

**THE RENIN ANGIOTENSIN ALDOSTERONE AXIS:
RELATIONSHIPS WITH OTHER HORMONE SYSTEMS,
AND NOVEL APPLICATIONS FOR ANGIOTENSIN
CONVERTING ENZYME INHIBITORS.**

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ABSTRACT:

The aim of this thesis was to look at the renin angiotensin system both in clinical heart failure, and in relation to other physiological systems where there may be an interaction. Furthermore, to address new areas where a potential for benefit with Angiotensin Converting Enzyme (ACE) inhibitors might exist. To this end there are five studies discussed within the thesis.

It is shown that in a group of heart failure patients, stabilised on maximum tolerated ACE inhibitor dose, mean levels of plasma neurohormones were remarkably stable over 18 months. Reactivation of aldosterone occurred in 13/97 samples (13.5%), in 5/22 (23%) individuals, and reactivation of angiotensin II occurred in 8/102 samples (8%), in 6/22 (27%) individuals. These appear to be sporadic phenomenon, and contrary to previous dogma, they do not herald disease progression.

Using endothelial function as a surrogate marker for cardiovascular events in hyperlipidaemic patients, it is shown that interruption of the renin angiotensin system using ACE inhibition, causes increases in both endothelial dependant and endothelial independent vasodilation. This could lead to the use of ACE inhibitors in hyperlipidaemic patients to reduce cardiovascular events, over and above traditional therapy such as statins.

In addition to the above, the effect of lisinopril on nitrate/nitrite excretion as a marker of nitric oxide metabolism in hypercholesterolaemia was assessed. The levels of plasma nitrate/nitrite after an eighteen hour fast in twenty of the hyperlipidaemic volunteers were taken, and contrasted with values in normal volunteers. Lisinopril was found to have no effect on nitrate/nitrite levels in the hyperlipidaemic patients.

Oestradiol is a vasodilator and has been shown to have effects on the vasculature of both men and women. Evidence in the literature has suggested interactions between oestradiol and the renin angiotensin system, at various sites. Using forearm plethysmography no evidence was found that either orally administered oestradiol, or medroxy-progesterone affected vascular responses to angiotensin II, or altered the activity of vascular ACE activity.

Finally, looking for a direct interaction between nitric oxide and the renin angiotensin system, it is shown that nitric oxide inhibition reduces renin release in human volunteers. However this renin suppressive effect was also seen with an equipotent vasopressor agent and it is felt that the effect, while present, is non specific and relates to pressor effects rather than specific nitric oxide inhibition at the renal level.

“The basis of medicine is sympathy and the desire to help others, and whatever is done with this end must be called medicine.”

-Frank Payne (1840-1910)

English Medicine in Anglo Saxon Times.

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DECLARATION:

This thesis is a record of work conducted by myself at the Department of Clinical Pharmacology, University of Dundee between February 1995 and February 1997. It has not previously been submitted for a higher degree.

I have personally consulted all the references cited, and am the sole author of this thesis.

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Publications Derived From The Work Within This Thesis:

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PRESENTATIONS.

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Lee AFC, Dick JBC, Struthers AD. Can Lisinopril improve endothelial function in hyperlipidaemics? Caledonian Clinical Pharmacology Society Meeting October 1996, Dundee, UK.

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Lee AFC, Dick JBC, Struthers AD. Can lisinopril improve endothelial function in hyperlipidaemics? American College of Cardiology 46th Annual Session, March 1997 Anaheim, California, USA.

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Lee AFC, Dick JBC, Bonnar C, Struthers AD. Angiotensin converting enzyme inhibitors (ACE) inhibitors in hyperlipidaemia: A novel therapeutic approach? Heart Failure '97, May 1997, Cologne, Germany.

Lee AFC, MacFadyen RJ, Morton JJ, Struthers AD. Does Angiotensin II reactivate during chronic ACE inhibitor therapy in heart failure? British Cardiac Society meeting May 1998, Glasgow, Scotland.

Aims of the thesis:

This thesis, and the investigations within looked at the following questions.

Angiotensin Converting Enzyme (ACE) inhibitors in routine clinical situations:

- Does routine therapy with an ACE inhibitor adequately suppress angiotensin II and aldosterone over an eighteen month period?
- Are there identifiable correlations between neurohormones that could allow therapeutic interventions to avoid clinical deterioration?

Angiotensin Converting Enzyme inhibitors in novel clinical situations:

- Are ACE inhibitors beneficial in hyperlipidaemia, in addition to ongoing cholesterol lowering therapy?

Renin Angiotensin Aldosterone System: interactions with other hormone systems.

- Do ACE inhibitors affect the production of nitric oxide in hyperlipidaemics?
- Does nitric oxide inhibition affect renin release?
- Is the vasodilation seen with oestradiol in human models due to effects on either ACE activity, or vasoconstrictor responses to angiotensin II?

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CHAPTER 1: INTRODUCTION

The Renin Angiotensin Aldosterone Axis, and Angiotensin
Converting Enzyme Inhibitors.

1.1: The renin angiotensin axis-a historical perspective:

The discovery of renin dates back to Tigerstedt and Bergman (1898), who identified a pressor substance derived from renal cortex at the end of the nineteenth century. They believed this substance to be a protein, and it was called “renin”. The importance of renin was not really appreciated until the seminal work of Goldblatt on hypertension in the 1930’s. He created artificial hypertension in dogs by constriction of the renal arteries (Goldblatt 1934), which he felt may be due to the production, by the kidneys, of a pressor substance, which worked in an endocrine fashion.

It was realised that the pressor effect of renin took some time to appear and renin was later shown to be an enzyme. Subsequently, it was demonstrated that renin activated a plasma substance to produce vasoconstriction (Muñoz 1939). This mediator was then identified as angiotensin II, one of the most potent vasoconstrictors known to man (Braun-Menendez 1940). Following this discovery there was much interest in renin and angiotensin II in the pathogenesis of hypertension. Hence angiotensin II sequencing as an octapeptide was achieved in the mid fifties, with synthesis of a pure peptide performed in 1956 (Schwyzer 1956). This allowed an explosion of research into the effects of this peptide, and it’s importance not only in hypertension, but also in heart failure.

Although aldosterone had also first been identified in the 1950’s as a separate entity from the glucocorticoids (Simpson 1952), the association of renin, angiotensin II and aldosterone did not immediately happen, and took several more years.

Most of the work on linking the three hormones, was conceived by Franz Gross (1968) and colleagues, who believed that while renin and angiotensin II were important in pathological hypertension they must also have a role in normal physiology. They observed that in salt loaded rats, the kidneys did not have renin like pressor ability, and that the response to administered renin was exaggerated. This effect was similar to that of renin in studies of bilateral nephrectomised animals (Gross 1968). He then connected this renin sensitivity with the observation that granules seen in the renal juxtaglomerular cells were reduced in salt replete states, and increased in salt depletion. After a series of investigations Gross believed that the renin content of the kidneys was inversely related to the salt loading of the animal. In the late 1950's he put forward the concept that renin via angiotensin II was responsible for the control of aldosterone release from the adrenal cortex, which controlled salt homeostasis. He continued to work on this theory which was eventually proved correct, largely by the work of Davis et al (1961).

In a series of experiments between 1958-1961 James Davis and colleagues (1961) showed that in dog models of heart failure sodium retention was dependant on either administration of corticosteroids, or on the presence of the adrenal glands. He went on to show that aldosterone levels were high in the heart failure model, and this related to increased production rather than reduced degradation. In a complex series of experiments using transplanted adrenal glands it was shown that aldosterone secretion is stimulated by a plasma hormone. It was noted that while ACTH is important in the secretion of aldosterone even with hypophysectomy a state of secondary hyperaldosteronism due to heart failure could be maintained. The second hormone stimulating aldosterone secretion was termed "ASH" or aldosterone

stimulating hormone. There followed an extensive series of experiments that eventually established that ASH was dependant on the kidneys, as nephrectomised, hypophysectomised dogs could not increase aldosterone in response to haemorrhage. Angiotensin II was not identified specifically by Davis, but he established that ASH did not have the time course of renin in stimulating aldosterone, and that an extract termed “hypertensin II” found in renal extracts seemed the most likely cause. This was later identified as angiotensin II (Gross 1968).

The interruption of the renin angiotensin aldosterone axis via drugs followed rapidly on the heels of its physiological elucidation.

Saralasin- a competitive antagonist of angiotensin II:

Saralasin is an octapeptide with substitution of different amino acids in position 1 and 8 of bovine angiotensin II. It was widely used to further characterise the effects of the renin angiotensin axis, but had to be given parenterally, hence was of limited clinical use (Pals 1971). Furthermore it had partial agonist effects, and could actually cause hypertension. In the late eighties early/nineties orally available angiotensin II receptor antagonists have become available. Losartan (DuP 753) was the first drug to be widely used (Chiu 1990), and recent developments have led to an exponential increase in this family, which are now available clinically both for hypertension, and now for heart failure.

Captopril- the first orally available ACE inhibitor:

The angiotensin converting enzyme inhibitors were firstly derived from the venom of a Brazilian snake, Bothrops Jararaca (Ondetti 1971), which contained peptides that

inhibited the enzyme. Initial extracts were only parenterally available, however SQ 14 225 (captopril) was developed and found to be effective in reducing the pressor response to angiotensin I in normal humans (Ferguson 1977). Captopril was extensively tested clinically in the late seventies/early eighties, and found to be effective for symptom relief (Cleland 1984), prognosis in heart failure (Garg 1995), and in blood pressure reduction in hypertension (VA co-op study group 1982). There are now fifteen orally available ACE inhibitors, all of which have been shown to have therapeutic efficacy.

1.2: Renin angiotensin system: Structure and function.

The renin angiotensin aldosterone axis is one of the major hormonal influences on blood pressure and vascular tone (Reid 1978).

Renin is an enzyme that is stored in granules in a variety of cells within the kidney, prior to release. It is concentrated in the juxtaglomerular cells, although not localised to these cells (Hackenthal 1990). Like angiotensin II it is a ubiquitous molecule which is also found in brain, pituitary, heart, and adrenals. Renin release is determined by a variety of stimuli, the main three being neurally mediated adrenergic stimulation, reductions in renal perfusion pressure, and tubular control based on the sodium and chloride delivery to the distal nephron (Hackenthal 1990).

Renal perfusion pressure is accepted to affect renin, with doubling of renin secretion for minor falls in pressure (Hackenthal 1990). Haemorrhage is a common experimental stimulus for renin release, believed to act through both alterations in pressure (Chiu 1995), and via the sympathetic nervous system.

The tubular control of renin secretion is based on the macula densa hypothesis, which was initially considered because of the proximity of the juxtaglomerular apparatus, and the macula densa. It has now been established that as sodium chloride concentration in the distal tubule rises, passing the macula densa cells, renin is inhibited and vice versa. Many aspects of the control of renin are still under debate, one of which is the mechanism by which the macula densa signals the juxtaglomerular cells. Several molecules have been implicated, one of which is nitric oxide. Tubular control is believed to be mechanistic in the undesirable rises in renin and angiotensin seen with diuretic therapy in heart failure (van Zwieten 1994).

One further important control on the secretion of renin is angiotensin II whose negative feedback is abolished by ACE inhibitors leading to reciprocal rises in both angiotensin I and renin (Cleland 1984, 1985, Juillerat 1990).

There are numerous additional factors which have been shown to affect renin release; for an authoritative review see Hackenthal (1990).

Renin, having been released from the kidney then cleaves angiotensinogen, which is a circulating plasma protein into angiotensin I. Angiotensinogen is a glycoprotein synthesised and stored mainly in the liver, and released into the circulation in response to several stimuli one of which is angiotensin II (Hackenthal 1990).

Angiotensin I is then converted via Angiotensin Converting Enzyme (ACE) to angiotensin II (Guyton 1982). ACE is membrane bound, particularly on endothelial cells, is concentrated in the lungs, and is not seen generally in parenchyma except in the kidneys (Caldwell 1976). It is a non-specific chymase, which can cleave other peptides (Hackenthal 1990), and initial investigation into ACE inhibitors were aimed

at potentiation of bradykinin rather than blockade of angiotensin II production (Ondetti 1971).

Angiotensin II acts at specific membrane receptors to effect vasoconstriction (Cody 1997), and stimulate aldosterone release from the adrenal medulla. These are just two functions of this molecule, and appear to represent the results of circulating angiotensin II. Angiotensin II also has effects in the brain and stimulates thirst centres (Hirsch 1987). However, there is now mounting evidence on the importance of angiotensin II as a mediator of vascular intimal metabolism. Interest in this has been stimulated by the localisation of ACE on the vascular endothelium, and the realisation that it can stimulate fibrosis and vascular smooth muscle cell proliferation of both myocardium, and vascular wall (Cody 1997, Kato 1991, Daemen 1991). The activity of angiotensin II in the vascular wall may also mediate a variety of other destructive processes in the vessel such as thrombosis (Cody 1997), hence the improvement in ischaemia seen with the use of ACE inhibitors.

Angiotensin II stimulates the release of aldosterone which is a steroid molecule, and is stored in the zona glomerulosa of the adrenals; it is stimulated not only by angiotensin II but also by serum potassium, and by ACTH. Recently the literature has suggested that a variety of other factors such as dopamine, atrial natriuretic peptide (Atarashi 1985), or nitric oxide (Nakayama 1996, Natarajan 1997) may affect release of aldosterone.

Aldosterone action is via intracellular receptors, and again appears to have humoral and local actions. Early work showed that aldosterone caused salt and water retention by the kidneys at the distal tubular epithelium by facilitating the loss of potassium and hydrogen and retaining sodium and water. It is now accepted that aldosterone also has

local effects in myocardial fibrosis and also controls magnesium homeostasis (MacFadyen 1997). These factors have led to the recent recognition that aldosterone may be of importance in sudden death in heart failure, as it has been shown that spironolactone reduces the prevalence of ventricular arrhythmias, and alters heart rate variability (Barr 1995, MacFadyen 1997), confirmed by the recent RALES study which showed that spironolactone reduces sudden death in this population (Pitt 1999).

1.3: Interaction of the renin angiotensin aldosterone axis with other hormone systems:

Natriuretic peptides and catecholamines:

Physiological response of the neurohormones to heart failure.

