	4.3	26.1	11.3	3.4	25.1	10.4	3.9
	(4.6)	(2.1)	(0.1)	(4.4)	(1.9)	(0.1)	(8.5)	(2.6)	(0.1)
45	18.3	8.8	4.1	25.3	10.5	3.5	32.1	16.8	3.5
	(6.1)	(1.9)	(0.1)	(4.5)	(2.1)	(0.2)	(10.9)	(6.2)	(0.2)
60	19.4	9.6	4.1	23.4	8.4	3.5	28.0	11.6	3.4
	(5.5)	(2.0)	(0.1)	(4.2)	(1.8)	(0.2)	(7.6)	(3.5)	(0.2)
90	17.7	9.6	4.2	25.6	10.2	3.4	26.3	13.9	3.3
	(5.3)	(1.6)	(0.1)	(4.4)	(3.7)	(0.1)	(3.6)	(2.9)	(0.1)
120	14.2	11.0	4.3	21.9	8.5	3.4	28.6	12.0	3.3
	(2.1)	(2.3)	(0.2)	(2.9)	(1.9)	(0.1)	(4.2)	(1.9)	(0.1)
150	12.6	9.8	4.1	21.5	8.0	3.5	21.1	11.2	3.0
	(1.4)	(2.0)	(0.1)	(3.3)	(1.8)	(0.2)	(3.1)	(2.2)	(0.1)

Table 14A: Mean (SEM) plasma renin (µU/ml), angiotensin II (ANG II) (pg/ml) and serum potassium [K+] (mmol/l) after inhalation of P/P (placebo + placebo), S/P (salbutamol 5mg + placebo) or S/S (salbutamol 5mg + salbutamol 5mg) in asthmatic patients (n = 8), Chapter 5.

Time (mins)	SALBUTAMOL Renin	SALBUTAMOL ANG II	SALBUTAMOL [K+]	PLACEBO Renin	PLACEBO ANG II	PLACEBO [K+]
0	21.7	6.7	4.4	19.0	7.5	4.3
	(4.5)	(2.3)	(0.1)	(0.3)	(2.3)	(0.1)
30	22.6	5.3	4.3	18.8	6.5	4.3
	(5.8)	(1.3)	(0.2)	(5.8)	(2.1)	(0.1)
60	27.7	6.1	4.1	18.5	6.9	4.4
	(7.2)	(2.0)	(0.1)	(5.6)	(2.4)	(0.1)
90	26.8	6.1	3.8	16.3	5.7	4.4
	(5.9)	(2.6)	(0.2)	(4.4)	(1.4)	(0.2)
120	24.7	7.4	3.8	11.8	5.1	4.4
	(4.6)	(2.2)	(0.2)	(4.0)	(1.2)	(0.1)

Table 15A: Mean (SEM) plasma renin (μU/ml), angiotensin II (ANG II) (pg/ml) and serum potassium [K+] (mmol/l) after intravenous infusion of salbutamol (5, 10 and 20 μg/min) or placebo in asthmatic patients (n = 8), Chapter 5.

PLASMA RENIN (μU/ml)

Time (mins)	0	15	30	45	60	90	120
P/P	27.5 (5.1)	23.8 (5.6)	20.2 (4.7)	20.2 (4.7)	20.5 (4.5)	19.8 (5.4)	17.4 (4.9)
P/S	35.6 (8.3)	61.7 (15.6)	52.3 (11.1)	47.7 (9.8)	40.2 (7.9)	40.2 (7.9)	39.1 (7.3)
L/S	160.1 (20.6)	198.4 (18.9)	186.8 (17.9)	163.1 (13.9)	170.8 (14.3)	181.4 (17.8)	162.5 (18.4)
L/P	150.6 (3.0)	136.1 (21.5)	135.5 (29.3)	145.5 (32.1)	156.3 (3.5)	128.1 (34.5)	124.3 (27.1)

Table 16A: Mean (SEM) plasma renin levels in normal subjects (n=8) after P/P placebo/placebo, P/S placebo/salbutamol (5mg), L/S lisinopril (20mg)/salbutamol (5mg) and L/P, lisinopril (20mg)/placebo, Chapter 6.

