

**STUDIES ON THE ANGIOTENSIN CONVERTING  
ENZYME GENE POLYMORPHISM  
AND ACE INHIBITORS**

A Thesis submitted for the degree of

**DOCTOR OF MEDICINE**

by Dr Ian George Chadwick

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Dedicated to my wife Helen

-for her love and support given to me during this work

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Finally, I thank my family for persisting with my efforts and absences whilst I prepared this thesis.

## Declaration of Authorship

This is to declare that I, Ian George Chadwick, have composed this thesis and am the author and primary investigator of the data within it. I have not previously submitted this work for a degree or professional qualification.

In all the studies herein I have taken a major part in design, undertaking and analysis.

However, I also state that other researchers within our group have assisted me during different stages of the work for this thesis, mainly providing assistance with recruitment and in performing the studies. Specifically assistance was given by Dr L O'Toole in chapter 3, Dr G Todd in chapter 4, and Dr M Krasciwicz in chapter 5, 6 and 8.



## Abstract

Angiotensin converting enzyme (ACE) converts angiotensin I to angiotensin II, an important step in the control of blood pressure. The gene encoding for ACE is subject to an insertion/deletion (I/D) polymorphism which is associated with different levels of the enzyme in serum. This polymorphism accounts for 47% of the variability in serum ACE concentrations between subjects but its relevance to tissue ACE is unknown. ACE inhibition increases kinin level, for example bradykinin. Kinins have been proposed be involved in the pathogenesis of cough due to ACE inhibitors, a common adverse effect in those prescribed these drugs. A genetic link was proposed to explain the differing susceptibility of subjects to develop cough. Those susceptible may differ in the cough reflexes initially or perhaps have different degrees of tissue ACE activity. In this thesis I examined healthy subjects using substrates and inhibitors of ACE to identify any possible differences in tissue ACE between those of different ACE genotype. I also examined the natural history of ACE inhibitor cough in particular changes in cough reflex and the possible roles of kinins in its aetiology. The frequency of ACE genotype amongst those who developed cough was also examined.

- a) The pressor response to angiotensin I was examined in 16 healthy subjects (DD, n=8; and II, n=8). The R(d)25 was the rate of angiotensin I infusion which caused a 25 mmHg rise in diastolic blood pressure. The geometric mean rate of infusion of angiotensin I required to achieve the R(d)25 was 2.53µg/min in II subjects and 2.67µg/min in DD subjects (ratio of doses (II:DD) = 0.95; 95% CI 0.44 to 2.02, p>0.05).
- b) The hypotensive response and serum ACE responses to an ACE inhibitor drug were examined in 27 healthy subjects grouped according to ACE genotype. Although changes after enalapril paralleled those differences seen between the different genotypes before no differences were seen between the groups when hypotensive responses were examined.
- c) A prospective controlled study comparing subjects taking ACE inhibitors (n=21) with those taking other anti-hypertensives (n=12) examined the cough reflex changes that occur during prolonged treatment using capsaicin challenge. A new cough developed in 8 subjects. There were no differences in capsaicin sensitivity between groups at baseline. At one month a significant increase in capsaicin potency from baseline was observed for patients with enalapril cough (relative potency 4.7, 95% CI 1.2-18.5, p<0.025).
- d) A prospective study examined different subjects (n=8) with cough as the ACE inhibitors were discontinued and confirmed the change in cough sensitivity at day 28, this time to lie in the opposite direction. (relative potency 0.2, 95% CI 0.1-0.7, p<0.05)
- e) The dermal responses to bradykinin were examined in 105 healthy subjects (DD genotype, n=30; ID genotype, n=51; and II genotype n=24). Using parallel line analysis the potency of bradykinin in subjects of II genotype relative to those of DD genotype was 1.25, with a 95% confidence interval of 0.83 to 1.88.
- f) The cough response to bradykinin was examined in 27 healthy subjects (DD, n=9; ID, n=9; and II, n=9). Bradykinin produced cough in 74% of subjects but there were no differences in the cough responses between genotypes.
- g) The distribution of ACE genotypes was examined in 31 patients with cough due to ACE inhibitor. No increase in frequency of one genotype over another was identified. (DD, n=12; ID, n=13; II, n=6)

In conclusion the I/D polymorphism in the ACE gene could not predict responses to either substrates of ACE or ACE inhibitor drugs in healthy subjects. The cough due to ACE inhibitors leads to reversible changes in cough reflex sensitivity. Individuals who develop cough do not seem to differ in their initial cough reflexes nor in their genotype in the I/D polymorphism of the ACE gene.

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Declaration of authorship

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## Chapter 1

### INTRODUCTION

## 1.1 HISTORY OF THE RENIN-ANGIOTENSIN SYSTEM

It is almost 100 years since Tigerstedt and Bergman initiated research into the renin-angiotensin system. In 1898 whilst trying to isolate the substance thought to cause uraemia they identified a substance from rabbit renal cortex, named renin, which when injected caused a rise in blood pressure (Tigerstedt 1898). These findings were not easily reproducible and their importance not appreciated at the time. It was many years later before interest in renin was rekindled. The focus of circulatory research at that time was to establish a cause for hypertension, with particular emphasis on renal causes. Goldblatt's group proposed that narrowing of the renal artery caused hypertension and demonstrated this by becoming one of the first to develop a stable model for experimental hypertension (Goldblatt 1934).

Renin was active only when mixed with plasma suggesting that there was a co-factor within plasma which facilitated vasoconstriction. Later isolated by Page and Helmer this co-factor was named angiotonin (Page & Helmer 1940) and is now known as angiotensin. In 1939 Braun-Menendez et al purified a pressor substance from ischaemic kidneys which was active without the presence of renin. He called this substance hypertensin and it was subsequently renamed angiotensin (Braun-Menendez et al 1939).

During the 1950s there was intense competition to isolate the angiotensins. Skeggs showed that two smaller molecular weight proteins named by him hypertensins I and 2 (later renamed angiotensins I and II) were formed from angiotensinogen (Skeggs et al 1954). Angiotensin converting enzyme (ACE), the enzyme producing angiotensin II, was characterised by Skeggs as a chloride-dependant metallopeptidase (Skeggs et al 1956).

The development of specific inhibitors of the renin-angiotensin system in the 1970s led to opportunities for therapeutic intervention and manipulation in this area. Peptides derived from the venom of the snake *Bothrops Jararaca* were found to inhibit an enzyme involved in bradykinin metabolism, kininase II (Ferreira 1965), now considered identical to ACE (Yang et al 1970). The major stimulus for fractionation of this mixture was the demonstration that these peptides also had a potent inhibitory action on the conversion of angiotensin I to II. Among the peptides present in the venom a nonapeptide, teprotide, was the most thoroughly studied (Antonaccio & Cushman 1981). In man teprotide was apparently safe and effective as an antihypertensive drug but its peptide structure necessitated parenteral administration. Teprotide raised the awareness of pharmacologists to the potential of manipulation of the renin-angiotensin system as a method of blood pressure control. This heralded a systematic search for oral inhibitors of angiotensin converting enzyme. The search for a simple non-peptide that would interact with high affinity at the active site of ACE led to the development of captopril by Cushman et al in 1977. Captopril resembles the carboxyl terminal dipeptide residue of teprotide (Cushman et al 1977). The presence of a sulphhydryl group contributed greatly to its enzyme binding affinity. Enalapril was the next ACE inhibitor developed by

Patchett et al in 1980 differing from captopril in that the zinc ligand by which it binds to the active site is a carboxyl group. ACE inhibitors have now become established therapy for hypertension and heart failure. Tolerated well by most who take them they are now in widespread use. A troublesome cough developing during treatment is probably the most common side-effect now seen, first recognised in 1985 by Sesoko.

In 1990 Rigat et al described a polymorphism of the ACE gene consisting of the presence or absence of an insertion. This insertion/deletion (I/D) polymorphism accounted for 47% of the variance of serum ACE activity when studied within healthy volunteers (Rigat 1990). Further interest in the possible phenotypic relevance of this polymorphism and its role in the renin-angiotensin system was provoked by the work of Cambien et al in 1992 who noted certain genotypic relationships of this polymorphism with the risk of ischaemic heart disease.

## 1.1 AIMS OF THIS THESIS

The renin-angiotensin system is now recognised as one of the most important regulatory systems in blood pressure homeostasis. This thesis aims to examine various aspects of the renin-angiotensin system, in particular angiotensin converting enzyme (ACE) and its inhibition; whether a genetic variation in the ACE gene might affect the response to substrates of ACE; and the propensity of ACE inhibition to cause cough.

The specific aims are outlined below.

1. To determine whether phenotypic differences exist between individuals of different genotype for the I/D ACE gene polymorphism with regard to substrates of ACE as indicated by:

- a) The pressor and neurohormonal responses to angiotensin I
- b) The hypotensive responses to ACE inhibitor drugs

2. To study prospectively aspects of the cough caused by ACE inhibitor drugs and relationship to the I/D ACE gene polymorphism, in particular:

- a) The effect of treatment with ACE inhibitors on the cough reflex in hypertensive subjects
- b) The natural history of ACE inhibitor cough when treatment is withdrawn.
- c) The effects of ACE inhibitors on the dermal wheal responses to bradykinin.
- d) The dermal wheal response to bradykinin and relationship to ACE genotype .
- c) The cough response to bradykinin and relationship to ACE genotype.
- c) The I/D allele frequency in those with ACE inhibitor cough.

## 1.2 BIOCHEMISTRY OF THE RENIN-ANGIOTENSIN SYSTEM

The renin-angiotensin system (RAS) is composed of a series of peptides and enzymes which together are involved in maintaining vascular tone. The combination of the pressor effects and the effects on electrolytes and fluid balance exert a major influence on blood pressure control. This system depends on a sequence of enzymatic reactions which result in peptide activation. The initial step is the conversion of angiotensinogen to angiotensin I by renin. Thereafter angiotensin I is converted to the active peptide angiotensin II by angiotensin converting enzyme (ACE). Angiotensin II acts through a membrane bound receptor leading to a variety of responses depending on the target tissue (Table 1.1). The main actions are vasoconstriction and release of aldosterone.

**Angiotensinogen** is a large glycoprotein composed of approximately 400 amino acids with a molecular weight of 47,000 to 66,000 (Eggena et al 1976). Most is synthesised in the liver but other tissues such as the kidney and brain are capable of producing angiotensinogen. Production is enhanced by oestrogens and glucocorticoids (Peach 1984).

**Renin** is a glycoprotein with a molecular weight of 35,000 to 42,000. This acid aspartyl protease cleaves the leucine-10-valine-11 bond from the aspartic (amino) end of angiotensinogen to release angiotensin I (Yukosawa et al 1980). It has high substrate specificity with angiotensinogen its only known substrate (Peach 1977). This is considered the rate-limiting step of the RAS and the rate of renin secretion ultimately determines the activity of the renin-angiotensin system (Skeggs et al 1967). Renin is synthesised, stored and secreted mainly within the juxtaglomerular cells of the kidney. Stimulants to renin release include vascular stretch of the afferent arteriole, the electrolyte content within the distal tubule (sensed by the macula densa), and the sympathetic nervous system. Most circulating renin is composed of inactive renin which has a higher molecular weight than its active counterpart but has identical enzymatic characteristics when activated. The conversion of renin into its active form in-vivo remains undetermined.

**Angiotensin I** is a decapeptide pro-hormone released from angiotensinogen. Its cleavage, with the subsequent release of angiotensin II, is catalysed by angiotensin-converting enzyme (ACE) or kininase II and occurs mainly on vascular endothelium throughout the body but probably chiefly in the lungs (Ng & Vane 1968).

Angiotensin I is unlikely to have any pharmacological activity, whereas its product, angiotensin II, is a potent vasoconstrictor, and also stimulates the release of aldosterone with consequent actions on sodium, potassium and water homeostasis.

**Angiotensin converting enzyme (EC 3.4.15.1; ACE)** or kininase II is a glycoprotein with a molecular weight of 130,000 - 140,000. It cleaves the dipeptide histidyl-leucine from the carboxyl terminal of the decapeptide angiotensin I and is classified as a dipeptidyl carboxypeptidase (Kokubu & Takada 1987).

**Angiotensin II** is the physiologically active octapeptide of the renin-angiotensin system. It acts through specific membrane bound receptors which are coupled to second messenger systems such as the phospholipase C-phosphatidylinositol and the adenylate cyclase-cyclic AMP system (Timmermans 1993). The development of antagonists to angiotensin receptors has led to identification of several subtypes of receptor with different actions. At present all the effects discussed below are considered to be due to mediated through the AT<sub>1</sub> receptor, with the role of the AT<sub>2</sub> less clear (Timmermans 1993).

Angiotensin II produces an increase in blood pressure mainly through vasoconstriction having a potency approximately 40 times greater than that of noradrenaline. When infused intravenously it elevates arterial pressure within seconds (Reid 1985), as a direct consequence of an increase in systemic vascular resistance. Contraction of vascular smooth muscle occurs by two mechanisms: (i) direct action of angiotensin II on smooth muscle and (ii) stimulation of the sympathetic nervous system. Angiotensin II may also cause increases in blood pressure via a central effect as angiotensin II receptors have been located in the area posterior to the medulla oblongata which lacks a blood-brain barrier (Ganong 1984).

Angiotensin II has a strong stimulatory effect on the adrenal glands causing the secretion of aldosterone (Gann et al 1964) with subsequent effects of sodium retention and water homeostasis. Within the kidney angiotensin II causes vasoconstriction of the efferent arteriole and subsequent effects at the juxtaglomerular apparatus inhibit the secretion of renin. It may also have a direct effect on the proximal tubule to promote sodium retention. Other physiological effects of angiotensin II include a direct action on atrial and ventricular cardiac muscle producing a pronounced plateau of the action potential and an increased force of contraction, but no direct effect on heart rate.

Although most angiotensin II is produced by ACE alternative production of angiotensin II occurs by non-ACE enzymatic pathways, notably human cardiac chymase (Urata 1993). Angiotensin II is degraded by aminopeptidase-N which cleaves an amino acid to form angiotensin III, a much less potent vasoconstrictor.



Table 1.1      Actions of angiotensin II

---

1. Vasoconstriction (Gunther 1982)
  2. Aldosterone secretion from adrenal zona glomerulosa (Douglas 1979)
  3. Sympathetic activity noradrenaline release from renal sympathetic nerves  
central enhancement (Summers 1983)  
peripheral facilitation (Zimmerman 1981)
  4. Vagal inhibition (Ajayi 1985)
  5. Cardiac stimulation (Holubarsch 1993)
  6. Vasopressin release from posterior pituitary
  7. Renal effects  
Inhibition of renin release (negative feedback) (Menard 1991)  
Vasoconstriction (efferent > afferent) (Myers 1975)  
Increased tubular sodium absorption (Wald 1991)  
Glomerular mesangial constriction (Brenner 1982)
  8. Stimulation of proto-oncogene *c-fos* (Kawahara 1988)
  9. Dipsogenic actions (Fitzsimmons 1969)
-

### 1.3 ANGIOTENSIN CONVERTING ENZYME (ACE)

Skeggs established that ACE is a chloride dependent metallopeptidase with dipeptidyl carboxypeptidase activity (Skeggs et al 1956). ACE is primarily involved in two important physiological reactions; i) the conversion of the inactive decapeptide angiotensin I to the potent vasoconstrictor angiotensin II, and ii) the degradation of the bradykinin.

#### 1.3.1 DIFFERENT FORMS OF ACE

Three forms of ACE are found in man. Most angiotensin converting enzyme is bound to the membrane of endothelial cells. However there is also a soluble form of the enzyme found in plasma, and a smaller isoenzyme found in testicular tissue (Soubrier 1993).

Immunohistochemical techniques have demonstrated that ACE is present within the vascular endothelium of most organs, located principally on the luminal surface and in the caveolae of endothelial cells (Caldwell et al 1976). ACE is attached to the surface membrane at its carboxyl terminus so that the active sites are directed extracellularly (Figure 1.1). ACE is also expressed on the membrane of renal epithelium, human placental microvilli, choroid plexus, prostatic tissue and the monocyte-macrophage system (Vuk-Pavlovic et al 1989, Caldwell et al 1976, Ng & Vane 1968). The membrane form of ACE is often referred to as tissue ACE however this term is often used rather loosely. The most common usage of the term tissue ACE refers to *all* activity of ACE within a certain organ or tissue preparation, for example reference to cardiac ACE would mean the activity of the membrane bound form and plasma ACE (Samani 1991, Morgan 1994). Some in-vitro research involves isolated preparations of tissue and in this context tissue ACE refers to the membrane form only (Morgan 1994).

ACE was first discovered in horse serum (Skeggs et al 1956), and ever since, the measurement of ACE has largely been of the serum or soluble form. Soluble ACE is believed to be derived by enzymatic release from the membrane bound form by an enzyme known as secretase (Oppong 1993). Studies of this form of ACE have shown that the soluble form lacks the carboxyl terminus which is considered to be the membrane anchor (Soubrier 1993). At present the relationship between the membrane bound form and the soluble serum ACE remains uncertain.

The function of the structurally smaller form of ACE, testicular ACE, remains unknown.

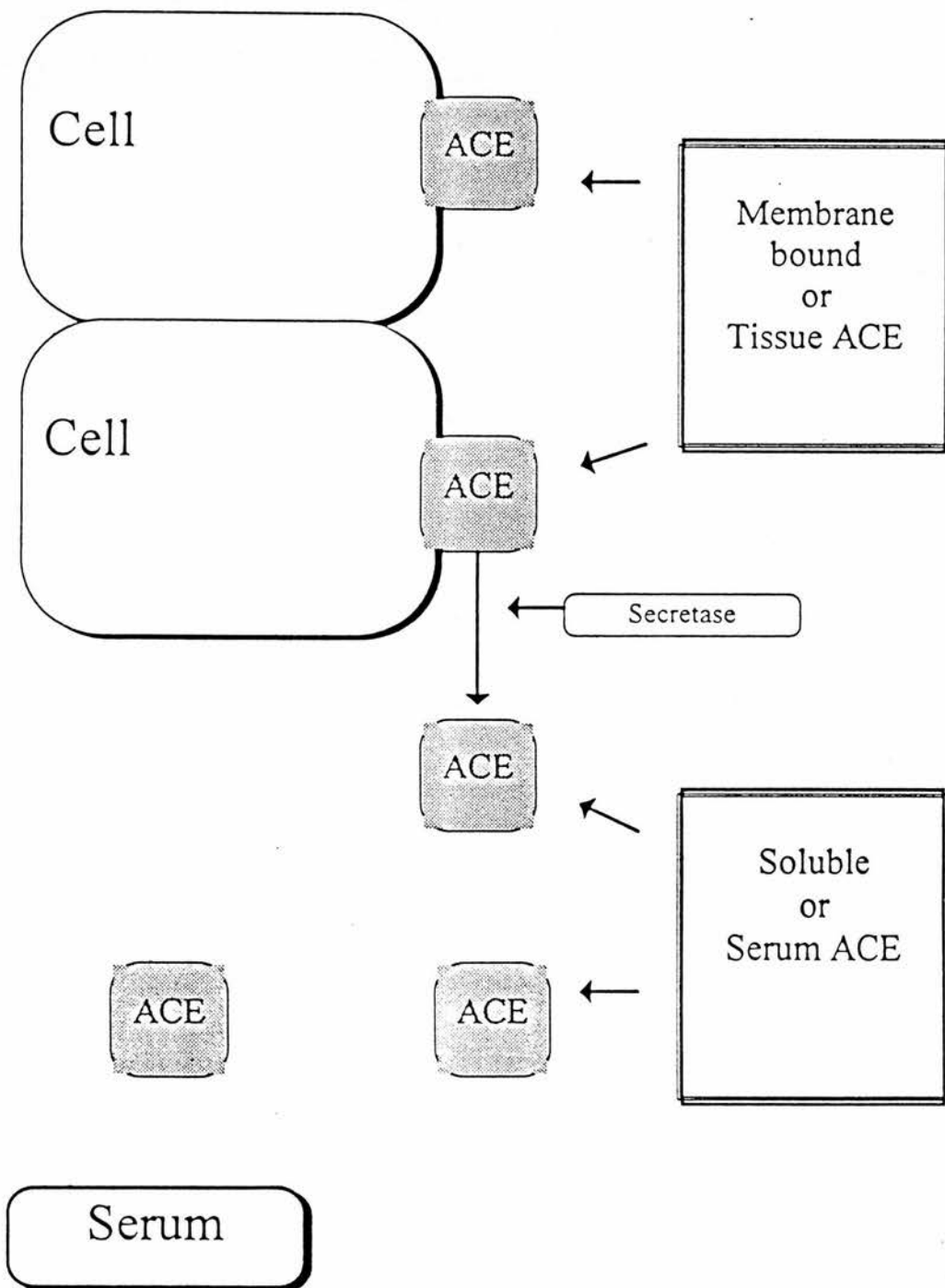


Figure 1.1 The different location and nomenclature of ACE.

### 1.3.2 STRUCTURE AND ENZYMATIC PROPERTIES

The structure of human ACE has been established by a combination of biochemical and cDNA sequencing (Soubrier et al 1988). The endothelial ACE molecule is composed of two large domains with a high degree of internal homology between them, suggesting an origin by gene duplication. Within each of these homologous domains are found short amino acid sequences identical to those at the active sites of other metallopeptidases such as neutral endopeptidase (Soubrier et al 1988). Thus each domain has in its structure a potentially active site. Each active site contains one zinc atom. The two sites may differ in substrate specificity (Soubrier 1993). A highly hydrophobic region close to the carboxyl-terminal end of the molecule probably acts as the anchor to the plasma membrane (Sen et al 1991).

### 1.3.3 SUBSTRATES OF ACE

It was the specific cleavage of a dipeptide residue from the COOH-terminal of angiotensin I which first brought the attention of biochemists to ACE. We now know that a large variety of peptides with different C-terminal amino acid sequences can act as a substrate to ACE (Table 1.2). The necessary requisites for an ACE substrate are: (i) an amino acid with a free carboxylic acid function at the C-terminal end and (ii) an amino acid other than proline on the penultimate position. Other endogenous peptides hydrolysed by ACE are enkephalins, substance P and substance K (Theile et al 1985), neurotensin (Skidgel et al 1984),  $\beta$ -lipotrophin, and luteinizing hormone releasing hormone (Skidgel & Erdos 1985). Phylogenetic studies link ACE to the RAS as a secondary association (Lipke & Olson 1988). Mammalian ACE degrades bradykinin more efficiently than it activates angiotensin I (Ryan 1983) indicating that bradykinin may be the primary substrate of ACE.

**Table 1.2** The principal natural and synthetic substrates of angiotensin converting enzyme.

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Principal natural substrates:

angiotensin I	[(N)Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu]
bradykinin	[(N)Arg-Pro-Pro-Gly-Phe-Ser-Pro-Arg]

Synthetic substrates:

hippuryl-histidyl-leucine
benzoylphenylalanyl-alanyl-proline
hippuryl-glycyl-glycine
phenylalanyl-glycyl-glycine

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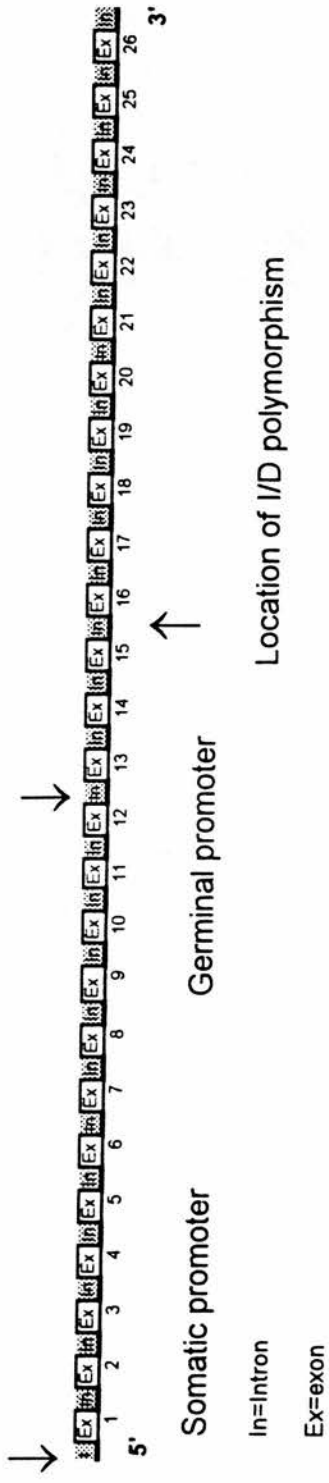
ACE is activated by the presence of chloride ions and inhibited by a variety of natural and synthetic compounds. Chloride concentrations exert considerable influence on the catalytic activity of ACE (Shapiro 1983) with differing concentrations being optimal for the two active sites (Soubrier 1993).

Binding data suggest ACE from different tissues may differ with respect to which of the two sites is the most active, as the equilibrium dissociation constants appear uniform within an organ but varied between organs (Jackson et al 1987). Differences are found in the binding dissociation constants for ACE in different parts of the heart and lungs:  $K_a$  atria >  $K_a$  lungs >  $K_a$  ventricles (Yamada et al 1991). These differences may be due to varying local chloride concentrations between tissues (Soubrier 1993).

#### *1.3.4 THE ACE GENE*

In man a single gene codes for both membrane-bound, soluble and testicular ACE (Figure 1.2, Hubert et al 1991). Located on chromosome 17q23 (Rigat et al 1992) it is transcribed as a 4.3kb mRNA species in endothelial cells or 3kb transcript in testicular cells (Hubert et al 1991).

The structure of the human ACE gene was determined by using overlapping genomic clones, which together contained the entire sequence of the ACE gene, isolated from a lambda-phage human DNA library (Hubert et al 1991). The human ACE gene contains 26 exons interrupted by 25 introns and spans approximately 21kb of DNA. The theory that the ACE gene originated as a result of gene duplication is confirmed by the presence of two homologous clusters of eight exons (exons 4-11 and 17-24). These exons have similar sizes and codon phases at the exon-intron boundaries. The ACE gene is unique in having two alternate promoter regions which vary in activity according to cell type. The somatic promoter region is active in various tissues whereas the testicular promoter is active only in male germinal tissue (Nadua 1992).



**Figure 1.2** The basic organisation of the human ACE gene.

### 1.3.5 GENETIC CONTROL OF ACE

In 1990 Rigat et al described a polymorphism of the ACE gene which is located within intron 16 of the ACE gene and consists of the presence or absence of an insertion which is 287 base-pairs in length and codes for an *alu* repeat sequence. This insertion/deletion polymorphism accounted for 47% of the variance of serum ACE activity when studied within healthy volunteers (Rigat 1990). The possible phenotypic relevance of this polymorphism and its role in the renin-angiotensin system is discussed later in this chapter and throughout the thesis.

### 1.3.6 ACE IN HEALTH AND DISEASE

Serum ACE activity is relatively constant within healthy individuals but can vary considerably between subjects, differing up to six times (Alhenc-Gelas 1991). The factors responsible for this variability have until recently remained unknown. Within subjects no temporal variation or circadian rhythm has been demonstrated in serum ACE activity (Lieberman 1975) and it does not appear to be affected by orthostatic position, modest physical work, salt depletion, or food intake (Dzau 1988, Jackson 1986).

Elevated activity of serum ACE is seen in a number of granulomatous conditions, with sarcoidosis being the most well recognised (Lieberman et al 1979). Increased activities may be used both as an aid to diagnosis and to follow disease progression. The increased ACE activity may reflect stimulation of the monocyte macrophage system (Friedland et al 1977, 1978). The percentage of patients with sarcoidosis with elevated activities of serum ACE varies in different studies from 60 - 95% (Studdy & Bird 1989, Lieberman et al 1979, Eklund & Blaschke 1986). Silicosis, asbestosis (Gronhagen-Riska et al 1978), active tuberculosis (Studdy & Bird 1989), Gauchers disease, leprosy (Lieberman & Beutler 1976) and hypothyroidism (Smallridge 1985) are all associated with increased ACE activity. ACE activity is raised during pregnancy and the puerperium (Dux 1984). ACE within human monocytes is induced, in the presence of glucocorticoids in-vitro (Vuk-Pavlovic 1989). Increased urinary ACE activity has been proposed as an index of renal tubular damage (Baggio 1981) with a raised serum ACE activity correlating strongly with the presence of severe retinopathy in diabetes mellitus (Lieberman 1980).

A decrease in serum ACE has been reported in vascular pathologies involving endothelial damage or disruption such as deep vein thrombosis (Drouet et al 1988), and endothelial dysfunction related to cancers, leukaemias, and transplants (Romer & Schmitz 1984). Reduced activity is seen following acute and chronic lung injury (Bedrossian et al 1978). Iatrogenic causes of reduced serum ACE activity include chemotherapy and radiotherapy

(Sorensen et al 1984; Rubin et al 1984), and of course treatment with ACE inhibitors. Treatment with ACE inhibitors has no effect on serum ACE *concentrations* as measured by RIA (Alhenc-Gelas et al 1983). Serum ACE activity is not altered in hypertension or heart failure (Dzau 1990).

#### 1.4 WHAT IS THE TISSUE RENIN-ANGIOTENSIN SYSTEM?

Until recently it was considered that the RAS existed largely within the vascular space composed of individual peptides produced from different tissues, each regulated by different stimuli and under feedback control. The discovery that many tissues or organs are capable of forming all the components of the RAS, and indeed are able to generate angiotensin II, has led to interest in a concept of a tissue RAS. The circulating or intravascular and tissue systems share a common function in producing angiotensin II. However the tissue system is self-contained and may not be influenced by the same factors as the intravascular RAS. The tissues or organs which independently produce AII that acts locally are the heart, blood vessels, brain and kidney. Evidence for the existence of these local tissue RAS comes from the detection of individual peptides and their mRNA in isolated tissue preparations (Admiraal 1990, Campbell 1987, Symonds 1970, Ganten 1971, Ryan 1976, Deschepper 1986, Dzau 1987). Several enzymes can cleave angiotensinogen, such as cathepsin D (Hackenthal et al 1978) and pepsin (de Fernandez et al 1965), and renin is not therefore essential when such enzymes are present.

Pharmacological studies have shown that pharmacodynamic effects of ACE inhibitors are not clearly related to serum ACE concentrations, suggesting that these drugs may be affecting the membrane bound enzyme which may be the most important for maximal effect.

In the vascular tree ACE has been localised to the endothelium, vascular smooth muscle, adventitia and perivascular structures (Johnston et al 1992b). Webb et al (1988) perfused a human forearm with angiotensin I to demonstrate that ACE in the blood vessels was functionally active. Even ACE is not essential for a functioning RAS since several enzymes including tonin (Boucher 1977), cathepsin G (Tonnesen 1982), trypsin (Arakawa 1980) and kallikrein (Maruta 1983) can cleave angiotensinogen to produce angiotensin II directly. Thus the emerging concept is that the major site of angiotensin production is within the peripheral tissues and that local angiotensin production may in fact exceed plasma production (Campbell 1987, Lees 1990, Samani 1991). It has been suggested that plasma concentrations of angiotensin actually represent a spill-over from the tissues and that the function of the circulating RAS is not to deliver angiotensin II to target organs but to deliver renin or angiotensinogen to the local RAS's (Campbell 1985).