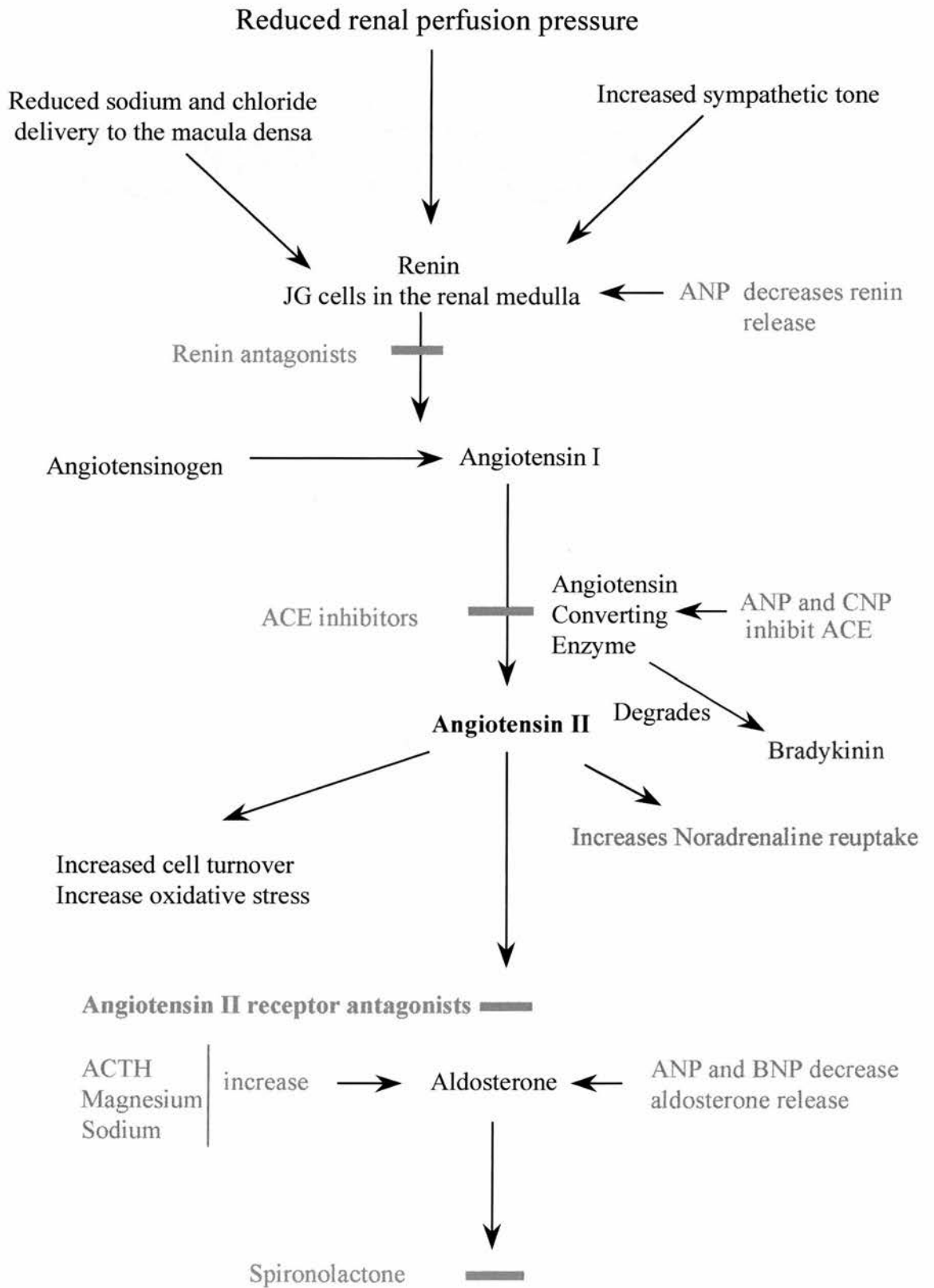
Neurohormonal activation in heart failure is a physiological response to the reduction in cardiac output due to myocardial insults such as infarction. The initial fall in cardiac output causes activation of the baroreceptors which cause a reflex rise in catecholamines, which increase contractility, and heart rate (Hirsch 1987). The sympathetic activation increases renin release, which leads to increases in angiotensin II and aldosterone, causing vasoconstriction and fluid retention, among other more specific effects at cellular level. This in turn causes higher left ventricular filling pressures so that in theory, cardiac output is increased according to Starling's law (Guyton 1982). There are complex interrelations between these systems. In heart

failure the normal control mechanisms appear to fail with continued vasoconstriction and fluid retention, causing deterioration (Hirsch 1987).

Catecholamines, adrenaline and noradrenaline stimulate the release of renin, and hence activation of the renin angiotensin aldosterone system. Angiotensin II is also believed to interact directly with sympathetic nervous tone by enhancing the release of noradrenaline from sympathetic nerve endings via a pre synaptic receptor (Dzau 1988, Lee 1991, Zimmerman 1981). These two hormone systems appear to potentiate one another, and the reflex control of their activation is of paramount importance to avoid escalating vasoconstriction and fluid retention in heart failure, which will lead to increased myocardial work load, and increased myocardial oxygen demand. In heart failure it appears that the baroreceptor control of b

appear to be important in the direct control of the renin angiotensin system and catecholamines. ANP can reduce the release of renin from the macula densa (Burnett 1984), inhibit the release of aldosterone from the adrenal gland (Atarashi 1985), and also suppress the activity of angiotensin converting enzyme inhibition. BNP is also known to reduce aldosterone levels, but does not appear to interact directly with renin (Yoshimura 1991). C-type natriuretic peptide is less well characterised, and is mainly located in the brain and vascular endothelium (Suga 1992). There is evidence that C-type natriuretic peptide interacts directly with the renin angiotensin system as it is believed to inhibit ACE enzyme in the vascular endothelium (Davidson 1996a).

Figure 1.1 Renin angiotensin aldosterone axis, and interrelations with other systems



Renin Angiotensin System And Nitric Oxide:

There are several other hormone systems which influence blood pressure and fluid balance homeostasis.

Nitric oxide is believed to be the most important local paracrine effector of vasodilatation, and its release is the end result of the application of a variety of vasodilators such as acetylcholine, and substance P (Newby 1996). In many experimental models, nitric oxide has the opposite effect to angiotensin II, as angiotensin II causes vasoconstriction (Guyton 1982), cellular hypertrophy (Geisterfer 1988), and intimal hyperplasia (Farhy 1993), in contrast to nitric oxide (Garg 1989). As yet there is only limited experimental evidence to link the renin angiotensin system directly with nitric oxide, rather than merely having antagonistic roles in organism homeostasis.

Nitric oxide and renin:

Nitric oxide may be a paracrine signalling molecule for renin release. This suggestion first arose when isoforms of nitric oxide synthase were found in the macula densa cells around the renal tubules (Mundel 1992) i.e. the same cells that are believed to control the secretion of renin from the juxtaglomerular cells (Hackenthal 1990). Nitric oxide synthase has also been localised in a variety of other sites within the kidney including vascular and tubular elements (Reid 1995a). Furthermore, activation of nitric oxide synthase in the macula densa appears to occur under conditions which would be expected to increase renin secretion, e.g. frusemide stimulation (Reid 1995b). The question has been addressed in animals, and in isolated cell culture, but not directly in

man. A difficulty in looking at renin secretion in man relates to pressure sensitivity of renin release, i.e alterations in renal perfusion pressures in the order of 2-3mmHg will double renin secretion (Hackenthal 1990). The issue has been not previously been addressed in intact man and chapter 7 of this thesis is a report of the effect of nitric oxide synthesis inhibition on renin secretion.

Nitric oxide and angiotensin II.

Angiotensin II may influence tonic NO levels, although indirectly, by altering the balance of superoxide radicals, which degrade NO and hence reduce the bioavailability. Angiotensin II at physiological concentrations can stimulate superoxide anion production (Griendling 1994, Rajagopalan 1996), which could reduce the half life of available nitric oxide. This phenomenon may be of biological significance, as the two processes occur in the vascular endothelium and intima where reductions in bio-availability of nitric oxide could be important. Nitric oxide inhibition also causes increases in angiotensin receptor expression in the adrenal gland (Usui 1998), which would increase angiotensin II induced release of aldosterone.

As with renin, nitric oxide may also be a signalling molecule for ACTH and aldosterone release, although even in isolated cell culture, the results are equivocal, with inhibition of aldosterone release seen with both NO liberators (Natarajan 1997) and NO inhibitors (Nakayama 1996). In whole animal experiments nitric oxide inhibition has caused reduction in plasma aldosterone (Usui 1998). These equivocal results again fail to demonstrate a direct link between the renin angiotensin system and nitric oxide. In chapter 5 of this thesis, a study is reported which attempts to link the

two systems by looking at the effect of ACE inhibition on nitrite and nitrate excretion in humans.

Renin Angiotensin Aldosterone System, Oestrogen And Progesterone.

Oestrogens are vasodilators in normal woman (Pines 1991). The mechanism by which this occurs is still obscure, however in highly oestrogenic states such as pregnancy, it is known that the vasoconstrictor effects of angiotensin II are blunted (Magness 1994).

There are several potential areas where oestrogens and progestogens could influence the effectiveness of the renin angiotensin system. In normal premenopausal women plasma renin and aldosterone do not appear to be affected by administration of oral contraceptives (Harvey 1999). However in postmenopausal women on hormone replacement, renin tends to be suppressed, and to a lesser extent aldosterone (Schunkert 1997). However there is mounting evidence to suggest that chronic oestrogen may influence the activity of circulating and bound angiotensin converting enzyme activity in monkeys and rats (Gallagher 1999, Brosnihan 1997). Proudler et al (1995) found that HRT causes a reduction in serum ACE activity in a population of postmenopausal women, and followed that by observing that both oestrogens and progestogens can reduce serum ACE in this population (Proudler 1996). However no one has yet shown whether acute dosing with oestrogens can influence ACE activity directly.

The evidence suggests that acute oestrogens in males will alter coronary vasodilation, (Reis 1998, Blumenthal 1997), this could be mediated by effects on the renin angiotensin system, in particular angiotensin converting enzyme. ACE not only converts angiotensin I to angiotensin II but also degrades bradykinin which is known to release nitric oxide, hence potentially enhancing coronary vasodilation.

Other studies had suggested that female hormones may act at a later stage in the renin angiotensin system by reducing the pressor responses to angiotensin II, noted in man (Mabe 1992) sheep (Magness 1994), and in vitro (Cheng 1992) which may involve influencing receptor expression. In chapter 6 a study addressing whether oestrogen or progesterone can affect ACE activity is discussed.

1.4: Therapeutic Interventions in the Renin Angiotensin Aldosterone Axis:

There are now numerous drugs developed to inhibit various steps in the renin angiotensin system. Those that have proved clinically useful are the angiotensin converting enzyme inhibitors, the angiotensin II receptor antagonists, and spironolactone.

Angiotensin converting enzyme inhibitors:

Angiotensin Converting Enzyme Inhibitors (ACE Inhibitors) are now widely used in many fields of medicine. They are established forms of treatment in hypertension, in reducing mortality in heart failure (Garg 1995, Opie 1994), and following myocardial infarction (ISIS-4 1995, Latini 1995, Pfeffer 1992, Swedberg 1992).

In heart failure, ACE inhibitors are now accepted as first line therapy (Clinical Quality Improvement Network Investigators 1996) and although prescription rates appear to fall short of the believed extent of the clinical problem (Eccles 1998), they are widely used. Mortality benefit in all clinical grades of heart failure is not disputed for the class, and there are placebo controlled trials for no less than 8 of the available 15 drugs (Garg 1995). On morbidity criteria, there is evidence that ACE inhibitors improve exercise tolerance in heart failure (Cleland 1984, 1985, Powers 1987) and they may also have beneficial effects on arrhythmias in heart failure (Fletcher 1993).

Angiotensin II receptor antagonists:

The angiotensin II receptor antagonists are as yet largely unproved in reducing mortality both in heart failure and in myocardial infarction. There are only two presently available studies in the literature which have mortality end points, although there are presently many studies ongoing. Given the accepted benefit of angiotensin converting enzyme inhibitors, it is difficult to randomise heart failure patients to placebo, hence the ongoing studies are comparing against ACE inhibitors, and combination of ACE inhibitors and angiotensin receptor antagonists. The Elite study (Pitt 1997a) compared losartan with captopril, and found significant mortality benefit of losartan over captopril, although there has been some criticism due to the different dosing schedules, and the suggestion that captopril three times daily compliance may have been poor. The other available study is the RESOLVD study, (McKelvie 1999) which compared enalapril with candesartan and the two in combination, and showed similar effects of candesartan and enalapril on morbidity endpoints, with a better result in the group on both therapies together. The Elite II study, looking at losartan vs

captopril in heart failure, has yet to be published, but has been presented at the American Heart Association meeting in November 1999, and showed similar effects on mortality in the captopril, and losartan groups. At present there are a large number of trials ongoing to look at angiotensin II receptor antagonists, both head to head with routine therapy and in combination with routine therapy in heart failure, hypertension and post infarct e.g. OPTIMAAL (Dickstein 1999) and VALUE (Mann 1998) and their results are eagerly awaited.

Aldosterone antagonists-spirolactone:

Spirolactone is the only commonly used aldosterone antagonist. It has demonstrated benefits in morbidity and cardiovascular fibrosis as discussed above. However, the recent results of the RALES study (Pitt 1999) have demonstrated that aldosterone suppression in addition to ACE inhibition produces significant mortality benefits, both in all cause mortality and also in sudden death. This suggests that despite adequate ACE inhibition there is still excess circulating aldosterone causing deleterious effects. This may reflect the multiple stimuli for the release of aldosterone, and the failure in some cases for ACE inhibition to fully suppress aldosterone (Zannad 1995).

1.5: Renin Angiotensin Aldosterone Axis And Mortality In Heart Failure:

Renin, angiotensin II, aldosterone, natriuretic peptides, and catecholamines have all been noted to correlate with mortality in heart failure. This correlation does not

automatically suppose a mechanistic role for any or all of the hormones in the pathogenesis of deterioration, but may merely reflect coexisting phenomenon as the myocardium fails.

In the CONSENSUS I trial (Swedberg 1990), where patients were randomised to receive either enalapril or placebo, positive correlations were seen between mortality and angiotensin II and aldosterone values in the placebo group. Furthermore, in the enalapril treated group, mortality was 7% in those whose reduction in angiotensin II was greater than 16pg/ml, in contrast to those in whom angiotensin II fell by less than 16pg/ml, where the mortality was 21%. In a smaller trial Pouleur et al (1993) divided enalapril treated patients into two groups, in one group mean ejection fraction fell by 10 %, in contrast to the other group whose ejection fraction was stable over time. In those noted to have deteriorating left ventricular function angiotensin II levels were un-suppressed (49 ± 15 vs 18 ± 5 pg/ml) in contrast to those with stable left ventricular function who had suppressed angiotensin II levels (Pouleur 1993). These suggest that high angiotensin II may have deleterious consequences in heart failure.

Catacholamines have also been correlated with higher mortality in heart failure. In the VeHeft II database of heart failure patients (Francis 1993) both log plasma renin and log plasma noradrenaline correlated with all cause mortality at baseline, but in a multivariate model the association only persisted with log noradrenaline. After thirteen weeks of treatment with either enalapril or hydralazine-isosorbide dinitrate, those in the enalapril group had stable plasma noradrenaline, whereas those in the hydralazine-isosorbide dinitrate group had significant elevations in plasma noradrenaline. Those on enalapril had significantly lower mortality than those in the

hydralazine-isosorbide group, and looking at quartiles of noradrenaline level there was a significant increase in mortality in the upper quartile, compared to the lowest quartile.

Natriuretic peptides also have been shown to correlate with mortality in post infarct patients (Hall 1994), but as infusions of these peptides results largely in beneficial effects on the haemodynamics of heart failure (Yoshimura 1991) the cause and effect relationship is yet to be established. It is likely, as their release relates to myocardial stretch (Yoshimura 1993), the increase in these peptides may reflect the failing heart rather than important pathogenic effects of these hormones. Their clinical use may relate more to diagnosis than to prognosis in heart failure (McDonagh 1998).