	PLASMA ANG II (pg/ml)						
Time (mins)	**0**	**15**	**30**	**45**	**60**	**90**	**120**
P/P	9.5 (2.2)	6.4 (1.1)	5.4 (0.6)	5.6 (0.1)	6.9 (1.4)	4.9 (0.6)	4.7 (0.9)
P/S	8.2 (2.2)	17.7 (5.4)	13.9 (3.0)	12.8 (2.7)	9.9 (1.9)	10.7 (2.5)	9.3 (2.0)
L/S	1.4 (0.1)	1.5 (8.4)	1.9 (0.4)	1.3 (0.4)	1.4 (0.4)	1.7 (0.4)	1.8 (0.5)
L/P	1.3 (0.4)	1.5 (0.4)	1.5 (0.4)	1.2 (0.3)	1.3 (0.3)	1.5 (0.3)	1.7 (0.3)

Table 17A: Mean (SEM) plasma angiotensin II (ANG II) levels in normal subjects (n=8) after P/P placebo/placebo, P/S placebo/salbutamol (5mg), L/S lisinopril (20mg)/salbutamol (5mg) and L/P, lisinopril (20mg)/placebo, Chapter 6.

	SERUM POTASSIUM (mmol/l)						
Time (mins)	**0**	**15**	**30**	**45**	**60**	**90**	**120**
P/P	4.2 (0.1)	4.2 (0.1)	4.3 (0.1)	4.2 (0.1)	4.3 (0.0)	4.3 (0.0)	4.2 (0.1)
P/S	4.3 (0.1)	3.4 (0.2)	3.1 (0.2)	3.0 (0.1)	3.2 (0.1)	3.2 (0.1)	3.4 (0.2)
L/S	4.2 (0.1)	4.3 (0.2)	4.2 (0.0)	4.4 (0.2)	4.3 (0.1)	4.3 (0.1)	4.2 (0.1)
L/P	4.7 (0.1)	3.5 (0.5)	3.4 (0.1)	3.5 (0.1)	3.6 (0.2)	3.3 (0.6)	3.5 (0.1)

Table 18A: Mean (SEM) serum potassium levels (mmol/l) in normal subjects (n=8) after P/P placebo/placebo, P/S placebo/salbutamol (5mg), L/S lisinopril (20mg)/salbutamol (5mg) and L/P, lisinopril (20mg)/placebo, Chapter 6.

			SERUM A.C.E. (U/l)							
	Time (mins)	**Subject**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
P/P	0		30	45	28	41	48	56	33	29
	120		25	41	30	44	52	56	32	22
P/S	0		42	39	-	-	53	25	22	27
	120		41	36	-	-	55	29	27	27
L/S	0		<5	<5	<5	<5	5	14	<5	5
	120		<5	<5	<5	<5	5	<5	<5	<5
L/P	0		<5	<5	<5	10	13	20	9	<5
	120		<5	<5	<5	12	12	10	8	<5

Table 19A: Individual serum angiotensin converting enzyme (A.C.E.) levels (U/l) in normal subjects (n=8) after P/P placebo/placebo, P/S placebo/salbutamol (5mg), L/S lisinopril (20mg)/salbutamol (5mg) and L/P, lisinopril (20mg)/placebo, Chapter 6.

PLASMA RENIN (μU/ml)

Time (min)	0	10	20	30	45	60	90	120	150
Hyp/Plac	30.7 (6.9)	28.1 (7.0)	29.7 (6.6)	31.8 (5.8)	24.1 (4.6)	28.5 (5.0	23.0 (5.50)	22.0 (5.6)	19.0 (5.8)
Air/Plac	23.0 (5.0)	23.0 (4.5)	25.8 (5.3)	27.6 (7.7)	19.3 (4.5)	21.6 (3.5)	20.1 (3.6)	21.8 (4.3)	17.0 (3.7)
Hyp/Sal	29.0 (7.0)	28.2 (7.5)	28.7 (9.0)	44.7 (12.1)	41.4 (9.0)	39.0 (11.2)	31.7 (8.5)	31.8 (8.6)	28.7 (8.5)
Air/Sal	23.3 (1.8)	18.2 (5.2)	21.8 (6.2)	30.8 (7.1)	26.1 (4.2)	26.3 (4.8)	23.0 (5.0)	24.7 (4.6)	23.1 (4.0)