## 1.5 WHAT IS THE FUNCTION OF THE TISSUE RAS?

The concept of a tissue RAS means that concentrations of circulating renin and angiotensin may not represent true activity of the system in either health or disease (Samani 1991).

Although there is a large body of evidence supporting the presence of a local tissue RAS the actual function of such a system remains unknown. Angiotensin II generated by tissue RAS's may feed back and affect nearby cells (paracrine function) or influence the same cell to which the ACE is attached (autocrine function). The function of tissue RAS's and angiotensin II production may thus vary according to the site of production. ACE and angiotensin II could be involved in regulation of regional blood flow and may play a crucial role in the local tonic control of vascular resistance, modulation of local sympathetic activity and stimulation of hyperplasia and hypertrophy (Dzau 1988). Tissue responses to endothelial damage may also involve ACE and a local RAS (Johnston et al 1992). There are links between angiotensin II and cell growth suggesting an association between local angiotensin production and tissue hypertrophy. In Goldblatt experimental one kidney one-clip hypertension in the rat, serum ACE does not increase but ACE within the hypertrophied mesenteric vessels and enlarged left ventricle are significantly increased (Johnston 1991). Also, in a rat model of experimental left ventricular hypertrophy, mRNA for ACE is increased within the myocardium (Schunkert et al 1990; Nagano et al 1991).

### 1.5.1 INHIBITION OF TISSUE ACE

The hypothesis that inhibition of tissue ACE (membrane-bound ACE) rather than serum ACE explains the clinical effects of ACE inhibitors stems from a discrepancy in time-course between serum ACE inhibition and haemodynamic changes. Inhibition of serum ACE correlates with simultaneous plasma drug concentration (Ajayi et al 1987). However the maximum blood pressure fall lags behind the peak plasma drug concentration (Wenting 1987). This lag phase may be due to equilibration between serum and tissue sites, with subsequent delayed inhibition of tissue RAS. Some single dose studies suggest a close relationship between fall in blood pressure and serum ACE inhibition (Jackson 1984) but others find no relationship (McFadyen 1993). Pharmacokinetic studies in rats show blood pressure decreases correlating better with tissue ACE than serum ACE (Unger et al 1994). During long term ACE inhibitor therapy, when steady state equilibrium has been reached, hyperreninaemia develops and angiotensin II is found despite continued hypotension. (Mooser 1990, McFadyen 1991).

The time course of tissue ACE inhibition appears to vary from one organ to another (Johnston 1988, 1989) but these findings are difficult to interpret and could be the product of tissue bioavailability rather than true differences in enzyme affinity. Tissue differences are more likely to be due to differences in penetration of the tissue, for example, if the lipid

concentration of the local ACE environment is high then tissue penetration may be delayed. The distribution of a drug between serum and tissue is likely to be a dynamic equilibrium and depending on quantitative availability of binding sites and the properties of the individual ACE inhibitor or tissue site.

Thus tissue ACE could be more important than inhibition of circulating ACE when discussing the clinical effects of ACE inhibition. Problems arise on further investigation of this concept because although serum ACE concentrations are relatively easy to measure accurate measurement of tissue ACE in man is difficult.

## 1.6 THE RENIN-ANGIOTENSIN AND BRADYKININ-KALLIKREIN SYSTEMS

Bradykinin is a nonapeptide component of the kallikrein-kinin cascade, active in mediating inflammatory responses. The bradykinin-kallikrein system and the RAS are linked by angiotensin converting enzyme which is considered identical to kininase II (Yang et al 1970). ACE is responsible for the majority of the degradation of bradykinin within the lung and a proportion of bradykinin degradation within the plasma (Erdos 1975, Ryan 1968). Kallikrein circulates in an inactive form until activated by many noxious stimuli. It cleaves a kinin called kallidin which is converted to bradykinin by tissue enzymes. Bradykinin has actions throughout the body including relaxation of vascular smooth muscle, plasma exudation and mediation of inflammatory pain. Bradykinin also stimulates histamine release. When injected intradermally bradykinin causes a dose-dependent wheal and flare response (Fuller 1987). The effects of bradykinin on the airways have been related to its ability to cause release of neuropeptides from sensory nerves such as C-fibres. Subsequent activation of cholinergic pathways can lead to bronchoconstriction in laboratory animals. In small studies bradykinin had a bronchoconstrictor response in those with asthma but not in healthy individuals (Simonsson 1973, Fuller 1987). Cough, unrelated to any change in airways calibre, was observed to varying extent in these and other studies (Dixon 1987, Choudry 1989, Ichinose 1992). However these studies were not designed to look for cough specifically and gave little information on its nature or severity.

## 1.7 COULD THE ACE GENOTYPE BE OF IMPORTANCE?

Angiotensin converting enzyme (ACE) converts angiotensin I to angiotensin II, an important step in the control of blood pressure and sodium balance. As discussed above the gene encoding for ACE is subject to an insertion/deletion (I/D) polymorphism associated with different concentrations of the enzyme in serum (Rigat 1990). Subjects homozygous for the deletion allele (DD) have serum ACE concentrations 48% higher than those homozygous for

the insertion allele (II), while heterozygotes (ID), have intermediate concentrations (Rigat 1990). This polymorphism accounts for 47% of the variability in serum ACE concentrations between subjects.

The phenotypic significance of the ACE gene polymorphism had been studied little at the outset of my work. As discussed above ACE is distributed widely throughout the tissues, largely bound to cell surfaces, especially on the vascular endothelium (Caldwell 1976).

Knowledge about the role of the I/D polymorphism in determining ACE bound to cells and thus tissue ACE was much less certain with only a single study examining this area when my research began (Costerousee 1993). In this study the I/D polymorphism was shown to influence the distribution of ACE concentrations in human T lymphocytes (Costerousse 1993) but the relationship was not as strong as with serum ACE.

To my knowledge its effect on the expression of ACE at the vascular endothelium had not been examined. The I/D polymorphism is confined to humans and limits potential research pathways thus models of tissue ACE were developed from previous work.

Further impetus to examine the ACE gene polymorphism came following several reports of a possible link between it and vascular disease. A case-control study from Europe suggested that the presence of the deletion allele may be a risk factor for myocardial infarction (Cambien 1992). Thus emerged a genetic marker which might help explain why individuals free of recognised risk factors develop ischaemic heart disease. Further case-control studies suggested that the deletion allele was a risk factor in those with ischaemic and dilated cardiomyopathy (Raynolds 1993), and for the development of left ventricular hypertrophy (Schunkert 1994). The mechanism by which the D allele may exert these detrimental effects is unknown, but could be through an enhanced rate of production of angiotensin II resulting in increased pressor and trophic cardiovascular responses. This enhanced rate of production might occur within the serum or in localised tissue systems such as the heart. Alternatively of course it could be acting as a marker in linkage disequilibrium with another as yet unknown gene. Identifying those at risk might in the future identify people who may benefit from treatment. If the ACE genotype could be linked to phenotypic differences in the RAA system then perhaps susceptible individuals might be targeted with drugs such as ACE inhibitors (Swales 1993). The responses to ACE inhibitors probably relate to inhibition of both serum and tissue ACE. The evidence as to the relationship between the initial serum ACE and the hypotensive response is conflicting with some finding a relationship (Johnston 1983, Jackson 1984) and others not (McGregor 1981, McFadyen 1993).

Through its strong association with serum ACE activity the ACE genotype may predict not only antihypertensive effects but possibly also adverse effects such as hypotension in heart failure. This might allow targeted treatment to those who would respond optimally and also recognition of those who might develop severe hypotension. One might argue that we can

already increase the chances of response to ACE inhibitor simply by adding a diuretic to activate the RAS and that it is not necessary to know who might respond most. However knowledge of genotype might also be helpful in determining those who may become susceptible to other adverse effects such as cough. Indeed it has been suggested that the reason why cough should only affect 20% of those given ACE inhibitors may be linked to ACE genotype (Yeo 1991). Those individuals with lower ACE concentrations (II subjects) may develop increased concentrations of bradykinin and substance P with alteration in cough threshold.

Thus the ACE genotype could give further insight into the complex nature of the renin-angiotensin system in health and disease and possibly predict both beneficial and adverse responses to ACE inhibitors.

## 1.8 ANGIOTENSIN CONVERTING ENZYME INHIBITORS

### 1.8.1 ACE INHIBITION AND THE RENIN-ANGIOTENSIN SYSTEM

It is generally considered that the effects of ACE inhibitors are largely due to inhibition of by inhibition of serum and membrane bound ACE resulting in a decrease in ACE activity and fall in angiotensin II (Edwards & Padfield 1985). ACE inhibition blocks the vasoconstriction induced by infusion of angiotensin I (Given 1984). Following administration of an ACE inhibitor there are reductions in angiotensin II and aldosterone (Atlas 1984, Lijnen 1982, De Leuw 1983) and increases in angiotensin I and renin concentrations. Angiotensin II concentrations drop within 30 mins to 4 hours depending on the ACE inhibitor (Atlas et al 1984). ACE inhibition also results in inhibition of bradykinin metabolism with consequent vasodilatation. Activation of the arachidonic acid cascade by ACE inhibitors results in the production of vasodilatory prostaglandins (Swartz & Williams 1982). Both these additional effects are additive to the vasodilatation produced by reduction in angiotensin II levels.

The resulting arterial vasodilatation leads to a reduction in systemic vascular resistance which is more pronounced in the hepatorenal vascular beds than those of the brain or skeletal muscle (Ibsen et al 1984). A single dose of ACE inhibitor can reduce the systemic vascular resistance by up to 15% and long term therapy reduces it a further 15% to 30% (Dunn 1984). The majority of this effect is seen in resistance vessels, arterioles, but larger conduit vessels are thought to dilate also.

The decrease in systemic vascular resistance is the predominant mechanism by which ACE inhibitors reduce blood pressure. In normotensive human subjects a single dose of an ACE inhibitor can reduce the blood pressure by 5 to 20 mm Hg (Brunner 1981, MacGregor 1981). If the RAS is activated, for example in sodium depletion then the hypotensive response is accentuated (MacGregor 1992, Jackson 1984, Swartz 1982, Atkinson 1979, Man in't Veld 1980). Whether the hypotensive effect of ACE inhibitors is initially proportional to the inhibition of the enzyme remains uncertain with Johnston et al (1983) showing a close relation between reduction in serum ACE activity and the fall in blood pressure in hypertensive subjects after a single 10 mg dose of enalapril and other authors showing no relationship with serum ACE activity reduction achieved. Unlike other arterial vasodilators used in hypertension administration of an ACE inhibitor does not cause a reflex tachycardia (Weinberger 1982) possibly due to alteration in the baroreceptor reflexes caused by reduced AII (Guidicelli 1985).

Difficulty in measuring plasma concentrations of the components of the RAS has led to variable results when trying to predict the effects to ACE inhibitors. The determination of

plasma renin activity by radioimmunoassay has been used most commonly (Sealey & Laragh 1977) correlating well with angiotensin II-mediated vasoconstriction. The hypotensive effects persist longer than suppression of serum ACE concentrations (Gavras et al 1981) suggesting that ACE inhibitors may mediate their effects via a tissue RAS as discussed earlier.

### *1.8.2 ACE INHIBITION AND THE BRADYKININ-KALLIKREIN SYSTEM*

As discussed above, the enzyme associated with degradation of bradykinin, kininase II, is identical to ACE, ACE inhibition would therefore be expected to increase concentrations of bradykinin. This is not easy to demonstrate as the bioassay of kinins is difficult and not particularly accurate or reproducible (van Leeuwen 1983). Furthermore kinins are generally considered local mediators and measuring plasma kinins may not necessarily reflect changes at local sites. They are also inactivated by many different enzymes apart from ACE and these may easily counter the effects of ACE inhibition. Reports of the responses of circulating bradykinin levels to ACE inhibition are variable with some groups reporting increases (Crantz 1980, Swartz 1982) and others failing to show any change (Given 1984, Ody 1983). ACE inhibition enhances the contractile effects of bradykinin on guinea-pig ileum and the vasodilation of guinea pig coronary artery (Zusman 1984, Yiang 1985). In man, Lindgren (1988) demonstrated a dose-response relationship for dermal wheal production using intradermal bradykinin during ACE inhibition. Wheal thickness increased after enalapril treatment in healthy volunteers (Ferner et al 1987, Li Kam Wa 1993) and hypertensive patients (Ferner 1989). Thus the wheal response to intradermal bradykinin may be used as an indirect measure of membrane ACE activity. Benjamin et al (1989) suggested that the effects of enalaprilat infusion on vasodilatation are mediated through both angiotensin II reduction and bradykinin potentiation. Indeed administration of competitive antagonists of bradykinin can partially reverse hypotensive effects of ACE inhibition (Seino et al 1989). Bradykinin has been found in increased serum concentrations in those with cough on ACE inhibitors compared with a control group, and it may be a mechanism for this side effect (Puolijoki 1992). As discussed above however bradykinin concentrations are difficult to measure and this work has not been reproduced.

### *1.8.3 ACE INHIBITORS IN HYPERTENSION*

ACE inhibitors are effective antihypertensive agents causing falls in blood pressure similar to those of other classes (Pool 1989, TOHMS 1991). Hypotensive effects of ACE inhibitors are additive to those of the thiazide diuretics (Wing 1987) and dihydropyridine calcium antagonists (Salveti 1987). Initial studies examining side effects compared them to outdated agents such as methyl-dopa and it was not surprising that they resulted in a better "quality of



life" (Croog 1986). A number of studies since have failed to show important positive benefits of ACE inhibitors on quality of life, and they have not proved superior to beta-blockers such as atenolol (Steiner 1990, Fletcher 1990, Herick 1989, THOMS 1994, Jones 1995). Overall evidence suggests that ACE inhibitors are as well tolerated as the other main classes of antihypertensive drugs and have similar effects on quality of life (TOHMS 1991, 1994, Maclean 1990).

The side effects of ACE inhibition are described in more detail below. In general, ACE inhibitors are relatively free of contraindications, and can be used safely in patients with coexisting disease states such as diabetes, chronic obstructive airway disease, and heart failure. ACE inhibitors have a particularly invaluable role when hypertension is complicated by heart failure due to the both the beneficial effects found in the treatment of heart failure with ACE inhibitors and the relatively negative inotropic effects of other agents with the exception of diuretics. They may also have some benefit on renal function in insulin-dependent diabetic patients where proteinuria and the decline in renal function observed in diabetic nephropathy is reduced (Lewis 1993).

In summary, ACE inhibitors are comparable to the thiazide diuretics and beta-blockers as regards their efficacy in lowering blood pressure, simplicity to prescribe, and tolerability. Their efficacy in preventing cardiovascular and cerebrovascular end-points in hypertensive subjects remains to be established with respect to older agents but studies are underway and recent favourable results in those with heart failure support the view that they are likely to prove beneficial.

#### *1.8.4 TREATMENT OF HEART FAILURE WITH ACE INHIBITORS*

The use of ACE inhibitors for the treatment of heart failure soon followed their use in hypertension and the combination of a diuretic and ACE inhibitor is now standard treatment for chronic heart failure. Several large randomised controlled studies showed that ACE inhibitors reduced mortality and morbidity in heart failure of varying degrees of severity. The CONSENSUS trial study group (1987) showed that ACE inhibitors significantly increased survival in patients with severe heart failure when compared to placebo. VeHFT II evaluated ACE inhibitor compared to the combination of hydralazine and isosorbide dinitrate in those with moderate congestive heart failure (Cohn 1991). Enalapril significantly improved survival at two years compared to the combined treatment which was previously the treatment of choice in this condition. Recently, the results of several large mortality studies investigating the value of ACE inhibitor treatment in terms of heart failure prevention and improved survival have been published (The SOLVD investigators 1991; 1992; Pfeffer 1992). They

demonstrated that the use of ACE inhibitors as therapy in left ventricular dysfunction in patients reduces not only mortality, but also the progression of heart failure, the number of hospitalisations and the rate of cardiovascular events. Following myocardial infarction ACE inhibitors reduce mortality when patients have left ventricular dysfunction which is either clinically apparent (AIRE study 1993) or detected by measurement of left ventricular ejection fraction (SAVE study 1992).

#### *1.8.5 SIDE EFFECTS OF ACE INHIBITORS*

Considerable experience of long-term use of ACE inhibitors has accrued, and it seems clear that they are generally safe drugs. Neutropenia, proteinuria, skin rashes, loss of taste, and angio-oedema were all documented with the initial use of captopril. These effects, with the exception of angio-oedema, seemed to be associated with the high doses initially recommended and are now rare. With the doses currently prescribed the main adverse effects seen are cough, renal impairment, hypotension and angio-oedema. Persistent dry cough is the most frequent side effect of ACE inhibitor therapy and is discussed below.



Table 1.3 Summary of the most common subjective side effects of ACE inhibitors.

Subjective side-effects-approximate incidence in controlled studies (Yeo 1990, THOMS 1994)

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dry cough	15%-20%
taste disturbance	2 - 4%
sore mouth	2 - 4%
rash	1 - 4%
dizziness	1 - 4%
diarrhoea	1 - 4%
headache	1 - 3%
fatigue	1 - 3%

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Some effects such as taste disturbance, and rash are related particularly to the sulphhydryl group in certain ACE inhibitors and therefore are less commonly seen when using an ACE inhibitor containing a different zinc ligand. The more important potential side effects are discussed in brief below.

*Angioedema* is a rare but potentially serious side-effect with an incidence estimated at 0.05-0.1% (Hedner et al 1992; Slater et al 1988) and occurs following the first dose or within 48 hours of initiating therapy (Ferner et al 1987). Angioedema commonly affects the face, lips, tongue, hands, feet and scrotum. Usually it is mild and subsides on withdrawal of ACE inhibitor treatment. In a few patients it may involve the pharynx or larynx with serious consequences. Treatment is usually by giving corticosteroids, adrenaline or even tracheostomy. The cause is unknown but may be due to potentiation of the subcutaneous effects of bradykinin in those susceptible.

*Hypotension* is more of a problem in patients with heart failure than hypertension. In patients treated on high doses of diuretics, or with bilateral renovascular disease, who may have high plasma renin activity before treatment, falls in systolic blood pressure of up to 50 mmHg may occur, this may cause syncope or even very rarely stroke or myocardial infarction (Webster 1987). Venodilation is the probable cause of the hypotension and the absence of a compensatory tachycardia suggests a parasympathetic action similar to vasovagal syncope (Capewell 1991). Simple ways to reduce the occurrence of this side effect include stopping diuretic therapy for a 1-2 days on initiation of ACE inhibitor therapy. If a patient is considered at high risk admission to hospital is recommended. There has been a suggestion of differences between ACE inhibitors in their potential to cause first dose hypotension

(McFadyen et al 1993) but this probably relates to half-life. Perindopril has a relatively long half-life and causes significantly less hypotension than captopril following a single dose (McFadyen et al 1993).

*Renal failure* due to ACE inhibitors may be severe and progressive in patients with advanced renovascular disease, particularly those with a tight stenosis of the artery supplying a solitary kidney, unilateral renal artery thrombosis and contralateral renal artery stenosis, or bilateral renal artery stenosis (Verbeelen 1984). In these cases glomerular filtration is maintained by angiotensin II-induced constriction of the efferent arterioles. ACE inhibition abolishes this constriction, and dilates the efferent arterioles causing filtration pressure to fall to such low levels that renal failure develops. The effect of ACE inhibitors on advanced renovascular disease is of considerable practical importance. Patients "at risk" are relatively common among those with resistant hypertension, the elderly, and those with peripheral vascular disease. Renovascular disease even of such a severe degree can often be clinically "silent", therefore, it is essential when prescribing an ACE inhibitor to measure serum creatinine before starting treatment and remeasure it during treatment in order to observe for any decline in renal function.

## 1.9 ACE INHIBITOR-INDUCED COUGH

### 1.9.1 PHYSIOLOGY OF COUGH REFLEX

The cough reflex protects the respiratory tract from a variety of irritants and clears associated secretions. Its control is by a complex interplay of receptors, neuronal pathways and central nervous system modulation.

A normal cough response begins with an initial deep inspiration and closure of the glottis, followed by contraction of the expiratory muscles causing a rapid increase in intra-thoracic pressure. The glottis then opens as muscular contraction continues to increase the pressure, forcing air out at high velocities. There are many variations on this basic pattern, with cough occurring as a single episode or in paroxysms with each cough occurring at lower lung volumes (Langlands 1967).

Many different mechanical and chemical stimuli can induce cough. The larynx and carina are particularly sensitive to cough (Fillenz & Widdicombe 1970, Sant'Ambrogio 1987) but cough can be produced by stimulation at several other sites in the upper airways. Central nervous control modulates the basic cough pattern producing a complex and often varied response to different stimuli. A discrete cough centre has not been identified in man but integration and modification of sensory data probably occur in an area distinct to the respiratory centre. In the cat specific electrical stimulation of the midbrain can cause pure cough with virtually no effect on respiration or blood pressure (Kase 1970). That cough can be voluntarily generated or suppressed suggests higher centres have a modifying role.

### 1.9.2 COUGH RECEPTORS AND AFFERENT FIBRES

The airways and lung are derived embryologically from the forepouch of the gut, and share the same nerve supply - the vagus nerve. Stimulation of the alveoli, external auditory meatus, pharynx and diaphragm, can initiate the cough reflex as a result of their common embryological origin and shared nerve supply (Todisco 1982). Afferent impulses along the vagus nerve travel in either myelinated or non-myelinated fibres. *Myelinated fibres* can be subdivided according to electrophysiological properties of the receptor stimulated into *rapidly adapting (RAR's)* and *slowly adapting receptors (SAR's)*. **Non-myelinated fibres** are classified as either **bronchial** or **pulmonary C-fibres**. *RAR's* are found predominantly in the larynx, trachea and carina and SARs within the bronchial smooth muscle. **Pulmonary C-fibres** are located within the alveoli. Both stimulation of *RARs* and **C-fibres** may result in cough in humans (Barnes 1986).

*Rapidly adapting receptors (RARs)* are located superficially within the airway mucosa, either below or between the epithelial cells (Davis et al 1982). They are particularly numerous at

branch points of the tracheo-bronchial tree. In general they respond to light mechanical stimulation and weak chemical stimulation, for example, citric acid (Banner 1986, Pounsford 1985). *RARs* adapt quickly and fire irregularly (Karlsson 1988) and are stimulated by many tussive stimuli. Partial block of vagal conduction limited to myelinated fibres inhibits the cough reflex (Widdicombe 1954, 1980, Karczewski & Widdicombe 1969). *RARs* may play an important role in the generation of cough, particularly cough arising from the upper airway. **C-fibres** are found in the airway epithelium close to the basement membrane and nearer to the airway lumen (Barnes 1986). They outnumber the myelinated fibres by estimates from 3-11:1 (Sant'Ambrogio 1982, Jammes et al 1983) in the cat vagus. Substance P and other sensory neuropeptides are released as part of a local axon reflex (Barnes 1986). Tussive chemical agents such as capsaicin (Coleridge et al 1964; Winning et al 1986), bradykinin (Kaufman et al 1980), histamine, PGE<sub>2</sub> (Choudry et al 1989), PGF<sub>2a</sub> (Nichol et al 1990), and sulphur dioxide (Coleridge & Coleridge 1984) all stimulate these nerves.

### *1.9.3 COUGH INDUCED BY ACE INHIBITORS*

First described ten years ago, persistent dry cough is now recognised as the most common subjective side-effect during long-term treatment with ACE inhibitors (Fletcher 1994, DTB 1994). Many studies have confirmed its high incidence and that it is the most frequent reason for stopping treatment (Lernhardt & Ziegler 1988, Israeli et al 1992, Yeo et al 1991). Important gaps remain in the knowledge of the natural history of the cough, its pathogenesis, and factors which predispose subjects to the cough.

### *1.9.4 HOW COMMON IS ACE-INHIBITOR COUGH?*

Different studies have shown a wide range of estimates for frequency of cough with ACE inhibitor ranging from about 3% in postmarketing surveillance studies to 22% by direct enquiry in controlled trials. That cough was not immediately considered as a potential side effect of drugs was apparent from post-marketing surveillance studies which provided estimates of 2-3% when ACE inhibitors were initially studied (Coulter and Edwards 1987, Inman 1988). Relying heavily on spontaneous reporting of symptoms will underestimate the incidence of cough as demonstrated by these studies. As cough is such a common symptom that it was clearly not considered relevant by patients. Likewise their medical attendants did not predict this unique adverse effect would occur given the pharmacological properties of this class. When they began to be used in clinical practice case reports of patients taking ACE inhibitors who had developed cough provoked further study (Sesoko 1985). Uncontrolled studies in hospital clinics (Gibson 1989, Strocchi 1989) suggested an incidence of cough between 6 - 15 %. Controlled studies using validated methods of ascertainment show that the true incidence of ACE inhibitor cough is about 15-20 % (Fletcher et al 1990, Yeo et al 1991a,

Yeo et al 1991b). The incidence of cough as measured by spontaneous reporting was 16% in a study which was uncontrolled but analysed by a life-table method. This was in addition to the 6% of patients who had already stopped treatment because of cough (Yeo & Ramsay 1990). In a similar study the prevalence of cough was 10% with captopril or enalapril treatment, when compared to hydrochlorothiazide (Sebastian et al 1991).

A later study showed that over two thirds of patients with cough detected by questionnaire, but not volunteering it when seen in clinic described their cough as moderate or severe (Yeo et al 1991a). In controlled studies direct enquiry using methods such as verbal questioning, self-administered questionnaires or visual analogue scales were used. Even here the assessment of symptom severity may be difficult because such trials are usually of short duration. Patients will often continue taking treatment for a pre-determined period, enduring symptoms which would otherwise make them stop the drug. Methods of direct enquiry will overestimate the incidence of cough in uncontrolled studies because of the high prevalence of chronic cough related to smoking and chronic respiratory disease (Barbee et al 1991), and are therefore reliable only in controlled comparative trials, even then only when corrected for baseline responses. Visual analogue scales (Yeo et al 1991b) are also of value in the setting of controlled trials, but are probably inferior to the use of simple questions because of variability within subjects in scoring.

When assessed by visual analogue scale the prevalence of cough with enalapril, compared to nifedipine, was 22% after six months in a double-blind controlled trial (Yeo et al 1991). The most reliable estimate of the incidence of cough comes from random prospective controlled trials using questionnaires (Fletcher et al 1990), and is about 20%. These estimates are all in general agreement with the figure of 15% derived by Just (1989) from an overview of published data.

#### *1.9.5 WHO GETS ACE INHIBITOR COUGH?*

Why only 20% of the patients treated with ACE inhibitors develop dry cough is unknown. The cough is probably a consequence of the non-specific pharmacological action of ACE inhibitors, related to their inhibition of the kininase action of ACE rather than interruption of the conversion of angiotensin I to angiotensin II. Kininase inhibition may cause accumulation or imbalance of putative mediators of cough such as bradykinin, substance-P and prostaglandins at the cough receptors served by C-fibres (Choudry et al 1989b). Changes in the cough reflex have been shown with individuals developing a marked increase in sensitivity during ACE inhibitor treatment (McEwan et al 1989). Subjects who develop ACE inhibitor cough seem to have a normal cough reflex initially, but develop a marked shift to the left of their cough responses to capsaicin (McEwan et al 1989) indicating an effect on C-fibres

(Berkin & Ball 1988). The limited evidence available suggests that ACE inhibitors affect the cough reflex only in these patients (McEwan et al 1989, Morice et al 1987), and that there is no change in the remainder of those treated. Sex and smoking habit are the only factors identified at present related to development of ACE inhibitor cough. Many studies have confirmed a preponderance of women among those who develop cough, with a female:male ratio of about 2:1 (Yeo et al 1991a; Yeo et al 1991b; Yeo & Ramsay 1990; Just 1989; Os et al 1992). Even these may not be true risk factors as other reasons may lead to over or under reporting of this side effect in these groups. Women might report cough more readily (Os 1992), and have a lower threshold to tartaric acid (Fujimura 1990). A large double-blind placebo-controlled study with lisinopril (Os et al 1992) confirmed previous suggestions (Yeo et al 1991) that non-smokers are more likely than smokers to develop cough as a side-effect. The incidence of cough was 16% in non-smokers compared to 7% in smokers (Os et al 1992). In clinical practice this difference may be exaggerated as smokers and their attendants are more likely to ascribe the cough to the effects of tobacco rather than a drug. There does not seem to be any relationship between ACE inhibitor cough and pre-existing airways obstruction, age, body weight, renal function, duration of treatment, or use of other antihypertensive drugs (Yeo et al 1991a). There is no convincing evidence that different ACE inhibitors differ in their propensity to cause cough (Ravid 1994).



### *1.9.6 CLINICAL FEATURES*

Most, perhaps 85%, of those who develop cough do so within the first month ranging from 2 days to several months (Fletcher 1990, Lacourcière 1994). Specific features of the cough and associated symptoms are largely derived from research that examined multiple symptoms in detail by questionnaire in a non-random but controlled comparison with nifedipine (Yeo et al 1991a). It was shown that cough with ACE inhibitors is characteristically dry or non-productive, and in this respect differs significantly from the cough related to respiratory disease, which is usually productive of sputum. It is persistent, repetitive, occurs in bouts, and is prominent at night (Webb 1986). Other symptoms associated with cough are voice change (hoarseness or huskiness) in 14% and throat discomfort in 10% of patients. Any one of these "laryngeal" symptoms (cough, voice change or throat discomfort) is present in 24% of patients after two years of treatment (Yeo et al 1991a).

In about 25% of patients with cough it is severe enough to necessitate withdrawal of the ACE inhibitor. (Yeo et al 1991a, Yeo et al 1991b). If treatment is continued most consider their cough to be moderate or severe, and in the majority it disturbs sleep and is a nuisance to others (Yeo et al 1991a). It may remit spontaneously if treatment is continued, but this is probably uncommon (Reisin and Shneeweiss 1992) and may suggest an alternative aetiology such as post viral cough. In some patients the bouts of coughing cause vomiting (Yeo et al 1991a). If it is considered important that an ACE inhibitor is given then a re-challenge with either the same drug or an alternative ACE inhibitor might be worth attempting. During the entry phases of a study examining the effects of an AII antagonist on cough, patients with a history of ACE inhibitor cough were re-challenged with ACE inhibitor. It is of note that 28% of these subjects failed to develop cough again after eight weeks treatment (Lacourcière 1994).

### *1.9.7 DIAGNOSIS AND TREATMENT*

When a cough with typical features develops in a patient taking an ACE inhibitor it should be assumed that the drug is the cause. The cough of asthma, oesophageal reflux, bronchial carcinoma or post-nasal drip may have similar features (Puoloki 1989). However a chest X-ray and spirometry are probably the only investigations required initially. If diagnostic doubt remains it should be resolved by withdrawing the ACE inhibitor.

Several treatments have been suggested for ACE inhibitor cough. There is some relation between the dose of ACE inhibitor and development of cough as demonstrated in patients with enalapril-induced cough. In this study halving or doubling of the dose of enalapril under controlled conditions altered the frequency and severity of coughing. Curiously there was no effect on the cough reflex as assessed by responses to inhaled capsaicin however this may have reflected a small sample size (Yeo et al 1995).