1.6: ACE Inhibitors And Mortality In Heart Failure:

ACE inhibitors remain the mainstay of therapy in heart failure, based on good mortality data, however there is still room for improvement in heart failure. The assumption that much of the benefit of ACE inhibitors is derived from reduction in circulating angiotensin II and aldosterone, is widely held. The available mortality data is of great interest, especially because there are reductions not just in death from heart failure, but also there are significant reductions in angina and myocardial infarction, which is not readily explained by effects on sodium and water homeostasis. As discussed above there is mounting evidence that both these hormones exert significant effects at local level. In the major post infarct trials there is evidence that the active

groups show overall reductions in mortality, due frequently to deterioration in heart failure, however there are also reduction in the ischaemic burden, with falls in myocardial infarction and stroke. The SOLVD trial (Yusef 1992) was a large double blind randomised controlled trial which looked at enalapril versus placebo, in patients with ejection fraction less than 35%. The additional analyses showed a 27% risk reduction for all myocardial infarction, and 23% risk reduction for hospitalisation for angina in those on enalapril compared to placebo. The curves demonstrating this effect show separation around 6 months with further separation at 48 months suggesting continued benefit. This benefit may relate to the observed reductions in blood pressure, however the study population were normotensive, and the fall while statistically significant was only 4mmHg. The other possibility is that reduction in angiotensin II improves the coronary arterial morphology or function, and that this effect takes some time to appear. There is mounting evidence that angiotensin II is important in vascular biology and remodelling, and these effects may represent the benefit of reducing vascular angiotensin II.

1.7 Chronic Effects Of Angiotensin Converting Enzyme Inhibition:

Studies have shown that the renin angiotensin aldosterone axis is not uniformly suppressed during chronic ACE inhibitor therapy either for hypertension (Van den Meiracker 1992, Staesson 1981) or heart failure (MacFadyen 1999). These phenomenon have been referred to as “angiotensin II reactivation” and “aldosterone

escape” and their appearance is believed to herald a poorer prognosis (Struthers 1995).

Therefore, neurohormonal elevation despite adequate treatment is believed to associate with a poor prognosis.

At present it is assumed that when reactivation of the neuroendocrine axis occurs in congestive heart failure it is progressive, i.e. when reactivation of angiotensin II or aldosterone occurs, despite an ACE inhibitor, then the patient enters a downward spiral with further angiotensin II and aldosterone reactivation, leading to clinical deterioration. One would imagine that angiotensin II reactivation would be progressive if due to the generation of angiotensin II by reactive rises in renin and angiotensin I (Aldigier 1993) or via non ACE pathways such as tissue chymases. On the other hand, if angiotensin II reactivation was occasionally due to intermittent non compliance with ACE inhibitor (Vinson 1990), then angiotensin II reactivation may be temporary and reversible, and of less clinical significance. It is important therefore to identify whether or not the neurohormones are adequately suppressed by ACE inhibition, and whether reactivation is a significant problem that should produce an adjustment of therapy to add in either an angiotensin II antagonist, or spironolactone.

1.8: Renin Angiotensin Modulation In Other Disease States:

Hypertension:

ACE inhibitors and angiotensin II receptor antagonists are now widely used in hypertension, they reduce blood pressure and also improve left ventricular

hypertrophy (Gottendiener 1997, Thurmann 1998). Furthermore, renin has been correlated with both myocardial infarction and stroke in hypertensives (Brunner 1972, Alderman 1991). The initial belief that ACE inhibitors produce their beneficial effects by reducing angiotensin II induced vasoconstriction and aldosterone induced water and salt retention, is now accepted as only one aspect of the several modes of action of this class of drugs. Much of the benefit does appear to relate to reductions in angiotensin II and aldosterone, but not merely their effects on sodium and water homeostasis. Angiotensin II has many effects at cellular level in excess of receptor mediated vasoconstriction, as discussed above. In particular, it has been noted that treatment with ACE inhibitors alters not only function of the artery (Benetos 1990, Clozel 1989), but also arterial structure (Dzau 1988, Chobian 1990, Lee 1991).

Hypertension and hyperlipidaemia cause alterations in cell turnover and metabolism of endothelial factors, which leads to significant changes in the vessel wall components and in vascular structure and function (See Table 1.1).

Much of the experimental work into arterial damage has been in rat models of hypertension where medial hypertrophy and collagen deposition appear to occur in a linear fashion according to the height of the blood pressure (Wolinsky 1970). Interest has focused on differentiating the vascular effects of reducing the blood pressure *per se* from additional effects of various anti hypertensives. The difficulty in separating the benefit derived from the reduction in pressure, compared to the specific effect of ACE inhibitors, has stimulated a plethora of studies.

Purely a pressure effect?

Harrap et al (1993) studied the spontaneously hypertensive rat model and demonstrated that if perindopril was administered, but the blood pressure was re-elevated by concomitant administration of aldosterone or saline, arterial morphometry showed that the media to lumen ratio of the mesenteric arteries studied was least in those rats given perindopril only. However, despite the treatment with perindopril, both the saline treated and the aldosterone treated rats showed significant medial hypertrophy consistent with the blood pressure that had been observed during the study. This study showed a linear relationship with blood pressure and medial thickening. Of note, angiotensin II levels were lowest not in the perindopril only group, but in the perindopril and aldosterone group. These results suggest that the observed reduction in medial hypertrophy in this study relates to neither ACE inhibition, nor the circulating angiotensin II level, but purely to the blood pressure level itself. Owens (1987) also found that in spontaneously hypertensive rats the effect of captopril reflected its blood pressure lowering ability, and was similar to other anti-hypertensives.

Or a specific effect of Angiotensin Converting Enzyme inhibition?

In contrast, many other studies have shown that ACE inhibitors are more effective than other anti-hypertensives in reducing arterial hypertrophy, despite similar pressure reductions (Christensen 1989, Pepine 1994). Wang and Prewitt (1990) studied the one kidney, one clip rat model, which is not renin/angiotensin dependant, hence the blood pressure does not fall on ACE inhibitor treatment. In this model, the rats

treated with captopril did not demonstrate a fall in pressure, but did show a significant reduction in medial thickness of both large (aortic) and small (cremasteric muscle) arteries over a 4 week period. They concluded that captopril can be shown to reduce vascular media/intimal area independent of the blood pressure.

There is growing evidence to suggest that angiotensin II itself is a significant factor in vascular hypertrophy and that reducing its production both locally and systemically not only reduces the pressure but also the detrimental vascular response.

Despite the mounting evidence that angiotensin II is a mediator of cellular turnover, the beneficial effects of angiotensin converting enzyme inhibitors may be mediated by bradykinin and subsequent endothelial nitric oxide release. Enalaprilat potentiates the effects of bradykinin in the forearm (Benjamin 1989) and the beneficial effects of ramipril on arterial damage in response to balloon injury are inhibited by HOE 140, a bradykinin antagonist (Farhy 1993). As discussed above, one of the major effects of ACE inhibition may be to increase nitric oxide availability in the vessel wall, either by increasing bradykinin, or by reducing angiotensin II. Angiotensin II promotes superoxide formation in the vessel wall, which is believed to degrade endogenous nitric oxide (Griendling 1994).

Table 1.1 Arterial structure and function:

Arterial Layer	Normal Function and Structure	In hypertension/hyperlipidaemia
Endothelium	<p>Monolayer of metabolically active cells on basement membrane.</p> <p>These cells release vasodilator and vasoconstrictor substances and regulate vascular smooth muscle growth</p>	<p>Reduced release of vasodilators, causing a tendency to imbalance the vasoregulatory control towards vasoconstriction</p> <p>Increased paracrine stimulus to VSMC growth</p>
Intima	<p>Sub endothelial layer involved in mechanical support of the endothelium.</p> <p>Site of the internal elastic lamina which allows the vessel to smooth pressure waves and mediate a persistent perfusion pressure, furthermore through which metabolites, growth factors and vasoactive compounds permeate.</p>	<p>VSMC migrate from the media into the intima, increasing connective tissue density, and reducing diffusion.</p> <p>Reduplication of the internal elastic lamina reduces the compliance of the vessel, increasing peripheral resistance.</p> <p>Cholesterol deposition in plaques which stimulate intense inflammatory response, of macrophages and monocytes.</p>
Media	<p>A combination of smooth muscle cells and their supporting matrix, mainly proteoglycans, and collagen. In large arteries the elastin content in the media controls the pulse pressure, smoothing this to maintain flow at an even pressure</p> <p>Raises in cytosolic calcium causes contraction of the smooth muscle cells reducing vessel diameter causing an increase in peripheral resistance and raising blood pressure</p>	<p>Hypertension causes cellular hypertrophy and cellular proliferation in the media. There is an increase in the medial collagen content and fibrosis causing reduction in arterial compliance.</p> <p>In larger arteries there is reduction of the elastin due to increased degradation, and replacement with collagen</p>
Adventitia	<p>Separated from the media by the external elastic lamina the adventitia is a mainly fibrous layer through which runs blood vessels, autonomic nerves and lymphatics.</p>	

Derived from Lindop et al (1992)

Hyperlipidaemia:

The beneficial effects of ACE inhibitors in hypertension has lead to studies into the effects of this family of drugs in other high risk groups. It is known that hyperlipidaemia per se, in the absence of hypertension, produces atherosclerosis and its precedent, endothelial dysfunction (Gilligan 1994d). A study by Zeiher et al (1991) showed that even in angiographically normal coronary arteries endothelial responses to acetylcholine, were abnormal in those patients with hyperlipidaemia. Acetylcholine is believed to cause vasodilation in normal coronary arteries and forearm resistance vessels by release of vasodilators including nitric oxide (Casino 1993). Zeiher et al (1991) also demonstrated that as the coronary arteries became more diseased, the arterial vasodilation to an increasing range of stimuli e.g. cold pressor test, flow mediated dilatation, diminished. This suggests that prior to angiographic evidence of disease there are alterations in the arterial function due to the concomitant risk factors.

Becker et al (1991) showed that New Zealand rabbits fed a hyperlipidaemic diet, and concomitantly given ramipril, had well preserved arterial relaxation in response to acetylcholine, while those animals receiving only vehicle showed poor relaxation to acetylcholine. This suggests that the angiotensin converting enzyme inhibitor altered arterial responses, suggesting a role for angiotensin II in the vascular effects of hyperlipidaemia. These responses were not based on changes in either blood pressure or lipids and are supported by other similar studies in rabbits (Schuh 1993).

Aberg et al (1990) found in a hyperlipidaemic cynomolgus monkey model, that captopril reduced blood pressure and the percentage of the intima involved by

atherosclerosis in aorta, coronaries and carotid arteries. Previously, in an identical study they had shown nifedipine in similar hypotensive doses had no effect on atherosclerosis. As neither drug had any effect on serum lipids, the intimal improvement was not due to altered lipid concentrations.

Similar results have been shown in the Watanabe heritable hyperlipidaemic rabbit, which is an animal model of familial hyperlipidaemia. Captopril prevented development of atherosclerosis, in contrast to propranolol and verapamil, despite comparable blood pressure reductions (Chobian 1990, Lichenstein 1989, Tilton 1985). These studies suggest that ACE inhibitors might be specific in reducing the arterial damage due to hyperlipidaemia.

There are as yet few studies looking at angiotensin II receptor antagonists in hyperlipidaemia. However losartan has been shown to reduce the degree of atherosclerosis in the rabbit model, but this was less effective than an ACE inhibitor (Li 1999), although in this study both drugs appeared to alter serum lipids which complicates the results, as changes in cholesterol affect vascular responses (Harrison 1987).

These studies raised the possibility that ACE inhibitors could have a place in remodelling of human atherosclerotic arteries. Hence, another area of interest is the possibility that ACE inhibitors might reduce restenosis after angioplasty. This is because intimal hyperplasia is a key process in restenosis following angioplasty. Powell et al (1989), using normotensive rats, demonstrated that cilazepil administration caused an 80% reduction in intimal hyperplasia following internal carotid endothelial denudation via balloon angioplasty. Similarly, Daeman et al (1991)

showed that angiotensin II infusion following balloon injury provoked both a significant blood pressure rise, and a dramatic increase in the intimal response to the carotid injury in comparison to the uninjured carotid exposed to the same blood pressure. They suggested that, in the absence of the regulatory influence of the endothelium, angiotensin II stimulates vascular growth and facilitates arterial injury. Unfortunately these exciting animal studies have not been confirmed in clinical trials. In the Mercator trial (The MERCATOR study group 1992), cilazapril was administered orally to 700 patients for six months following coronary balloon angioplasty. Treatment with the ACE inhibitor did not reduce cardiac events, total mortality, or need for surgical or medical intervention for symptoms in the 600 in whom follow up was available. This was a disappointing result, especially in the context of improvements seen in ischaemic burden in the post MI patients. It may be that the animal experiments do not accurately reflect the human situation, since in the animal experiments the pre-injury endothelium is normal, with a normal balance between vasoconstrictors and vasodilators whereas the human vessel is already damaged by atherosclerosis with less endothelial vasodilatory potential even before the injury takes place.

1.9: Potential Mechanisms for the benefits of ACE inhibitors on the Vascular Wall.

Assuming that ACE inhibitors have actions above the effect of blood pressure on the vascular wall, these could be mediated either by the effects on angiotensin II or by the effects on bradykinin (Carbonell 1988). Furthermore, angiotensin II could have direct

effects via cellular receptors (Kato 1991), or via potentiation of the sympathetic nervous system (Zimmerman 1981).

Ace Inhibitors And Bradykinin:

Bradykinin is believed to induce vasodilatation by the release of nitric oxide (Gilligan 1994c) and prostaglandins (Mombouli 1991, Horning 1997). It is known that, in part, the hypotensive effects of ACE inhibitors are due to the reduced degradation of bradykinin, as inhibition of bradykinin reduces, but does not completely abolish the ACE inhibitor induced reduction in blood pressure (Carbonell 1988, Cachofeiro 1992). Farhy et al (1993), using Sprague-Dawley normotensive rats, demonstrated that both ramipril and losartan could reduce neointimal formation in balloon injured common carotid arteries, but interestingly ramipril was significantly more effective. The difference between losartan and ramipril could be abolished by concomitant administration of either a bradykinin antagonist or by inhibition of nitric oxide synthesis using L-NAME. These results suggest that part of the neointimal response in this model is mediated by reduced bradykinin degradation causing increased levels of nitric oxide. It is interesting to note that Carbonell et al (1988) showed that circulating levels of kinins were not increased in rats, suggesting that the effects occur at tissue level.

Although the effects of ACE inhibitors are at last partially mediated by alterations in the metabolism of bradykinin, direct angiotensin II receptor antagonists have beneficial effects on intimal hyperplasia (Ledingham 1996) and left ventricular hypertrophy (Thurman 1998).

Angiotensin II and the sympathetic nervous system:

Lee et al (1987) showed that destruction of the sympathetic nervous system in prenatal SHR causes regression of the vascular hypertrophy in both large and medium sized arteries. This suggests that the sympathetic nervous system may control early vascular growth in this model. Furthermore, Yamori et al (1980) provoked hypertension, via noradrenaline infusion, in Wistar Kyoto (WK) rats and administered both α and β blockers and found that both drugs reduced the aortic mass despite limited reductions in blood pressure, suggesting a direct sympathetic stimulation of growth. They went on to contrast hydralazine with propranolol in SHR looking at protein synthesis and found that despite better reduction in blood pressure, hydralazine had no effect on vascular protein synthesis.