Table 20A: Mean (SEM) plasma renin in normal volunteers (n=8) after hyp/plac; hypoxia/placebo, air/plac; air/placebo, hyp/sal; hypoxia/salbutamol (5mg) and air/sal; air/salbutamol (5mg), Chapter 7.

	PLASMA ANG II (pg/ml)								
Time (min)	**0**	**10**	**20**	**30**	**45**	**60**	**90**	**120**	**150**
Hyp/Plac	19.2 (2.7)	17.9 (2.6)	18.7 (3.1)	18.9 (2.6)	14.8 (1.7)	17.0 (2.9)	13.8 (1.6)	12.1 (1.8)	12.8 (2.3)
Air/Plac	7.4 (2.6)	6.7 (1.8)	8.0 (2.6)	9.0 (2.3)	9.9 (3.1)	6.8 (2.0)	9.3 (3.3)	5.8 (1.5)	6.0 (2.2)
Hyp/Sal	7.5 (1.6)	8.4 (2.4)	7.9 (2.6)	14.7 (5.9)	12.8 (4.6)	13.1 (4.1)	10.9 (4.3)	8.0 (1.6)	8.7 (1.7)
Air/Sal	5.6 (1.2)	5.9 (1.6)	10.6 (4.7)	11.2 (2.6)	14.2 (4.7)	13.9 (5.1)	8.3 (1.8)	9.1 (2.4)	5.8 (8.7)

Table 21A: Mean (SEM) plasma angiotensin II (ANG II) levels in normal volunteers (n=8) after hyp/plac; hypoxia/placebo, air/plac: air/placebo, hyp/sal; hypoxia/salbutamol (5mg) and air/sal; air/salbutamol (5mg), Chapter 7.

PLASMA NORADRENALINE (nmol/l)

Time (min)	0	10	20	30	45	60	90	120	150
Hyp/Plac	2.60 (0.41)	2.88 (0.37)	3.29 (0.44)	3.45 (0.51)	3.94 (0.47)	3.40 (3.40)	3.74 (0.66)	3.40 (0.46)	3.40 (0.55)
Air/Plac	2.60 (0.21)	2.20 (0.21)	2.20 (0.25)	2.10 (0.30)	2.41 (0.18)	2.74 (0.34)	2.54 (0.33)	2.54 (0.19)	2.60 (0.55)
Hyp/Sal	2.60 (0.36)	2.40 (0.27)	2.68 (0.32)	3.43 (0.32)	3.40 (0.4)	2.91 (0.28)	2.80 (0.29)	2.60 (0.35)	2.50 (0.43)
Air/Sal	2.60 (0.40)	2.89 (0.52)	4.25 (0.49)	4.34 (0.49)	4.20 (0.56)	4.43 (0.76)	3.72 (0.58)	3.60 (0.53)	3.71 (0.79)

Table 22A: Mean (SEM) plasma noradrenaline levels in normal volunteers (n=8) after hyp/plac; hypoxia/placebo, air/plac; air/placebo, hyp/sal; hypoxia/salbutamol (5mg) and air/sal; air/salbutamol (5mg), Chapter 7.

	PLASMA NORADRENALINE (nmol/l)								
Time (min)	**0**	**10**	**20**	**30**	**45**	**60**	**90**	**120**	**150**
Hyp/Plac	2.60 (0.41)	2.88 (0.37)	3.29 (0.44)	3.45 (0.51)	3.94 (0.47)	3.40 (3.40)	3.74 (0.66)	3.40 (0.46)	3.40 (0.55)
Air/Plac	2.60 (0.21)	2.20 (0.21)	2.20 (0.25)	2.10 (0.30)	2.41 (0.18)	2.74 (0.34)	2.54 (0.33)	2.54 (0.19)	2.60 (0.55)
Hyp/Sal	2.60 (0.36)	2.40 (0.27)	2.68 (0.32)	3.43 (0.32)	3.40 (0.4)	2.91 (0.28)	2.80 (0.29)	2.60 (0.35)	2.50 (0.43)
Air/Sal	2.60 (0.40)	2.89 (0.52)	4.25 (0.49)	4.34 (0.49)	4.20 (0.56)	4.43 (0.76)	3.72 (0.58)	3.60 (0.53)	3.71 (0.79)