When the ACE inhibitor is stopped the cough usually disappears within 2 - 10 days, but in some patients, perhaps those who have had the cough for a long time, it may take several weeks for the cough to disappear (Reisin and Shneeweiss 1992). A case report of resolution with sulindac led to further study of non-steroidal anti-inflammatory drugs (Gilchrist et al 1989), but claims that this group of drugs or others such as theophylline (Pomari et al 1989) abolish or improve ACE inhibitor cough have not been substantiated, and until recently no treatment was proved effective. Classes of drug which block the renin-angiotensin system more selectively, such as angiotensin antagonists or renin-inhibitors, may prove free of this side-effect. This hypothesis is supported by a recent study with losartan, a specific antagonist of angiotensin II at AT<sub>1</sub> receptors. When examined in a group of subjects who coughed with ACE inhibitors on 2 separate challenges the incidence of cough was indeed significantly less than ACE inhibitors (Lacourciere 1994) however about 29% of subjects given losartan still did develop cough again for reasons that are not immediately apparent. The incidence was also similar in the group randomised to bendrofluzide (34%) a drug not recognised as causing cough.

When faced with a patient with cough due to ACE inhibitors there are several newer treatment options which have been recently examined. The first option would be to change from an ACE inhibitor to an angiotensin II antagonist such as losartan. If it is considered that the ACE inhibitor has to be continued then the use of inhaled sodium cromoglycate (Hargreaves 1995) has been demonstrated to have some effect. The efficacy of this drug also lends further support to the involvement of kinins. Clinical experience suggests that once the cough has developed patients are often reluctant to continue with treatment particularly in those with hypertension. Other groups have persuaded their patients to continue for longer periods and identified that the cough may resolve or diminish in intensity in up to 25% of patients. Whether this is affected by adaptation of the cough response or the fact that the cough was related to other causes for example respiratory viruses is unknown (Reisin 1992, Ravid 1994).

In summary, Dry cough leads to withdrawal of ACE inhibitor treatment in about 5% of patients and is a persistent nuisance in a further 15%. It is often accompanied by voice change and throat discomfort, and affects women and non-smokers particularly. There is no effective treatment. It is related to inhibition of kininases rather than the action of ACE inhibitors on angiotensin conversion.



## 1.10 AIMS OF THIS THESIS:

1. To determine whether phenotypic differences exist between individuals of different genotype for the I/D ACE gene polymorphism with regard to substrates of ACE as indicated by:
  - a) The pressor and neurohormonal responses to angiotensin I
  - b) The hypotensive responses to ACE inhibitor drugs
  
2. To study prospectively aspects of the cough caused by ACE inhibitor drugs and relationship to the I/D ACE gene polymorphism, in particular:
  - a) The effect of treatment with ACE inhibitors on the cough reflex in hypertensive subjects
  - b) The natural history of ACE inhibitor cough when treatment is withdrawn.
  - c) The effects of ACE inhibitors on the dermal wheal responses to bradykinin.
  - d) The dermal wheal response to bradykinin and relationship to ACE genotype .
  - c) The cough response to bradykinin and relationship to ACE genotype.
  - c) The I/D allele frequency in those with ACE inhibitor cough.

## Chapter 2

### MATERIALS AND METHODS

## 2.1 INTRODUCTION

In this chapter I describe some of the techniques used in studies within this thesis. Three main areas are covered:

- i) the genotyping of an individual,
- ii) the measurement of serum and tissue ACE activity,
- iii) the method of cough reflex testing.

## 2.2 POLYMERASE CHAIN REACTION (PCR) METHOD FOR THE DETECTION OF THE ACE GENE INSERTION/DELETION POLYMORPHISM

Polymerase chain reaction(PCR) involves amplification of a specific sequence of DNA by specific sequences of nucleotide called primer, and the use of an enzyme that polymerises DNA. The reaction requires relatively small amounts of genomic DNA and is considerably faster than Southern blotting. First described by Rigat et al (1992) PCR detection of the ACE gene polymorphism has now been performed in many laboratories. The region of the ACE gene containing the variable site is amplified with two possible products. A 190 base pair sequence in the absence of the insertion (ie a deletion) and 490 base pair sequence in the presence of insertion.

This result thus allows three genotypes; homozygotes for the insertion (II), homozygotes for the deletion (DD) and heterozygotes (ID). It has been suggested by Fogarty et al that occasional mistyping might occur with those of genotype ID being classified as DD, and that the addition of 5% dimethylsulphoxide prevents this (Fogarty 1994). My work had been performed prior to this observation, and I retyped individuals retrospectively. There were no changes in genotype allocation. Since I have completed my research, techniques such as genotyping an individual have become considerably faster. In 1991 it would have taken several days to genotype a few individuals whereas in 1995 several thousand samples can be processed within a few hours (O'Dell 1995)

### 2.2.1 *Purification of Oligonucleotides*

1. Oligonucleotides were synthesised on an Applied Biosystems 394 DNA/RNA synthesiser, using phosphoramidite chemistry, in the University of Sheffield Department of Medicine and Pharmacology.
2. Protecting chemical side groups were removed by incubation at 55°C for 8 - 15 hours in ammonia.
3. Ammonia was subsequently removed by drying overnight at 55°C.
4. Oligonucleotides were re-suspended in 1 ml of de-ionised water and then quantitated by measuring UV absorbance (Beckman SP 64 spectrophotometer and quartz cuvettes), using 25µg/ml as OD of 1.0 at 260 nm.

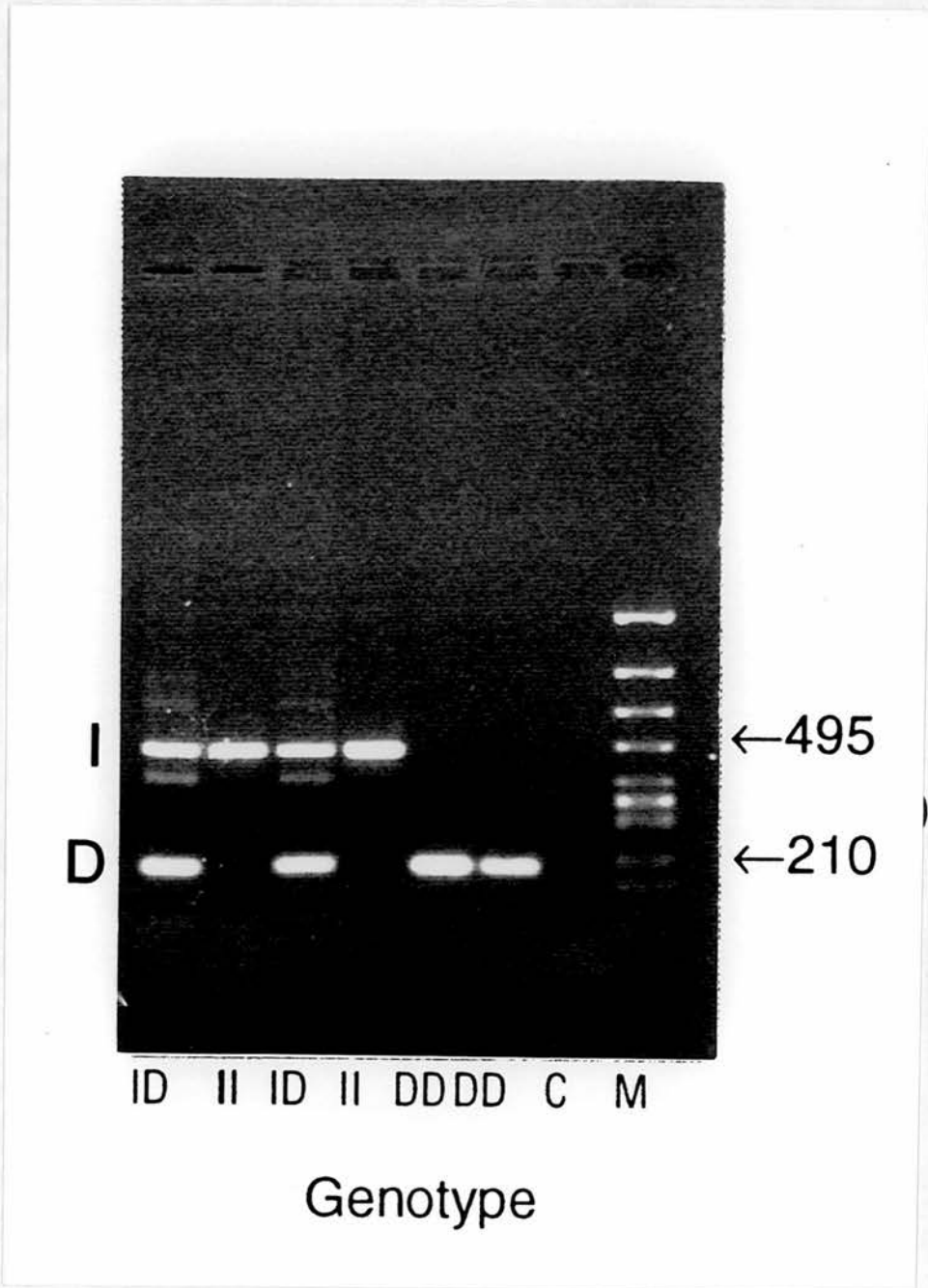
### 2.2.2 *Rapid preparation of DNA from whole blood (Innis et al 1990)*

1. 50µl of whole blood (EDTA) was added to 0.5 ml TE buffer (10mM Tris HCL pH 8, 1mM EDTA)
2. Samples were then centrifuged for 10 seconds at 10,000g (Beckman microfuge 11) and the resulting pellet was re-suspended in 0.5 ml TE buffer.
3. Stage 2.above was repeated twice. Thereafter the final pellet was re-suspended in 100µl of K buffer (K buffer is 100µg/ml proteinase K, 2.5µl Tween 20 and stock buffer [50mM potassium chloride, 15mM Tris-base, 2.5 mM Mg Cl<sub>2</sub> pH 8.3 with HCL]). Incubation was at 56°C for 45 min in order to digest the cells, then 10 min at 95 °C to inactivate the process.
4. The final solution was used at 1/10 of the final PCR volume.

### 2.2.3 Polymerase chain reaction (PCR) method for the detection of the ACE gene insertion/deletion polymorphism

The method of PCR detection of the ACE gene polymorphism described below was developed in our laboratory from methods described by Saiki et al (1988) and Rigat et al (1992).

1. The sense oligonucleotide primer was 5'CTG GAG ACC ACT CCC ATC CTT TCT 3' and the antisense primer was 5' GAT GTG GCC ATC ACA TTC GTC AGA T 3'.
2. Reactions were performed in a final total volume of 50ml containing 10 pmol of each primer, 5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl pH 8.4, 0.1 mg/ml gelatin, 0.5 mM of each dNTP (Pharmacia), 1 unit of *Taq* polymerase (Advanced Biotechnologies, UK), 5 ml rapid extracted DNA and overlaid with 50 ml filter-sterilised mineral oil. A water blank (control) was included.
3. Amplification was carried out in a Perkin-Elmer/Cetus DNA Thermal Cycler 480 with an initial 7 min denaturation followed by 30 cycles with steps of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. Samples were then stored at 4°C until removed from the machine.
4. PCR products were electrophoresed in 1% agarose submarine gels using fx174RF/HincII as a size marker. DNA was visualised by ethidium bromide staining (0.5mg/ml) and UV transillumination (UVP, Inc). Figure 2.1



**Figure 2.1** Example of an electrophoresis gel result following Polymerase chain reaction for ACE gene polymorphism. I= allele containing insertion, D=allele without insert. Results are shown for six individuals; ID (heterozygote), II (homozygote), and DD (homozygote). C= blank control. M=molecular size marker.

## 2.3 MEASUREMENT OF SERUM ACE

Angiotensin converting enzyme may either be measured as absolute levels or as activity of the enzyme. The activity is more relevant when the effects of ACE inhibitor drugs are being investigated.

### 2.3.1 Determination of ACE activity

Assays of ACE activity now involve chemical substrates, most commonly synthetic aryl-oligonucleotides:

**Hippuryl-glycyl-glycine (HGG)** or benzoglycyl-glycyl-glycine is the original chemical substrate. Several assays using HGG were developed involving dialysis of the sample and are not often used now (Ryan et al 1977).

**Hippuryl-histidyl-leucine (HHL)** is the most commonly used substrate. Both histidyl-leucine and hippuric acid are released on hydrolysis by ACE and may be estimated using various techniques:-

*Spectrophotometric determination* (Cushman and Cheung 1971) is time consuming with relatively poor sensitivity and precision.

*Colorimetric method* (Kwarts et al 1982) involves the condensation of hippuric acid with p-dimethylamino-benzaldehyde and quantification.

*Fluorimetric analysis* (Friedland and Silverstein 1976) The release of HL is measured by spectrofluorimetry following addition of o-phthaldialdehyde. This method is rapid, simple and very sensitive, and requires as little as 1ml of test serum. The assay has high reproducibility and is more sensitive than spectrophotometric methods.

*Radiochemical assay* (Rohrbach 1978) using radiolabelled HHL.  $^{14}\text{C}$ -hippuric acid is measured following incubation of substrate with enzyme. This assay provides good sensitivity but is not suited to determination of ACE activity in serum or plasma.

**FAPGG (furylacryloylphenylalanyl-glycyl-glycine)** (Holmquist et al 1979) - the original method was developed using enzyme purified from tissue. Ronca-Testoni (1983) adapted the method for use with serum and several automated assays based on this method have been described since. The use of FAPGG has advantages in that the change in absorbance may be measured kinetically and hydrolysis is three times greater than with other substrates.

Therefore the assay time is shorter and the assay is more sensitive.

*2.3.2 Measurement of the ACE Molecule* Direct measurement of ACE has been possible since the preparation of specific anti-ACE antibodies. Alhenc-Gelas et al (1983) developed a direct RIA for the determination of ACE levels in human serum using antisera to human lung ACE raised in rabbits. Their method is sensitive enough to measure immunoreactive enzyme molecules in human plasma in volumes of 50ml or less. Enzyme linked immunosorbent assay (ELISA) techniques have been described but not used in clinical investigation (Lanzillo & Fanburg 1982).

### *2.3.3 Measurement of human serum ACE activity*

The assay used in this thesis was that of Friedland & Silverstein (1976).

1. 20ml of test serum was added to 480ml buffered substrate at 37°C. Following vortexing this was incubated for exactly 15 minutes. Buffered substrate was prepared fresh as follows: 0.2 ml substrate (1.25mM Hippuryl-histidyl-leucine (HHL) in methanol) 1.0 ml phosphosaline buffer (0.5M di-potassium hydrogen phosphate, 1.5M sodium chloride, pH 8.3) 3.6 ml dH<sub>2</sub>O
2. Simultaneously, 480 ml buffered **standard** was allowed to rest in the water bath for the same period without serum. Buffered standard was prepared fresh as follows: 0.2 ml working standard (21.5mM, final conc, Histidyl-leucine (HL) in methanol) 1.0 ml phosphosaline buffer, 3.6 ml dH<sub>2</sub>O
3. Buffered **substrate** was also allowed to rest for the fifteen minutes without samples added to act as blanks.
4. The reaction was then stopped by adding 2.9ml 0.28N NaOH to test samples, standards, and blanks.
5. Serum was then added to both standards and blanks.
6. Then 200ml of the fluorescent reagent o-phthalaldehyde (15mM in methanol) was added to all test tubes, and incubated at 37°C for 10 minutes.
7. This reaction was stopped by the addition of 400ml 3.0N Hydrochloric acid.
8. Serum proteins bound to o-phthalaldehyde are eliminated from the sample by centrifugation at 1500 g for 10 minutes (International Equipment Company, USA, Centaur-7R).



**Fluorimetry** After 40 minutes the fluorescence was read with excitation at 368 nm and emission at 500 nm (Perkin-Elmer Fluorescence spectrophotometer 1000, quartz cuvettes). The fluorescence reaches a maximum after 20-30 minutes and is stable for about 1.5 hours (Friedland & Silverstein 1976)

#### 2.3.4 Calculation of ACE activity

Results were interpreted by the following formula:

$$\frac{(\text{Test} - \text{Blank}) \times \text{nmol HL in Std} \times \text{Vol. sample} \times \text{time}}{(\text{Std} - \text{Blank})}$$

Results are thus in nmolHL/ml/min.

Test, Blank, Standard = the fluorescent reading for each sample.

Volume sample = 20 ml for the routine assay.

Time = 15 minutes, for the routine assay.

Std = 10.32 nmol Histidyl-leucine for the normal control standard

A standard serum was produced from pooled serum from 50 healthy volunteers. This was stored at -20°C, and at each assay a sample of this standard serum was run. The average value of this serum had been determined by at least 10 previous assays to be 31.3 nmol/ml/min, and at each procedure the final result of sample activity was adjusted for variation in the result of standard serum activity.

## 2.4 DETERMINATION OF TISSUE ACE

### 2.4.1 *Direct Measurement of Tissue ACE*

Measurement of the biological activity of ACE in-vivo has been largely confined to studying plasma ACE. It is much harder to examine membrane-bound (or thus tissue) ACE activity due to the problem of dissociating the plasma component whilst maintaining live tissue. Until recently there was also difficulty in accurately measuring angiotensin II levels. The dermal responses to bradykinin, the cough response to bradykinin and the pressor responses to angiotensin I provide biological models of ACE which include the main forms of the enzyme, that is the membrane-bound form and the plasma form.

### 2.4.2 *Dermal Responses as an Indirect Measure of Tissue ACE*

Subcutaneous injection of bradykinin produces a wheal and flare skin response (Basran 1982). When the ability of ACE to metabolise bradykinin is blocked by ACE inhibitor treatment the response to bradykinin given intradermally increases (Ferner et al 1987, 1990). Thus intradermal bradykinin administration has become an accepted method of assessing dermal ACE and thus membrane bound ACE activity *in vivo* (Ferner et al 1987). It is of course not possible to separate the effects of dermal ACE from serum ACE *in-vivo*. ACE, apart from inactivating bradykinin, also catalyses the metabolism of substance P (Lindgren et al 1987). Bradykinin stimulates sensory neurones containing substance P, thereafter this stimulates mast cells resulting in histamine release (Widdicombe 1980, Lindgren 1989). A sufficient dose will send orthodromic impulses to the spinal cord to relay sensory information such as itching, pain and burning via C fibres. Antidromic impulses produce a flare, and the increased permeability in the widely dilated blood vessels result in the local oedema called the wheal. The wheal can be measured according to its area or thickness (Ferner 1987, Ferner 1989, Chapman 1977). The metabolism of histamine is non-kininase dependent and the dermal responses to histamine are not affected by ACE inhibitor treatment (Anderson 1990). The dermal responses to bradykinin and histamine were examined in chapter 8, when examining possible differences between subjects of different ACE genotype. The responses to bradykinin and substance P were examined in chapter 6 to determine the pattern of changes in tissue ACE when an ACE inhibitor drug was withdrawn.

#### *2.4.3 Wheal and flare response to intradermal vasoactive agents (Bradykinin, Histamine and Substance P)*

1. Solutions were prepared fresh from stock solutions on each test day and stored on ice. Sodium chloride (0.9%) was used to make up each dilution.
2. A wheal and flare response was produced by a single 0.1ml intradermal injection in a grid pattern of eight injection sites on the anterior aspect of the right forearm, using a 0.4 mm diameter needle. Injections were made at least 3 cm apart.
3. The cutaneous reaction induced by intradermal injection of the vasoactive agents was evaluated by measurement of the wheal area.
4. The response was measured at 15 minutes by drawing round the wheal in ink and transferring the image to graph paper using adhesive cellophane tape. The area of response was measured by digitalised computer planimetry using a Cherry computer bit pad, using software designed by P. Armstrong (Computing Services, University of Sheffield) using the mean of three measurements.

#### *2.4.4 Intravenous infusion of angiotensin I*

Angiotensin I causes a dose-dependent pressor effect when infused intravenously (Belz et al 1987, Wellstein et al 1987, Essig et al 1989) with steady-state levels of blood pressure achieved within 3 minutes (Belz et al 1987). Factors important in determining the blood pressure response are race and sodium balance (Joubert et al 1990). I examined the pressor response to angiotensin with respect to ACE genotype in chapter 3 with the purpose of establishing whether vascular tissue ACE might differ according to genotype.

## 2.5 ASSESSMENT OF COUGH AND COUGH REFLEX TESTING

2.5.1 *Measurements of Subjective Cough.* In chapters 5 and 6 the symptom of cough is measured by the use of questionnaires and visual analogue scales.

2.5.1.1 **Questionnaire in assessment of cough** The self-administered questionnaire used in the studies on cough was validated by Fletcher and consists of a list of symptoms which are graded by the subject (Appendix 1). The symptoms examined were: dry mouth, cramps, dry cough, racing heart, heartburn, headache, sore throat, nocturia, facial flushing, voice changes, diarrhoea and stuffy nose. The severity of these symptoms was graded as: *not at all* (0), *a little* (1), *moderately* (2), *quite a bit* (3) or *extremely* (4) (Fletcher 1990, Yeo 1991, Lacourcière 1994).

The second part of the questionnaire invited further details regarding the presence or absence of two symptoms: **headache** and **cough**. Questions on headache preceded those on cough. The inclusion of detailed questions on headache and other symptoms aimed to reduce the bias towards reporting of cough.

In the study examining the cessation of cough a diary relating to the nature of the cough was completed by the patient each day. This consisted of six questions concerning nocturnal severity, frequency, daytime severity, throat soreness, husky voice and stuffy nose. These were graded in the same way as the questionnaire above. A similar questionnaire was used to measure night time cough (Appendix 2).

### 2.5.1.2 **Visual analogue scales in assessment of cough**

Visual analogue scales have been used in several previous studies examining cough with ACE inhibitors (Fletcher 1990, Lacourcière 1994, Yeo 1991). Severity and frequency of cough were measured by two of these validated visual analogue scales. They consisted of 10 cm lines with the following statements at opposing ends: (I never cough - I am always coughing); (Cough has been absolutely no trouble to me- my cough has been as bad as it could possibly be) (Fletcher 1990, Appendix 3).

### 2.5.1.3 Definition of ACE inhibitor cough

The criteria for diagnosis of ACE inhibitor cough during the prospective study (Chapter 5) were (a) a new onset of cough during ACE inhibitor treatment (b) persistence of the cough for longer than three weeks and (c) no other identifiable cause. These clinical criteria have a positive predictive value of 95% for ACE inhibitor cough (Yeo 1991). The same criteria were applied to the patients who were examined with cough and in who the ACE inhibitor was discontinued (chapter 6). They also applied to those patients with ACE inhibitor cough genotyped for the I/D ACE polymorphism (chapter 9).

### 2.5.2 *Methods of assessing the cough reflex*

Several methods have been used to study the cough reflex. There is general agreement that capsaicin and citric acid are the most useful tussive agents for cough reflex testing but less agreement exists about dose and the method of administration. Capsaicin (8-methyl-N-vallenyl-6-nonenamide), a purified extract of chilli peppers, is the most commonly used tussive agent directly stimulating bronchial C-fibres (Winning et al 1986). Stimulation of C-fibres by capsaicin induces cough and transient bronchoconstriction lasting less than one minute (Collier & Fuller 1984) as shown by single nerve fibre recordings (Coleridge & Coleridge 1984). Citric acid (Pounsford & Saunders 1985, Morice 1987), and continuous inhalation of distilled water (Morice 1987) have also been used to stimulate cough. They suffer from the disadvantage of tachyphylaxis, and it is thus difficult to construct dose-response curves (Morice 1992). The delivery systems used to administer the tussive agent have varied considerably. Initial methods consisted of continuous inhalation from a one litre dead space supplied via a nebuliser with subjects inhaling for between 30 and 60 seconds, whilst measuring the cough response (Collier & Fuller 1984, Pounsford & Saunders 1985, Morice 1987). Recently single dose inhalation has been used, either via a Mefar breath-activated dosimeter, or De Vilbiss No. 40 nebuliser. Both of these methods have been extensively validated (Fuller & Choudry 1987, Morice 1987, Foster 1991). The method of administering doses of tussive agents either as incremental doses (Pounsford & Saunders 1985, Morice 1987), or in random order (Fuller & Choudry 1987, Foster 1991), appear to produce equally valid and reproducible results for cough reflex testing.

### 2.5.3 *Interpretation of cough data*

The cough response is not linear. Doubling the stimulus causes less than a doubling of the response (Widdicombe 1980), and analysis of cough dose-response curves to stimuli such as capsaicin poses interesting mathematical problems. The raw cough response data derived by tussive challenge are not normally distributed, and nonparametric tests such as the Wilcoxon signed rank test and Friedman's non-parametric analysis have been used. These statistical tests are useful when comparing related or matched samples to examine whether cough response changed significantly within subjects under the test condition (Pounsford 1985).

The measurements usually taken when cough testing is done include the following:

- i) The number of coughs per minute.
- ii) The total number of coughs for all doses, the total cough response (**TCR**). This is an approximation of the area under the curve of the dose response and is a measure of the overall sensitivity during cough challenge (Foster 1992).
- iii) The lowest dose to cause 1, 2 or 5(or more) coughs; the **D1** (Foster 1992), **D2**, or **D5** (Collier & Fuller 1984, Morice 1987) respectively. The D2 and D5 have been used as measures of the cough threshold and sensitivity (Collier & Fuller 1984, Choudry 1990).

Another method of analysing cough responses is to determine whether there is a shift in the dose-response curve to the left or right on the x-axis, or to examine the dose required to attain a particular cough response, rather than analysing cough scores. In these techniques the dose used is log transformed and then parametric tests can be performed. Parallel line bioassay is an example of this method of analysis and is now described.

### 2.5.4 *Parallel line bioassay in cough reflex testing*

In this thesis the capsaicin dose response curves and skin responses to bradykinin were analysed using a parallel line bioassay method (Armitage and Berry 1987). This technique estimates the relative potency, with 95% confidence intervals, of capsaicin on the cough response in subjects at different timepoints. The relative potency is a measure of shift of the dose-response curve to left or right when a paired comparison is made. Thus lower relative potency estimates indicate reduced sensitivity to the capsaicin. For each analysis the slope should be significant and the model has to satisfy rules for parallelism and linearity. This technique is a form of analysis of variance and when used the doses of drug given are log-transformed.

### 2.5.5 *Cough testing with capsaicin* (Foster et al 1991).

This method involves the inhalation of single doses of capsaicin solution administered in a random order.

#### *Preparation of capsaicin solutions*

0.125 ml of the concentrated stock solution was diluted with 2.375 ml sodium chloride (0.9%) to give a working solution of 5 mmol/ml. This was made into solutions for inhalation by further serial dilutions with saline. The doses delivered per 0.0025 ml delivery were thus in log incremental doses from 0.025 - 6.25nmol.

#### *Method of administration*

1. One ml of each solution was placed in a separate nebuliser.
2. Subjects inhaled slowly and deeply over 1-2 secs. One delivery from the nebuliser was administered at the beginning of inspiration.
3. The number of coughs produced during the one minute after challenge were recorded.
4. Challenges were given at two minute intervals.
5. The number of coughs at each dose were recorded by an examiner blinded to either the subjects genotype in chapter 7 or to the drug taken in chapter 5.

### 2.5.6 *Cough reflex testing with bradykinin*

The cough response to bradykinin is examined in chapter 7. When previously studied in man bradykinin caused a bronchoconstrictor response in those with asthma but not in healthy individuals (Simonsson 1973, Fuller 1987). Cough unrelated to any change in airways calibre, was observed, to a varying extent in these and other studies (Dixon 1987, Choudry 1989, Ichinose 1992).

Bradykinin (Sigma,UK) in 10% ethanol in saline, was administered in the following doses: 0.125 mg/ml, 0.25 mg/ml, 0.5mg/ml, 1mg/ml, 2mg/ml, 4mg/ml. Dilutions were made with 0.9% sodium chloride.

### 2.5.7 *Measurement of respiratory function*

Respiratory function including forced expiratory volume (FEV1), forced vital capacity (FVC) and peak expiratory flow rate (PEFR) was measured using a Vitalograph-compact (Vitalograph Ltd, UK). Three readings were taken on each occasion and the highest value recorded was used.

Chapter 3

ACE GENE POLYMORPHISM  
AND  
PRESSOR AND HORMONAL RESPONSES  
TO ANGIOTENSIN I INFUSION  
IN HEALTHY SUBJECTS



### 3.1 INTRODUCTION

An individual's genotype for the I/D polymorphism in the ACE gene gives a strong indication of the serum ACE for that person. The clinical relevance of this finding was uncertain until it was suggested that the ACE genotype was linked to vascular disease. The deletion (D) allele has been suggested as a risk factor for myocardial infarction (Cambien 1992), ischaemic and dilated cardiomyopathy (Raynolds 1993), and the development of left ventricular hypertrophy (Schunkert 1994). The mechanism by which the D allele may exert such detrimental effects is unknown but could be through an enhanced rate of production of angiotensin II resulting in increased pressor and trophic cardiovascular responses. The enhanced production of angiotensin could be related to the concentration of serum ACE or the activity of ACE bound to the vascular endothelium (Swales 1993).

To my knowledge the expression of ACE at the vascular endothelium, another form of tissue ACE had not been examined. I have studied this indirectly by examining the response to intravenous infusion of angiotensin I. Angiotensin I causes a dose-dependent pressor effect when infused intravenously (Belz et al 1987, Wellstein et al 1987, Essig et al 1989) with steady-state levels of blood pressure achieved within 3 minutes (Belz et al 1987). Factors that might be important in determining the blood pressure response are race and sodium balance (Joubert et al 1990).

### 3.2 AIMS

1. To determine whether pressor or neurohormonal responses to angiotensin I infusion differ in healthy subjects homozygous for either the I or D allele.

### 3.3 METHODS

#### 3.3.1 *Subjects*

Sixteen healthy Caucasian men aged 26 years (range 19-36) were selected to provide equal numbers of the two genotypes DD (n=8) and II (n=8). Supine blood pressure was measured on three occasions over one week and those with a mean diastolic greater than 80 mmHg were excluded. All had normal serum creatinine, electrolytes and electrocardiograph. Subjects gave written informed consent to the study which was approved by the district ethics committee.

### 3.3.2 *Study design*

(Figure 3.1) Subjects attended at 0900h having avoided added salt and foods with a high salt content for three days prior to the study, and having refrained from food, smoking, caffeine and strenuous exercise for 12 hours. Urinary sodium excretion over 24 hours was measured immediately prior to the infusion. Sodium chloride 0.9% was infused intravenously for a 30 minute baseline period at 48ml/hr via an IVAC p1000 pump whilst subjects lay supine. Angiotensin I was prepared in the hospital pharmacy as a sterile solution containing 50 µg/ml in 0.9% sodium chloride then diluted to concentrations of 1.2 µg/ml and 4µg/ml. Angiotensin I was infused at an initial rate of 0.1µg/min and increased to 0.3, 0.9, 1.8, 3.0, 4.0, 5.0 and 6.0 µg/min at 3 minute intervals, or until a rise in diastolic pressure of 25 mmHg was achieved. Blood pressure and heart rate were measured 30, 15, 10, 5 and 0 minutes before the angiotensin infusion, during the final 30 seconds of each 3 minute infusion period, and at 10 minute intervals after the infusion was discontinued. Blood was taken for serum ACE activity and plasma renin, angiotensin II and aldosterone concentrations before and at the end of the angiotensin I infusion. Identical syringes were used during the baseline and angiotensin I infusion periods so that subjects were unaware when the angiotensin infusion began. The investigators were blind to the genotype of the subject.