If we accept that the sympathetic nervous system is important in vascular growth in response to pressure, then ACE inhibitors may interact with the sympathetic nervous system, mediating the beneficial structural changes observed. Angiotensin II is believed to augment the release of noradrenaline from sympathetic nerve endings (Zimmerman 1981), and therefore reducing the production of angiotensin II via ACE inhibitors could, via the sympathetic nervous system, mediate the suppression of vascular growth observed in the animals treated.

Owens (1987) found that propranolol did not reduce vascular growth in Spontaneously Hypertensive Rats (SHR), unlike captopril which produced a greater reduction in vascular smooth muscle cell hypertrophy than would be expected from the observed reduction in blood pressure. Furthermore, α adrenoceptor blockade does not improve

endothelial function in man (Panza 1990). It is unlikely that the beneficial effects of ACE inhibitors on vascular structure in hypertension are mediated by their effects on the sympathetic nervous system, as the results appear to be in excess of those seen with full beta blockade. Furthermore in the rat model, the new angiotensin II receptor antagonists appear to have only limited effects on the prejunctional receptor on the sympathetic nerve ending (Olhstein 1997).

Angiotensin II As A Growth Modulator:

There is now substantial evidence to suggest that angiotensin II can provoke protein synthesis via specific receptor mediated effects. In cell culture angiotensin II can stimulate vascular smooth muscle cell synthesis of collagen and fibronectin (Kato 1991). Furthermore, this effect was blocked by a specific receptor antagonist, Saralasin. These proteins are the basis of the extra cellular matrix of the vascular media and increased collagen in the media reduces the elasticity of the arterial walls in hypertension (Dzau 1988). Black et al (1993) have shown in a rat model that infusing angiotensin II causes more vascular damage, and left ventricular hypertrophy than an equipressor dose of norepinephrine. This suggests that angiotensin II has specific growth promoting properties.

In vitro angiotensin II can provoke cellular hypertrophy via protein synthesis, in vascular smooth muscle cells (Geisterfer 1988, Schelling 1991), since these effects are blocked by Saralasin. Cell hypertrophy can be induced by angiotensin II in cell culture in many different cell lines (For Review see Schelling 1991). It is well known that not

only do the renin angiotensin components exist within the vascular tree but also that they appears to function in a manner which is largely independent of circulating ACE activity (Dzau 1988). Thus, it is apparent that, as receptors exist on smooth muscle cells, these could then stimulate cell turnover.

Whether angiotensin II is truly mitogenic in vivo is still a matter for debate. In vivo, McEwan et al (1998) have shown that angiotensin II stimulates proliferation of vascular smooth muscle cells which was inhibited by losartan, however they did not show a similar effect on myoendothelial cell proliferation. Similarly, Paquet et al (1990) have demonstrated mitogenesis stimulated by angiotensin II in cell lines derived from SHR but not in normotensive rat lines, suggesting differences in control of cell turnover between the two cell types. Finally, in hypertensive arteries the incidence of cellular polyploidy is increased and this effect is reduced by ACE inhibitors (Black 1989). It is possible therefore that angiotensin II can stimulate cellular replication under certain conditions.

Conclusion:

The renin angiotensin aldosterone axis is important both in salt and water homeostasis, and in vascular function. The widespread use of ACE inhibitors and the newer angiotensin II receptor antagonists has demonstrated that manipulation of this system can produce both short and long term benefits in heart failure and hypertension. The mechanisms behind this are as yet unknown. They appear to improve arterial function both by a reduction in blood pressure and by inhibiting receptor mediated effects of angiotensin II. A variety of potential mechanisms have been suggested, and evidence so far gathered suggests the beneficial effects of ACE inhibitors are due to a combination of bradykinin and nitric oxide potentiation, in addition to inhibition of angiotensin II stimulated protein synthesis and mitogenesis.

There are still many questions to be answered in relation to the renin angiotensin aldosterone axis, particularly in physiological inter-relations with other hormone systems. Furthermore, there is mounting evidence that ACE inhibitors may have therapeutic benefits in other disease states such as diabetes, and in particular hyperlipidaemia. This thesis addresses some of these questions in a series of clinical studies.

Summary points:

- The renin angiotensin aldosterone axis is an important hormonal system for the control of blood pressure, and sodium and water balance. The activation of this system is under careful control to maintain organism homeostasis.
- Angiotensin II is an important mediator of vasoconstriction, but also has effects at cellular level that could cause vascular damage.
- The renin angiotensin aldosterone axis interacts with several other hormone systems. There may be direct interactions with nitric oxide but at present there is no evidence of a direct interaction and they may represent two opposing factors in the maintenance of physiological balance.
- The renin angiotensin system may interact with female ovarian hormones, and may in part mediate the benefits seen with HRT in the post-menopausal population.
- ACE inhibitors improve mortality post infarct and in heart failure, some of which benefit may relate to the reduction in neurohormonal activation, although the efficacy of suppression in daily practice is unknown.
- ACE inhibitors are believed to improve vascular function in hyperlipidaemia and in hypertension in animal models, but the effects in man are not proven. Hence potential novel therapeutic uses may exist for these drugs in vascular protection in man.

CHAPTER 2:

Subjects, Materials And Methods.

Introduction:

This chapter is a description of the experimental details in relation to the studies that constitute this thesis. To avoid duplication this chapter explains methods of blood sampling, blood analysis, forearm plethysmography, and the test solutions used within the subsequent chapters. For completeness, each chapter will have a summary of the protocol specific to that study but will refer to this chapter for details.

For all studies written informed consent was obtained from the subjects to each protocol. All protocols were previously approved by the Tayside Committee for Medical Research Ethics and all investigations conformed with the principles outlined in the Declaration of Helsinki.

Subject recruitment:

Subjects were recruited for each study either from specialised clinics for patients or by word of mouth and press advertisement for the normal volunteers.

Each subject was screened initially for suitability for participation, which included a clinical history, physical examination, baseline biochemical and haematological tests, an electrocardiogram and in normal volunteers and heart failure patients an echocardiogram. Patients for chapter 3 also had nuclear scintigraphy depending on quality of echo pictures.

Blood sampling ;

Below are listed the methods of collection, storage, handling and analysis of the individual investigations. At baseline screening visits syringes and needles were used,

however if there was repeated sampling, then intravenous cannulae were inserted into antecubital fossa veins, and samples withdrawn into syringes.

Screening investigations:

Baseline bloods were taken at screening for all the studies. Samples were taken into standard auto-analyser tubes and analysed on the day of the sample. Electrolytes, lipids and ACE activity were collected into clotted tubes for routine processing on the same day. Full blood counts were taken into EDTA tubes. Fasted lipids were collected after an overnight fast.

Collection and Storage:

All blood samples were collected onto ice then spun in a refrigerated centrifuge (3500rpm (1480 G) for 15 minutes), and the supernatant removed and frozen for later batched analysis at -70°C (angiotensin II, ANP, BNP) or -20°C (aldosterone, renin, oestrogen, progesterone, testosterone, nitrite/nitrate).

Renin, angiotensin, aldosterone and natriuretic peptides were collected after 30 minutes supine rest.

Nitrite and nitrate were collected after 18 hours fasting as discussed below

Angiotensin II: Samples were collected into 10ml tubes with chilled dried inhibitor cocktail containing 20 μmolar human renin inhibitor H142, 25mmolar o-phenanthroline, 25mmolar EDTA and 0.25% neomycin sulphate, to prevent angiotensin I conversion or angiotensin II breakdown.

ANP/BNP: Natriuretic peptides were collected into 10ml EDTA tubes with Trasylol, (4000 kallikrein inactivation units per tube).

Nitrite/nitrate: Patients and volunteers were fasted overnight, with fasting starting at 7.00pm, and attending the department at 10.00am the next day. Subjects were allowed to drink only Milli Q+ water (Millipore, Bedford MA), and limited to 1L water. Cannulae were inserted into antecubital fossa vein at 10am and first samples were taken at 11.am with further sampling at 12.00pm and 1.00am. Samples were collected into needles and syringes rinsed x3 with Milli-Q+, and into lithium heparin tubes rinsed three times with Milli-Q+ water.

Oestrogen/progesterone/testosterone: All samples were collected into plain glass on ice for oestradiol, progesterone, and testosterone.

Renin/Aldosterone: Renin was collected into EDTA on ice, spun and plasma pipetted off and stored. Aldosterone was collected onto ice in lithium heparin tubes, spun and plasma stored.

Biochemical Analyses:

ACE activity: Assayed on the day of collection by automated analyser (Technicon Axon/Technicon Dax, Bayer Diagnostics, Basingstoke UK). Results in IU/L.

Angiotensin II: Preparative column chromatography was conducted according to the methodology of Nussberger (1988) prior to the determination of angiotensin II by conventional radio-immunoassay using anti-sera with minimal angiotensin I/II crossreactivity (Morton 1985). Inter and intra assay coefficients of variation were 4.5% and 9% for angiotensin II. Results in pg/ml.

ANP/BNP: Plasma was batch analysed, after peptide extraction via column chromatography, with radio-immunoassay kits (Peninsula Labs Ltd, Merseyside, UK). Inter and intra assay coefficients of variation are 11.8% and 4.23% (ANP) and 14.8% and 9.9% (BNP) and both are expressed as pmol/L.

Electrolytes: Batch assayed using standard automated analyser, results expressed as mmol/L. Urinary electrolytes were assayed as a batch using a similar system.

Lipids: Blood was analysed on the same day, using automated analysers (Technicon Axon/Technicon Dax, Bayer Diagnostics, Basingstoke, UK). At follow up, bloods were taken either on the day of the study, or within two days prior to the second study, as necessitated by fasting. Results in mmol/L

Nitrite/Nitrate: These were assayed using high performance capillary electrophoresis, a method that has been validated, (Leone 1994) and is discussed in detail in the literature (Leone 1995). The assay uses high voltages applied over silica capillaries, and separates the ions using mass to charge ratios. The intra-assay CV for nitrite is 5% at 50 μ M, 10% at basal nitrite, and for nitrate is 1.2% at 50 μ M, 6.4% at basal nitrate. Results are expressed in μ mol/L.

Oestradiol/progesterone/testosterone: Hormones were assayed using radio-immunoassay techniques: oestradiol and progesterone (INCSTAR Corp Stillwater Minnesota, USA), testosterone (Sorin Biomedica, Italy). Results are expressed in pmol/L (oestradiol) and nmol/L (progesterone and testosterone)

Renin/Aldosterone: Samples were batch assayed in the same laboratory. Renin levels were analysed by radioimmunoassay (Sorin Biomedica, Italy) and expressed as the generation of angiotensin I as ng/ml/hr. Aldosterone was also analysed by radio-immunoassay (Incstar Ltd, Wokingham, Berks, UK.) and expressed as pg/ml. Inter

and intra assay coefficients of variation are 7.5% and 4.9% (aldosterone) and 7.9% and 6.8% (renin).

QT Dispersion:

QT interval analysis was done on 12 lead electrocardiograms, recorded on each visit for the study in chapter 3. All ECGs were numbered randomly by a second investigator, and analysed blind by the author using an electronic pad and computer programme.

ECGs were taken at 25mm/sec speed, and three complexes in each lead were used, ECGs were rejected if there were less than eight leads available for analysis, there was less than three complexes per lead, or if left bundle branch block, or atrial fibrillation were present.

QT interval was taken from the onset of the QRS to the end of the T wave (i.e. return to the J point). Where U waves were present the QT was measured to the nadir of the curve between the U and the T wave. QT intervals were corrected for rate using Bazett's formula ($QTc = QT / \sqrt{RR}$ interval). QTc dispersion is defined as the difference between the maximum and the minimum QTc. Adjusted QTc dispersion was also measured to correct for the known dependence of the QTc dispersion on the available number of leads.

Forearm plethysmography:

Brachial artery endothelial function has been used as a surrogate marker for arterial damage at other sites in the vasculature such as the coronary arteries. Vasodilation or

vasoconstriction to mediators applied in the antecubital fossa can be assessed downstream in the forearm vascular bed which mainly supplies muscles. The advantages and problems of this methodology are discussed later in Chapter 8 of this thesis and in the literature by Benjamin et al (1995) and Chin-Dusting (1999). The justification for its use in the hyperlipidaemic population is discussed in the introduction to Chapter 4. The author has studied both vasodilator responses to acetylcholine and nitroprusside (Chapter 4), and vasoconstrictor responses to angiotensin I and II (Chapter 6) within this model.

Subjects were, where possible studied at the same time each day and had abstained from food, caffeine, alcohol and cigarettes for at least four hours before the study, which was performed in an air conditioned room controlled between 23-24 ° C.

On each study day the subject lay supine and a mercury-in-silastic strain gauge (Medosonics, Mountain View, California) was applied to each forearm at the point of maximal muscle bulk. The position of the gauge was determined by measuring the distance from the lateral aspect of the anterior elbow crease and was kept constant for each individual between study days. Cuffs were placed around each wrist and upper arm, and were attached to a rapid cuff inflator (Hokanson, Washington, Columbia). Measurements were taken from both arms over each period for two minutes. During measurements, the wrist cuff was inflated to 200mmHg to exclude hand circulation. Each measurement was taken as the mean of five readings which were obtained during periodic inflation of the upper cuffs to 30 mmHg (to occlude venous outflow) for two seconds in every 15 seconds. Data from the strain gauges were processed by a plethysmograph (Medisonics) and analysed using MacLab computer hardware and software. A 27G needle was inserted into the brachial artery of the non dominant arm

under local anaesthesia, and 0.9% saline was infused for 30 minutes prior to the establishment of baseline readings. Strain gauge measurements were taken at five minute intervals to establish three consecutive readings which were within 10% of each other. The mean ratio of measurements from both arms at these points was taken as the baseline ratio of forearm blood flow. After the establishment of baseline the test solutions were applied.

Infusion solutions:

Chapter 4: This study looked at the effect of lisinopril on endothelial dependent and independent responses in a group of hyperlipidaemic patients to see whether six month treatment with lisinopril altered arterial function.

Following the establishment of baselines, acetylcholine (Miochol CIBA Vision, Southampton, UK) was dissolved in 0.9% saline and infused at three concentrations; 7.5, 15 and 30 $\mu\text{g/ml}$, with the infusion rate kept constant at 1 ml/min throughout the study period (Grasby 310 syringe pump, Grasby Medical).

For endothelial independent responses, Sodium Nitroprusside (Nipride, Roche Welwyn Garden City, Herts UK) was dissolved in 0.9% saline which was infused at 0.8, 1.6, and 3.2 $\mu\text{g/ml}$ also with constant infusion rate. Due to known photosensitivity, nitroprusside was protected from light throughout the study. An identical protocol was followed on both days, before and after six months treatment with lisinopril. All solutions were made up within 30 minutes of the study starting.

Chapter 6:

Eight male volunteers underwent a randomised double blind crossover trial to look at the effects of a short course of oestrogen and progesterone on vascular ACE activity in the forearm.