Table 22A: Mean (SEM) plasma noradrenaline levels in normal volunteers (n=8) after hyp/plac; hypoxia/placebo, air/plac; air/placebo, hyp/sal; hypoxia/salbutamol (5mg) and air/sal; air/salbutamol (5mg), Chapter 7.

PLASMA ADRENALINE (nmol/l)

Time (min)	0	10	20	30	45	60	90	120	150
Hyp/Plac	0.15 (0.03)	0.15 (0.03)	0.15 (0.02)	0.15 (0.02)	0.17 (0.03)	0.16 (0.03)	0.17 (0.03)	0.15 (0.02)	0.16 (0.02)
Air/Plac	0.15 (0.02)	0.04 (0.12)	0.14 (0.02)	0.16 (0.02)	0.16 (0.02)	0.16 (0.02)	0.16 (0.02)	0.15 (0.02)	0.17 (0.03)
Hyp/Sal	0.34 (0.10)	0.34 (0.19)	0.51 (0.31)	1.18 (0.64)	0.6 (0.36)	0.95 (0.52)	0.74 (0.38)	0.30 (0.36)	0.30 (0.14)
Air/Sal	0.15 (0.03)	0.15 (0.02)	0.20 (0.02)	0.19 (0.03)	0.19 (0.03)	0.19 (0.03)	0.18 (0.02)	0.17 (0.01)	0.18 (0.03)

Table 23A: Mean (SEM) plasma adrenaline levels in normal volunteers (n=8) after hyp/plac; hypoxia/placebo, air/plac; air/placebo, hyp/sal; hypoxia/salbutamol (5mg) and air/sal; air/salbutamol (5mg), Chapter 7.

OXYGEN SATURATION (%)

Time (min)	0	10	20	30	45	60	90	120	150
Hyp/Plac	97.8 (0.7)	83.4 (2.0)	87.4 (2.3)	85 (1.9)	97.7 (0.9)	98.8 (0.5)	98.8 (0.4)	99.0 (0.3)	96.1 (1.6)
Air/Plac	96.4 (0.8)	96.4 (0.8)	98 (0.3)	97.4 (0.9)	96.1 (0.5)	98.8 (0.4)	98.8 (0.4)	98.8 (1.4)	97.0 (1.2)
Hyp/Sal	96.1 (1.6)	82.8 (1.5)	87.6 (3.9)	84.5 (3.0)	98.7 (8.6)	98.7 (0.4)	99 (0.4)	99.2 (0.3)	98.8 (0.3)
Air/Sal	97.8 (1.2)	97.8 (1.2)	97.8 (1.2)	98.0 (0.9)	98.4 (0.4)	98.6 (0.5)	99.0 (0.3)	99.0 (0.4)	97.4 (0.3)

Table 24A: Mean (SEM) oxygen saturations in normal volunteers (n=8) after hyp/plac; hypoxia/placebo, air/plac; air/placebo, hyp/sal; hypoxia/salbutamol (5mg) and air/sal; air/salbutamol (5mg), Chapter 7.

HEART RATE (beats/min)

Time (min)	0	10	20	30	45	60	90	120	150
Hyp/Plac	82 (3)	95 (4)	95 (5)	92 (5)	84 (4)	82 (4)	82 (4)	78 (3)	78 (6)
Air/Plac	85 (4)	89 (6)	84 (5)	83 (5)	80 (3)	79 (3)	76 (6)	78 (6)	80 (4)
Hyp/Sal	83 (5)	97 (7)	118 (7)	125 (5)	107 (4)	107 (4)	95 (4)	92 (4)	92 (4)
Air/Sal	82 (4)	86 (5)	105 (6)	111 (5)	102 (5)	96 (3)	92 (4)	88 (4)	80 (5)

Table 25A: Mean (SEM) heart rate in normal volunteers (n=8) after hyp/plac; hypoxia/placebo, air/plac; air/placebo, hyp/sal; hypoxia/salbutamol (5mg) and air/sal; air/salbutamol (5mg), Chapter 7.