### 3.3.3 *Measurements*

ACE genotype was determined by polymerase chain reaction as described in chapter 2. Serum ACE activity was measured by fluorimetric assay with units expressed as nmol of histidyl-leucine produced per ml of sample per minute as previously described. Plasma renin (Millar 1980), angiotensin II (Morton 1985) and aldosterone ('Coat-a-count' Kit, Diagnostic Products Corporation) concentrations were measured by radioimmunoassay. Blood pressure and heart rate were measured by a Dinamap semi-automated recorder.

### 3.3.4 *Endpoints*

The principal endpoints to be examined were the doses of angiotensin I which lead to an increase in blood pressure by 25 mmHg. These were the  $R_{(d)25}$ ,  $R_{(s)25}$ , and the  $(HR_{25})$ . The  $R_{(d)25}$  was the rate of angiotensin I infusion which caused a 25 mmHg rise in diastolic blood pressure; and the  $R_{(s)25}$  was the rate of infusion at which a 25 mmHg rise in systolic pressure occurred. Changes in heart rate ( $HR_{25}$ ) were analysed at the time the  $R_{(d)25}$  was attained.

### 3.3.4 *Statistical analysis*

Using a published standard deviation for the diastolic pressor response to angiotensin I infusion in Caucasian subjects (Joubert 1990) it was calculated that eight subjects were required in each group to detect a difference in  $R_{(d)25}$  between groups of  $1.75\mu\text{g}/\text{min}$  of angiotensin I with 80% power and  $\alpha=0.05$ . After logarithmic transformation of the rate of infusion of angiotensin I to approximate a linear dose response the  $R_{(d)25}$  and  $R_{(s)25}$  were interpolated from individual responses. Fifteen subjects achieved a 25 mmHg or greater increase in diastolic blood pressure, but one achieved a rise of only 21 mmHg and had the  $R_{(d)25}$  calculated by extrapolation. The  $R_{(d)25}$  and  $R_{(s)25}$  are expressed as geometric means of the responses for each genotype, and the difference between genotypes is expressed as the ratio of the geometric means with 95% confidence intervals for this ratio. Unpaired t-tests were used to investigate differences in blood pressure, heart rate and neurohormonal responses between the groups at baseline and after angiotensin I infusion. Paired t-tests were used to examine neurohormonal responses within groups.

### 3.4 RESULTS

#### 3.4.1 Comparability of groups

(Table 3.1). Age, weight, baseline blood pressure, heart rate and 24 urinary sodium excretion were similar in the two groups. As anticipated there was a significant relationship between ACE genotype and serum ACE activity at baseline, with mean serum ACE 25.0 nmol/ml/min in group DD and 13.1 nmol/ml/min in group II ( $p < 0.02$ ). There were no significant differences in baseline plasma renin and aldosterone levels between the genotypes. Baseline angiotensin II levels were higher in the DD subjects (7.0 pg/ml) than II subjects (4.7 pg/ml), and this difference approached significance (difference DD-II = 2.3 pg/ml, 95% C.I.: 0.0 to +4.6,  $p = 0.07$ ).

**Table 3.1** Baseline data for healthy male volunteers according to ACE genotype;  $n = 8$  for each group (mean  $\pm$  se).

	DD	II
Age (years)	26.7 (2.1)	24.9 (1.7)
Weight (kg)	70.7 (2.0)	76.0 (3.2)
SBP (mmHg)	123.5 (2.1)	121.0 (2.0)
DBP (mmHg)	70.8 (2.1)	69.2 (1.8)
Heart rate (bpm)	58.4 (2.0)	62.3 (2.1)
Urinary sodium (mmol/24h)	130.4 (17.7)	111.0 (16.1)
Plasma Renin Concentration ( $\mu$ /ml)	13.4 (2.5)	11.5 (2.7)
Plasma Aldosterone (ng/100ml)	11.7 (1.3)	11.0 (1.2)
Plasma Angiotensin II (pg/ml)	7.0 (1.0)	4.7 (0.4)
Serum ACE activity* nmol/ml/min	25.0 (1.7)	13.1 (0.6)

\*  $p < 0.02$  for difference between groups,

#### 3.4.2 Changes in blood pressure

(Table 3.2, Figure 3.1). The geometric mean rate of infusion of angiotensin I required to achieve the  $R_{(d)25}$  was 2.53  $\mu$ g/min in II subjects and 2.67  $\mu$ g/min in DD subjects (ratio of doses (II:DD) = 0.95; 95% CI 0.44 to 2.02,  $p > 0.05$ ). The equivalent rates of infusion for systolic blood pressure ( $R_{(s)25}$ ) were 4.47  $\mu$ g/min in II subjects and 3.39  $\mu$ g/min in DD subjects (ratio of doses (II:DD) = 1.32; 95% CI for ratio 0.49 to 3.56,  $p > 0.05$ ).



### 3.4.3 Changes in heart rate

(Table 3.2, Figure 3.2). There was a significant difference between the groups in the chronotropic response to angiotensin I infusion. At the time of the  $R_{(d)25}$  the mean change from baseline heart rate was +1.2 bpm for DD subjects and -9.5 bpm for II subjects (Diff II-DD= 10.7 bpm; 95% CI: 6.7 to 14.8;  $p=0.01$ ).

### 3.4.4 Changes in serum ACE activity and plasma renin, angiotensin II and aldosterone concentrations

(Table 3.2). There was a rise in serum ACE activity after angiotensin I infusion in all subjects but there was no difference in this response between subjects of differing genotypes. There was no change in the mean plasma renin concentration in response to angiotensin I infusion. Serum aldosterone and angiotensin II levels rose as expected but there was no difference in responses between groups. There were also no differences between the genotypes when these measurements were calculated as the unit change per  $\mu\text{g}$  angiotensin I infused (i.e. the sensitivity to angiotensin I).

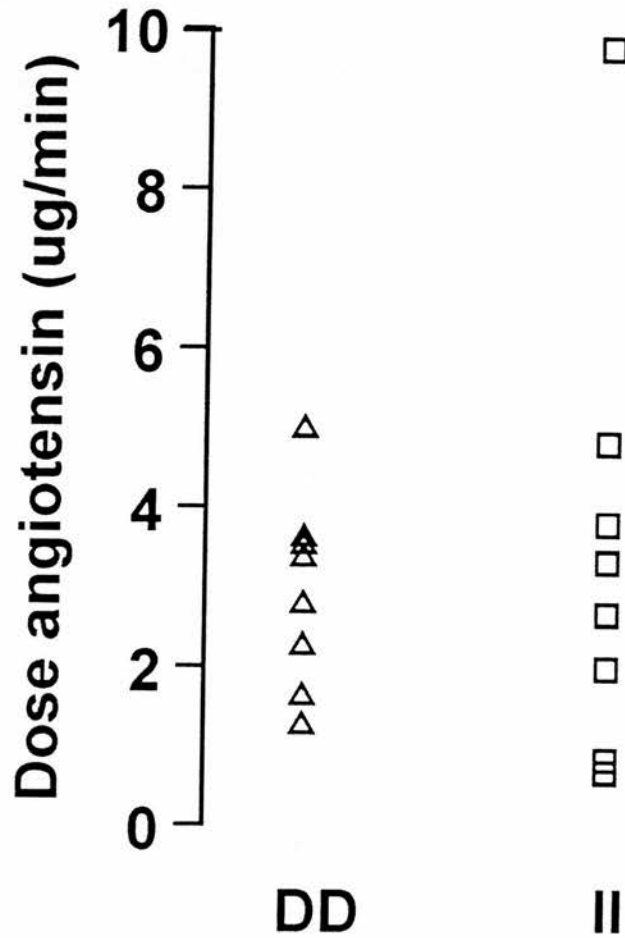
**Table 3.2.** (i) Rate of angiotensin I infusion to achieve a 25 mmHg rise in diastolic ( $R_{(d)25}$ ) and systolic ( $R_{(s)25}$ ) blood pressure and (ii) changes in heart rate ( $HR_{25}$ ) and neurohormonal parameters from baseline after a 25mmHg rise in diastolic blood pressure during angiotensin I infusion in DD and II subjects.

	II	DD		
(i)	Geometric mean	Geometric mean	Ratio of doses (II:DD)	95% C.I. of ratio
$R_{(d)25}$ ( $\mu\text{g}/\text{min}$ )	2.53	2.67	0.95	0.44 to 2.02
$R_{(s)25}$ ( $\mu\text{g}/\text{min}$ )	4.47	3.39	1.32	0.49 to 3.55
(ii)	Mean (se)	Mean (se)	Difference (II-DD)	95% CI for difference
$HR_{25}$ (bpm)	-9.5 (3.1)	+1.2 (2.1)	-10.7*	-6.7 to -14.8
Plasma Renin (uU/ml)	-2.1 (1.2)	-0.4 (1.0)	-1.7	-5.1 to +1.6
Serum ACE (nmol/ml/min)	+15.7 (0.7)	+30.1 (2.3)	-14.4*	-9.3 to -19.5
Angiotensin II (pg/ml)	+181.0 (24.0)	+180.0 (9.0)	+1.0	-53.6 to +55.6
Aldosterone (ng/ml)	+10.3 (2.3)	+11.4 (3.4)	-1.1	-9.8 to +7.6

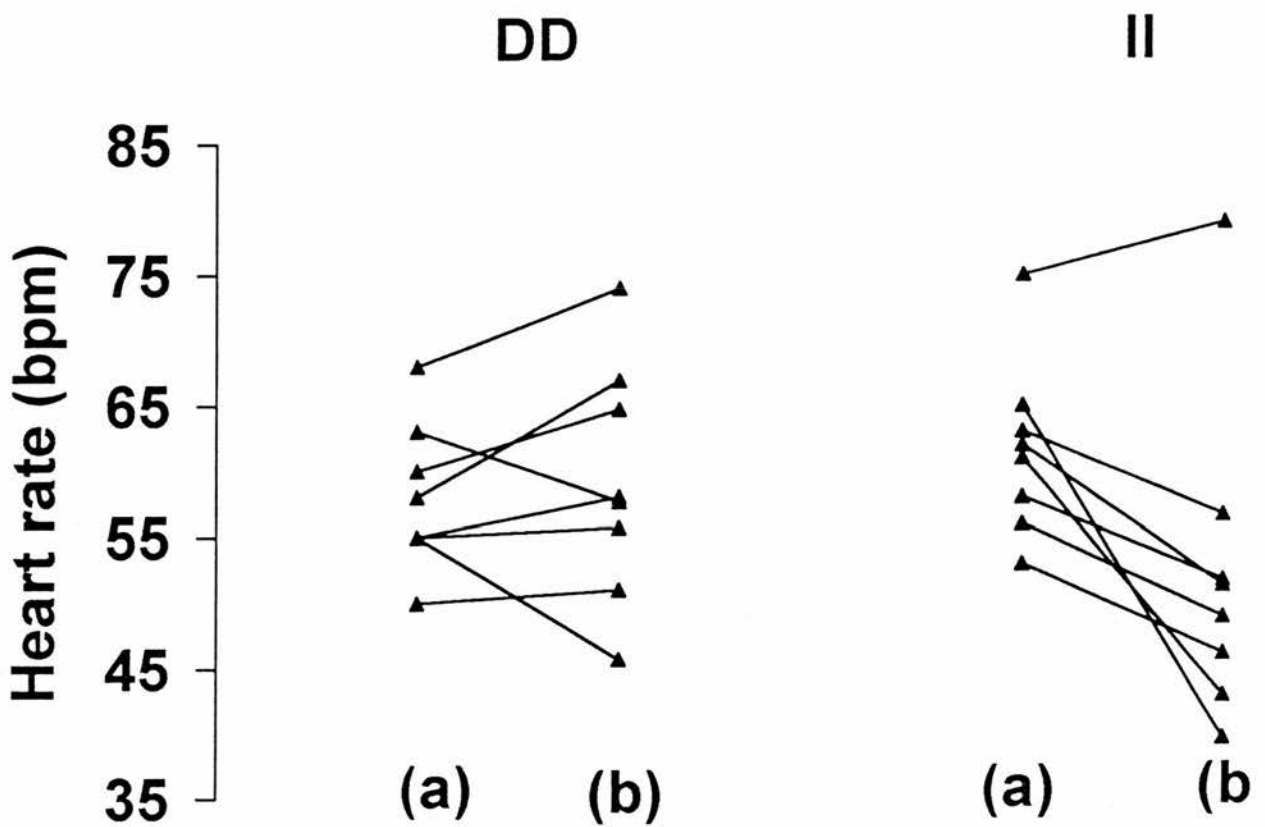
\* $p = 0.01$

3.4.5 Changes in blood pressure v initial serum ACE.

There was no relationship between the change in blood pressure as determined by  $R_{(d)25}$ ,  $R_{(s)25}$ , or  $(HR_{25})$  and the initial serum ACE activity.



**Figure 3.2** The dose of angiotensin I ( $\mu\text{g}/\text{min}$ ) required to increase diastolic bp by 25mmHg in healthy male subjects according to genotype ( $\Delta$  =DD, n=8;  $\square$  =II, n=8).



**Figure 3.3** The heart rate (bpm) at baseline (a) and at maximal angiotensin I infusion rate required to increase diastolic bp by 25mmHg (b) in healthy male subjects according to genotype (n=8 for each genotype).



### 3.5 DISCUSSION

The strong relationship between ACE genotype and serum ACE activity was confirmed in this study. As expected angiotensin I caused a pressor response, increased plasma angiotensin II and aldosterone concentrations, but caused no change in plasma renin concentration.

However, neither the pressor nor the hormonal responses differed significantly between the two genotypes despite a near fourfold higher mean serum ACE level in the DD subjects. The only significant difference in response between the genotypes was in heart rate. There was an increase in serum ACE activity shown in this study. This unusual and unexpected finding is difficult to explain but is most likely to represent some interference in the assay technique rather than enzyme induction.

Overall these results show no evidence that the sensitivity of the renin-angiotensin-aldosterone system to angiotensin I varies with serum ACE level or I/D genotype. However although the geometric means for the  $R_{(d)25}$  were similar, with a ratio of 0.95, the 95% confidence intervals show that the study had insufficient power to exclude ratios between 0.44 (DD>II) and 2.02 (II>DD). This relatively low power was not expected, and was due to much greater variability in the pressor response to angiotensin I infusion using this protocol (Figure 1) than was anticipated from the data of Joubert and Brandt (Joubert 1990). The reasons for this are not clear, but as shown in Figure 1 there was one outlying subject in the II group. However even if this subject was excluded the ratio of geometric means (II:DD) was 0.78, with 95% confidence intervals of 0.39 to 1.55, so that there was still no significant difference in the genotypes.

While my study was in progress a similar study of the pressor sensitivity to angiotensin I infusion in relation to ACE genotype was reported (Ueda et al 1994). Ueda et al showed a significant increase in pressor sensitivity to angiotensin I infusion in 10 healthy normotensive DD men compared to 10 II men. The II:DD ratio for the rates of infusion of angiotensin I required to achieve a 20mmHg rise in mean arterial pressure in their study was 1.68. I recalculated my data in an analysis similar to that of Ueda et al, correcting for body weight and using as the end point a 20mmHg rise in mean arterial pressure. Analysed thus the ratio of infusion rates II:DD was 1.12 with 95% CI 0.65 to 1.92. By contrast Menard et al found no relation between diastolic blood pressure response and genotype (Menard 1995) using similar methodology to my study. Thus this study does not confirm their positive finding, although it had insufficient power to exclude a difference of the magnitude reported.

The only significant difference between the two genotypes was in the chronotropic response to angiotensin I infusion. Subjects of II genotype exhibited bradycardia averaging 9 beats per minute associated with a 25 mmHg rise in diastolic pressure, whereas DD subjects showed no change in heart rate. That subjects of DD genotype exhibited less heart rate slowing than the

II subjects at a similar pressor response could imply altered baroreceptor sensitivity between the genotypes. Angiotensin II is known to inhibit the reflex slowing of heart rate to a rise in arterial pressure (Smyth 1969). In chapter 4 I will describe the finding of no difference in the chronotropic response to a single oral dose of enalapril in healthy subjects of differing ACE genotype. However as I found no evidence of other differences in haemodynamic responses in homeostasis or in the rate of production of angiotensin II, between II and DD subjects the difference between genotypes in chronotropic response to angiotensin I may be a chance observation, however further study of cardiovascular reflexes in relation to ACE genotype may be of interest.

The conversion of angiotensin I to angiotensin II is not the rate limiting step in the renin-angiotensin cascade (Danser 1992) and marked inter-individual differences in serum ACE levels may be of little or no importance.

In summary:

- The effects of incremental infusion of angiotensin I on pressor and hormonal responses in relation to the ACE genotype were compared in healthy men of genotype DD and II.
- Serum ACE activity differed significantly between the genotypes with significantly higher mean values in DD subjects than II subjects. Serum ACE also increased during the study but this did not differ between genotypes.
- The mean infusion rates of angiotensin I required to achieve a 25 mmHg rise in diastolic pressure [R(d)25] were 2.53  $\mu\text{g}/\text{min}$  in II subjects and 2.67  $\mu\text{g}/\text{min}$  in DD subjects (ratio of infusion rates 0.95; 95% CI 0.44 to 2.02,  $p>0.05$ ).
- At the time of R(d)25, changes in heart rate from baseline were +1.2 bpm for DD subjects and -9.5 bpm for II subjects (Diff II-DD= 10.7 bpm; 95% CI: 6.7 to 14.8;  $p=0.01$ ).
- There were no differences in plasma renin, angiotensin II and aldosterone responses to angiotensin I infusion between the DD and II genotypes.
- No differences in blood pressure or renin-angiotensin-aldosterone system responses to infusion of angiotensin I related to the ACE gene polymorphism were shown, but the study has insufficient power to exclude with certainty such differences. There was a significant difference between II and DD subjects in the chronotropic response to angiotensin I infusion.

CHAPTER 4

EFFECT OF A SINGLE DOSE OF ENALAPRIL  
ON SERUM ACE ACTIVITY  
AND BLOOD PRESSURE  
IN HEALTHY SUBJECTS  
OF DIFFERENT ACE GENOTYPE.

## 4.1 INTRODUCTION

ACE inhibitor drugs such as enalapril are used widely in the treatment of hypertension and heart failure. The initial response to ACE inhibition is probably due to a combination of factors including vagal responses and pre-existing activation of the renin-angiotensin system (Squire 1996). ACE inhibitors reduce serum ACE activity, and some studies have shown a relation between this reduction in serum ACE activity and falls of blood pressure (Johnston 1983). Through its strong association with serum ACE activity the ACE genotype could therefore influence or predict the response to ACE inhibitors.

## 4.2 AIMS

The purpose of this study was to examine:

1. The relation of ACE genotype to the acute effects of the ACE inhibitor enalapril on serum ACE activity and blood pressure
2. The relation between changes in serum ACE activity and changes in blood pressure in healthy subjects.

## 4.3 METHODS

### 4.3.1 *Subjects*

Twenty-seven healthy normotensive men of mean age 27 years (range 20-46) were selected to provide equal numbers of the three genotypes, ie n=9 for each of the genotypes DD, ID and II. They gave written informed consent to the study which was approved by the district ethics committee. Five of these subjects had also taken part in the study in chapter 3

### 4.3.2 *Study design*

Each subject was given a single oral dose of enalapril 10mg in an open parallel group comparison of the three genotypes. Subjects attended at 0830 h having refrained from smoking, caffeine or strenuous exercise for 12 hours. Otherwise they had followed their normal diet prior to the study, and specifically sodium intake was not controlled or perturbed. They lay supine for 30 min before receiving a single 10 mg dose of enalapril orally. They were semi-recumbent for 6h after taking enalapril and remained fasting except for a standard meal 4h after enalapril. Blood pressure and heart rate were measured at 30, 15, 10, 5 and 0 min before enalapril and at 20 min intervals for 6 h post-dosing. Serum ACE activity was measured at 0 (pre-enalapril), 2, 4, 6 and 24 h.

### 4.3.3 Measurements

ACE genotype was determined by polymerase chain reaction described in chapter 2.2. Serum ACE activity was measured by fluorimetric assay with units expressed as nmol of histidyl-leucine produced per ml of sample per minute (chapter 2.3.3). Blood pressure and heart rate were measured by a Dinamap semi-automated recorder (Silas 1980) taking the mean of duplicate measurements at each time point.

### 4.3.4 Statistical analysis

Changes in blood pressure and serum ACE activity from baseline (pre-enalapril) values were compared between the three genotypes by one-way analysis of variance, and 95% confidence intervals were calculated for differences between DD and II genotypes. The relationship between changes in serum ACE activity and blood pressure was examined by product-moment correlations. Blood pressure data were log transformed to normalise the distribution.

## 4.4 RESULTS

*Comparability of groups* (Table 4.1). Subjects from the three genotypes were of similar age but differed as regards weight, with ID subjects significantly heavier than those of DD or II genotype. Average baseline blood pressure was similar in the three groups. As anticipated (Rigat 1990) there was a significant relation between ACE genotype and serum ACE activity at baseline ( $p < 0.02$  between groups), with DD subjects having mean serum ACE activity 56% higher than that of II subjects (Figure 4.1). The ACE genotype accounted for 40% of the between-subject variance in serum ACE activity at baseline.

**Table 4.1.** Baseline data for 27 healthy male volunteers before enalapril dosing according to ACE genotype (Mean  $\pm$ SD )

	DD	ID	II
Age (yr)	23.8 (4.1)	27.3 (6.6)	28.4 (8.0)
Weight (kg) *	71.4 (5.5)	78.3 (8.5)	69.9 (6.5)
SBP (mmHg)	128.2 (5.6)	127.6 (10.0)	128.0 (5.6)
DBP (mmHg)	72.7 (7.4)	72.7 (9.3)	68.1 (9.1)
MAP (mmHg)	91.2 (5.7)	91.0 (8.7)	85.6 (7.4)
Heart rate (bt/min)	66.6 (8.3)	65.0 (10.5)	63.7 (8.0)
ACE activity** (nmol/ml/min)	40.7 (9.3)	31.8 (6.4)	26.1 (6.1)

\* p=0.04; \*\* p=0.017 for difference between groups

#### 4.4.2 Changes in serum ACE activity

(Table 4.2, Figure 4.1). In all subjects serum ACE activity was reduced substantially 2 h after enalapril, and remained below baseline levels at 24 h. The serum ACE responses to enalapril differed significantly between the ACE genotypes, with the reduction significantly larger in DD than II subjects at 2, 4, and 6 h (Table 4.2, Fig 4.1). The difference between genotypes was no longer present at 24 hours (Table 4.2). The ACE genotype continued to explain approximately 40% of the between-subject variance in serum ACE activity at each time point after enalapril. The *percent* reduction in baseline ACE activity after enalapril showed a different pattern, with larger proportional falls in II than DD genotype subjects, but differences between the groups were small (Table 4.2).

**Table 4.2** Mean (SE) changes at 2, 4 and 6 hours in serum ACE and mean arterial blood pressure (map) for each genotype

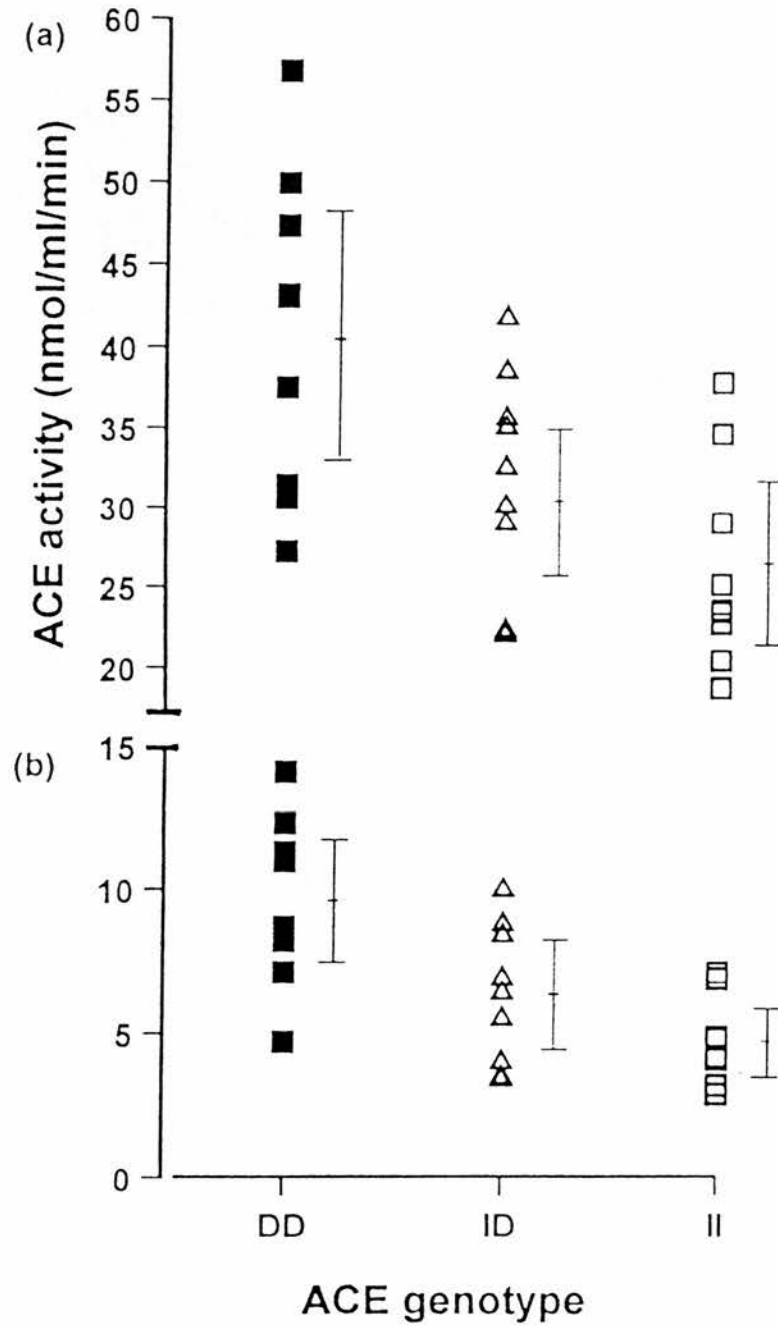
	DD	ID	II	p*	Diff. DD - II	95% CI DD - II
<b>Absolute ACE reduction (nmol/ml/min)</b>						
2 h	27.9 (3.5)	22.5 (2.9)	18.9 (1.7)	ns	9.0	0.7 - 17.2
4 h	32.3 (2.7)	26.1 (1.9)	21.6 (1.8)	0.01	10.7	3.8 - 17.6
6 h	31.2 (2.7)	25.5 (1.9)	21.5 (1.7)	0.02	9.7	2.8 - 16.6
24 h	14.1 (3.3)	17.1 (2.5)	15.2 (1.7)	ns	-1.1	-8.9 - (6.7)
<b>Percentage ACE reduction</b>						
2 h	67.5 (4.4)	68.6 (6.3)	72.9 (3.7)	ns	-5.4	-17.6 - (6.8)
4 h	79.3 (1.2)	81.8 (1.9)	82.8 (1.0)	ns	-3.5	-6.7 - (-0.3)
6 h	76.5 (1.8)	80.3 (1.9)	82.6 (1.1)	0.04	-6.1	-10.5 - (-1.7)
24 h	34.8 (6.3)	50.2 (4.9)	56.6 (5.1)	0.04	-21.8	-38.9 - (-4.7)
<b>MAP reduction (mmHg)</b>						
2 h	4.6 (3.0)	1.7 (3.0)	5.6 (3.5)	ns	-1.0	-10.7 - 8.7
4 h	8.8 (2.2)	4.9 (3.9)	9.1 (2.9)	ns	-0.3	-8.0 - 7.4
6 h	12.9 (2.9)	8.5 (3.6)	10.6 (3.5)	ns	2.3	-7.4 - 12.0

\* Significance of difference between the groups

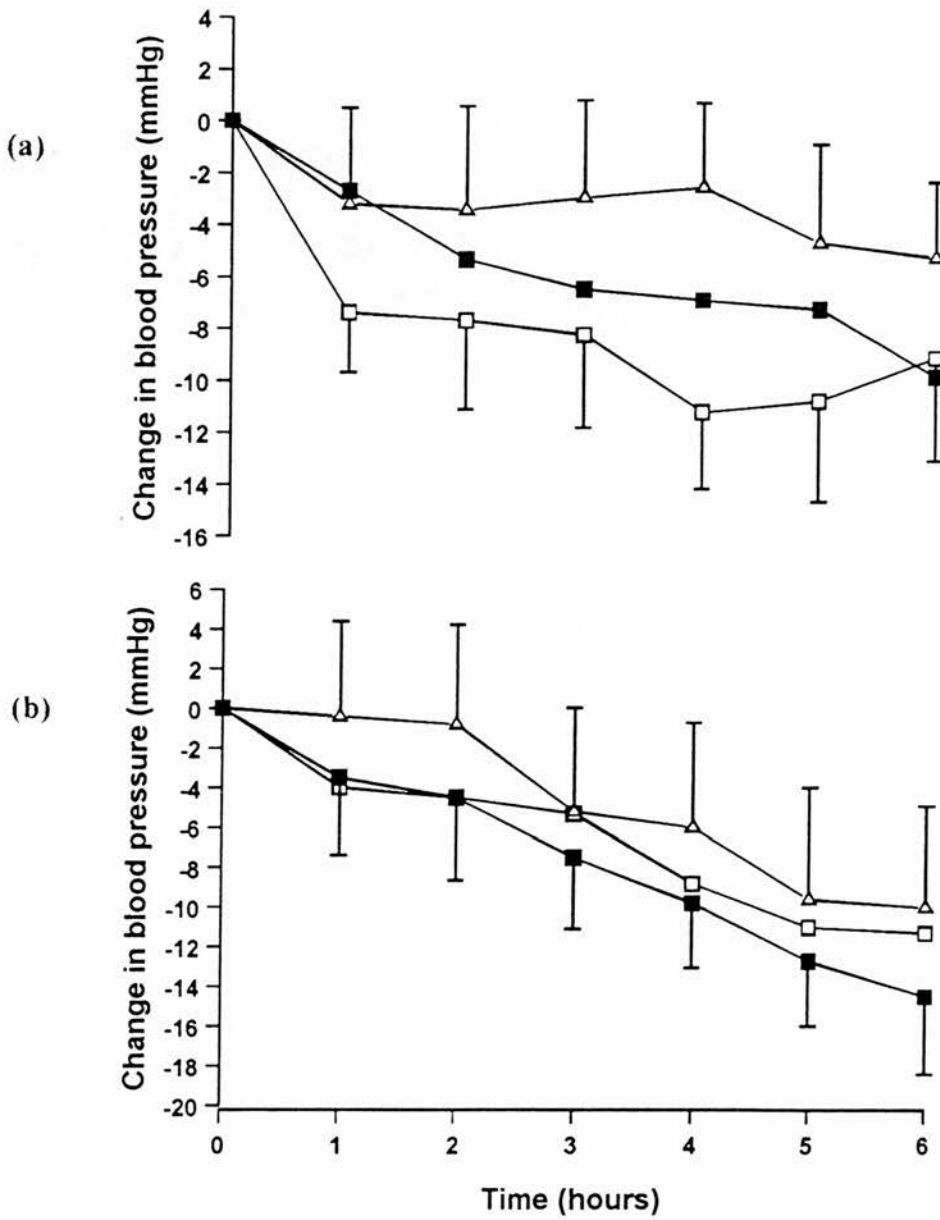
4.4.3 *Changes in blood pressure.* (Figure 4.2) The average fall in mean arterial pressure in all subjects was 7 mmHg (8%) in the 6 hours after enalapril. There were no significant differences between the genotypes for blood pressure response, and in particular no consistent trend for the enalapril response to be larger or smaller in DD compared to II subjects (mean difference DD-II: 0.7 mmHg). The 95% confidence interval makes unlikely differences in response between the genotypes larger than 4.1 mmHg (DD>II) or 5.5 mmHg (II>DD).