The activity of the ACE enzyme was assessed as the ability of angiotensin I to vasoconstrict the artery, on the basis that it requires conversion to angiotensin II by vascular ACE. This was compared with the vasoconstriction to angiotensin II.

After the establishment of baselines Angiotensin I was infused at three concentrations, 12 pmol/min, 24 pmol/min, and 48 pmol/min. These doses were expected to produce 25%, 35% and 50% reduction in blood flow.

0.9% saline was then infused until flow returned to baseline. Thereafter angiotensin II was infused at 4 pmol/min, 8 pmol/min, and 16 pmol/min.

Angiotensin I and II were supplied by Calbiochem/Novobiochem Nottingham, England, and the same batch were used for all studies, they were made into solution within 45 minutes of the study starting. Peptides were dissolved in 0.9% saline, and the infusion rate was kept constant at 1ml/min throughout the study period (Grasby 310 syringe pump, Grasby Medical).

Blood Flow Responses:

Blood flow is expressed as the ratio of blood flow in the infused arm over that in the control uninfused arm. These ratios have been used for statistical analysis for those experiments using forearm plethysmography in chapters 4 and 7.

There has been some discussion as to the correct way of expressing flow data from forearm plethysmography studies. In a recent authoritative review article on methodology it was felt that forearm vascular resistance was an inaccurate way of expressing this type of data, as Forearm Vascular Resistance (FVR) is calculated according to the following formula: $FVR = \text{Blood pressure}/\text{blood flow}$ and mathematically relies on Newtonian flow kinetics, which does not apply to blood as it is pulsatile (Benjamin 1995). The use of the ratio between blood flow in both arms allows for a continuous control to avoid potential confounding environmental factors (Benjamin 1995), and individual patient characteristics (Chowienczyk 1994), which is why the data is presented in this way.

Statistical analyses:

Haemodynamic and biochemical data:

For all chapters where two groups were compared but there was no crossover of subjects to the other treatment (i.e. chapters 3, 4, and 5) unpaired student's t-tests were used to compare baseline parameters, such as blood pressure, and biochemical parameters. Where subjects crossed over the treatment groups paired t-tests were used i.e. chapters 6 and 7.

Blood flow ratios:

In chapter 4 the blood flow data was analysed using a repeated measures ANOVA, using change in blood flow ratios pre and post treatment, and between the active group and the placebo group, this was done with the assistance of Dr Michelle

Robertson, and Dr Jan Love from the Robertson Department of Medical Biostatistics, University of Glasgow. For chapter 5 and chapter 6, the results were summarised using area under the curve analysis and a single ANOVA was used to compare the groups. Throughout, results are expressed as mean \pm SEM, and statistical significance was accepted for p values <0.05 .

CHAPTER 3:

Optimising ACE inhibitor therapy in heart failure:

Neurohormonal monitoring as a method for identifying those at risk of clinical deterioration.

The first investigation in this thesis looks at the prevalence of reactivation of angiotensin II and aldosterone in a group of heart failure patients followed over eighteen months. Furthermore, the initial aims included trying to correlate other more simple tests such as urinary sodium:potassium ratios as markers of neurohormonal escape.

Introduction:

Angiotensin converting enzyme inhibitors are now primary management in chronic heart failure, reducing both in hospital (Clinical Quality Improvement Network Investigators 1996) and out of hospital mortality (Yusef 1992). ACE inhibitors are believed to mediate this benefit by a variety of mechanisms, one of which is the reduction in circulating angiotensin II and aldosterone (Sigurdsson 1994,1995). However, it has been noticed that the renin angiotensin aldosterone axis is not completely suppressed by ACE inhibitor therapy either for hypertension (Lijnen 1982, Staesson 1981, van den Meiracker 1992) or heart failure (Cleland 1984). These phenomena have been referred to as “angiotensin II reactivation” and “aldosterone escape” and their appearance is thought to reflect detrimental disease progression (Struthers 1995).

The physiological mechanisms behind neurohormonal activation and the importance of its persistence despite appropriate therapy is discussed in chapter 1, section 1.5. Large randomised studies have shown that neurohormonal activation correlates with mortality, and failure to suppress angiotensin II appears to confer a higher risk despite therapy (Swedberg 1990). Similarly there is evidence that deterioration in left ventricular function associates with failure to suppress angiotensin II (Pouleur 1993).

At present it is assumed that when reactivation of the neuroendocrine axis occurs in congestive heart failure it is progressive, i.e. when reactivation of angiotensin II or aldosterone occurs, despite an ACE inhibitor, then the patient enters a downward spiral with further angiotensin II and aldosterone reactivation, leading to clinical deterioration. This chapter addresses this issue, and looks specifically at three areas.

1. Progression of angiotensin II and aldosterone when reactivation has occurred.

The assumption has been that angiotensin II reactivation would be progressive if due to the generation of angiotensin II by reactive rises in renin and angiotensin I (Aldigier 1993) or via non ACE pathways such as tissue chymases. Although the literature suggests that with adequate ACE doses angiotensin II levels fall to very low levels (Juillerat 1990). On the other hand, if angiotensin II reactivation was occasionally due to intermittent non compliance with ACE inhibitor, (Vinson 1990) then angiotensin II reactivation may be temporary and reversible.

2. Correlation of angiotensin II reactivation and aldosterone escape in individual patients.

As there are different therapies for each we wished to look at the relative frequency and importance of each. According to the physiological concepts, angiotensin II levels should be reflected by those of aldosterone. However there is evidence from previous studies that the relationship is not linear, and different mechanisms may be important in the reactivation of these two hormones.

3. Importance of the duration of action of specific drug.

As the shorter acting ACE inhibitors may produce less consistent blockade of plasma ACE (deGraeff 1987), it may be possible to see more reactivation of neurohormones with captopril than with other members of the group.

It was decided to study patients established on maximum tolerated therapy rather than those at treatment initiation to avoid all patients altering dose and possibly drug during the study period. It was accepted that some changes in ACE inhibitor therapy might occur due to clinical circumstances.

Methods:

Subject recruitment:

Twenty two patients were recruited from the specialised heart failure clinic, to be followed for eighteen months to assess the degree of reactivation of neurohormones despite maximum tolerated ACE inhibitor dosage. The heart failure clinic has been running for over ten years and caters for patients with symptoms of heart failure and reduced left ventricular ejection fractions. Patients are routinely assessed and their medication optimised. Most patients are on ACE inhibitors, and diuretics, unless they are unable to tolerate these drugs. The twenty two subjects enrolled to the study had a mean age of 72.5 ± 7 years (range 56-86 years), and there were 19 males and 3 females. Left heart failure was diagnosed on the basis of history and physical examination and then confirmed by transthoracic echocardiography (n=8) and/or radionuclide scintigraphy (n=14) mean ejection fraction = $33\% \pm 2$. In all patients medication had remained stable for a minimum of one month prior to inclusion in the trial. Baseline medication and haemodynamics are listed in Table 3.1. At the beginning of the study the patients general practitioner was informed of the study protocol and duration.

Patients had previously been titrated to the maximum tolerated dose of ACE inhibitor and therapy included a diuretic in 20 of the 22 cases. In three patients admission or clinic attendance resulted in conversion from captopril to a longer acting ACE inhibitor during the study.

Subjects attended the department on five separate occasions during the study period of 18 months having taken normal medication except diuretics which were withheld for the full 24 hour period. All patients attended between 9.00am and 1.30pm, the

majority of subjects (16/22) before 11.00am, hence samples were taken 3-6 hours after ACE inhibitor dose. The subjects then underwent a clinical review, weight and physical examination, followed by a 30 minute rest supine before blood sampling. Blood samples were collected into appropriate inhibitor cocktails, spun, and assayed as stated in Chapter 2. Standard 12 lead ECGs were taken, after blood testing. The ECGs were analysed for QT dispersion, QTc dispersion, and adjusted QTc.

After supine blood sampling subjects voided a urine sample which was used to look at urinary Na^+/K^+ ratios off diuretics (Brunner 1972), and then completed a full 24 hour collection off diuretics. The following day they took their diuretics in the morning, performed a spot urine, and completed a further 24 hour urine collection while on diuretics. The 24 hour urinary Na^+/K^+ ratios and 24 hour excretions were assessed using these specimens.

Statistical analysis:

Haemodynamic, hormonal, and ECG data were compared between visits using paired t-tests, comparisons between captopril takers and non captopril takers were assessed using ANOVA, and correlations were established using standard regression analysis on a Statgraphics programme. All regression analyses were done initially on each visit, then data was graphed together for brevity, provided the correlations were present at each timepoint. All data are expressed as mean \pm SEM, and statistical significance was accepted where $p < 0.05$.

Table 3.1: Baseline clinical and haemodynamic parameters of the patients (mean \pm SEM).

N=	22
Cardiac medications:	
Beta Blockers	4
Nitrates	9
Aspirin	16
Calcium channel antagonist	13
Digoxin	7
Mean ACE Inhibitor dose in Enalapril equivalent	14.6 \pm 1.6mg
Diuretic dose (in mg of Frusemide)	
Total	68 \pm 9.8mg
<i>Captopril takers</i>	72 \pm 29 mg
N=	5
<i>Non Captopril</i>	56 \pm 9 mg
N=	17
Ejection fraction	33% \pm 2.24
NYHA class	
I	6
II	11
III/IV	5
Blood pressure (mmHg)	
systolic	141 \pm 6.7
diastolic	81 \pm 2.7
Heart rate (bpm)	81 \pm 3.0
Weight (kg)	79 \pm 3
Biochemistry:	
Potassium (mmol/L)	4.31 \pm 0.1
Urea (mmol/L)	8.41 \pm 0.7
Creatinine (mmol/L)	122 \pm 8

Results:

Of the 22 subjects that were initially recruited, 19 completed the full 18 months. One subject died suddenly in the community after 12 months and a second died in hospital at nine months, post mortem showed congestive heart failure. One subject was withdrawn after one month by his family because of progressing dementia.

Haemodynamic data:

Table 3.2 shows the mean values of haemodynamic data over the period for all subjects.

There was a trend downwards in both systolic and diastolic blood pressures, and heart rate over the period, although only diastolic reached statistical significance between baseline and visit five. Overall there was a trend towards worsening NYHA classification, although the NYHA class I group generally did well.

Overall medication remained stable although in the two subjects that died additions of anti-anginals (nitrate and amlodipine) and increases in diuretics did occur. Two other subjects commenced amlodipine during the period. Three patients were converted to a long acting ACE inhibitor from captopril during the period.

Urine data:

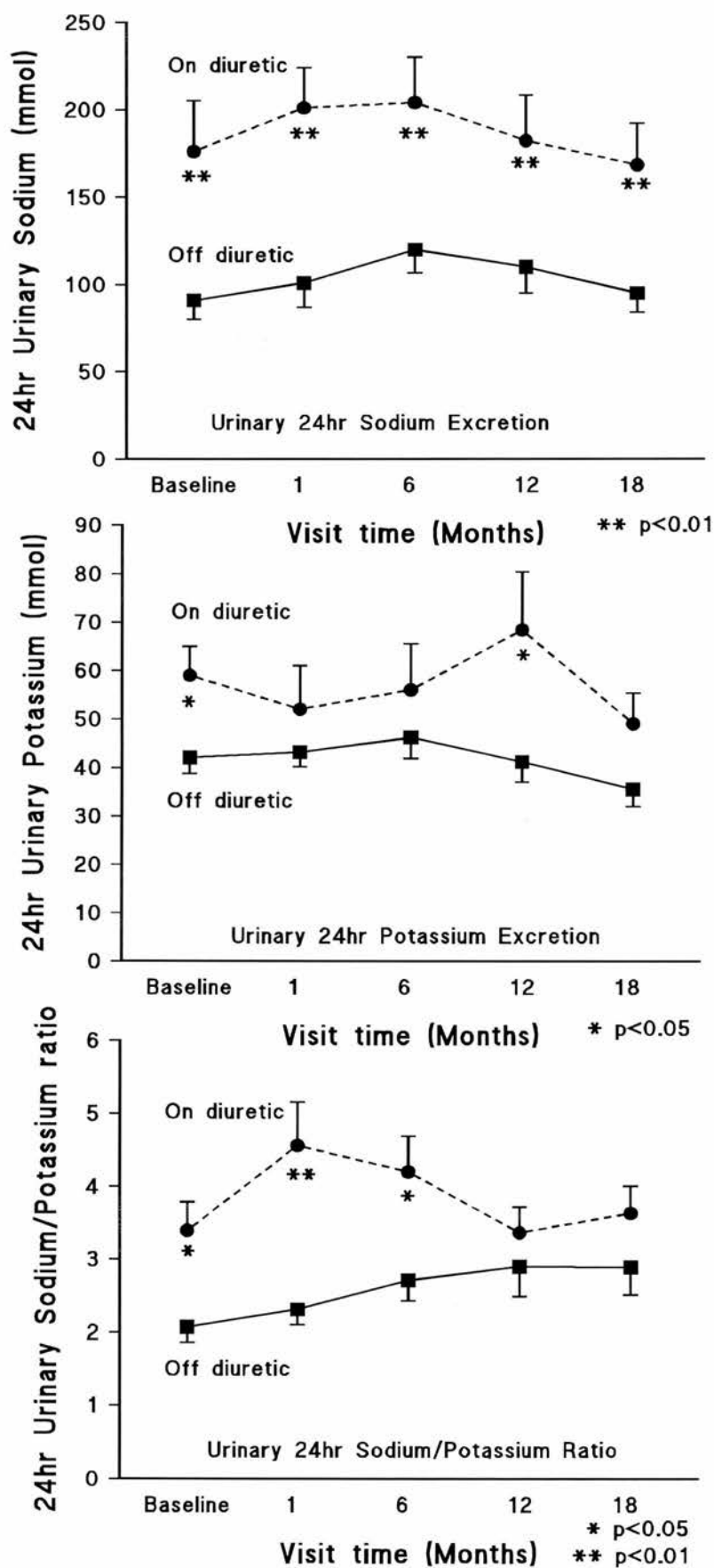
As expected, twenty-four hour urine volumes were statistically greater on diuretics (Table 3.2). 24hr total sodium excretion was significantly greater on diuretics than off throughout, whereas 24 hour potassium excretion was lower off diuretics but not consistently significant. The ratio of sodium to potassium was higher on diuretics than off, consistent with the effect of loop diuretics. Spot urine results did not add anything to those detailed for 24 hour collections, seen in figure 3.1.

Table 3.2: Clinical, haemodynamic and biochemical results during the 18 months, (mean \pm SEM).