PLASMA ANG II (pg/ml)

		Placebo				**ANG II**			
Subject	**Time (mins)**	**0**	**30**	**60**	**90**	**0**	**30**	**60**	**90**
1		9	12	10	12	13	19	44	53
2		8	6	6	6	7	35	91	115
3		7	5	8	9	11	45	54	221
4		7	8	7	5	24	22	62	96
5		19	17	15	15	12	16	18	128
6		17	10	8	10	7	26	29	89
7		30	30	27	25	13	29	72	147
8		11	10	8	10				
MEAN		**12.8**	**12.2**	**11.1**	**11.5**	**12.4**	**25.1**	**55.9**	**121.3**
(SEM)		(7.9)	(8.8)	(6.9)	(6.3)	(5.0)	(4.0)	(10.2)	(20.2)

Table 26A: Plasma angiotensin II (ANG II) levels in asthmatic subjects after infusion of angiotensin II (ANG II) (2, 4 and 8 ng/kg/min) or placebo, Chapter 8.

SYSTOLIC BLOOD PRESSURE (mmHg)

		Placebo				ANG II			
Subject	**Time (mins)**	**0**	**30**	**60**	**90**	**0**	**30**	**60**	**90**
1		130	139	140	130	138	142	168	160
2		140	130	139	141	130	152	192	-
3		122	127	125	110	108	120	118	134
4		124	122	119	122	131	130	142	96
5		138	128	134	134	136	154	158	170
6		120	108	114	110	116	126	136	155
7		126	126	113	120	144	165	182	180
8		162	170	169	170	148	148	150	163
MEAN		**132**	**131**	**131**	**131**	**130**	**142**	**154**	**154**
(SEM)		(5)	(6)	(6)	(6)	(5)	(5)	(9)	(7)

Table 27A: Systolic blood pressure responses to infused angiotensin II (ANG II) (2, 4 and 8 ng/kg/min) and placebo in asthmatic subjects, Chapter 8.

		FEV$_1$ (litres)							
		Placebo				ANG II			
Subject	**Time (mins)**	**0**	**30**	**60**	**90**	**0**	**30**	**60**	**90**
1		2.00	2.00	2.01	1.98	1.90	1.69	1.53	1.53
2		3.28	3.14	3.29	3.22	3.23	3.03	3.01	2.65
3		2.08	1.87	1.90	1.92	1.93	1.83	1.84	1.73
4		2.72	2.70	2.72	2.70	2.01	1.93	1.86	1.75
5		4.89	4.80	4.98	4.95	5.22	5.03	4.74	4.51
6		4.22	4.32	4.19	4.15	4.56	4.58	5.01	4.87
7		3.81	3.81	3.86	3.80	3.01	3.0	2.84	2.48
8		3.15	3.30	3.27	3.30	2.79	2.70	2.34	-
MEAN		3.27	3.24	3.26	3.25	3.08	2.97	2.89	2.79
(SEM)		(0.30)	(0.37	(0.37)	(.57)	(0.44)	(0.40)	(0.40)	(.52)

Table 28A: Spirometric response (FEV$_1$) to infusion of angiotcnsin II (ANG II) (2, 4 and 8 ng/kg/min) and placebo in asthmatic subjects, Chapter 8.