4.4.4 *Changes in serum ACE activity v blood pressure.* In all 27 subjects the fall in mean arterial pressure averaged over 6 hours after enalapril did not correlate with initial ACE activity, or with percent or absolute changes in serum ACE activity.





**Figure 4.1** Serum ACE activity at baseline (a) and 6 hours after treatment with enalapril 10 mg (b), related to ACE genotype. Note different scale between baseline and treatment values. Vertical bars show mean and 95% confidence intervals for each genotype.



**Figure 4.2** Changes in systolic blood pressure (2a) and diastolic blood pressure (2b) after treatment with enalapril 10 mg related to ACE genotype, ■=DD genotype, □ = II genotype and Δ =ID genotype (mean ± se)

## 4.5 DISCUSSION

This study confirms the relationship between ACE genotype and serum ACE activity (Rigat 1990), with the ACE genotype accounting for 40% of between-subject variance and DD subjects having serum ACE activity 56% higher than those of II genotype. As might be expected subjects with genotype DD, who had the highest serum ACE activity initially, had significantly larger falls in serum ACE activity at 2,4 and 6 hours after 10mg enalapril when compared to the other genotypes. This difference was no longer evident after 24 hours. In *percentage* terms subjects of genotype II, who had the lowest serum ACE activity initially, actually showed the largest fall in serum ACE activity, although the differences between the three genotypes were small. Enalapril did not abolish the difference between genotypes in serum ACE activity, with residual serum ACE remaining significantly higher in DD than II subjects at each time point. Despite enalapril treatment the ACE genotype continued to account for about 40% of the between-subject variance in serum ACE, and subjects of genotype DD had serum ACE values approximately twice those of genotype II.

Johnston et al. showed a close relation between reduction in serum ACE activity and the fall in blood pressure in hypertensive subjects after a single 10 mg dose of enalapril (Johnston 1983). In the present study blood pressure response did not correlate with percent or absolute reduction in ACE activity, or with initial serum ACE. There was no significant difference in hypotensive response between genotypes. Although subjects of DD genotype had higher serum ACE activity initially, and larger falls in serum ACE in response to enalapril, their blood pressure response was not different from those of the other genotypes. Shown by the 95% confidence intervals the study had sufficient power to exclude differences, in mean arterial pressure responses larger than 5.5 mmHg (II>DD) or 4.1 mmHg (DD>II). I examined healthy subjects in normal sodium balance, who have only small blood pressure reductions with enalapril (Todd 1986), and these results do not preclude the possibility that the ACE genotype may predict the response of hypertensive patients or those with heart failure to ACE inhibitor treatment. In those with chronic heart failure the chronic response does not appear to differ to the acute response to ACE inhibitor (Squire 1996).

Some recent work published at present in abstract form also examined whether there was a relationship between genotype and blood pressure responses. O’Kane et al (1996) examined the responses in salt-deplete healthy subjects using 5mg of enalapril.

No significant difference between the groups was found however confidence intervals are not given to allow determine the certainty of this conclusion. Using different methodology Ueda et al (1996) examined the responses to enalaprilat in healthy subjects who had been infused with angiotensin I. Their work suggests that subjects of genotype II might have greater responses to enalaprilat however the data published shows wide confidence intervals for difference from placebo. The confidence intervals for the difference in response between the genotypes are not shown in the abstract form and it will be interesting to study the full published work. Thus this area of research has attracted more attention since my initial study but as yet it remains uncertain as to whether true conclusions can be drawn.

In summary:

- The ACE genotype influences serum ACE activity under normal conditions and after a single oral dose of enalapril, and also predicts the acute responses of serum ACE activity to a single dose of enalapril.
- I found no evidence that the ACE genotype predicts the acute pharmacodynamic response to enalapril.

Chapter 5

PROSPECTIVE CONTROLLED STUDY OF COUGH WITH  
ENALAPRIL:  
MEASUREMENT BY QUESTIONNAIRE, VISUAL ANALOGUE  
SCALES,  
AND  
CHANGES IN RESPONSE TO INHALED CAPSAICIN.

## 5.1 INTRODUCTION

As discussed in chapter 1 cough due to ACE inhibitor is much more common than initially thought. Most estimates of its prevalence were from retrospective studies or cross-sectional surveys and a prospective study had not been undertaken (Yeo 1990).

ACE inhibitor cough is probably related to inhibition of the metabolism of kinins which facilitate the cough reflex at sensory fibres in the airways (Choudry et al 1989b). The sensory nerves involved are thought to be C-fibres because patients with ACE inhibitor cough have an enhanced response to inhaled capsaicin, a potent stimulant of sensory C-fibres (Fuller 1987, McEwan 1989). Capsaicin inhalation causes cough in a dose-dependent manner (Morice 1992, Foster 1991). It is unknown whether *all* patients treated with ACE inhibitors have a change in the cough reflex or *only* those who go on to develop cough. It is also unknown if those who develop cough have a different cough reflex initially, prior to treatment.

In this next section I describe a prospective controlled study of the effect of the ACE inhibitor enalapril on symptoms of cough and the cough reflex as determined by response to capsaicin.

## 5.2 AIMS

The aims of this study were:

1. To determine the natural history of the development and persistence of ACE inhibitor cough.
2. To quantitate serially the development of cough by the use of questionnaires and visual analogue scales.
3. To record changes in the response to inhaled capsaicin over time in those with ACE inhibitor cough, by comparison with patients treated with enalapril but with no cough, and control patients on alternative antihypertensive treatment.

## 5.3 METHODS

### 5.3.1 *Subjects*

This was a prospective controlled study over twelve months of symptoms and the cough reflex in patients who commenced a new drug for hypertension. Twenty-one patients (11 women, 10 men; mean age 62 years) starting enalapril, and 12 controls (5 women, 7 men; mean age 56 years) starting any other antihypertensive drug were recruited from the Sheffield Hypertension Clinic. Treatment allocation was by the doctor in the clinic who also invited them to participate in the study. The treatment chosen was not randomised however the assessment of cough symptoms and sensitivity to inhaled capsaicin were by an independent observer blinded to the nature of treatment taken.

The criteria for diagnosis of ACE inhibitor cough during the study were (a) a new onset of cough during ACE inhibitor treatment (b) persistence of the cough for longer than three weeks and (c) no other identifiable cause. These clinical criteria have a positive predictive value of 95% for ACE inhibitor cough (Yeo 1991). An additional criterion used for this study was resolution of the cough after stopping enalapril treatment. Patients gave written informed consent to the study which was approved by the hospital ethics committee.

### 5.3.2 *Study design*

Patients attended on six occasions; at recruitment (baseline) and 1, 3, 6, 9 & 12 months after starting treatment. At each visit subjective cough was measured by a self-administered questionnaire (Appendix 1,2; Fletcher et al 1990) and visual analogue scales; VAS (Chapter 2; Appendix 4); cough reflex testing was performed as described below; and FEV<sub>1</sub>, FVC and PEF<sub>R</sub> were recorded.

### 5.3.3 *Measurements*

Capsaicin challenge was performed by single inhalations via a De Vilbiss no 40 nebuliser of four doses of capsaicin, 0.05, 0.2, 0.8, and 3.2 nmol in random order with three minute intervals between doses. These doses have been previously shown to lie on the steeper part of the dose-response curve for cough response to capsaicin (Foster et al 1991). The number of coughs occurring during one minute after each dose was recorded.

### 5.3.4 *Measurements of subjective cough.*

The severity of cough was measured by a validated self-administered questionnaire grading cough as not at all [0], a little [1], moderately [2], quite a bit [3] or extremely [4] (Fletcher et al 1990). Severity and frequency of cough were measured by two validated 10 cm visual analogue scales (Chapter 2; Fletcher et al 1990).

The primary endpoints were (1) the development of persistent cough during treatment, and (2) comparison of changes in cough symptoms and capsaicin response at one month in three groups of patients: (a) Those who developed ACE inhibitor cough, (b) those on enalapril who did *not* develop cough, and (c) controls on alternative antihypertensive therapy.

### 5.3.5 Statistical analysis

Questionnaire and VAS scores for cough were analysed by Kruskal-Wallis non-parametric ANOVA. This provides an analysis of variance for the three groups combined, and also pairwise comparisons between groups with appropriate corrections for ties and multiple comparisons (Siegel & Castellan 1988). Changes from baseline in response to capsaicin for all groups combined were examined by ANOVA. Absolute capsaicin dose-response curves were examined by analysis of variance appropriate to parallel line bioassay, giving estimates of relative potency with 95% confidence intervals (Armitage & Berry 1987). In each analysis the slopes (or common slope) were significant, and did not deviate from linearity or parallelism.

## 5.4 RESULTS

The baseline characteristics for 21 patients starting enalapril and for 13 controls are shown in Table 5.1. The groups were comparable with respect to age, sex, smoking habit, severity of hypertension, and other concomitant antihypertensive drugs.

Table 5.1 Baseline characteristics (SD) for thirty-three patients at recruitment.

Baseline characteristic	Enalapril (n=21)	Controls(n=12)
Age (years)	62 (14)	56 (15)
Sex	10M:11F	7M:5F
Body mass index	27 (4)	26 (5)
Cigarette smoking (%)		
- current	24%	17%
- ex-smoker	29%	33%
- non-smoker	48%	50%
Respiratory disease	2 (10%)	3 (25%)
Blood pressure (mmHg)		
- <u>systolic</u>	<u>184 (24)</u>	<u>184 (25)</u>
- diastolic	99 (13)	105 (19)
Number of anti-hypertensive drugs (before study)	2.3 (0.9)	1.9 (0.8)
$\beta$ -blocker	13 (62%)	7 (58%)
Diuretic	14 (67%)	6 (50%)
Calcium antagonist	2 (10%)	5 (42%)
Aspirin or NSAID	8 (38%)	4 (33%)



#### 5.4.1 Development of ACE inhibitor cough

Eight of 21 patients (38%, 95%CI 16 - 60%) who started on enalapril fulfilled the criteria for ACE inhibitor cough (Table 5.2), while 13 patients did not subsequently develop cough when followed for one year. The mean time to the onset of cough in the eight patients was 2.4 weeks (range 1-6 weeks). This was based on recall of the onset of cough by patients at one month, or at three months if the cough took longer than 4 weeks to develop. In the six patients who stopped enalapril early due to cough it subsided completely after withdrawal of the drug. In most the cough occurred both day and night, usually in bouts. In one case it caused vomiting with the cough. Two patients of those taking other anti-hypertensive medication withdrew early from the study because of treatment changes. At the end of the 12 months of observation all but two of the enalapril-cough patients had withdrawn from the study. By contrast 10 of 13 patients in the group on enalapril who did not cough, and 10 of 12 patients in the control group completed the study.

**Table 5.2** Baseline characteristics and time to the onset of cough in the eight patients treated with enalapril who developed ACE inhibitor cough.

Patient	Age	Sex	Smoking habit	Aspirin NSAID	History of respiratory disease	Onset of cough (weeks)
1	57	F	None	NSAID	no	2
2	73	F	None	no	no	6
3	46	M	Ex-smoker	no	no	2
4	72	F	None	Aspirin	no	2
5	30	M	Smoker	no	no	2
6	61	F	Smoker	Aspirin	no	2
7	32	F	None	no	no	2
8	75	F	Ex-smoker	Aspirin	no	1

#### 5.4.2 Subjective assessment of cough

Changes in cough questionnaire score at one month were significantly different for patients who developed enalapril-induced cough (median change +2), those on enalapril with no cough (0), and controls (0; KW 12.9,  $p < 0.01$ ). In absolute terms these changes were the equivalent of patients who had reported no cough at all coughing moderately at one month. Similar changes were observed for cough severity measured by VAS in patients who developed enalapril-induced cough (median +39 mm), those on enalapril with no cough (+3 mm), and controls (+5 mm; KW 6.64,  $p < 0.05$ ). Changes for cough frequency by VAS showed the same trend but did not achieve statistical significance (+29, 0, +1 mm respectively; KW 3.87,  $p = \text{NS}$ ; table 5.3).

**Table 5.3** Changes in cough at one month measured by self-administered questionnaire and visual analogue scales, and by the response to inhaled capsaicin in 33 patients starting enalapril or an alternative drug for hypertension.

Change at 1 month	Enalapril cough (n=8)	Enalapril no cough (n=13)	Controls(n=12)	p*
Cough score by questionnaire	+2	0	0	0.01
Cough severity VAS (mm)	+39	+3	+5	0.05
Cough frequency VAS (mm)	+29	0	+1	ns
Capsaicin potency (ratio)	4.7	0.9	0.5	0.0005
[95% CI]	[1.2 - 18.5]	[0.4 - 2.1]	[0.3 - 0.9]	

\* p values cited are for the appropriate analysis of variance for the three groups combined.

#### 5.4.3. Capsaicin challenge

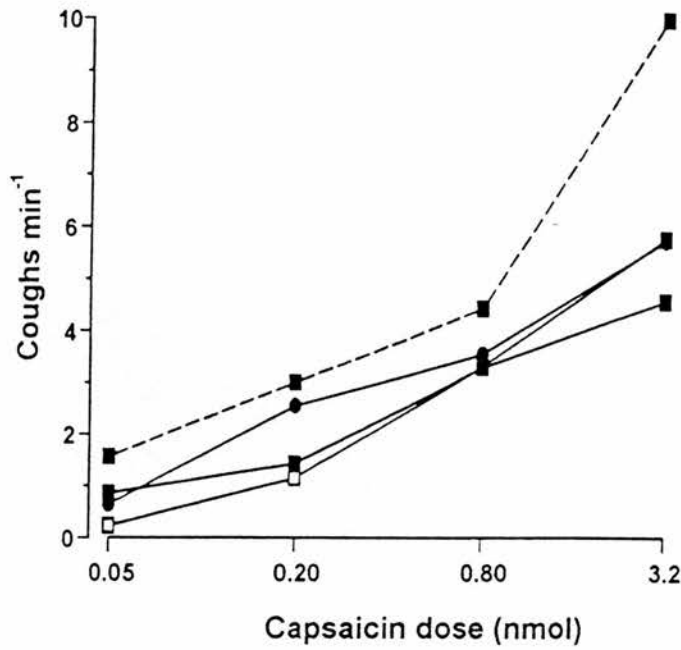
The sensitivity to inhaled capsaicin at baseline did not differ between groups (Figure 5.1), but at one month a significant *increase* in capsaicin potency from baseline was observed for patients with enalapril cough (Figure 5.1, 5.2; relative potency 4.7, 95% CI 1.2-18.5,  $p < 0.025$ ). No significant change from baseline was observed for the enalapril non-cough group (relative potency at one month 0.9, 95% CI 0.4 - 2.1), but a small and significant *decrease* in capsaicin potency was seen for the control group (relative potency 0.5, 95% CI 0.3 - 0.9; Figures 5.3,5.4).

#### 5.4.4 Relation between subjective cough measures and capsaicin response

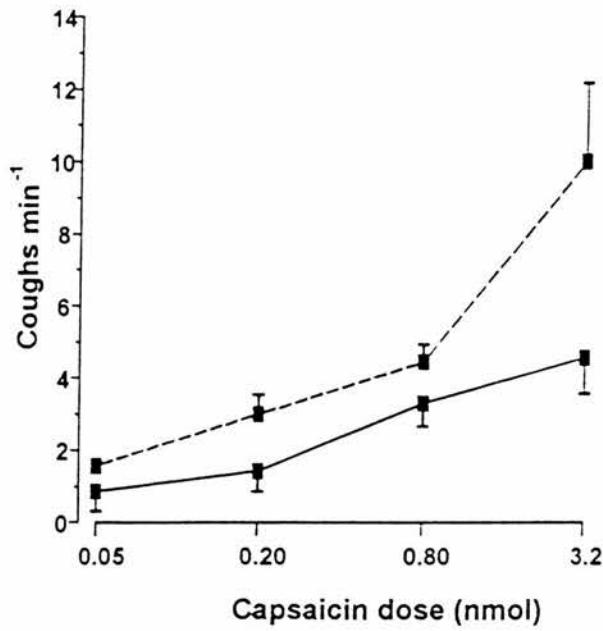
There was a significant correlation between increased VAS scores measuring cough frequency and severity at one month, and more severe cough by questionnaire ( $r_s = 0.43$ ,  $p < 0.02$ , and  $r_s = 0.37$ ,  $p < 0.05$  respectively; Figures 5.5a and 5.5b). Similarly more severe cough scores by questionnaire and by VAS were reflected by increases in the capsaicin response at one month (Figure 5.5c and 5.5d). For this purpose the capsaicin response was defined as the total cough response - the sum of the coughs obtained for all doses of inhaled capsaicin (Foster et al 1991).

#### 5.4.5 Effect of time on capsaicin-response in enalapril-treated patients without cough.

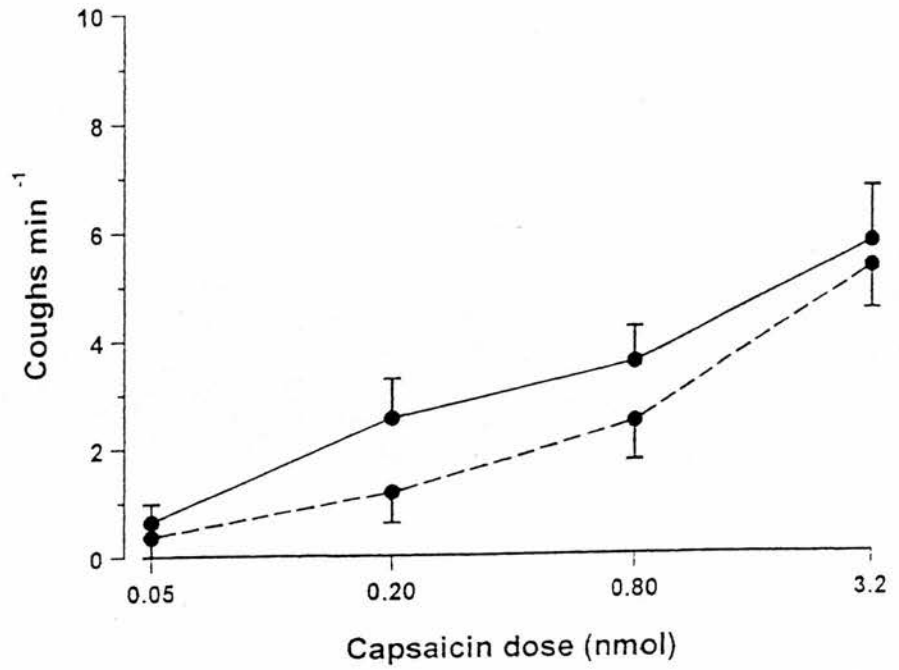
This effect was examined by using the data at baseline, 1, 6 and 12 months for the ten patients in the enalapril group who did not experience cough and were able to complete the study. ANOVA for three factors: time, patients, and doses of capsaicin revealed that only a small proportion of the total variance observed (1.6%) was due to time ( $F=1.52$ ,  $p=0.21$ ). The estimated means for the cough response to capsaicin by ANOVA for patients on enalapril, but with no spontaneous cough, were 2.4, 2.1, 2.2 and 1.6 coughs  $\text{min}^{-1}$  for baseline, 1, 6 and 12 months respectively. This trend towards a slightly diminished response with time was similar to that seen in control patients.



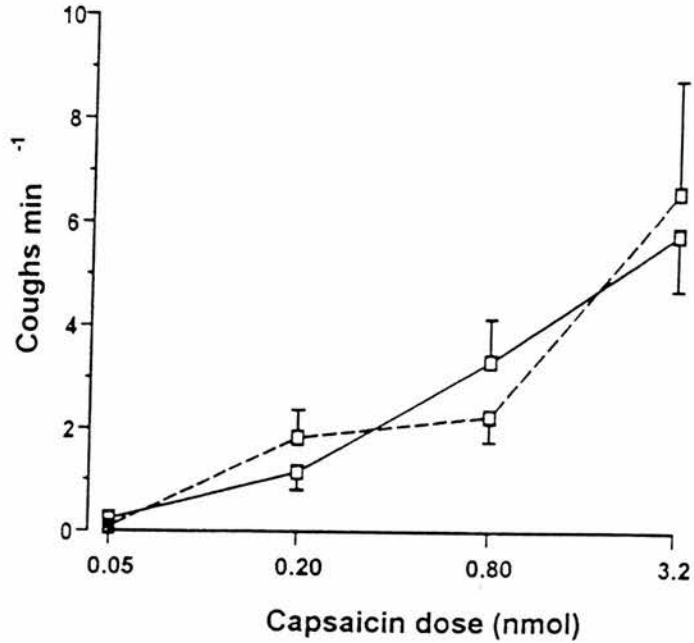
**Figure 5.1** Dose-response curves for 33 patients (enalapril-induced cough, n= 8, ■, enalapril and no cough n=13, □, and control group, n=12, ●) before treatment (solid line) and 8 patients with enalapril-induced cough, ■ (broken line) after 1 month of treatment. Mean ( $\pm$ se)



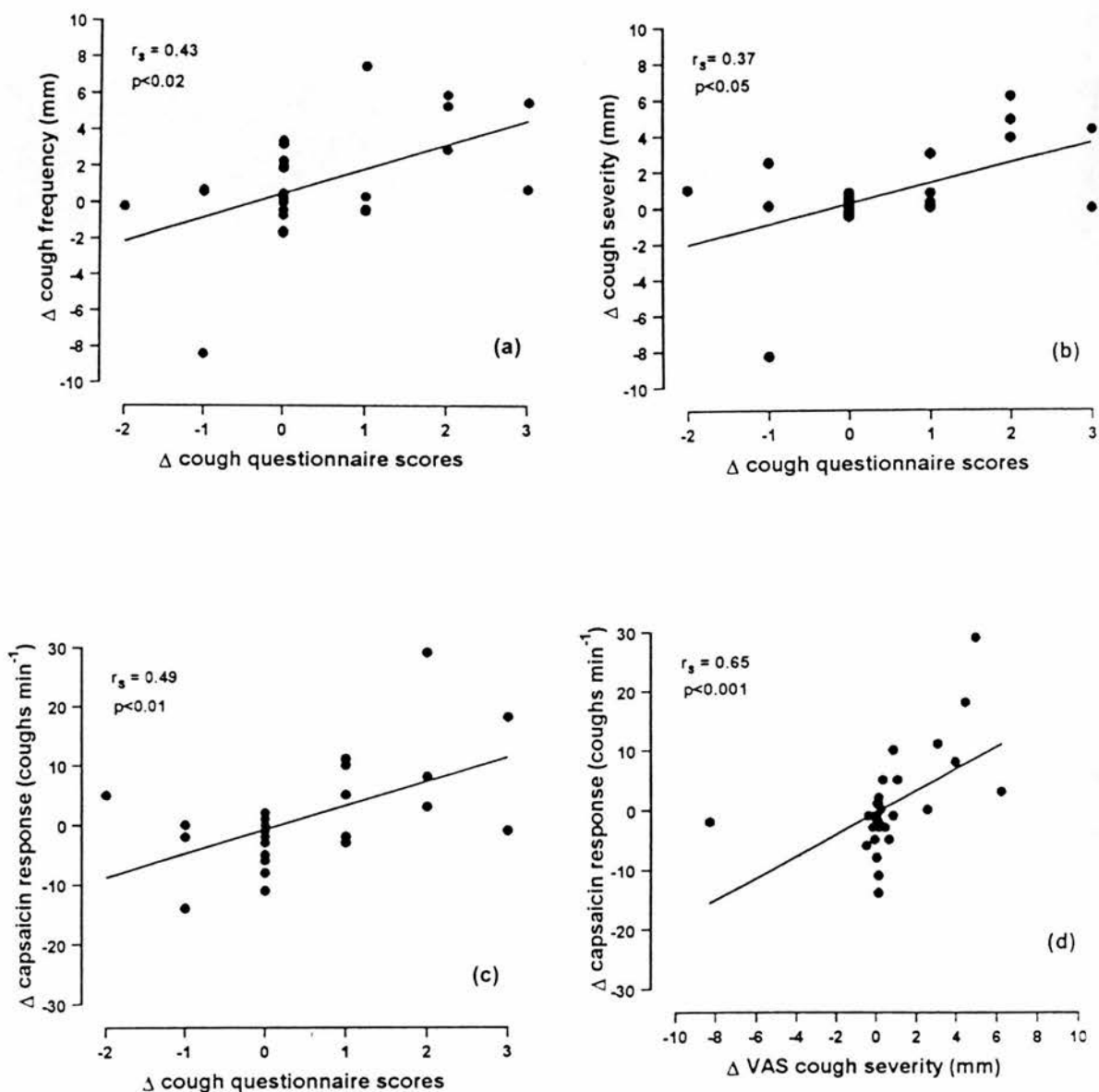
**Figure 5.2** Dose-response curves for 8 patients, ■, before treatment (solid line) and after 1 month of treatment when they had developed cough (broken line) with enalapril. Mean ( $\pm$ se)



**Figure 5.3** Dose-response curves for 12 control patients, ●, before treatment (solid line) and after 1 month of antihypertensive drug treatment (broken line) Mean ( $\pm$ se)



**Figure 5.4** Dose-response curves for 13 patients, □, before treatment (solid line) and after 1 month of treatment (broken line) with enalapril. These subjects did not develop cough. Mean ( $\pm$ se)



**Figure 5.5** The changes from baseline in VAS scores (cough frequency and severity), the changes in cough score by questionnaires and in response to inhaled capsaicin displayed as correlations between these measurements. The plots are raw data. Correlation coefficients and p values cited correspond to Spearman rank correlation's.

## 5.5 DISCUSSION

Eight of 21 patients starting enalapril (38%) developed ACE inhibitor cough as defined by pre-set clinical criteria known to have a positive predictive value of 95% (Yeo 1991). Six of these patients could not complete a full year of observation because of the severity of the cough. By one month these eight patients recorded significantly increased scores for cough by questionnaire and by visual analogue scales when compared to patients who did not cough with enalapril, and patients starting other antihypertensive treatment (Table 5.2).

In this study patients with persistent cough had normal sensitivity to inhaled capsaicin initially. By one month the cough sensitivity had increased five-fold. This magnitude of increase in potency is identical to the five-fold difference in relative potency observed between patients with ACE inhibitor cough and control subjects in a previous study (Yeo et al 1991). By contrast capsaicin sensitivity in patients with no cough and in controls was slightly reduced, with the majority of the reduction occurring between the first and second measurement.

In the study by McEwan et al [1989] three of twenty patients treated with enalapril and ramipril for one week developed cough, and this was accompanied by an increase in capsaicin sensitivity to lie beyond the 95% confidence interval for the whole group. However the changes in capsaicin sensitivity were convincing only for enalapril, and the shift was not significant because only three patients developed cough (McEwan et al 1989). The present study demonstrates a substantial and statistically significant shift in capsaicin sensitivity in those who developed cough. The small non-significant reduction in sensitivity in the group taking enalapril who did not develop cough suggests that ACE inhibitor treatment increases the capsaicin dose-response only in those who develop spontaneous cough.

The observations in those who did not cough and the control group are new findings as previously there was little information on changes in capsaicin sensitivity over time *within* patients. There has been no comparative data from other research for capsaicin sensitivity in patients who do not experience spontaneous cough while taking an ACE inhibitor, or in control patients not on ACE inhibitors. The responses to inhaled capsaicin were reproducible within patients accounting for only a very small part of the total variance of between 1.5 and 4.2% in enalapril treated patients with no cough, and control patients. When significant differences did occur in the control groups it tended to be a small decrease in potency of capsaicin with time, and this was most pronounced between the first and second measurements.

When the cough measurements were examined there were significant associations between cough responses to capsaicin and measurements of severity and frequency of cough by visual analogue scales and by questionnaire. This supports the notion that the abnormality

underlying ACE inhibitor cough is an increase in C-fibre mediated sensitivity of the cough reflex (Fuller & Choudry 1987, McEwan et al 1989).

The association between cough measured by questionnaire and capsaicin sensitivity ( $r_s=0.49$ ,  $p<0.01$ ) was somewhat weaker than that for visual analogue scales for cough severity and capsaicin sensitivity ( $r_s=0.65$ ,  $p<0.001$ ) suggesting that visual analogue scales may be more sensitive than the questionnaire for measuring cough severity.

The incidence of cough in this study is higher than is expected from larger studies (Yeo 1990). In an open uncontrolled prospective study Ravid et al found 18.6% of patients developed cough, beginning after an unusually long time, a mean 24.7 weeks of treatment. They found that the cough disappeared quite rapidly when treatment was stopped in about 2-7 days (Ravid 1994). Possible reasons for the higher incidence found in my study include chance or even the introduction of bias. The study was open and non-randomised. Although the assessor was blinded to the treatment the patient was aware of what they were taking and that we were examining side-effects of drugs with a particular emphasis on headache and cough. An improved design would include double-blinding as regards the nature of the medication and less emphasis on cough in the questionnaire. Whatever these biases might be, they would not be expected to explain the highly significant findings of changes in cough sensitivity.

In summary:

- Eight of twenty-one patients starting enalapril developed spontaneous cough during one year.
- The time to onset of cough in these patients was 2.4 weeks (range 1 - 6 weeks)
- These patients had significantly increased scores for subjective cough by questionnaires and visual analogue scales by one month.
- The sensitivity to capsaicin was increased by a factor of five by one month in those who developed cough.
- By contrast patients who were started on enalapril but did not develop cough did not shift their sensitivity to capsaicin significantly over a year of observation.
- Control patients on other treatments had a small reduction in capsaicin sensitivity which occurred mostly between the first and second measurement.
- ACE inhibitor cough appears associated with a five-fold increase in sensitivity to inhaled capsaicin which is present by one month after treatment is started, and these changes in capsaicin sensitivity are significantly associated with changes in subjective cough scores.