	Visit 1	Visit 2 1month	Visit 3 6 months	Visit 4 12 months	Visit 5 18 months
N=	22	22	21	20	19
Cardiac medications:					
Beta Blockers	4	4	4	4	4
Nitrates	9	9	9	8	8
Aspirin	16	16	16	15	15
Ca channel blocker	13	13	13	12	11
Digoxin	7	7	6	5	4
Mean ACE Inhibitor dose in Enalapril equivalent	14.6 \pm 1.6mg	14.8 \pm 1.6mg	16.0 \pm 1.6mg	16.1 \pm 1.6 mg	15.3 \pm 1.8mg
Diuretic dose (in mg of Frusemide)					
Total	68 \pm 9.8mg	64 \pm 12mg	74 \pm 14mg	68 \pm 12mg	70 \pm 12mg
<i>Captopril takers</i>	72 \pm 29 mg	88 \pm 43 mg	80 \pm 23 mg	80	80
N=	5	5	3	1	1
<i>Non Captopril</i>	56 \pm 9 mg	57 \pm 10mg	73 \pm 17mg	67 \pm 17mg	69 \pm 14mg
N=	17	17	18	18	17
Ejection fraction	33% \pm 2.24				
NYHA class					
I	6	6	6	7	7
II	11	12	10	8	4
III/IV	5	4	4/1	4/1	6/2
Blood pressure mmHg					
systolic (mean \pm sem)	141 \pm 6.7	141 \pm 5.9	141 \pm 6.2	132 \pm 8.2	133 \pm 6.0
diastolic	81 \pm 2.7	80 \pm 2.5	76 \pm 2.3	74 \pm 3.08	71 \pm 2.5
Heart rate (bpm)	81 \pm 3.0	78 \pm 4	75 \pm 3	75 \pm 4	73 \pm 3
Weight (kg)	79 \pm 3	79 \pm 3	79 \pm 3	78 \pm 3	79 \pm 3
Urine vol mls/24hr					
off diuretic	1236 \pm 125	1135 \pm 112	1450 \pm 230	1304 \pm 119	1270 \pm 124
on diuretic	2217 \pm 320*	2090 \pm 270*	2337 \pm 248*	2450 \pm 260*	2043 \pm 215*
Biochemistry:					
Potassium	4.31 \pm 0.1	4.35 \pm 0.1	4.36 \pm 0.15	4.4 \pm 0.1	4.3 \pm 0.1
Urea	8.41 \pm 0.7	8.81 \pm 1.0	8.5 \pm 1.0	8.52 \pm 0.9	9.22 \pm 1.0
Creatinine	122 \pm 8	123 \pm 6	121 \pm 7	119 \pm 7.4	128 \pm 8

*=P<0.01 vs off diuretic

Figure 3.1: Urinary sodium and potassium concentrations for 24 hour urine collections.



Renin angiotensin aldosterone axis:

Basic blood biochemistry remained constant throughout the study period (Table 3.2). Overall no trend to increases in neurohormones were seen, suggesting that there is no general trend to aldosterone and angiotensin II reactivation in chronic dosing of heart failure patients. We did find however that the levels of neurohormones in those on captopril (n=5 at Visit 1) compared to those on longer acting ACE inhibitors (Lisinopril n=6, Enalapril n=11) showed different profiles, as seen in figure 3.2. No differences in baseline renal function were observed between those on captopril, and those on other ACE inhibitors. During the 18 months, five subjects initially on captopril reduced to only one after one year (visit three); two died and two were converted during hospital admission to longer acting drugs. Hence the first three visits only are plotted, where there is adequate data to allow comparison. Angiotensin II was significantly higher in those on captopril compared to those on longer acting preparations (Figure 3.2, panel 1). Surprisingly, aldosterone (Figure 3.2, panel 2) was lower in those on captopril, although due to large inter individual variability leading to corresponding variance, this did not reach statistical significance ($p=0.1$). Renin activity (Figure 3.3 panel 1) was similarly lower, and again failed to reach statistical significance. Captopril failed to suppress ACE activity in vitro, (Figure 3.3 panel 2) which is noted in the literature, and may be due to poor in vitro affinity for the enzyme (Unger 1981, Rodriguez 1986). In this study, captopril was taken three times daily in 4/5 subjects, the other taking the drug BD. There were no differences in baseline characteristics of the captopril takers from those on longer acting preparations (Table 3.3).

Table 3.3: Clinical and haemodynamic results of the heart failure subjects at baseline in those taking captopril against longer acting preparations.

	Non Captopril	Captopril
N=	17	5
Cardiac medications:		
Beta Blockers	3	1
Nitrates	5	4
Aspirin	13	3
Diuretics	15	5
Mean dose in Frusemide equivalent	56 ± 8 mg	72 ± 14 mg
Biochemistry		
Urea mmol/L	6.26± 0.5	9.09± 0.8
Creatinine mmol/L	106± 7	127± 10
Mean ACE Inhibitor dose	14.8 ± 2mg	90 ± 10mg
Ejection fraction	32±2%	36±5%
NYHA class I	5	1
II	9	2
III/IV	3	2

FIGURE 3.2: Mean \pm SEM values for angiotensin II and aldosterone, for both captopril and long acting preparations. Note only one subject is on captopril after the third visit therefore data after this is not plotted

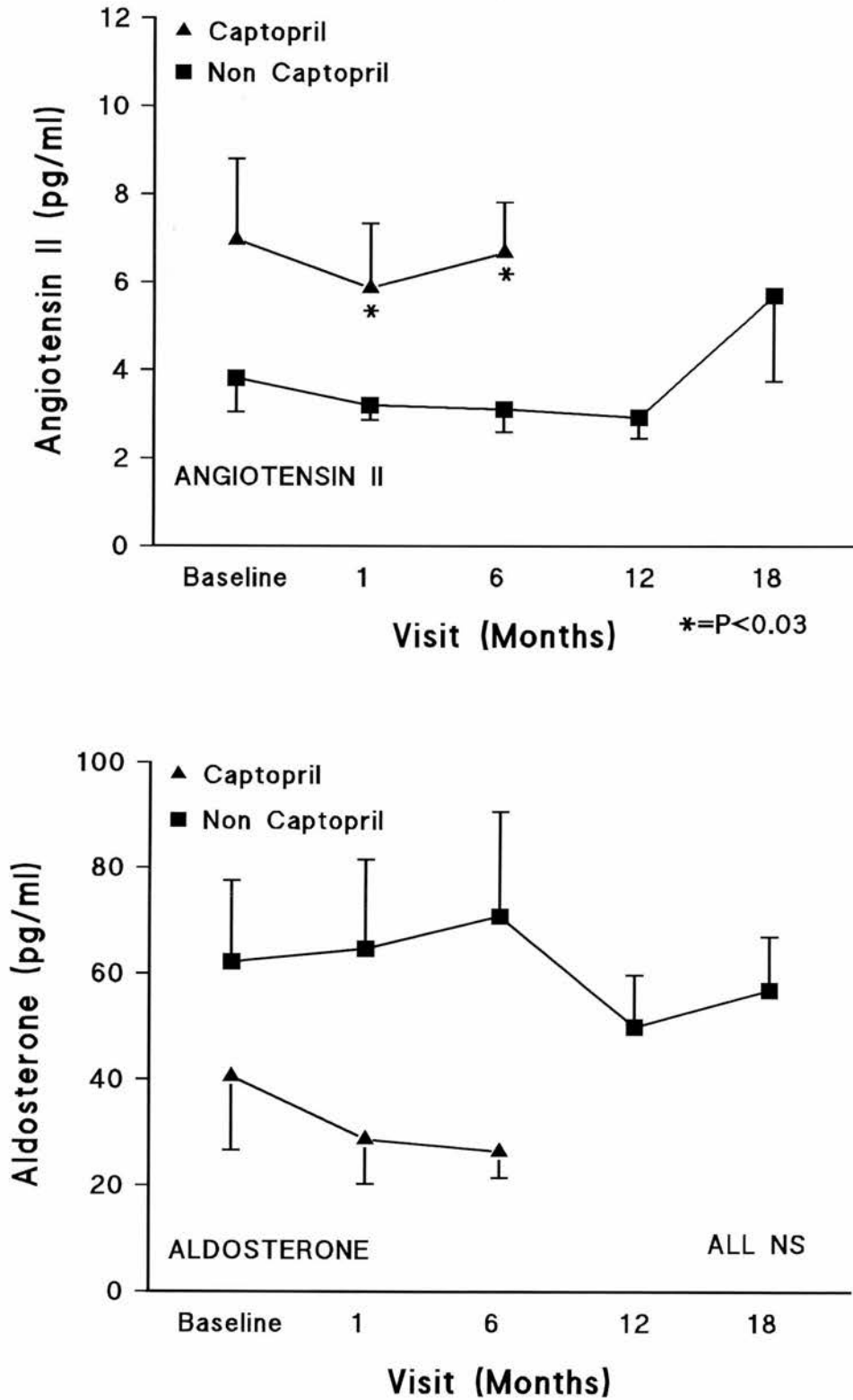
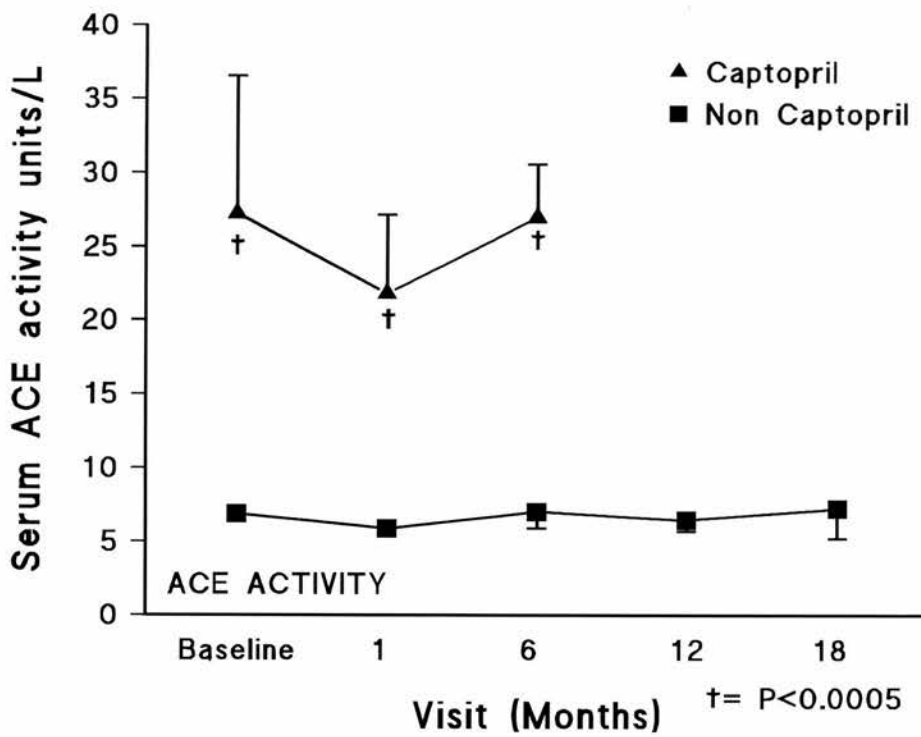
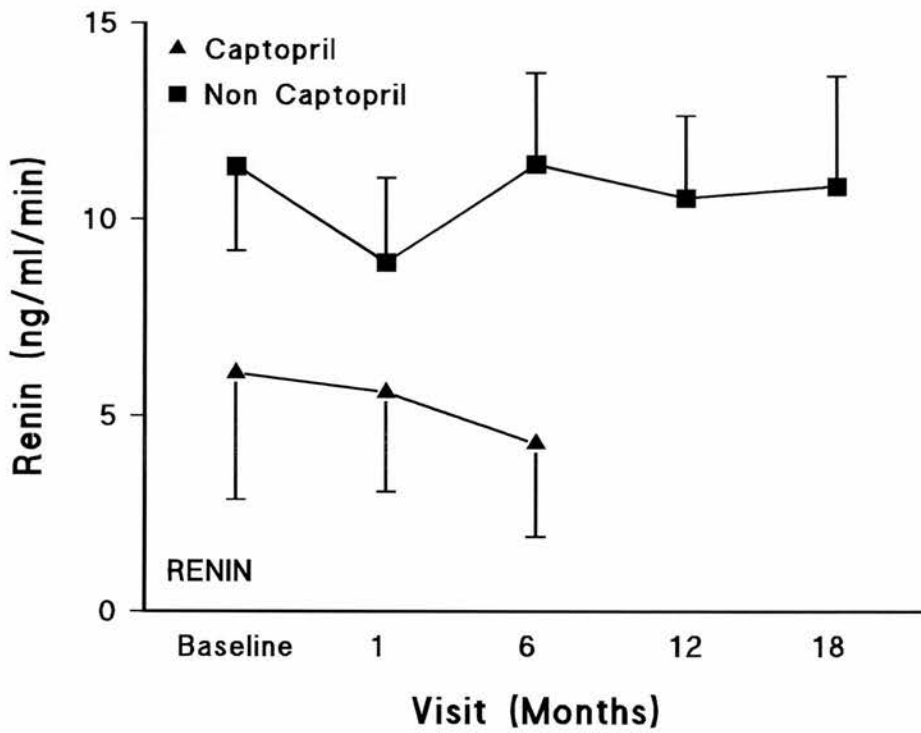


FIGURE 3.3: Mean \pm SEM values for renin, and ACE activity, for both captopril and long acting preparations. Note only one individual on captopril after visit 3.



Angiotensin II and aldosterone reactivation:

In several individuals angiotensin II and aldosterone could be seen to “escape” above specified cut off points of 10pg/ml for angiotensin II, and 80pg/ml aldosterone, prior to returning to suppressed levels. These cut off points are inevitably arbitrary as there is simply no quantitative definition of how suppressed each hormone should be in the presence of an ACE inhibitor. In normal patients without heart failure and not on an ACE inhibitor, using the present assay method, the mean angiotensin II level is 5.8 ± 2.3 (SD) (Morton 1985), hence we have used mean +2SD for our cut-off. Aldosterone is accepted to have wide inter-individual variation, leading to large standard deviations (Swedberg 1990). Looking at normal volunteers studies, baseline mean aldosterone levels are 80pg/ml (Nussberger 1988, Richer 1987), thus this level was chosen as the cut-off for aldosterone. Figure 3.3 shows the results for angiotensin II for individuals whose levels rose at one point above 10pg/ml, occurring in six of 22 subjects for one or more visits. In the sixteen remaining subjects angiotensin II levels remained constant below 7pg/ml and stable, and they are left off the graph for clarity. These results have also been expressed as percentage changes from baseline at each visit and show the intra-individual variability during the study period. Figure 3.4 shows that five individuals developed levels above an arbitrary cut off of 80pg/ml of aldosterone, with the other seventeen being well suppressed below 70pg/ml, which has also been expressed as percentage change from baseline.

FIGURE 3.4: Individual subject data for angiotensin II for those individuals whose levels rose above an arbitrary cut off value of 10pg/ml. Panel 2 shows the same data for these individuals as a percentage change from baseline.