		PC_{20} to methacholine (mg/ml)	
Subject	**Placebo**	**ANG II 1ng/kg/min**	**ANG II 2ng/kg/min**
1	2.49	1.77	0.46
2	2.56	0.86	0.80
3	6.0	3.77	2.08
4	3.53	3.45	1.36
5	4.14	1.71	0.84
6	1.14	2.33	2.00
7	4.14	2.12	2.00
Geometric Mean	**0.49**	**0.33**	**0.08**
(Range)	**(0.06 - 0.78)**	**(-0.77 - 0.58)**	**(-0.34 - 0.32)**

Table 29A: Individual PC_{20} valves for inhaled methacholine in asthmatic subjects, after infusion of placebo, angiotensin II (ANG II) 1ng/kg/min and 2ng/kg/min., Chapter 9.

	Baseline (t = 0 mins)			Before MCh Challenge (t = 30 mins)			After MCh Challenge (t = 60 mins)		
				← INFUSION OF DRUG →					
Subject	Placebo	ANG II 1ng/kg/min	ANG II 2ng/kg/min	Placebo	ANG II 1ng/kg/min	ANG II 2ng/kg/min	Placebo	ANG II 1ng/kg/min	ANG II 2ng/kg/min
1	-	2.7	8.6	11.2	16.5	36.6	9.2	46.2	23.1
2	12.9	10.7	2.3	14.0	15.4	40.1	14.3	27.8	73.9
3	8.4	4.0	6.0	12.1	17.3	21.4	12.4	17.4	32.3
4	4.3	4.0	4.1	3.9	14.9	28.3	5.4	28.7	21.0
5	8.0	5.3	2.5	10.2	19.6	32.2	-	16.5	-
6	7.5	3.7	11.8	3.0	13.2	38.6	7.2	31.7	48.3
7	14.1	5.6	6.6	12.2	16.5	-	10.9	22.8	27.6
MEAN	**9.2**	**5.1**	**6.0**	**9.5**	**16.2**	**32.9**	**9.9**	**27.3**	**37.7**
(SEM)	**(3.8)**	**(1.2)**	**(1.5)**	**(2.0)**	**(0.9)**	**(5.1)**	**(1.8)**	**(5.3)**	**(9.5)**

Table 30A: Individual plasma angiotensin II (ANG II) levels (pg/ml) at baseline, after 30 minute infusion of placebo, ANG II, 1 or 2 ng/kg/min (before methacholine (MCh) challenge), and after MCh challenge, Chapter 9.

Concentration of MCh (M)	% Control Maximum Response			
	MCh		MCh + ANG II 10^{-7} M	
10 - 9	0.17	(0.17)	0.67	(0.67
3 x 10 - 9	1.59	(1.03)	1.28	(0.81)
10 - 8	3.60	(1.74)	4.53	(1.7)
3 x 10 - 8	6.66	(2.25)	8.77	(2.90)
10 - 7	10.74	(3.98)	16.44	(5.99)
3 x 10 - 7	24.38	(6.12)	33.4	(9.95)
10 - 6	34.27	(6.35)	50.5	(11.17)
3 x 10 - 6	53.15	(6.84)	77.8	(11.52)
10 - 5	70.78	(6.61)	108.76	(19.59)
3 x 10-5	87.03	(3.42)	126.42	(16.29)
10 - 4	93.5	(2.36)	133.89	(15.55)
3 x 10 - 4	98.19	(1.81)	137.51	(14.99)
EC_{50}	**5.6± 0.17**		**6.06±0.23**	

Table 31A: Mean (SEM) cumulative contraction to methacholine (MCh) in human bronchi in the presence and absence of angiotensin II (ANG II) 10^{-7} M. Results expressed as % control maximum response, $pD_2 = -\log EC_{50}$, Chapter 9.

PUBLICATIONS ARISING FROM THE WORK IN THIS THESIS.

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3. Millar EA, Angus RM, Nally JE, Clayton R,Thomson NC. The effect of hypoxia and ß2 agonists on the renin-angiotensin system. Clin Science 1995; 89: 273-276.

4. Millar EA, Nally JE, Thomson NC. Angiotensin II potentiates methacholine induced bronchoconstriction in mild asthmatic patients. Eur Resp J 1995;8 (11):1838-1841.

5. Millar EA, Connell JMC, Thomson NC. The effect of nebulised albuterol on the activity of the renin-angiotensin system in asthma. Chest 1996 (submitted).

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