CHAPTER 6

RESOLUTION OF ACE INHIBITOR COUGH:

CHANGES IN SUBJECTIVE COUGH,

AND RESPONSES TO

INHALED CAPSAICIN, INTRADERMAL BRADYKININ AND SUBSTANCE-P.

## 6.1 INTRODUCTION

About 25% of patients with cough due to ACE inhibitors have to stop treatment (Yeo 1990, Yeo 1991a) and in the remainder continuing with treatment the cough is moderate to severe, disturbs sleep, and a nuisance to others (Yeo 1991a). Although much is known about the epidemiology of the cough its mechanism remains uncertain. As discussed in the previous chapter it is thought that kinins such as bradykinin or substance P, normally metabolised by the kininase II action of angiotensin-converting enzyme (Choudry 1989), may act on sensory nerves to facilitate the cough reflex and cause spontaneous coughing. The dose response to capsaicin is shifted to the left in those with ACE inhibitor cough (Chapter 5, Fuller 1987, McEwan 1989). In the preceding chapter I found that most of the change occurred within one month. ACE inhibitor cough is said to disappear rapidly, within one week of stopping treatment (Coulter 1987, Hume 1989, Berkin 1989) but the time-course of resolution of cough has not been examined in detail. This knowledge may be important when designing trials examining the effects of ACE inhibitors or other drugs on cough, and one aim of this study was to examine formally the time-course of resolution of ACE inhibitor cough.

The wheal response to intradermal bradykinin is potentiated by ACE inhibitor treatment (Ferner 1987). This reflects ACE or kininase II activity in the skin and thus provides a surrogate measure of tissue ACE activity. Substance-P is also metabolised in part by ACE (Yokosawa 1985), and changes in perineuronal substance-P have been proposed as having a role in ACE inhibitor cough (Thysell 1988, Morice 1987). I was interested to study responses to intradermal bradykinin and substance-P relating these to symptoms of cough and changes in the cough reflex. The association or dissociation of these measures of response to ACE inhibition might cast light on the mediators of ACE inhibitor cough.

## 6.2 AIMS

The aims of this study were:

1. To examine the time-course of resolution of ACE inhibitor cough.
2. To examine the relations between changes in subjective cough, changes in the cough reflex to capsaicin, and responses to intradermal bradykinin and substance-P.

## 6.3 METHODS

### 6.3.1 *Subjects*

Patients taking enalapril who had ACE inhibitor cough were recruited from the Sheffield Hypertension Clinic, using as the criteria for diagnosis (a) a new onset of cough during ACE inhibitor treatment (b) persistence of the cough for longer than three weeks and (c) no other identifiable cause. These clinical criteria have a positive predictive value of 95% for ACE inhibitor cough (Yeo 1991). Patients gave written informed consent to the study which was approved by the hospital ethics committee. Two of the subjects from the study in chapter 5 were recruited into this study.

### 6.3.2 *Study design*

This was a prospective observational study over four weeks of symptoms, cough reflex, and skin responses to bradykinin and substance-P in patients with ACE inhibitor cough who stopped the ACE inhibitor. Patients attended on five occasions; at recruitment (day 0) and then 3, 7, 14 and 28 days after stopping the ACE inhibitor. At each visit subjective cough was measured by a self-administered questionnaire and visual analogue scales (VAS; chapter 2); cough reflex testing and skin testing were performed as described in chapter 2; and FEV<sub>1</sub>, FVC and PEF<sub>R</sub> were recorded.

### 6.3.3 *Measurements*

**Capsaicin cough challenge** was performed as described in Chapter 2. The number of coughs occurring during one minute after each dose was recorded.

**Intradermal bradykinin and substance P tests** were performed at each visit as described in chapter 2. These intradermal tests were performed in 6 subjects but completed in only 5 because one developed a generalised macular rash at day 3.

**Subjective cough.** Severity of cough was measured by a validated self-administered questionnaire described in chapter 2. The severity of these symptoms was graded as not at all (0), a little (1), moderately (2), quite a bit (3) or extremely (4).

A diary relating to the nature of the cough was completed by the patient each day (Appendix 3). This consisted of six questions concerning nocturnal severity, frequency, daytime severity, throat soreness, husky voice and stuffy nose. These were graded in the same way as the questionnaire above.

#### 6.3.4 *Statistical analysis*

The scores from the self-administered questionnaire and visual analogue scales were analysed by the non-parametric Friedman's two-way analysis of variance. The capsaicin dose-response curves were analysed using a method for parallel line bioassay. The dose-responses for the wheal areas to bradykinin and substance-P were significantly non-parallel, and these variables were analysed by repeated measures ANOVA. Changes in FEV<sub>1</sub>, FVC and PEFr were also examined by ANOVA. Multiple regression analysis was used to explore (i) the relations between changes in subjective measures of cough and the cough response to inhaled capsaicin; and (ii) the relations between four separate measures of cough (capsaicin response, questionnaire-severity, VAS-frequency, VAS-severity) and the changes in wheal areas to substance-P and bradykinin. For those variables measured as dose-response curves the value used for this analysis was the mean for all doses at each time point. This single summary measure which represent the dose-response curve at each time point was regressed against single measures for VAS and questionnaire at each time point. Multiple regression with stepwise subtraction of non-significant predictors was performed with each of the four measures of cough regressed separately against the responses to substance-P and bradykinin, and with variance due to time-course and between-patients the additional factors included in the regression equation.

### 6.4 RESULTS

#### 6.4.1 *Patients*

The baseline characteristics of the eight patients (7 women) are shown in Table 6.1. All had been taking enalapril at doses between 2.5 and 40 mg daily for a median period of 8 months (range 1.5-24 months), and had developed a dry cough which required discontinuation of the drug. Five patients were non-smokers and three continued to smoke between 10 and 30 per day (Table 6.1).

**Table 6.1.** Baseline characteristics of eight patients before stopping the ACE inhibitor enalapril.

Patient	Age (years)	Sex	BMI	BP(mmHg)	Enalapril dose (mg/day)	Duration of treatment (months)	Smoking habit
1	50	F	29.5	116/90	2.5	1.5	Never
2	29	F	40.2	146/86	5	3	10 cigs/day
3	71	F	37.3	170/98	5	24	Ex-smoker
4	75	F	21.9	180/70	2.5	1.5	Ex-smoker
5	73	F	29.5	218/100	20	4	Never
6	60	F	30.7	180/82	5	12	10 cigs/day
7	54	F	25.3	154/100	40	24	30 cigs/day
8	65	M	28.6	140/72	20	24	Ex-smoker

#### 6.4.2 Subjective symptoms of cough

All patients recorded a subjective improvement in cough as measured by the questionnaire scores for severity and night time waking, and by visual analogue scales for severity and frequency of cough (Table 6.2). The median questionnaire score for severity fell from 3 ("quite a bit") to 0.7 ("not at all" to "a little") over the 28 days ( $p < 0.01$ ). The change in severity score was significant by day 7, with further reductions by day 14 and 28 (Table 6.2). Night time cough was significantly reduced from 2.2 ("moderately") to 0.7 by 14 days ( $p < 0.05$ ) with a further decrease to 0.1 ("not at all") by 28 days ( $p < 0.01$ , Table 7.2). The frequency of cough measured by visual analogue scale fell from 6.2 cm to 4.8 cm by day 3 with further declines to 0.8 cm ( $p < 0.01$ , Table 6.2) at day 28. The severity of cough by visual analogue scale was significantly reduced from its starting value of 6.5, to 2.9 cm by day 7 ( $p < 0.05$ ), 1.6 cm by day 14 ( $p < 0.05$ ) and 0.8 cm at day 28 ( $p < 0.01$ ; Table 6.2).

The diary showed resolution in night time cough in seven subjects after 4 weeks, but the cough was still present in three subjects (subjects 5,6, and 7) to some degree by the end of the study.

**Table 6.2.** Subjective and objective measures of cough: estimated median scores by self-administered questionnaire (SAQ) for severity of cough and night time cough, visual analogue scales (VAS) for severity and frequency of cough, and relative potency estimates for capsaicin, with 95% CI. The potency at day 0 is set at 1.0 and potency estimates for capsaicin at subsequent time points are relative to this value.

Date	SAQ score		V.A.S. (cm)		Capsaicin relative potency (95% C I)	
	Severity ¶	Night time cough ¶	Severity ¶	Frequency ¶		
day 0	3.0	2.2	6.5	6.2	1.00	-
day 3	2.5	2.0	4.8	4.8*	0.34	(0.09 - 1.03)
day 7	1.8*	1.0	2.9*	3.1*	0.46	(0.16 - 1.35)
day 14	1.5*	0.7*	1.6*	1.4**	0.25*	(0.07 - 0.87)
day 28	0.7**	0.1**	0.8**	0.2**	0.18**	(0.06 - 0.67)

¶ P < 0.0005 for trend from day 0 to day 28      \* P < 0.05, \*\* P < 0.01 versus day 0

#### 6.4.3 Respiratory function

There were no changes in FEV<sub>1</sub>, FVC and PEFR over the period of the study.

#### 6.4.4 Capsaicin challenge

The sensitivity to inhaled capsaicin fell over the 28 days of study after stopping enalapril. The potency of capsaicin relative to day 0 was significantly reduced to 0.25 (95% CI 0.07-0.87) by day 14, and there was a further reduction in relative potency to 0.20 (95% CI 0.06-0.67) by 28 days (Table 6.2). There was therefore a significant shift to the right in the dose response curve to inhaled capsaicin after patients stopped the ACE inhibitor (Figure 6.1).

#### 6.4.5 Wheal area to intradermal bradykinin and substance-P

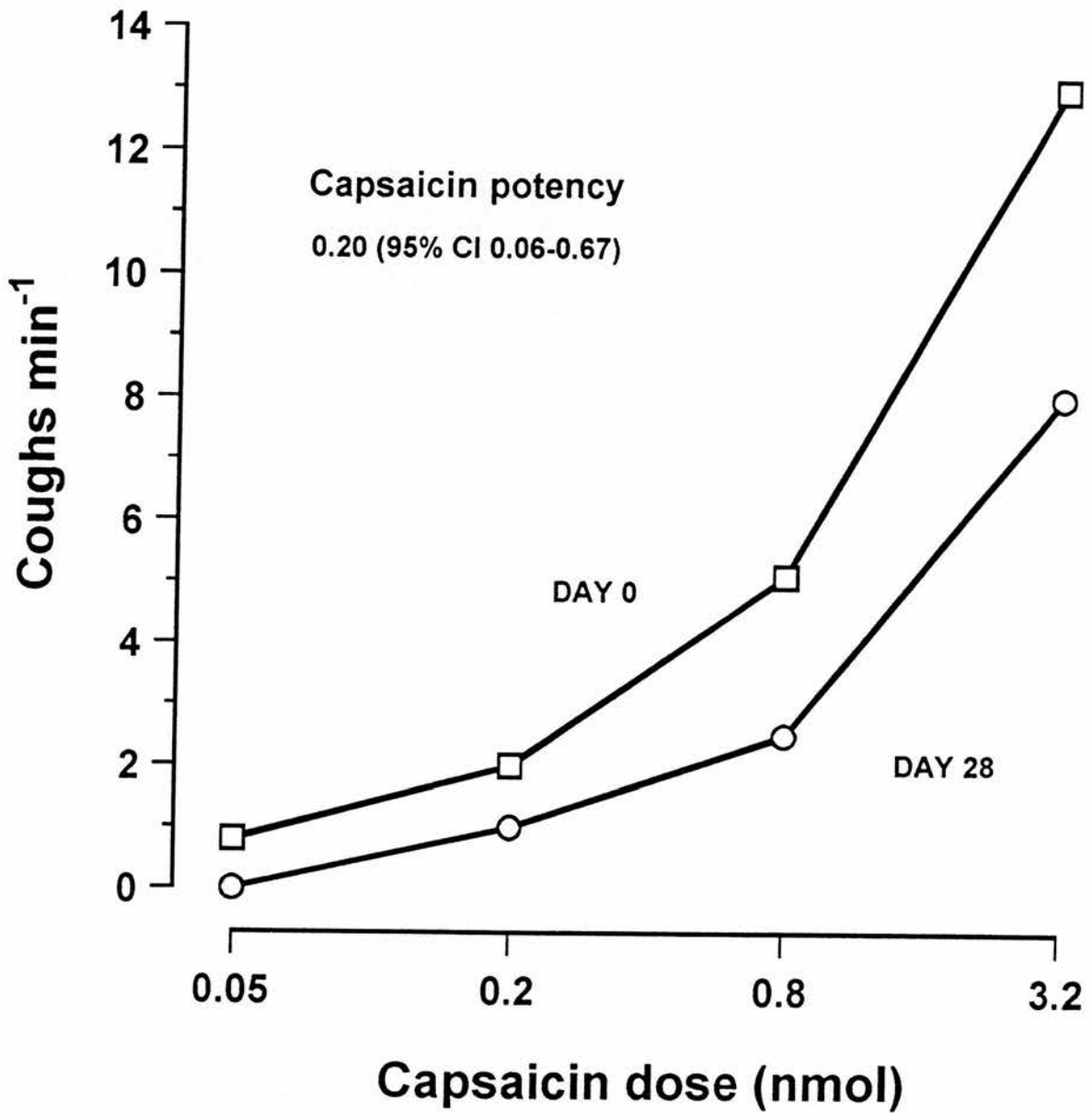
After stopping enalapril there was a highly significant reduction in wheal area produced by intradermal bradykinin, with the majority of the effect seen by day 3 (Table 6.3, Figure 6.2). The wheal area to intradermal substance-P also declined with time after stopping enalapril, but significant changes were not observed until 14 days, with a further reduction by 28 days (Table 6.3, Figure 6.2).

**Table 6.3** Mean wheal area (mm<sup>2</sup>) 15 minutes after a 0.1 ml intradermal injection of bradykinin and substance-P in five patients from day 0 to day 28 after stopping ACE inhibitor treatment..

Date	Bradykinin dose (µg/ml) ¶				Substance-P (µg/ml) ¶			
	0.1	1.0	10	100	0.001	0.01	0.1	1.0
day 0	99	137	227	343	73	83	126	218
day 3*	68	86	118	201	60	87	127	199
day 7*	68	96	130	163	52	80	105	180
day 14*†	61	82	104	160	49	68	103	176
day 28*‡	52	82	114	145	43	64	89	139

¶ P<0.0005 for trend day 0 to day 28.

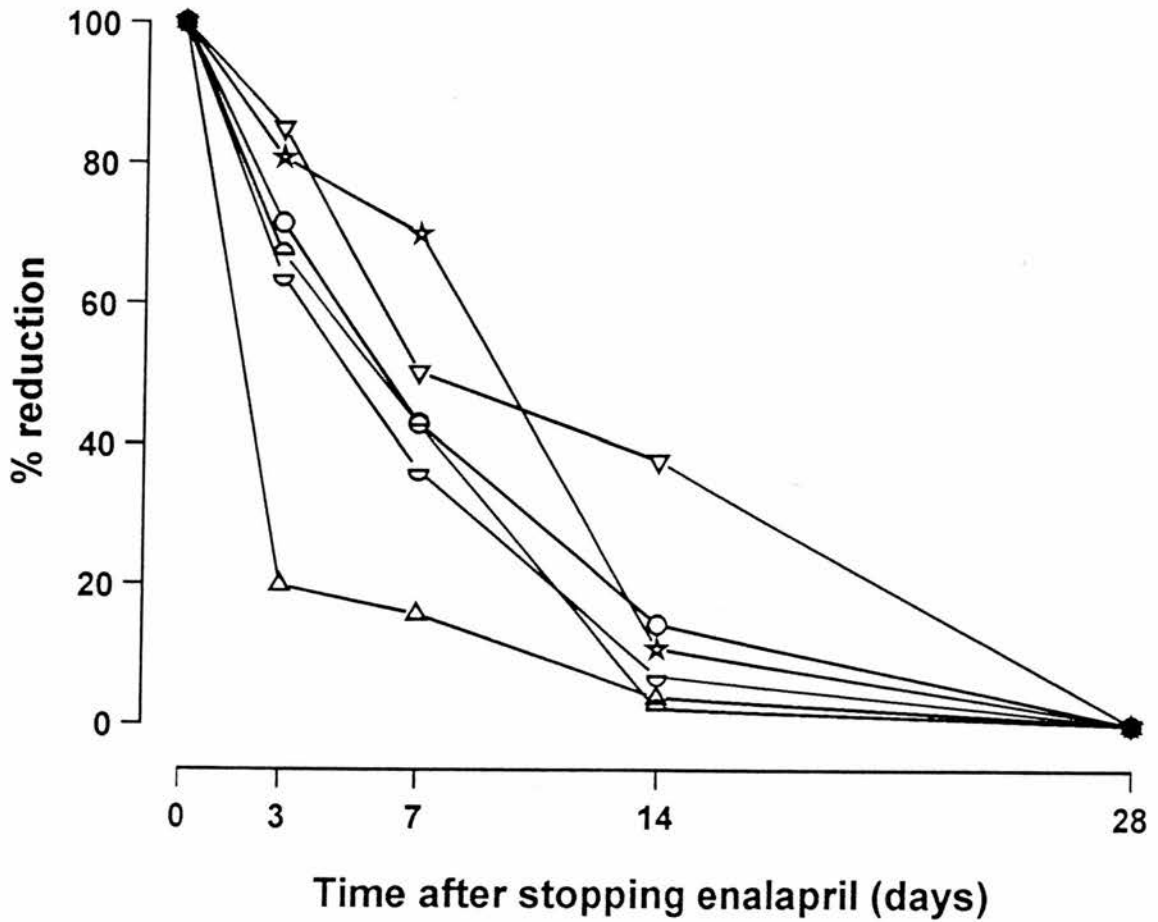
\* P<0.0005 *versus* day 0 for bradykinin; † P<0.01, ‡ P<0.001 *versus* day 0 for substance-P



**Figure 6.1** Dose-response to capsaicin in eight patients at day 0 and day 28 after stopping ACE inhibitor treatment. The shift to the right in dose-response represents a reduction in relative potency of capsaicin at 28 days of 0.2 compared to day 0,  $p < 0.005$ .



**Figure 6.2.** Relation between offset of ACE inhibitor cough as assessed by visual analogue scales for cough severity and frequency (VAS, open half circles), self administered questionnaires for severity of cough (SAQ, O) and capsaicin challenge (☆) with the intradermal responses to bradykinin (Δ) and substance-P (▽). The changes are represented as a percentage reduction between day 0 and day 28 in the five patients who had all variables recorded.



#### *6.4.6 Relation between subjective cough and capsaicin response*

The rates of decline for the subjective and objective measures of cough, and for responses to intradermal bradykinin and substance-P, are shown in figure 2 for the five patients who had all the tests performed. The time-course of changes in questionnaire cough severity, VAS scores for severity and frequency of cough, and responses to inhaled capsaicin were broadly similar. Multiple regression analysis showed significant associations of the response to inhaled capsaicin with the VAS score for severity of cough (regression coefficient 0.13,  $p=0.005$ ) and the VAS score for frequency of cough (regression coefficient 0.11,  $p=0.011$ ). Capsaicin response was not related significantly to the severity of cough measured by self-administered questionnaire.

#### *6.4.7 Relation between measures of cough and responses to intradermal bradykinin and substance-P*

The changes in wheal area to bradykinin appeared to occur more rapidly after stopping enalapril than did changes in measures of cough, while those for substance-P appeared to diverge at day 14 (Figure 6.2). Multiple regression analysis showed a significant association between bradykinin response and VAS scores for frequency of cough (regression coefficient 0.021,  $p<0.04$ ) and severity of cough (regression coefficient 0.022,  $p<0.05$ ), but not with cough by questionnaire or the capsaicin response. The response to substance-P did not relate significantly to any of the measures of cough. When the response to substance-P was removed from the regression equation there was no longer a significant relation between bradykinin response and measures of cough. However in all analyses bradykinin explained more of the variance in cough responses than did substance-P. For example bradykinin explained 23% of the variability in VAS scores for frequency of cough, 22% for VAS scores for severity of cough, 8% for questionnaire responses for severity of cough, and 11% for the response to capsaicin, after removing variance related to time-course and between-patients. The corresponding figures for substance-P were 0.0%, 0.1%, 2.6% and 0.0%.

## 6.5 DISCUSSION

Before discussing the findings, possible limitations of this study should be mentioned. The study was an open observational study with no control group, and it is therefore not possible to exclude some element of error due to patient or investigator bias. As there was no control group the study relies on the responses measured being relatively reproducible and stable over time. I believe these factors have not greatly influenced the results presented for three reasons. Firstly there is internal consistency across all patients within the study; secondly the changes observed for the responses are much greater than the within-subject coefficient of variation for these responses when performed under controlled conditions; and lastly these findings are consistent with the findings of a double-blind study as will be discussed below.

ACE inhibitor cough is thought to resolve rapidly, within one week of stopping treatment (Coulter 1987, Hume 1989, Berkin 1989), but these impressions predate quantitative methods of assessing ACE inhibitor cough (Yeo 1991a, Fletcher 1990, Yeo 1991b, Yeo 1995). In this study cough related to enalapril resolved after the ACE inhibitor was stopped, with reductions in measures of the severity and frequency of cough and night-time cough, but resolution took longer than one week. There was a significant improvement 14 days after stopping enalapril, but complete disappearance of the cough during the daytime was observed only after 28 days. This is in agreement with a recent controlled study in which patients with ACE inhibitor cough were challenged, de-challenged and re-challenged with lisinopril (Lacourciere 1994). The median time to complete resolution of the cough caused by lisinopril was 26 days (Lacourciere 1994). The capsaicin cough sensitivity in those with chronic cough of various causes was examined by O'Connell et al (1990). In a small group which included four subjects taking ACE inhibitors successful identification and treatment of the cause of the cough was associated with not only resolution in the symptoms but a decrease in the cough sensitivity to inhaled capsaicin. Interestingly in those who continued to have symptoms with no cause identified the cough response to capsaicin did not change.

There was a highly significant association between cough responses to capsaicin and measurements of severity and frequency of cough by visual analogue scales. This supports suggestions that the abnormality underlying ACE inhibitor cough is an increase in C-fibre mediated sensitivity of the cough reflex (Fuller 1987, McEwan 1989, Yeo 1995). There was no significant association between cough measured by questionnaire and capsaicin sensitivity, suggesting that the visual analogue scales may be more sensitive than the questionnaire for measuring cough. This finding was also seen in the previous chapter where the visual analogue scores showed significant association with changes in cough sensitivity to capsaicin but the questionnaire only showed an association when cough was rated as severe.

While the significant increase in capsaicin sensitivity in patients with ACE inhibitor cough is now well established (Fuller 1987, Yeo 1995, chapter 5), there is little information on changes in capsaicin sensitivity over time within patients. In one study (McEwan 1989) three of twenty patients treated with enalapril and ramipril for one week developed cough accompanied by an increase in capsaicin sensitivity to lie beyond the 95% confidence interval for the whole group. However the change in capsaicin sensitivity was convincing only for enalapril, and the shift was not significant because only three patients developed cough (McEwan 1989). With hindsight the treatment and washout periods of one week were probably too short (McEwan 1989).

My study shows a substantial and highly significant reduction in sensitivity of the cough reflex to capsaicin after stopping enalapril. The relative potency of capsaicin was reduced by one week, fell significantly to 0.25 of the starting value by 14 days, and declined further to 0.20 by 28 days (Table 6.2, Figure 6.2). The five-fold fall in potency of capsaicin over 28 days after stopping enalapril in the present study is identical to the five-fold difference in relative potency observed between patients with ACE inhibitor cough and control subjects (Yeo 1995), and suggests that the cough reflex had returned to normal four weeks after stopping treatment. In chapter 5 the five-fold increase in capsaicin sensitivity found occurred during the first month of treatment. There were no other assessments at other time-points during this period and the initial rate of change could not be determined.

Recent evidence from a study comparing the specific angiotensin II antagonist losartan to lisinopril in patients with ACE inhibitor cough (Lacourciere 1994) indicates that kinins are likely to be involved in the cause of ACE inhibitor cough (Lacourciere 1994, Coulter 1987, Yokosawa 1985, Thysell 1988, Morice 1987). The wheal area in response to intradermal bradykinin and substance-P showed progressive and highly significant declines after stopping enalapril. For bradykinin this change occurred rapidly, with 80% of the reduction occurring by day 3 (Table 6.3, Figure 6.2), and this appeared to be dissociated from changes in cough over the first 14 days. The wheal response to substance-P paralleled changes in cough more closely (Figure 6.2), but appeared to diverge at day 14. Multiple regression analysis showed a weak but significant association between bradykinin response and visual analogue scale measurements but not with questionnaire responses or capsaicin sensitivity. Bradykinin accounted for 22-23% of the variance in VAS cough not explained by variability related to the time-course or patients. There was no association between the wheal responses to substance-P and measures of cough. These findings give some support to the hypothesis (Coulter 1987, Berkin 1989) that bradykinin may be a mediator of ACE inhibitor cough, but provide no positive support for substance-P as a likely mediator. However intradermal responses to these

peptides may not reflect events in the respiratory tract, and there may be type II error as the sample size was small.

I conclude that cough caused by enalapril improves markedly by 14 days but takes up to 28 days to resolve completely after stopping the ACE inhibitor, and the same is true for lisinopril (Lacourciere 1994). The offset of cough could be more rapid with ACE inhibitors that have a shorter half-life, but the offset for enalapril and lisinopril is far longer than would be predicted from their plasma half-lives or duration of action (McInnes 1993). One immediate practical implication is that studies examining ACE inhibitor cough are likely to require washout periods of at least 28 days. ACE inhibitor cough is associated with increased sensitivity to inhaled capsaicin which is reversed over 28 days after treatment is stopped.

Changes in capsaicin sensitivity parallel those in subjective cough supporting suggestions that ACE inhibitor cough is caused by facilitation of the C-fibre mediated cough reflex. Change in response to intradermal bradykinin was a better predictor of cough responses than that for substance-P, and accounted for about a quarter of unexplained variability in the changes in visual analogue scales for cough during the resolution of ACE inhibitor cough.

In summary:

- cough caused by enalapril improves markedly by 14 days but may takes up to 28 days or more to resolve completely after stopping the ACE inhibitor.
- changes in capsaicin sensitivity parallel those in subjective cough.
- change in response to intradermal bradykinin was a better predictor of cough responses than that for substance-P

Chapter 7

ACE GENE POLYMORPHISM  
AND  
RESPONSES TO INHALED BRADYKININ  
IN HEALTHY SUBJECTS



## 7.1 INTRODUCTION

Bradykinin has been implicated in the pathogenesis of cough due to ACE inhibitor treatment and increased serum levels have been recorded in those with this adverse effect (Puolijoki 1992). Bradykinin stimulates the C-fibres via specific receptors (Kaufman et al 1980, Simonsson et al 1973) probably through the release of sensory tachykinins such as substance P, neurokinin A, and calcitonin gene-related peptide (Polosa 1992). Subsequent activation of cholinergic pathways can lead to bronchoconstriction in laboratory animals. Bradykinin has a bronchoconstrictor response in those with asthma but not in healthy individuals (Simonsson 1973, Fuller 1987). Cough, unrelated to any change in airways calibre, was observed to varying extent in these and other studies (Dixon 1987, Choudry 1989, Ichinose 1992). However these studies were not designed to specifically look for cough and did not give much information on its nature or severity.

Only 20% of those taking ACE inhibitors develop cough, and this led to the hypothesis that there may be a genetic component in its development (Yeo et al 1991). The ACE gene polymorphism was a possible candidate in that those subjects of genotype II, ie low serum ACE activity, may have a lower threshold to bradykinin and its potential effects. I have tested this hypothesis by administering bradykinin to healthy subjects grouped according to ACE genotype.

## 7.2 AIMS

1. To examine the effects of bradykinin on the cough response and airways function in healthy subjects.
2. To examine the relation of the ACE genotype to these acute effects of bradykinin on the cough response and airways function.

## 7.3 METHODS

### 7.3.1 *Subjects*

Twenty-seven healthy subjects (11 males and 16 females) of mean age 28.4 years (range 20-51), were selected to provide equal numbers of the three genotypes (n=9 for each of the genotypes DD, ID and II). They gave written informed consent to the study which was approved by the district ethics committee. Those with asthma, eczema, multiple allergies, a previous reaction to bradykinin, a respiratory infection within the last 2 weeks and current cigarette smokers were excluded from the study. Seven of the subjects had taken part in the study in chapter 4.

### 7.3.2 Study design.

Each subject was given six doses of bradykinin in the order described below following an initial administration of 10% ethanol in saline in an open parallel group comparison of the genotypes. Subjects attended in the afternoon having refrained from caffeine containing drinks for 12 hours. Baseline readings of forced expiratory volume in one second (FEV<sub>1</sub>) were recorded on arrival and five minutes later. Subjects then underwent seven cycles of test substance inhalation from a compressed air nebuliser controlled by a breath activated dosimeter (Mefar, Bovezzo, Italy). A noseclip was applied to ensure consistent mouth breathing. During each cycle subjects took *three* separate breaths, each of the *same* dose, from the nebuliser at one minute intervals, breathing room air between inhalations. At the end of the cycle the FEV<sub>1</sub> was rechecked. After a further minute the cycle was repeated for a different dose.

Bradykinin (Sigma, UK) in 10% ethanol in saline, was administered in the following doses: 0.125 mg/ml, 0.25 mg/ml, 0.5mg/ml, 1mg/ml, 2mg/ml, 4mg/ml.

The order of administration of bradykinin was not randomised and followed the same order in each subject. This was as follows: saline → 3 → 4 → 5 → 1 → 6 → 2; where 1=lowest dose & 6=highest dose. This order was not known by the observer who was recording the cough. If a reduction of 20% in FEV<sub>1</sub> was found the test was stopped and FEV<sub>1</sub> recorded at five minute intervals until it returned to baseline.

### 7.3.3 Measurements

The cough response for each dose and the total cough response (TCR) for all doses were recorded by an observer blinded to the nature and dose of the test substance and genotype of the subject. Forced expiratory volume in 1 second was recorded by vitalograph. ACE genotype was determined by polymerase chain reaction as described in chapter 2. Serum ACE activity was measured by fluorimetric assay with units expressed as nmol of histidyl-leucine produced per ml of sample per minute (Freidland 1976).

### 7.3.4 Statistical analysis

Baseline differences and changes in FEV<sub>1</sub> were studied using one-way analysis of variance. The mean change in FEV<sub>1</sub>, that is the mean of the change from baseline for each dose for each subject, following administration of bradykinin, was calculated. Kruskal-Wallis tests were used to examine the difference between the cough scores between the genotypes. The total cough response was corrected for response to control. The relationships between cough response, changes in FEV and serum ACE were examined by Spearman rank correlation. Bradykinin produced a fall in FEV<sub>1</sub> of over 20% in one subject associated with symptomatic



wheeze. A further subject developed symptomatic wheeze associated with a fall in FEV<sub>1</sub> of 18%. These two individuals did not receive all doses of bradykinin however their results are included in subsequent analyses.