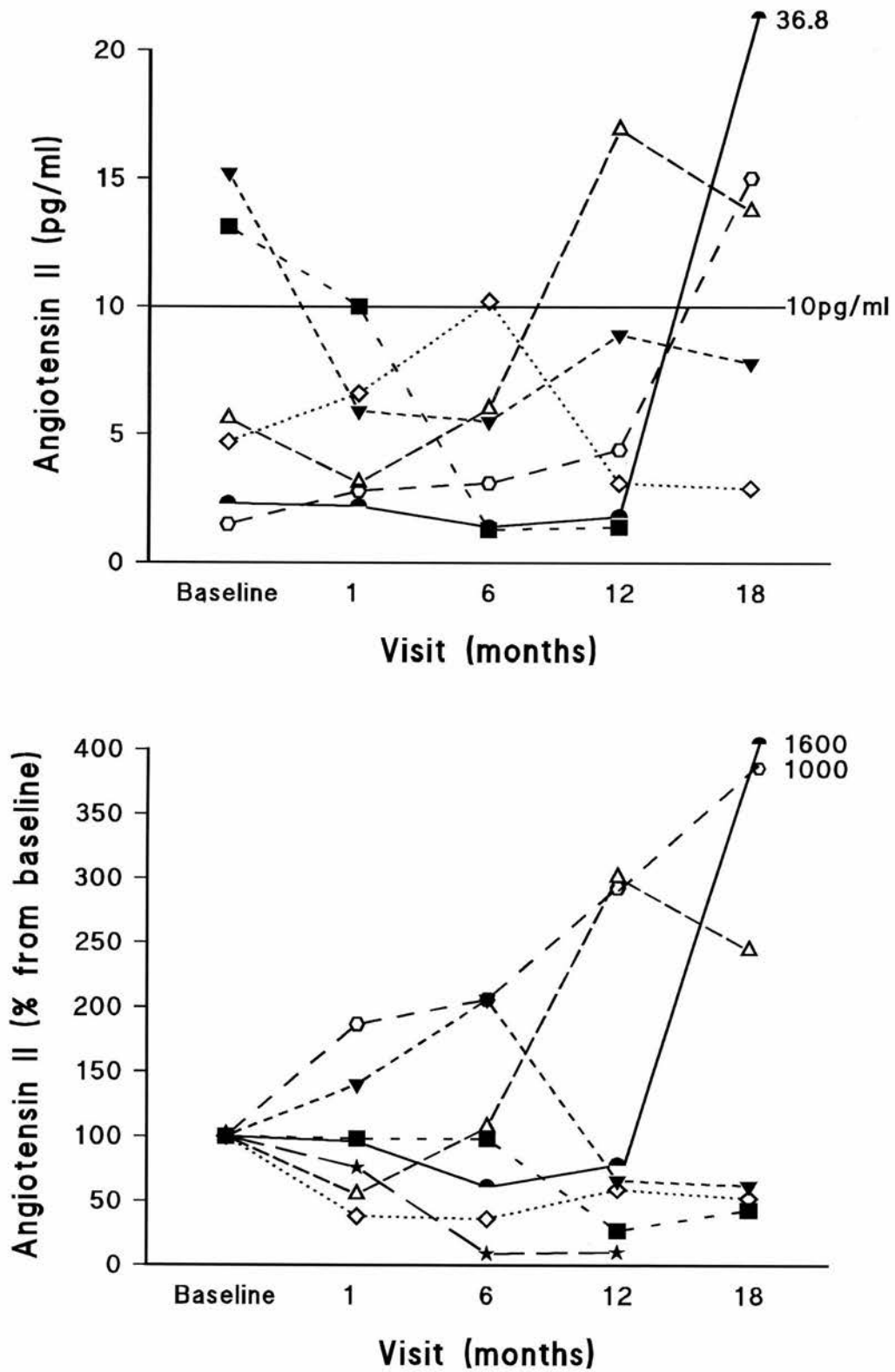
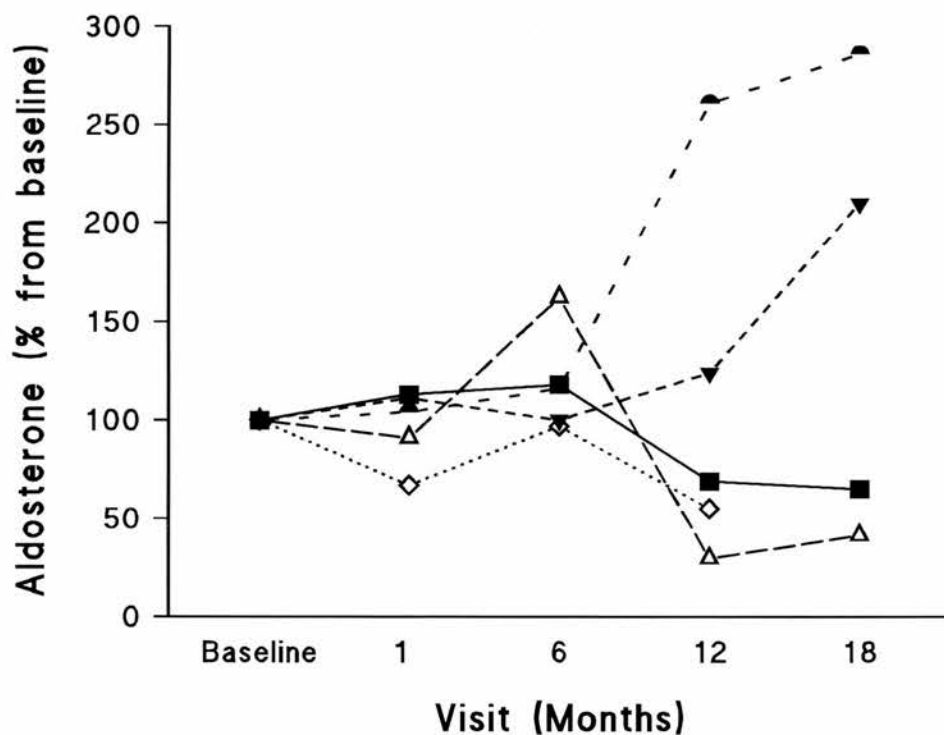
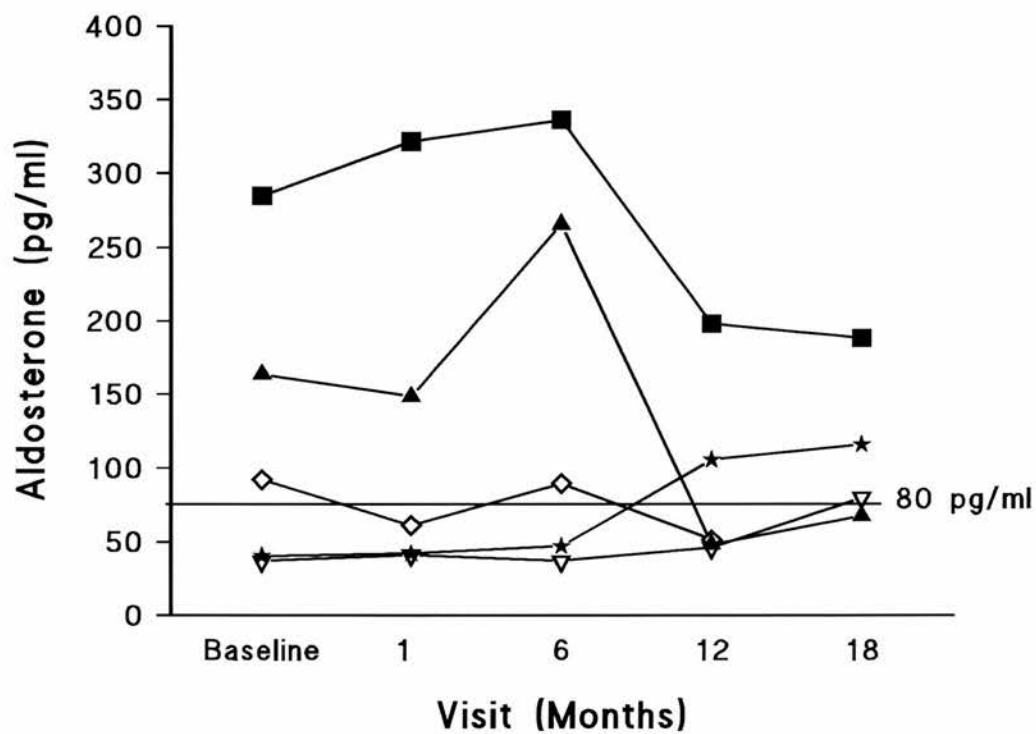


FIGURE 3.5: Individual subject data for aldosterone in individuals whose levels rose above an arbitrary cut off value of 80pg/ml. Panel 2 shows the same data for these individuals as a percentage change from baseline.



We went on to look at the correlations between the components of the renin angiotensin system. Those on captopril demonstrate significant correlations between renin and angiotensin II ($r=0.62$ $p<0.02$) (not shown), and angiotensin II and aldosterone (Figure 3.6 panel 2). Furthermore, although there are correlations between renin and aldosterone for both groups the relationship was much stronger for those on captopril (Figure 3.6 panel 1). Note for brevity the data from all five visits are grouped together, however the correlations were similar for each timepoint. These correlations suggest that in patients taking captopril the renin angiotensin aldosterone axis may not be fully suppressed and additionally all patients on ACE inhibitors have the potential for temporary reactivation of aldosterone and angiotensin II.

Natriuretic peptides:

Levels of both atrial natriuretic peptide and brain natriuretic peptide remained stable over the eighteen months and there was no evidence that the type of ACE inhibitor affected the levels. The mean level of natriuretic peptides were comparable to other studies of heart failure patients, (Davidson 1996b) and may reflect that the study group were overall in a good functional class. It has been shown that there is a correlation between the degree of left ventricular dysfunction and ANP values (Benedict 1994). While there was a correlation between ANP and BNP levels, we demonstrated no significant correlation between the natriuretic peptides, and angiotensin II or aldosterone in this group (Figure 3.7).

QT Dispersion:

Both QT, adjusted QTc and QTc dispersion were similar over time, and while consistent with other similar studies in heart failure subjects (Barr 1994) and post infarct (Moreno 1994, Glancy 1995) these variables did not correlate with reactivation of the renin angiotensin system in this population. Due to the small mortality in this group there is little that can be said of the association with risk of sudden death (Figure 3.8).

FIGURE 3.6: Regression lines correlating renin and aldosterone, and angiotensin II with aldosterone, for captopril and non captopril takers.

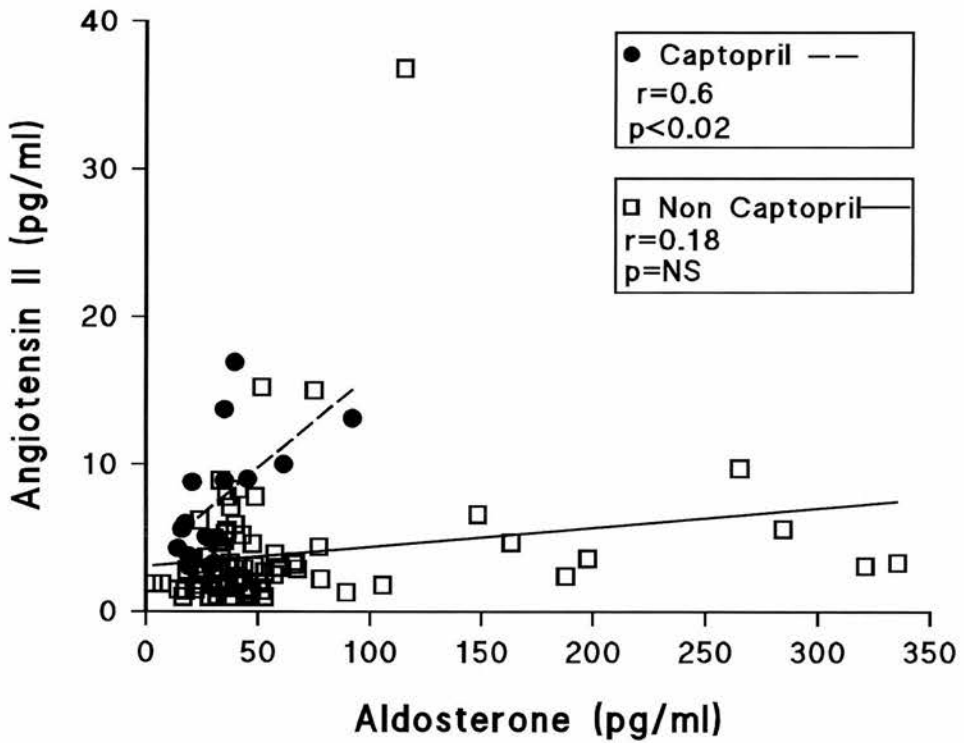
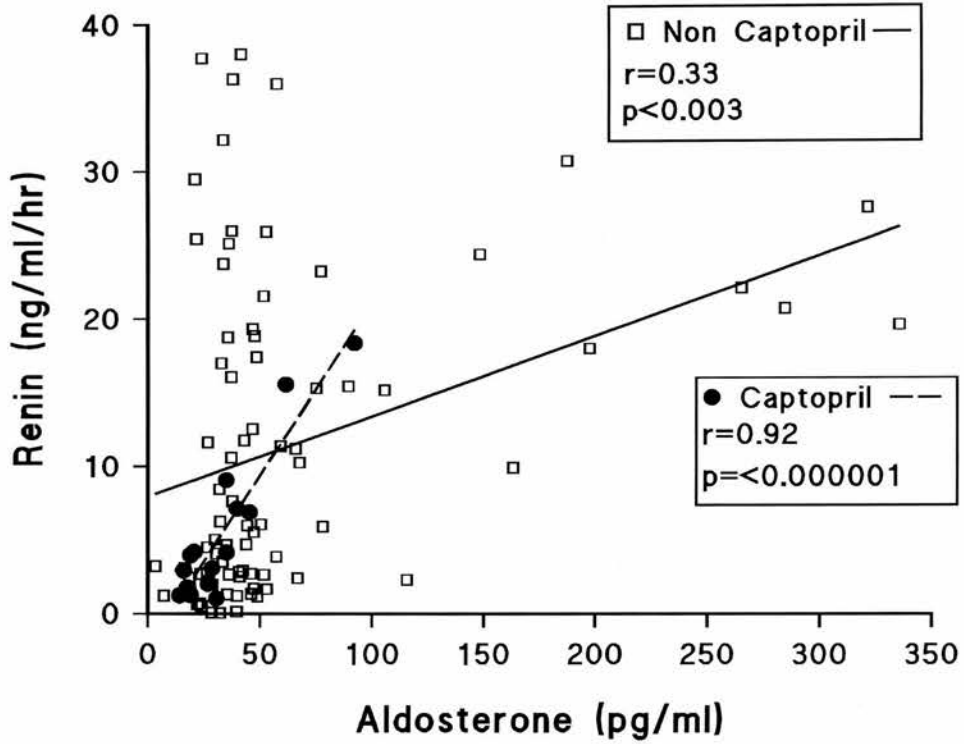


FIGURE 3.7: Mean levels of natriuretic peptides in the eighteen subjects over the eighteen month period, and regression analyses between BNP and ANP, angiotensin II and BNP.