## 7.4 RESULTS

### 7.4.1 Comparability of groups

(Table 7.1). Subjects from the three genotypes were of similar age but differed as regards the distribution of sexes with the ID group having fewer men. Average baseline FEV<sub>1</sub> as a percentage of that predicted for height and sex was similar in the three groups. In 15 subjects serum ACE was measured. There was a significant relation between ACE genotype and serum ACE activity at baseline (table;  $p < 0.03$  between groups), with DD subjects having mean serum ACE activity 65% higher than that of II subjects. The ACE genotype accounted for 38% of the between-subject variance in serum ACE activity at baseline.

### 7.4.2 Changes in respiratory function.

(Table 7.1, Figure 7.1) The mean change in FEV<sub>1</sub>, following administration of bradykinin, was 1.3% (se 1.4) compared with the control (10% ethanol in saline) which was 0.5% (se 0.7) [difference 0.8%; 95% CI -2.3 to 3.9%,  $p = 0.58$ ]. Those of genotype DD had a mean change in FEV<sub>1</sub> (over all dose) following bradykinin of -2.5% compared with those of genotype II in who it was 3.8% (Difference DD-II = -6.3%; 95%CI -15.0-2.4;  $p = 0.05$ )

### 7.4.3 Cough response to bradykinin.

(Table 7.1, Figures 7.2, 7.3) Bradykinin caused a cough in 20 subjects (74%) compared to the control (10% ethanol in saline) which caused cough in 4 subjects (15%;  $p < 0.05$ ). The response to bradykinin decreased after the initial administration suggesting tachyphylaxis (Figure 7.4). Thus a summary measure, the total cough response to all doses of bradykinin was used (Figure 7.2). This was corrected for changes from control (saline). This showed no significant difference between the genotypes (median number of coughs; DD = 6.0, ID = 4.0, II = 10.0;  $p > 0.05$ ). There were no significant differences between the genotypes for cough after the first dose (Figure 7.3; median coughs/min; DD = 0.0, ID = 2.0, II = 2.0 ;  $p > 0.05$ ).

### Total cough response v Changes in FEV<sub>1</sub>

(Figure 7.5) The total cough response did not correlate with the mean change in FEV<sub>1</sub> ( $r_s = -0.20$ ).

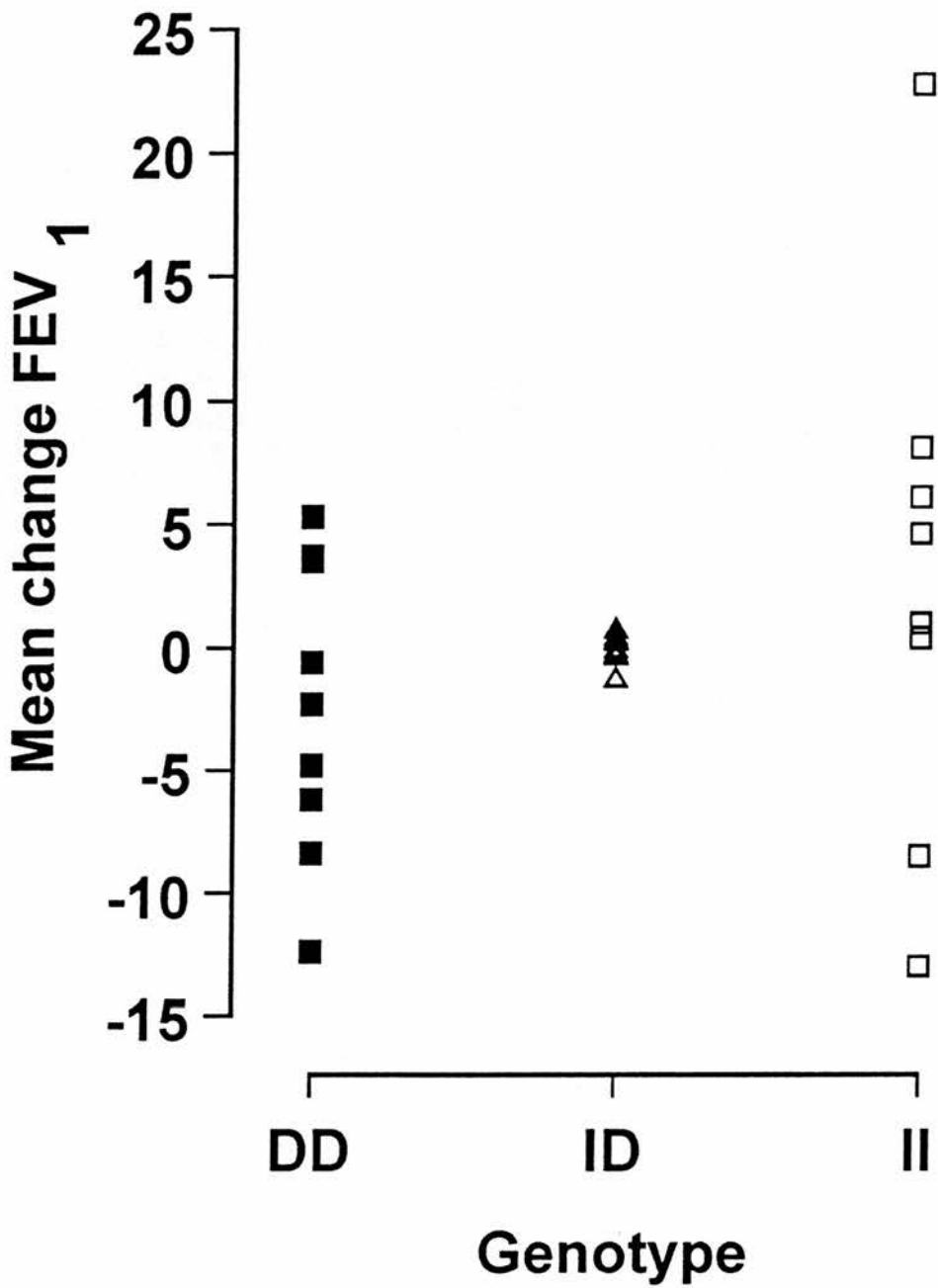
### Serum ACE activity v cough response.

The total cough response did not correlate with initial ACE activity ( $r_s = -0.21$ ).

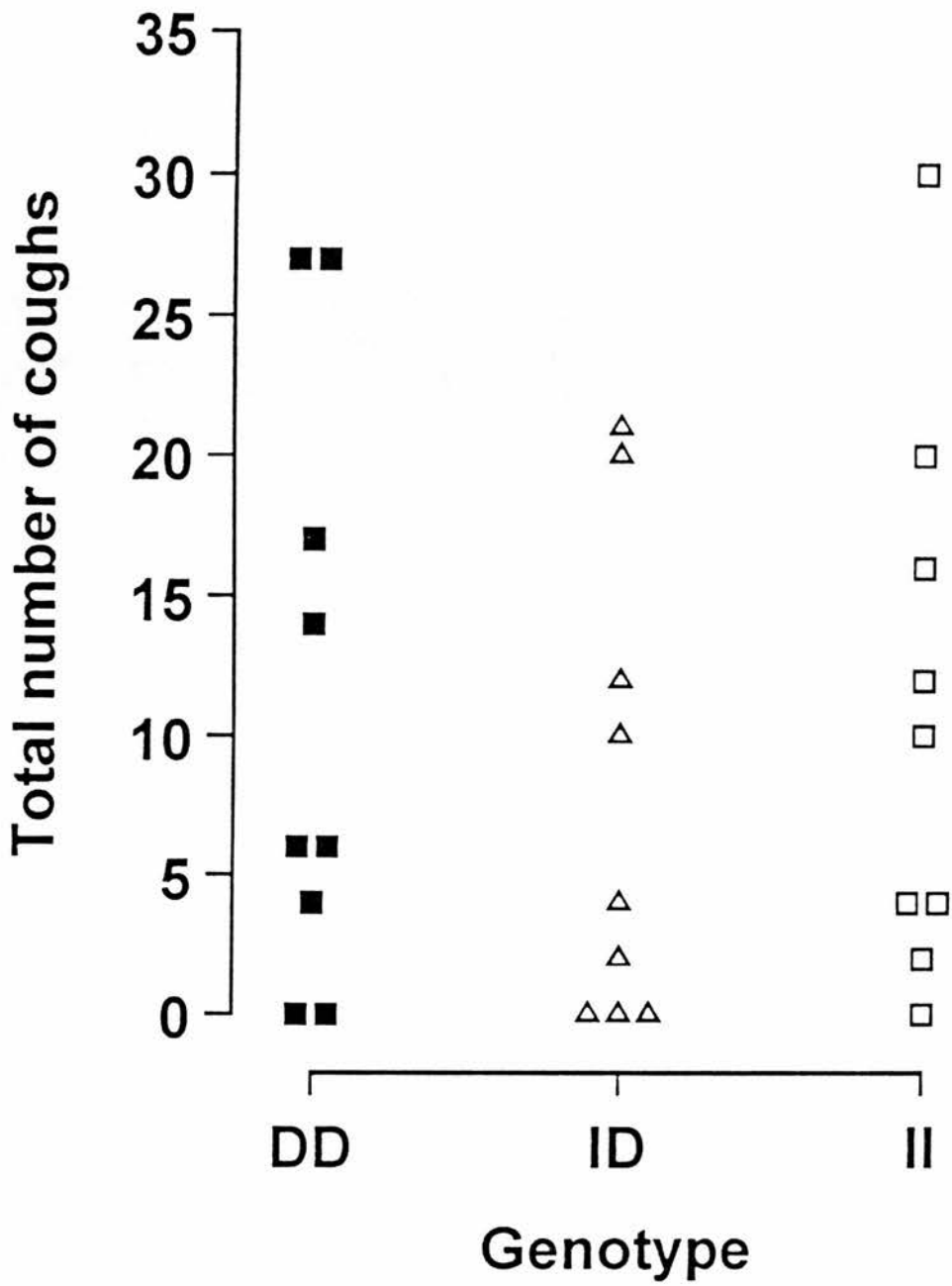
**Table 7.1.** Baseline data, cough response and changes in respiratory function following inhaled bradykinin for 27 healthy subjects according to ACE genotype

Genotype	DD	ID	II
Age (years; mean; sd )	25.9 (7.0)	32.2 (10.8)	27.0 (4.7)
FEV <sub>1</sub> (% predicted; mean; sd)	97.1 (16.3)	107.0 (17.1)	105.3 (14.7)
ACE activity* (nmol/ml/min; mean; sd)	23.7 (8.0)	20.8 (5.1)	14.3 (4.7)
Subjects who coughed after bradykinin	6	6	7
No. of coughs following 0.5mg/ml bradykinin (median; range)	0.0 (0-6)	2.0 (0-12)	2 (0-7)
Total no. of coughs (median; range)	6.0 (0-27)	4.0 (0-21)	10.0 (0-30)
Change in FEV <sub>1</sub> from baseline (%; mean; se)	-2.5 (2.0)	-0.1 (0.2)	3.8 (3.6)

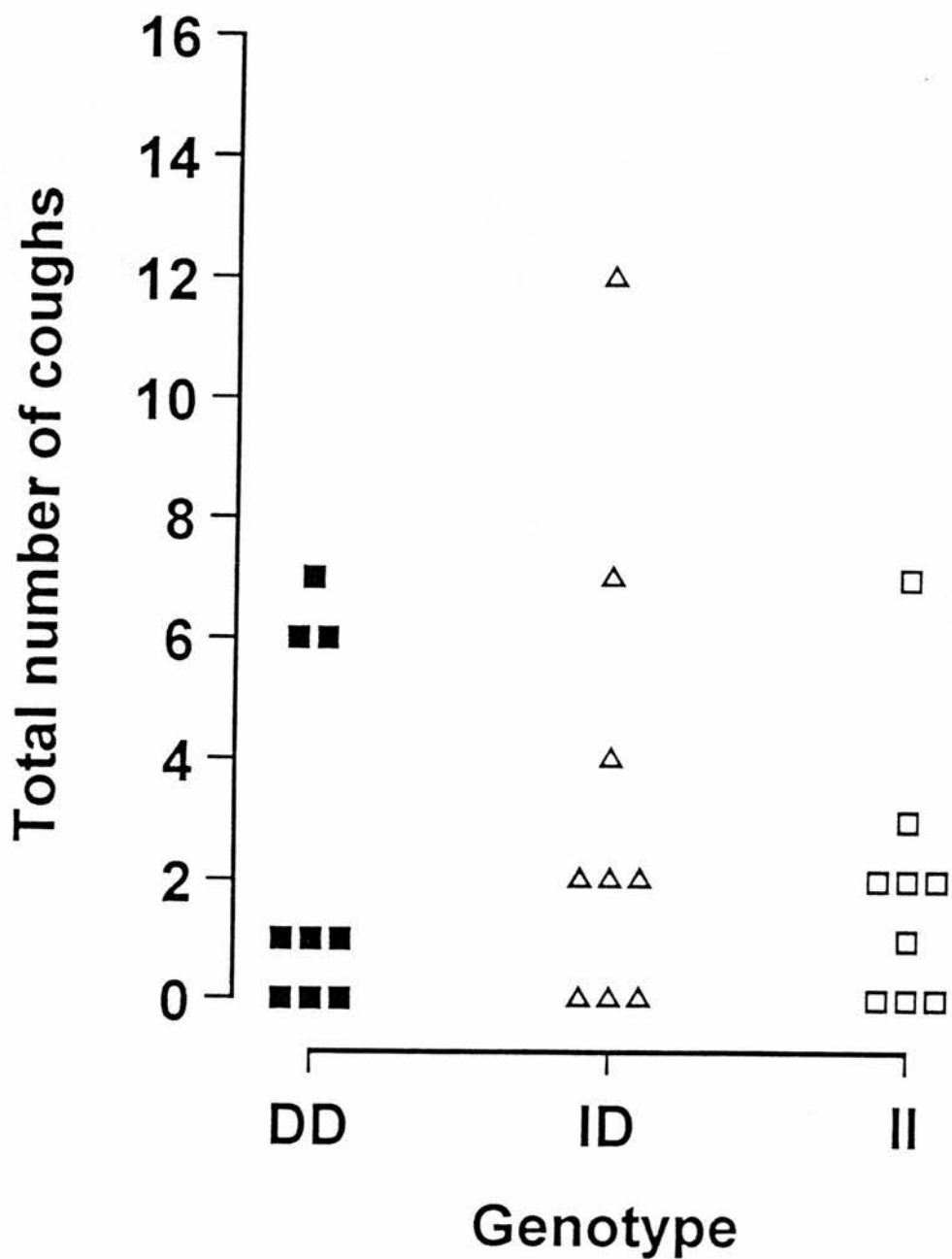
\* p=0.03 for difference between groups DD and II



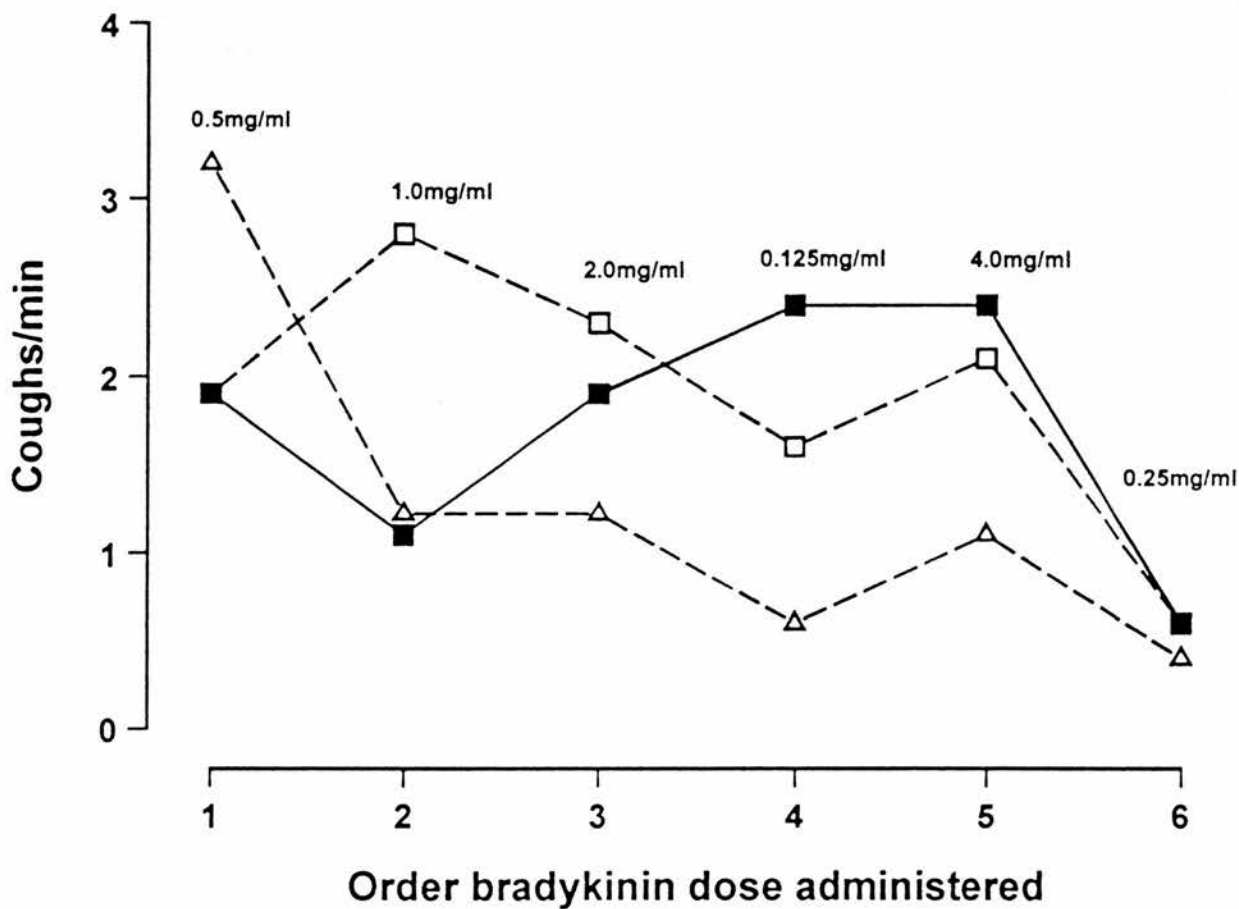
**Figure 7.1** The mean change in FEV<sub>1</sub> following inhaled bradykinin in 27 healthy subjects grouped according to ACE genotype, ■=DD genotype, □ = II genotype and Δ =ID genotype.



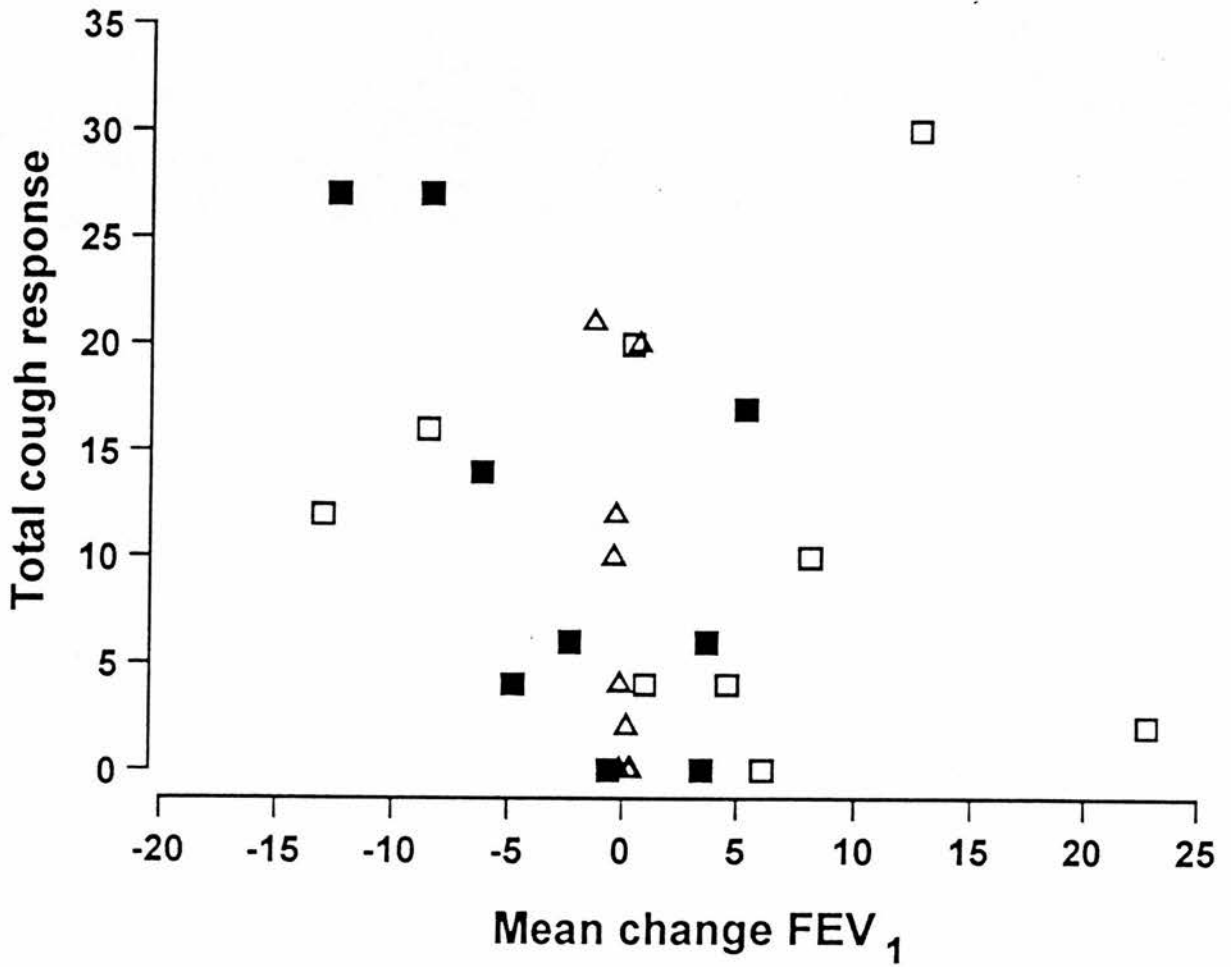
**Figure 7.2** The total number of coughs/min following all doses of inhaled bradykinin in 27 healthy subjects grouped according to ACE genotype, ■=DD genotype, □ = II genotype and Δ =ID genotype.



**Figure 7.3** The total number of coughs/min following the first dose of inhaled bradykinin in 27 healthy subjects grouped according to ACE genotype, ■=DD genotype, □ = II genotype and △ =ID genotype.



**Figure 7.4** The cough response to inhaled bradykinin in 27 healthy subjects grouped according to ACE genotype, in order that the doses were administered. ■=DD genotype, □ = II genotype and Δ =ID genotype.



**Figure 7.5** The total number of coughs/min  $\nu$  the mean change in FEV<sub>1</sub> following inhaled bradykinin in 27 healthy subjects grouped according to ACE genotype, ■=DD genotype, □ = II genotype and Δ =ID genotype.

## 7.5 DISCUSSION

Bradykinin administered by inhalation caused a cough in 74% of subjects compared to a control inhalation which caused cough in 15%. The changes in FEV<sub>1</sub> produced by bradykinin were not significantly different to that of ethanol in saline. However, despite the exclusion of asthmatic and atopic subjects there were significant falls in FEV<sub>1</sub> enough to cause clinical bronchoconstriction in two subjects. In one of these subjects a family history of atopy was subsequently found to be present. In view of the potential severity of bronchospasm following inhaled bradykinin it would probably be advised that not only those with a personal history but also those with a family history of atopy are excluded from further studies of healthy volunteers. The cough response did not appear to correlate with changes in airways function. The cough response did not differ between genotypes when examined either as initial response or as total cough response. This may indicate that ACE is not as important for bradykinin metabolism on the surfaces of the upper airways as other enzymes and thus differences in activity of ACE become unimportant. When ACE inhibitor drugs lead to cough this develops usually after 7-28 days of treatment and this acute model may not be suitable.

The design of the study was flawed by the use of the same order of administration of doses of bradykinin. When the cough response was related to the order of administration of the drug, (Figure 7.4), it appeared that tachyphylaxis occurred. The use of the same order of administration for each subject may exacerbate this phenomenon when the group as a whole are studied. Tachyphylaxis to bradykinin has been noted when bronchoconstrictor responses were studied in asthmatic subjects (Polosa 1992a) possibly due to depletion of the sensory neuropeptides. When Polosa et al examined mucociliary clearance following bradykinin in healthy subjects they noted that a dry cough occurred in some subjects. Some subjects noted a pharyngeal irritation which rapidly diminished during subsequent inhalations (Polosa 1992b). An alternative explanation for the failure to show a dose-response might be that the doses examined were on the plateau of the dose-response curve for cough. Bradykinin often caused only one or two coughs and thus using direct observation it may not be possible to conduct dose-response curves. The potential adverse effect of unexpected bronchoconstriction seen in some healthy volunteers would probably limit the use of higher doses in future research.

In conclusion although bradykinin caused or stimulated cough in healthy subjects this does not appear to be related to ACE genotype or to a reduction in FEV<sub>1</sub>.



In summary:

- Bradykinin administered by inhalation causes cough in healthy subjects.
- Bradykinin can produce significant bronchoconstriction associated with wheeze in non-asthmatic subjects.
- Cough in response to bradykinin does not appear to be related to ACE genotype.
- Cough in response to bradykinin does not appear to be related to changes in airways reactivity as determined by FEV<sub>1</sub>.

Chapter 8  
ACE GENE POLYMORPHISM  
AND  
DERMAL WHEEL RESPONSES TO BRADYKININ AND  
HISTAMINE  
IN HEALTHY SUBJECTS

## 8.1 INTRODUCTION

The insertion/deletion (I/D) polymorphism of the angiotensin converting enzyme (ACE) gene which consistently predicts differences in serum ACE activity has not been examined fully as regards tissue ACE. Costerousse et al examined ACE bound to T lymphocytes (Costerousse et al 1993), and reported that ACE genotype predicted lymphocytic ACE activity. The lymphocytic ACE activity differed between genotype in the same way as serum ACE but the relationship was not as strong (Costerousse et al 1993).

Bradykinin is a decapeptide involved in inflammatory reactions, and is metabolised by ACE. When injected intradermally bradykinin causes a dose-dependent wheal and flare response (Basran 1982), and this response is increased by treatment with ACE inhibitor drugs (Ferner et al 1990). In chapter 6 I was also able to demonstrate an increased response to bradykinin with ACE inhibitors. Thus the wheal response to intradermal bradykinin provides a measure of tissue ACE activity. In this chapter I examined the relationship between ACE genotype and the dermal wheal response to bradykinin in healthy subjects to find out if the ACE genotype might be related to tissue ACE activity.

## 8.2 AIMS

1. To examine the relationship between ACE genotype and tissue ACE activity using the dermal wheal response to bradykinin as a measure of tissue ACE activity

## 8.3 METHODS

### 8.3.1 *Subjects*

One hundred and five healthy subjects (50 men and 55 women) were recruited by open invitation and gave written informed consent to the protocol which was approved by the local ethics committee. Subjects with asthma or eczema were excluded. The 105 subjects comprised 30 subjects with DD genotype, 51 subjects with ID genotype, and 24 subjects with II genotype. Fifteen subjects, five of each genotype had taken part in the previous study (chapter 7).

### 8.3.2 *Study design*

Each subject was given four doses of bradykinin and of histamine intradermally, with both the investigator (M. Kraskiwicz) and subjects blinded to the genotype status. Histamine was included as a "negative control" because its metabolism, unlike that of bradykinin, is *not* influenced by ACE (kininase II) (Anderson 1990). Skin tests were performed as described in chapter 2. Bradykinin and histamine were injected intradermally in doses of 0.001, 0.01, 0.1 and 1 $\mu$ g.

### 8.3.3 *Measurements*

The wheal areas in response to intradermal bradykinin and histamine were measured at 15 minutes by drawing round the wheal in ink and transferring the image to graph paper using adhesive cellophane tape then measured using digitised planimetry.

ACE genotype was determined by polymerase chain reaction as described in chapter 2.

### 8.3.4 *Statistical analysis*

The sample size was calculated according to Pearson-Hartley Power Charts (Maxwell 1990) to have 80% power at a significance level of  $p < 0.05$  to detect a 50mm<sup>2</sup> difference between the groups in the response to bradykinin, using a standard deviation of 118 mm<sup>2</sup> derived from previous studies within our department. The bradykinin dose response curves were analysed using a parallel line bioassay method (Armitage and Berry 1987) which estimates the relative potency of bradykinin with 95% confidence intervals. The relative potency of bradykinin in subjects of different ACE genotype is a measure of shift of the dose-response curve to the left or right in relation to the ACE genotype.

## 8.4 RESULTS

*8.4.1 Comparability of groups* (Table 8.1). The subjects in the three genotypes were well matched for age, body mass index and blood pressure. The groups differed as regards sex distribution, with fewer female subjects in the ID group (37%) than in the DD and II groups (57%). This is ignored in the analysis as the sex distributions were similar in the DD and II groups, the comparison of prime importance.

**Table 8.1** Characteristics of subjects in relation to ACE genotype (mean  $\pm$  sd).

	DD	ID	II
number each group	30	51	24
age (years)	26 (7.4)	25(6.5)	25.8(6.8)
sex (m/f)	17/13	19/32	14/10
BMI	22 (2.3)	22 (3.1)	22 (2.7)
systolic BP (mmHg)	112 (10)	112 (10)	117 (11)
diastolic BP (mmHg)	72 (8)	71 (7)	71 (7)

8.4.2 *Dermal responses* (Table 8.2, Figure 8.1) Intradermal injection of bradykinin produced dose-dependent increases in wheal area, with dose-response curves which did not deviate from linearity and parallelism (both  $p > 0.05$ ), and were highly significant ( $p < 0.01$ , Figure 3.1a ). In the parallel-line analysis the potency of bradykinin in subjects of II genotype relative to those of DD genotype was 1.25, with a 95% confidence interval of 0.83 to 1.88. As the confidence interval bracketed unity the difference between II and DD groups was not statistically significant. Intradermal injection of histamine also produced dose dependent increases in wheal area which were linear, parallel and highly significant (Figure 3.1b). The potency of histamine in II subjects relative to DD subjects was 1.16 with a 95% confidence interval 0.71 to 1.88. Thus the relative potencies of bradykinin, which is metabolised by ACE, and histamine, which is not, were similar when comparing subjects of genotype II to those of DD genotype.

**Table 8.2** Wheal areas ( $\text{mm}^2$ ) in response to intradermal bradykinin and histamine related to ACE genotype (mean  $\pm$  se).

Bradykinin dose ( $\mu\text{g}$ )	DD (n=30)	ID (n=51)	II (n=24)	Potency (II relative to DD)
0.001	41.6 (3.2)	39.0 (1.7)	38.6 (2.3)	1.25
0.01	57.6 (3.5)	57.9 (2.4)	63.8 (3.7)	0.88-1.88 (95%CI)
0.1	78.6 (2.9)	81.4 (3.7)	84.7 (3.7)	
1.0	107.7 (4.9)	107.8 (5.0)	107.1 (5.6)	

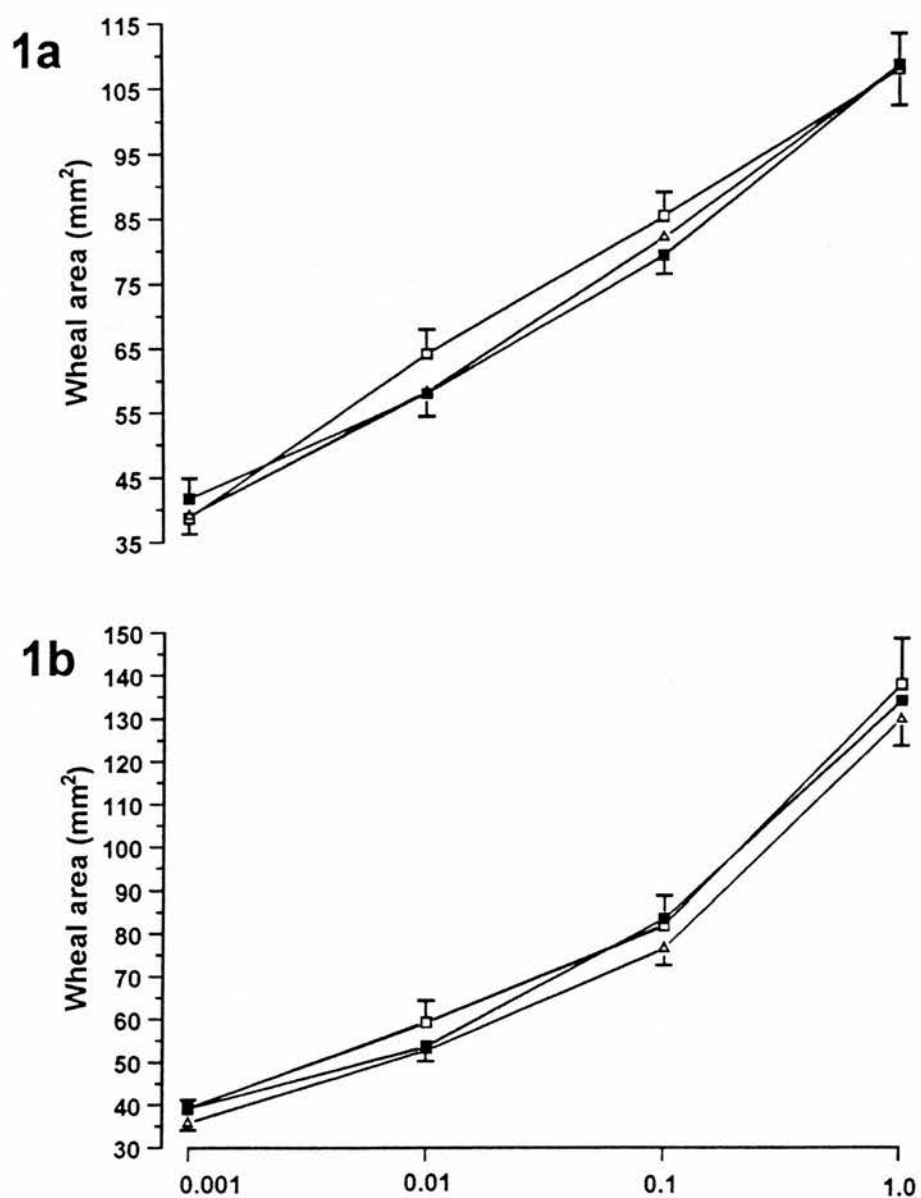
Histamine dose ( $\mu\text{g}$ )	DD	ID	II	Potency (II relative to DD)
0.001	39.0 (2.2)	35.7 (1.7)	39.2 (2.1)	1.16
0.01	53.7 (3.5)	52.8 (2.4)	59.3 (5.1)	0.71-1.88 (95%CI)
0.1	83.4 (5.4)	76.5 (4.0)	81.8 (4.5)	
1.0	134.0 (7.4)	129.7 (6.1)	137.8 (10.7)	

#### 8.4.3 Total area of wheal.

(Table 8.3) The total wheal area produced by bradykinin was calculated by summing the individual responses for each subject. The total wheal area produced by bradykinin was 285.6  $\text{mm}^2$  in DD subjects and 294.2  $\text{mm}^2$  in II subjects (diff DD-II 8.6 $\text{mm}^2$ ; 95%CI -39.5 to 22.3  $\text{mm}^2$ ). This difference was not statistically significant. For histamine the wheals were 310.0  $\text{mm}^2$  in DD subjects and 318.1  $\text{mm}^2$  in II subjects (diff DD-II 8.2 $\text{mm}^2$ ; 95%CI -50.0 to 33.8  $\text{mm}^2$ ).

**Table 8.3.** The total area of skin wheal produced from 4 doses of bradykinin and histamine (mean  $\pm$  se)

	DD	ID	II	Diff DD-II	95% CI
Bradykinin ( $\text{mm}^2$ )	285.6 (11.7)	286.2 (10.7)	294.2 (12.0)	-8.6	-39.5 to 22.3
Histamine ( $\text{mm}^2$ )	310.0 (14.6)	294.7 (11.9)	318.1 (19.4)	-8.1	-50.0 to 33.8



**Figure 8.1** The wheal responses to intradermal bradykinin (fig 8.1a) and histamine (fig 8.1b) in 105 subjects grouped according to ACE genotype. Figure shows mean ( $\pm$ se) for each genotype. ■=DD genotype, □ = II genotype and  $\Delta$  =ID genotype.

## 8.5 DISCUSSION

The relationship between ACE genotype and serum ACE has been observed consistently, with subjects of genotype II having a low serum ACE activity. If this was mirrored by lower tissue ACE activity they would be expected to have increased wheal responses to bradykinin.

Measuring tissue ACE activity in-vivo is difficult due to the close proximity of extra-cellular and intra-vascular fluids and thus serum ACE. The dermal response to bradykinin is a measure of ACE activity at the tissue level (Ferner 1989, Fuller 1989).

In my study the potency of bradykinin on wheal response did not differ significantly between the three ACE genotypes. In using dermal response to bradykinin I adopted a different approach to examine the relation between the ACE genotype and activity of ACE in tissue. It has been shown that treatment with ACE inhibitors increases the dermal response to bradykinin, indicating that the renin-angiotensin system has a readily measurable role within the dermal tissues (Chapter 6). My results show no significant difference in the potency of bradykinin in dermal wheal response between subjects of DD and II genotypes. There was a trend for subjects of genotype II to have larger responses to bradykinin, but the study had sufficient power to exclude a potency in II subjects greater than 1.88 relative to DD subjects. Moreover the findings for intradermal histamine, whose effect is independent of ACE, were similar to those for bradykinin with a trend towards a slightly higher potency in subjects of II genotype. Thus the polymorphism in the ACE gene that is associated with a marked difference in ACE activity in serum shows no relation to the activity of ACE in dermal tissues.

While the polymorphism in the ACE gene is associated with differences in ACE activity in serum, it is not related to the activity of ACE within the dermal tissues. I conclude that the skin responses to bradykinin as determined by wheal area are not related to ACE genotype and there is therefore no evidence for a relationship between the polymorphism in the ACE gene and membrane-bound ACE activity in this tissue.

In summary:

- The relations between ACE genotype and wheal responses to intradermal bradykinin and histamine were studied in 105 healthy subjects, 30 of genotype DD, 51 of genotype ID and 24 of genotype II.
- Bradykinin and histamine produced significant linear log dose - wheal area responses.
- The potency of bradykinin in II subjects relative to DD subjects was 1.25, with the 95% CI (0.71-1.98) indicating no significant difference between the genotypes
- The ACE gene polymorphism does not predict dermal bradykinin responses, which are a measure of tissue ACE activity.



## Chapter 9

### ACE GENOTYPE AND ACE INHIBITOR COUGH

## 9.1 INTRODUCTION

It remains uncertain why only about 20% of subjects develop a cough when taking ACE inhibitor treatment. Genetic factors certainly contribute to the susceptibility to develop adverse reactions to certain drugs and it may be that they are involved with this unusual effect. It has been hypothesised that the cough may be related to an accumulation of kinins due to reduced ACE activity (Yeo 1991). If this is the case one might expect the ACE genotype, a strong predictor of ACE activity, to be related to susceptibility to develop cough. In particular, those with a low ACE activity such as individuals of genotype II, might have greater accumulation of kinins and thus be more likely to develop cough.

I aimed to examine this theory by studying the distribution of genotypes amongst subjects who had developed a new cough when treatment with ACE inhibitors was commenced.

## 9.2 AIMS

1. To examine the distribution of I and D alleles in subjects with ACE inhibitor cough.

## 9.3 METHODS

*9.3.1 Study design and subjects* Records of the Sheffield hypertension clinic were examined and those patients with a cough which was considered to be due to ACE inhibitor drugs (as defined in chapter 2.5.1.3) were invited to attend for a blood test. A 5ml blood sample was obtained which was used for DNA testing of the ACE genotype as discussed in chapter 2. A control group consisting healthy subjects was obtained by open invitation. Ethics approval was obtained as before.

*9.3.2 Statistical analyses*

Groups were compared by Chi-square tests.

## 9.4 RESULTS

31 patients with a cough which could be attributed to ACE inhibitors were identified and agreed to be genotyped compared with 221 healthy controls. These patients consisted of 25 women and 7 men of mean age 58.7 years (sd 12.8) taking a mean 2.2 medications (sd 0.7). There were no significant differences between the distribution of genotypes (Table 9.1).

**Table 9.1** British patients with ACE inhibitor cough (B1, n=31), Healthy British control population (B2, n=221) according to ACE genotype.

Group		Genotype			Allele distribution	
		II	ID	DD	I	D
Those with cough	(B1)	6	12	13	0.39	0.61
Those without cough	(B2)	58	97	66	0.48	0.52

## 9.5 DISCUSSION

In this study I examined the distribution of genotypes for the ACE gene polymorphism in a group of patients with cough due to ACE inhibitors.

No significant difference in ACE genotype was observed between those with cough and either a healthy population or a group without cough due to ACE inhibitors. There was no excess of genotype II in the group who coughed; nor did DD protect against cough. This finding was confirmed in a French population (Kreft-Jais 1994) who also examined a group of those who developed cough with ACE inhibitors. The control groups in this study consisted of patients taking ACE inhibitors and not coughing and a population of hypertensive patients (Table 9.2). There has been no convincing relationship demonstrated between hypertension and ACE genotype in Western populations at present (Cambien 1988, Harrap 1993, Schmidt 1993). Interestingly in a Japanese population an excess of II was indeed found (Furuya 1994; Table 2). This may represent racial differences as not only has the ACE genotype been linked to hypertension in this racial group (Morise 1994) but a higher incidence of cough has been found in Chinese, among whom the population prevalence of the I allele is high (Chan 1993, Lee 1994). It has been suggested that the reasons for this difference between European and Asian populations may reflect differences in haplotype or expression of the gene between these populations of the angiotensin converting enzyme gene (Kreft-Jais 1994). Alternatively, and probably more likely, the findings may be due to chance particularly as these studies are often case-control in nature and of undetermined power.

In conclusion in a cohort of patients with hypertension who developed a cough whilst on ACE inhibitor treatment there was no relationship between ACE genotype distribution or frequency of the I allele. The ACE genotype is unlikely to be involved in the pathogenesis of ACE inhibitor cough in Western populations.

**Table 9.2:** \*British patients with ACE inhibitor cough (B1, n=31), Healthy British control population (B2, n=221), French patients with ACE inhibitor cough (F1, n=75), French patients without cough on enalapril (F2, n=71), Hypertensive French control (F3, n=206), Japanese patients with ACE inhibitor cough (J1, n=31), Japanese patients without cough on enalapril (J2, n=71).

Group*	Genotype			Allele distribution	
	II	ID	DD	I	D
B1	6	12	13	0.39	0.61
B2	58	97	66	0.48	0.52
F1	12	33	30	0.38	0.62
F2	15	29	27	0.42	0.58
F3	42	95	69	0.44	0.56
J1	19	12	0	0.81	0.19
J2	25	35	11	0.6	0.4

In summary:

- There was no relationship between ACE genotype distribution and cough due to ACE inhibitor.

## Chapter 10

### Discussion

It has taken 100 years to learn what we now know on the renin-angiotensin system. From what was a slow and uncertain path the rate of understanding has been exponential in recent years. The recent discovery of a genetic variation in the ACE gene that might affect responses to either substrates or inhibitors of ACE and also possibly act as a risk factor for cardiovascular disease (Cambien 1992) has been the focus for studies in this thesis.

I therefore aimed to study the responses of healthy subjects of different genotype for the I/D ACE gene polymorphism to establish whether the differences that occurred in serum ACE activity were mirrored by differences in tissue ACE activity. This was done by studying the vascular responses to angiotensin I and ACE inhibitors in subjects of different ACE genotype. ACE inhibitor drugs are now commonly prescribed for patients with hypertension and heart failure with little prospect of the frequency with which they are used diminishing (McInnes 1993). With an increase in use of any drug will follow an increase in adverse effects and it is important that we continue to examine these side effects. It would be hoped that we can learn not only more about the clinical pharmacology but that eventually through this knowledge better treatments might be developed. Thus I examined cough, a common side effect of ACE inhibitors, with particular emphasis on elucidating its effects on the cough reflex and the role of various peptide mediators in its pathogenesis. Thereafter I examined the hypothesis that responses to bradykinin might be determined by ACE genotype in healthy subjects. Finally I examined whether ACE genotype might be related to the development of ACE inhibitor cough.

### **10.1 Does an individual's ACE genotype determine responses to substrates of angiotensin converting enzyme?**

A strong relationship between ACE genotype and serum ACE has been observed consistently by others (Rigat 1990, Ueda 1994) and in our own laboratory. Subjects of genotype II have serum ACE activity that is approximately 50% that of DD subjects (Cambien 1994). At present it is unclear whether the relation between ACE genotype and serum ACE is paralleled by a relation of the genotype to tissue ACE, that is the membrane-bound form ACE, the predominant form in the heart, lungs and vascular endothelium. There is some evidence ACE genotype and tissue ACE might be related from *in-vitro* studies (Costerousse 1993), but studies in humans examining this question indirectly through the responses to substrates of ACE have only recently been performed. The relation of the ACE genotype to membrane-bound ACE activity in tissue which unlike serum ACE, is thought to effect the physiological and pathophysiological actions of ACE (Soubrier 1993), is important because of numerous reports linking the ACE genotype to various cardiovascular disorders (Cambien 1994, Tixeront 1993, Raynolds 1993, Schunkert 1994).

In this thesis I examined the vascular responses of subjects of different ACE genotype to angiotensin I and ACE inhibitors. If the relation of genotype to serum ACE was paralleled by a similar relation to tissue ACE one would anticipate an association between the ACE genotype and responses of angiotensin I or ACE inhibitor. For example if the higher serum ACE activity of DD subjects relative to II subjects was mirrored by an increase in tissue ACE activity in DD subjects, they would be expected to have greater responses to angiotensin I and smaller responses to ACE inhibitors.

#### *Vascular responses and the ACE genotype*

The pressor response to angiotensin I was not found to be related to ACE genotype. This agrees with the findings of Menard et al (1995) but contrasts with the results of Ueda et al who reported that subjects of genotype DD did have enhanced pressor responses to angiotensin I (Ueda et al 1995). All the studies were of similar magnitude and thus there remains doubt as to the true involvement in ACE genotype in determining pressor responses to Angiotensin I. I also examined some neurohormonal responses. Perhaps those of different genotype might have differing amounts of the end product of the renin cascade- angiotensin II. Differing effects on the downstream production of angiotensin II were not seen in my study or indeed in a large study where normotensive subjects of different ACE genotype had various parameters from the RAA system measured (Harrap 1993).

I examined the blood pressure and serum ACE responses to enalapril in healthy subjects to ascertain if there was any possible role of the ACE genotype. I found that the ACE genotype influences serum ACE activity under normal conditions and after a single oral dose of enalapril, and also predicts the acute responses of serum ACE activity to a single dose of enalapril. However I found no evidence that the ACE genotype predicts the acute pharmacodynamic response to enalapril in terms of blood pressure responses. Preliminary results from similar work agrees with my results (O'Kane 1996) My observations relate to a relatively low single dose of enalapril, and it will be of interest to examine whether higher doses or chronic treatment abolish serum ACE differences between the genotypes.

It would seem unlikely therefore that the ACE genotype can determine vascular responses either as pressor response to Angiotensin I or hypotensive responses to ACE inhibitors with possible reasons for this being discussed below.

### *Bradykinin responses and the ACE genotype*

When I examined the effect of inhaled bradykinin it produced cough in healthy subjects but this does not appear to be related to ACE genotype. Unfortunately this study was flawed in design in that the same order of administration was used for each subject. This design fault probably exaggerated the tachyphylaxis which seems to be seen with inhaled bradykinin (Polosa 1992a). However when the first dose response was examined there did not seem to be significant differences although the small sample size might conceal these.

In the study of dermal responses to bradykinin I adopted a different approach to examine the relation between the ACE genotype and activity of ACE in tissue. It has been shown that treatment with ACE inhibitors increases the dermal response to bradykinin, indicating that this response to bradykinin is a measure of ACE activity at the tissue level (Ferner 1989, Fuller 1987). If the relation of genotype to serum ACE was paralleled by a similar relation to tissue ACE one would anticipate an association between the ACE genotype and responses of intradermal injection of bradykinin. For example if the higher serum ACE activity of DD subjects relative to II subjects was mirrored by an increase in tissue ACE activity in DD subjects, they would be expected to have smaller wheal responses to bradykinin. Likewise dermal responses to histamine which is not metabolised by ACE (Anderson 1990) would be unrelated to the ACE genotype. My results show no significant difference in the potency of bradykinin in dermal wheal response between subjects of DD and II genotypes although there was a trend for subjects of genotype II to have larger responses to bradykinin. The study had sufficient power to exclude a potency in II subjects greater than 1.88 relative to DD subjects. Moreover the findings for intradermal histamine, whose effect is independent of ACE, were similar to those for bradykinin with a trend towards a slightly higher potency in subjects of II genotype. Thus the polymorphism in the ACE gene which is associated with a marked difference in ACE activity in serum shows no relation to the activity of ACE in dermal tissues. These findings do not support a role for the ACE genotype in the physiological action of ACE at the tissue level.

Unfortunately it is unlikely that individually my studies involving examination of ACE genotype had sufficient power to exclude with any certainty differences between genotype of a small magnitude. Most of them excluded differences of a large magnitude however. When taken together my studies lead me to doubt whether there is a role for the ACE genotype in the physiological action of ACE at the tissue level.



Why might the ACE genotype have no role to play in determining membrane-bound ACE or there being phenotypic differences in subjects of differing genotype? There could be several explanations for this. Firstly the experimental models of tissue ACE that I used may not be optimal and could include too many other variables. This would be best solved by using isolated tissue preparations but this is clearly difficult and to my knowledge has only been done by Costerousse (1993) using T lymphocytes and more recently in cardiac tissue (Jan Danser 1995). Vascular responses in-vivo show great variability and obtaining a suitable sample size may be problematic. Determining the responses of serum ACE to ACE inhibitors are limited by measurement of ACE activity in the presence of some ACE inhibitors ie captopril. This will mean long-acting agents are used with subsequent practical difficulties in recruitment for volunteer studies unless solely blood pressure response is examined.

Secondly my studies are relatively small and the variability in response quite large. My results would give others an idea of the variability and allow a more accurate estimation of sample size. Others have tried to minimise variation using sodium depletion or the use of an intravenous pro-drug (O'Kane 1996, Ueda 1996) however the variation in response remains considerable.

Finally, there may of course be no role of the ACE I/D polymorphism in determining tissue ACE responses. At most it is only a marker in linkage disequilibrium with the locus, as yet unidentified, responsible for regulating serum ACE levels (Lindpainter 1995). The true locus responsible for determining ACE level is considered to lie within the promoter region of the ACE gene and if identified then further work would need to be done to re-group subjects according to genotype. Even then it may be difficult to identify phenotypic differences apart from ACE level.

Until recently it was not appreciated how even simple gene defects might exhibit phenotypic complexity. Research into diseases such as cystic fibrosis and Huntingdon's chorea was considered to be advanced by the discovery of the gene defect. Unfortunately in both these diseases the genotype does not always predict the phenotype (Alper 1996). This may be due to reduced penetrance, variable expressivity, variable sets of symptoms (pleiotropy) or the effects of age on presentation (Alper 1996).

I would suggest that reports linking the ACE genotype to various cardiovascular and other (Ruiz 1994) disorders due to possible phenotypic variation in the RAA, often based upon rather unsatisfactory evidence (Teo 1995), should be viewed with caution until such times as the ACE genotype has been shown to have an association with some physiological activity. My view is strengthened by the recent publication of prospective studies that have failed to confirm a relationship between ACE genotype and cardiovascular disease (Harrap 1993, Lindpainter 1995, Lindpainter 1996).

### **10.3 What are the effects of ACE inhibitors on the cough reflex?**

#### *10.3.1 The cough due to ACE inhibitors*

Cough is now accepted as a common and troublesome effect of treatment with ACE inhibitors. I examined the effects on the cough reflex of patients starting treatment with ACE inhibitors. Patients starting enalapril who developed a new onset of persistent cough during a one year follow-up reported significantly more cough by questionnaire and visual analogue scales at one month than did controls. The patients had normal sensitivity to inhaled capsaicin initially but a five fold increase at one month. By contrast in those with no cough and in controls capsaicin sensitivity was reduced. ACE inhibitor treatment appears to shift the capsaicin dose-response only in those who develop spontaneous cough, not in all patients treated.

ACE inhibitor cough is thought to resolve rapidly, within one week of stopping treatment (Coulter 1987, Berkin 1989), but these impressions predate quantitative methods of assessing ACE inhibitor cough (Yeo 1991, Fletcher 1990, Yeo 1994). I studied the resolution of cough related to enalapril after the ACE inhibitor was stopped. The cough certainly improved on cessation of treatment with reductions in measures of the severity and frequency of cough and night-time cough, but resolution took longer than one week. There was a significant improvement 14 days after stopping enalapril, but complete loss of the cough was observed only after 28 days.

There was a highly significant association between cough responses to capsaicin and measurements of severity and frequency of cough by visual analogue scales. This supports both the findings of myself and others that the abnormality underlying ACE inhibitor cough is an increase in C-fibre mediated sensitivity of the cough reflex (Fuller 1987, McEwan 1989, Yeo 1994). With the exception of severe cough, there was no significant association between cough measured by questionnaire and capsaicin sensitivity, suggesting that the visual analogue scales may be more sensitive than the questionnaire for measuring cough. While the significant increase in capsaicin sensitivity in patients with ACE inhibitor cough is well established (Fuller 1987, Yeo 1994) there is little information on changes in capsaicin sensitivity over time within patients. My study shows a substantial and highly significant reduction in sensitivity of the

cough reflex to capsaicin after stopping enalapril. The five-fold fall in potency of capsaicin over 28 days after stopping enalapril in the study in chapter 6 is identical to the five-fold difference in relative potency observed between patients with ACE inhibitor cough and control subjects found in chapter 5, and suggests that the cough reflex had returned to normal four weeks after stopping treatment.

### *10.3.2 What is the role of kinins?*

It has been considered for some time that kinins have a role in the pathogenesis of ACE inhibitor cough (Yokosawa 1985, Thysell 1988, Morice 1987). This has been further confirmed by recent work where a specific angiotensin II antagonist losartan was compared to lisinopril in patients with ACE inhibitor cough (Lacourciere 1994). When I studied the discontinuation of treatment in those with cough I found the wheal area in response to intradermal bradykinin and substance-P showed progressive and highly significant declines after stopping enalapril. These findings give some support to the hypothesis (Coulter 1987, Berkin 1989) that bradykinin may be a mediator of ACE inhibitor cough. Although the dermal responses to substance-P were associated with ACE inhibitor withdrawal they were not linked to changes in cough response. However intradermal responses to these peptides may not reflect events in the respiratory tract, and there may be type II error as the sample size was small. Changes in capsaicin sensitivity parallel those in subjective cough supporting suggestions that ACE inhibitor cough is caused by facilitation of the C-fibre mediated cough reflex. Change in response to intradermal bradykinin was a better predictor of cough responses than that for substance-P, and accounted for about a quarter of unexplained variability in the changes in visual analogue scales for cough during the resolution of ACE inhibitor cough.

#### **10.4 Is the ACE genotype linked to ACE inhibitor cough?**

In subjects with ACE inhibitor cough I examined the distribution of ACE genotype. I found no evidence that the frequency distribution of alleles was any different in those who cough compared to healthy controls. Subsequently this has been confirmed by others examining other European populations (Kreft-Jais 1994) but interestingly data from Far Asian population showed an increase in those of genotype II (Furuya 1994). These geographical variations I feel have more to do with sampling differences than actual genetic reasons. It is unlikely that the ACE genotype has any role in determining who develops cough due ACE inhibitors.

#### **Conclusions of thesis**

In summary, this thesis has explored various aspects of the renin-angiotensin system with particular emphasis on ACE inhibitor cough and the ACE genotype. I have established that when a cough develops in a patient on ACE inhibitor treatment this is likely to have been accompanied by a change in the cough reflex probably mediated by kinins. I have also established that in healthy subjects their responses to both substrates of ACE (angiotensin I and bradykinin) and ACE inhibitors are not likely to be related to ACE genotype.

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Publications arising from work within this thesis:

“Relation between changes in blood pressure and serum ACE activity after a single dose of enalapril and ACE genotype in healthy subjects.” Todd GP, **Chadwick IG**, Higgins KS, Yeo WW, Jackson PR, Ramsay LE. *Br J clin Pharmac* 1995; 39:131-134

“Resolution of ACE inhibitor cough: Changes in subjective cough, And Responses To Inhaled Capsaicin, Intradermal Bradykinin And Substance-P.” Yeo WW, **Chadwick IG**, Kraskiewicz M, Jackson PR, Ramsay LE. *Br J clin Pharm* 1995; 40:423-429

“ACE genotype and dermal wheal response to bradykinin” **Chadwick IG**, Kraskiewicz M, Yeo WW, Ramsay LE, & Jackson PR *Clinical Science* 1996; 91:617-620

Appendix 1.

An example of the self-administered questionnaire used in the studies on cough, chapters 5 and 6 (Fletcher 1990, Yeo 1991, Lacourcière 1994).

**SHEFFIELD HYPERTENSION CLINIC SURVEY ON DRUG SIDE EFFECTS**

PLEASE ANSWER THE FOLLOWING QUESTIONS:-

DO YOU SUFFER FROM ANY OF THE FOLLOWING?

	YES	NO
Asthma	[ ]	[ ]
Chronic Bronchitis	[ ]	[ ]
Emphysema	[ ]	[ ]

Do you have any of the complaints listed below? Please complete for each question.

Symptoms	not at all	a little	moderately	quite a bit	extremely
dry mouth					
cramps in legs					
dry cough					
racing heart					
heartburn					
headache					
sore throat					
getting up at night to pass urine					
flushing of face					
voice changes (for example : husky or hoarse)					
diarrhoea					
stuffy nose					

**DO YOU GET HEADACHES REGULARLY?**

YES[ ]      NO[ ]

HOW OFTEN DO YOU GET THEM? \_\_\_\_\_

WHEN DID THEY START? \_\_\_\_\_

**TICK ONE OF THE BOXES IN EACH SQUARE WHICH BEST DESCRIBES YOUR HEADACHE**

---

Behind eyes	[ ]	Daytime	[ ]	Lasts	minutes	[ ]
Top of head	[ ]	Night-time	[ ]		hours	[ ]
Back of head	[ ]	Day & night	[ ]		days	[ ]
One side of head only	[ ]	Other	[ ]		Other	[ ]
Other	[ ]	give details			give details	
Severe	[ ]			Vomiting with headache		Sleep disturbed by headache
Moderate	[ ]			YES [ ]		YES [ ]
Mild	[ ]			NO [ ]		NO [ ]

Other people complain about you always having a headache?

YES [ ]

NO [ ]

Have you consulted your GP about the headache?

YES [ ]

NO [ ]

DO YOU HAVE A PERSISTENT COUGH? YES [ ] NO [ ]

HOW OFTEN DO YOU GET IT? \_\_\_\_\_

WHEN DID IT START? \_\_\_\_\_

TICK ONE OF THE BOXES IN EACH SQUARE WHICH BEST DESCRIBES YOUR COUGH

Dry cough	[ ]	Daytime	[ ]	
Intermittent	[ ]	Night-time	[ ]	
Produces phlegm	[ ]	Day & night	[ ]	Bouts of
Continuous	[ ]	Give details		
Blood with phlegm	[ ]			
cough	[ ]			
Other	[ ]			
Severe	[ ]	Vomiting with cough		Sleep disturbed by cough
Moderate	[ ]	YES [ ]		YES [ ]
		NO [ ]		NO [ ]
Mild	[ ]			

Other people complain about your cough?		Have you consulted your GP about the cough?
YES [ ]		YES [ ]
NO [ ]		NO [ ]

DO YOU SMOKE ? YES [ ] NO [ ]

IF YES:- Cigarettes [ ] Pipe [ ] Cigars [ ]

IF CIGARETTES:

How many cigarettes per day ?  
(please insert number in box ) [ ] cigarettes per day.

HOW OLD WERE YOU WHEN YOU STARTED SMOKING ? [ ] yrs

ARE YOU AN EX-SMOKER? YES [ ] NO [ ]

IF YES, AT WHAT AGE DID YOU STOP? [ ] yrs

HOW MANY DID YOU SMOKE EACH DAY? [ ] cigarettes

PLEASE LIST YOUR TABLET TREATMENT BELOW

*(Remember to include inhalers, oral suspensions and suppositories, etc)  
(You will find the name of the tablet, strength and dose on the bottle or packet from your chemist )*

## Appendix 2

An example of the diary used to collect information on cough following cessation of the ACE inhibitor.

### SYMPTOMS ASSESSMENT-COUGH REPORT FORM

PATIENTS NAME

DATE

#### INSTRUCTIONS TO PATIENT : COMPLETION OF THE CARD

In the morning ,please indicate the severity of any nighttime cough. In the evening indicate the severity of any symptoms throughout the day.

#### SCORING OF SYMPTOMS

On this page there is a list of words that determine symptoms people have. Please read each one carefully, then enter one number to represent the extent to which the symptom has bothered you. Be sure to mark one box for each day.

Not at all      A little      Moderately      Quite a bit      Extremely  
0                    1                    2                    3                    4

	<u>DAY</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>
Night-time cough (0-4)						
Frequency of cough (0-4)						
Severity of cough (0-4)						
Sore throat (0-4)						
Husky voice (0-4)						
Stuffy nose (0-4)						

Appendix 3

An example of the visual analogue scales used in chapters 5 and 6.

**VISUAL ANALOGUE SCALE**

PATIENTS NAME \_\_\_\_\_ DATE \_\_\_\_\_

**EXAMPLE:**

**Read the statements at each end of the line carefully, then mark the line, to describe how you have been, in the way shown below.**

I am never thirsty

I am always thirsty

I never cough

I am always coughing

Cough has been absolutely  
no trouble to me

My cough has been as  
bad as it could possibly  
be

I never have headache

I always have headache

Headache have been  
absolutely no trouble to me

My headaches have  
been as bad as they  
could possibly be