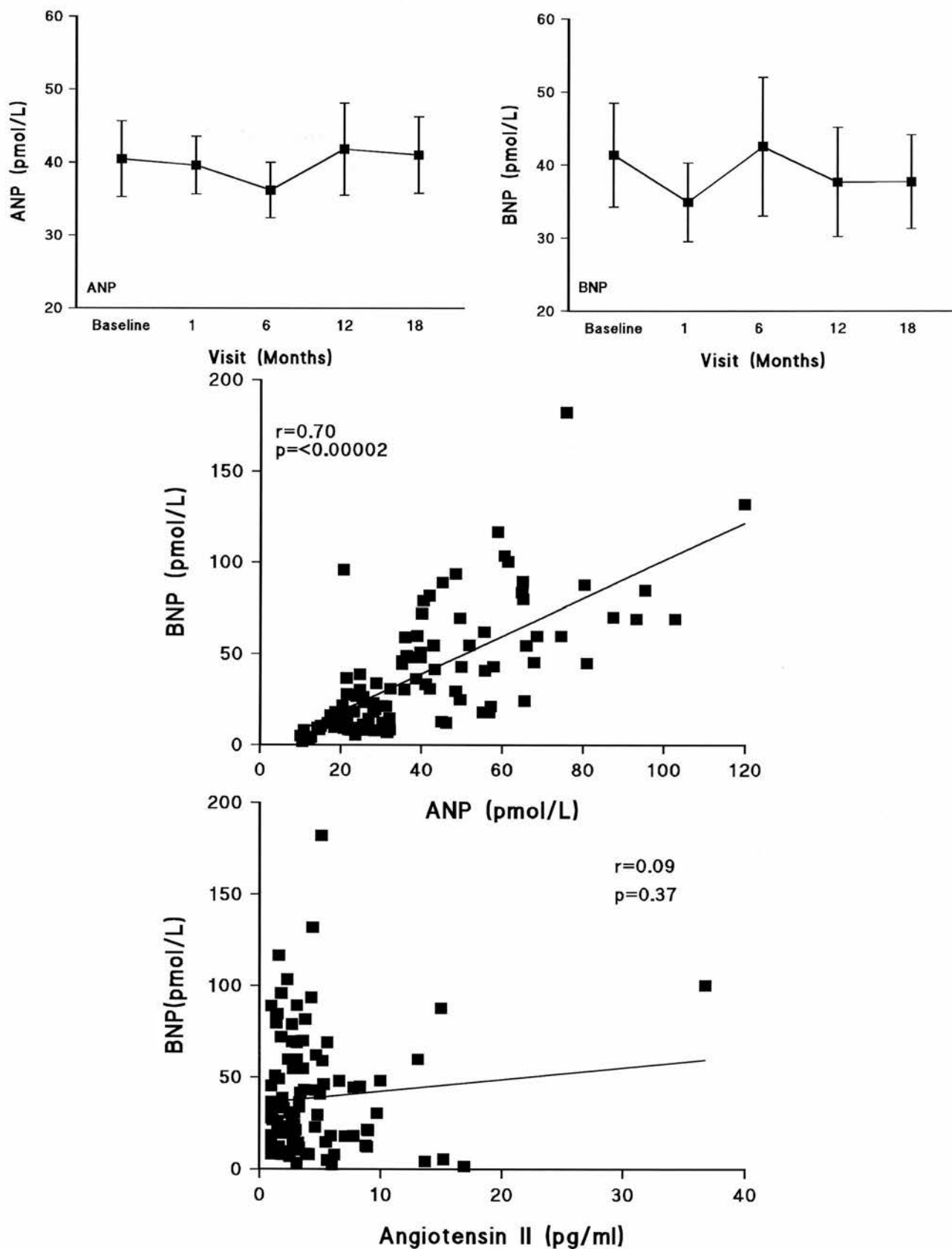
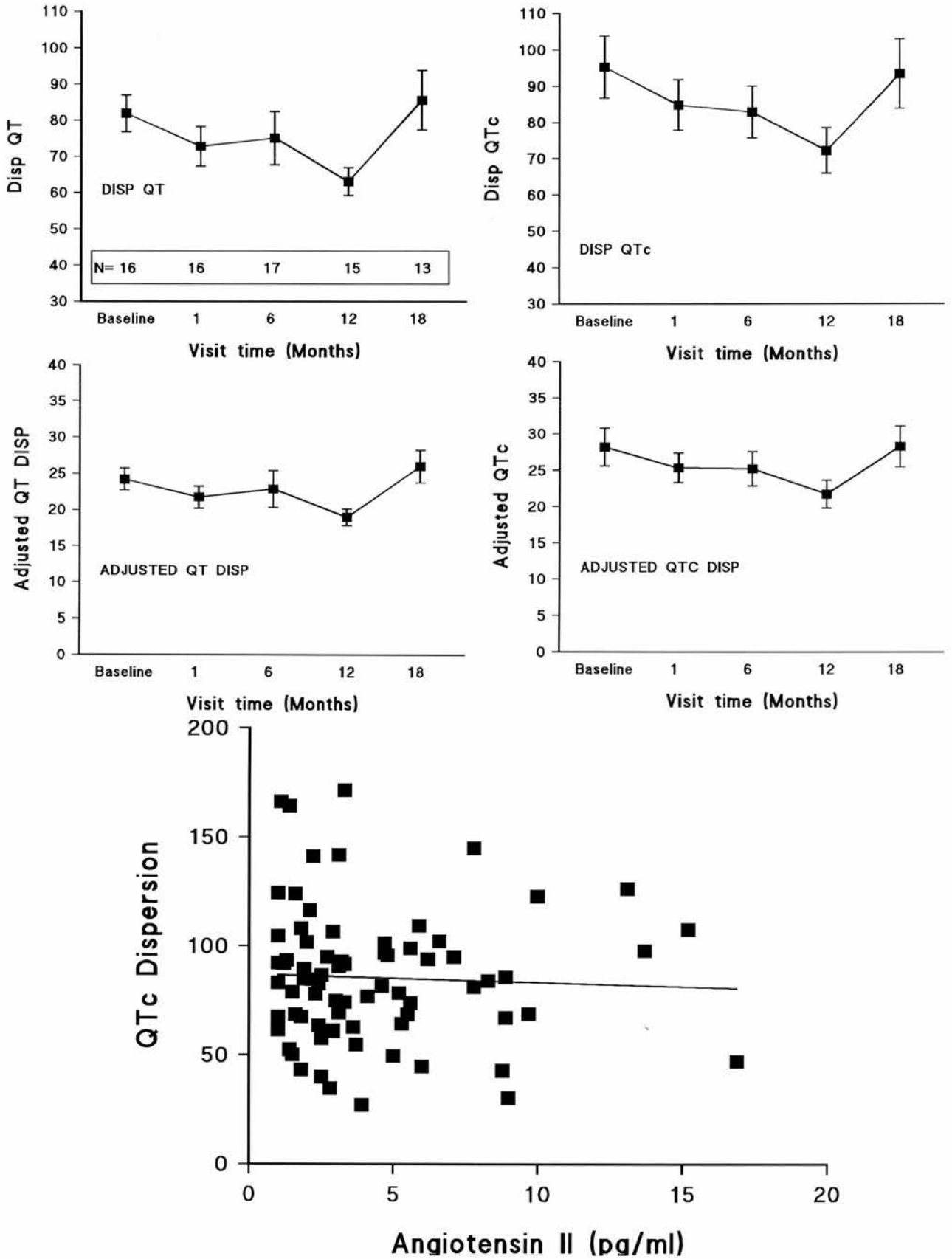


FIGURE 3.8: Mean levels of ECG parameters, and regression analysis between angiotensin II and QTc dispersion, which show no correlation.



DISCUSSION:

This study has three main findings about the pathophysiology of the renin angiotensin system. All go against traditional thinking that angiotensin II reactivation and aldosterone escape are linked and progressive. The first finding is that neither reactivation of angiotensin II or aldosterone is inevitably progressive. In practical terms this means that the finding of angiotensin II reactivation or aldosterone escape does not necessarily herald inevitable deterioration requiring the addition of an angiotensin II receptor antagonist or spironolactone. Secondly, reactivation of angiotensin II and aldosterone appear to occur independently, and do not necessarily occur simultaneously in the same patient. A third observation is that although the numbers are small, captopril takers had higher mean levels of angiotensin II than non captopril takers, but less hyperrenninaemia and lower aldosterone despite taking on average more diuretic (Table 3.2), which would tend to increase renin angiotensin system activation (van Zweiten 1994).

In major studies assessing neurohormonal data in subjects on ACE inhibitors, it has been noted that while most neurohormones are elevated in subjects with heart failure, they do not necessarily correlate well with ejection fraction, or functional class (Benedict 1994). Similarly, there is little evidence of a linear relationship between neurohormones and mortality in heart failure, although those with the highest concentrations tend to do worse (Francis 1993, Swedberg 1990). Interestingly, as discussed in chapter 1, angiotensin converting enzyme inhibitors are accepted to be beneficial, but the comparative importance of reduction in angiotensin II per se

against the less specific improvement in sodium and water balance via aldosterone is still under debate. Swedberg et al (1990) showed reductions in both aldosterone and angiotensin II due to ACE inhibitor therapy, with no correlation seen between ACE activity and angiotensin II and only a limited correlation ($r=0.37$) between angiotensin II and aldosterone in severe heart failure. In mild to moderate heart failure, ACE inhibition with ramipril significantly reduced mean aldosterone, but not mean angiotensin II (Sigurdsson 1994). This suggests that the interaction between the components of the renin angiotensin system is not simple and linear.

Our data suggest that angiotensin II reactivation and aldosterone escape appear to be virtually separate events. Taking the cut off at 100pg/ml for aldosterone and 10pg/ml for angiotensin II, there is only one subject who demonstrates high simultaneous levels of angiotensin II and aldosterone, which in that individual at that time point was associated with high ACE activity, suggesting that non-compliance with the ACE inhibitor was the underlying problem. All the other subjects who had high aldosterone levels had simultaneously suppressed angiotensin II. This is an important message since the therapeutic response for angiotensin II reactivation would be to change to an angiotensin II receptor antagonist and for aldosterone escape to change to spironolactone. The fact that each phenomenon appears to occur in isolation means that neurohormonal monitoring of individuals could provide useful information to direct therapeutic strategies. It is now recognised that aldosterone has many harmful effects in congestive heart failure independent of angiotensin II (Barr 1995, MacFadyen 1997). Furthermore, there are a variety of factors that influence aldosterone production over and above the prevailing levels of angiotensin II, including sodium, potassium and magnesium levels, ACTH, and atrial natriuretic

peptide (Zannad 1995), and more recently even bradykinin may have an effect in vitro (Roslowky 1992), although this observation has not been confirmed in vivo (Rudichenko 1993). Looking carefully at several studies, one obvious phenomenon is the large inter individual variance seen in aldosterone (Swedberg 1990, Sigurdsson 1994), which suggests that the above factors either alone or in combination exert important effects in individuals. The RALES study released recently, demonstrated a reduction in mortality from the addition of spironolactone to an ACE inhibitor in heart failure. This important data emphasises the benefit of enhanced suppression of aldosterone and indicates an area of therapy that has yet to be exploited in the majority of heart failure patients (Pitt 1999).

It is worth emphasising patient compliance will always be an important aspect in observational studies. In those patients taking non captopril ACE the serum ACE activity was fully suppressed in all but 4/88 samples, suggesting excellent compliance. The subjects were strictly instructed to swallow their tablets at the same time each day, and samples were always taken at <6 hours post dosing, with three to four hours being the commonest. The mean dose of captopril was similar to the 50 mg BD that produced mortality benefits in the ISIS-4 trial (1995). We were therefore sampling at the time of peak action of captopril which would suggest that plasma angiotensin II should be at its minimum in captopril takers (Nicholls 1982). This makes our finding that captopril takers had higher angiotensin II levels than those on longer acting ACE inhibitors particularly noteworthy. Compliance will always complicate observational data and we did not count tablets, so we have no direct evidence of individual compliance. However, serum ACE activity is a useful measure of compliance in ACE

inhibitor therapy. Unpublished data from our group showed that plasma ACE < 6.5 IU/L meant that 81% of patients have > 85% compliance with their ACE inhibitor while a plasma ACE > 12 IU/L means that 91% of patients have <100% compliance with a grey area between 6.5-12 IU/L. In this present group only one patient on non captopril ACE inhibitors showed simultaneously high levels of ACE, aldosterone and angiotensin II, consistent with poor compliance. Two other isolated ACE values in non captopril takers are >12 IU/L but in both cases plasma angiotensin II and aldosterone are low which suggests that non compliance can be excluded as a cause. This is useful information because it means that in this study where most patients had ACE <7 IU/L, angiotensin II reactivation, or aldosterone escape are not due to poor compliance.

It is difficult to reconcile the accepted mortality benefits of captopril both post infarct (ISIS-4 1995) and in heart failure (Garg 1995), with the possibility that it may produce less angiotensin II suppression. It is possible that captopril and long acting ACE inhibitors are equally beneficial clinically, as the former suppresses aldosterone while the latter will suppress angiotensin II more.

No randomised mortality comparison of captopril against longer acting ACE inhibitors has ever taken place and, with the exception of one observational study (Pouleur 1991), there is little to suggest that captopril and other ACE inhibitors are different in terms of mortality or morbidity from larger trials. Looking at Garg et al (1995) in an overview of heart failure trials, they combine all the results from 32 trials using 8 different ACE inhibitors, and a total of 7105 patients. Captopril appears to

perform as well as all the longer acting preparations, although the dosage regimes are likely to differ between these trials, as are the patient populations.

In the post infarct population, ISIS-4 (1995) looked at captopril in an un-selected group demonstrating a modest but significant 7% reduction in mortality, requiring 200 to be treated to save one life, with a failure of the benefit to remain statistically significant over one year (Hall 1997). In comparison, GISSI-3 (1994) using lisinopril in a similar population reduced mortality by 11% and comparatively only 125 patients treated to save one life (Latini 1995).

With direction of therapy towards those at high risk, post acute myocardial infarction, the SAVE data using Captopril showed a 19% reduction in mortality, requiring 24 to be treated to save one life compared to the AIRE data with ramipril where there was a 27% reduction in mortality, requiring only 17 people to be treated for one life saved (Latini 1995).

Additionally, there are several small trials which show benefits of longer acting ACE inhibitors over captopril in terms of exercise tolerance, and left ventricular function (Giles 1988, Powers 1987) and it may be that in the long term the failure of captopril to fully suppress angiotensin II both in our study, and in other small studies (Atlas 1984), may explain these minor differences.

Captopril may not suppress angiotensin II as effectively as it has a short duration of action, both intravenously (Nussberger 1988) and orally (deGraeff 1987, Nicholls 1982) which would logically be associated with a more fluctuating pattern of ACE inhibition over time. Fluctuating ACE inhibition with captopril could result in more angiotensin II with captopril than non captopril ACE inhibitors. The data presented above shows that in those on captopril there are significant correlations between renin

activity, aldosterone and angiotensin II, and suggest that the small percentage of un-suppressed ACE, especially towards the end of the dose interval, may drive through to angiotensin II production. It has been suggested that in the presence of high levels of renin and angiotensin II even small differences in ACE activity will produce large variations in angiotensin II levels (Mooser 1990, Nussberger 1994).

ACE genotype has recently been associated with increased cardiovascular risk (Cambien 1992), and may influence these results but probably only to a small extent. Chadwick et al (1997) infused angiotensin I into normal volunteers with DD and II genotypes, and failed to identify any difference in resulting plasma angiotensin II or aldosterone, or even in the blood pressure response to infused angiotensin I. In the presence of an ACE inhibitor, Davidson et al (1996b) found that plasma ACE varied by only 3 IU/l between the DD and the II genotype on lisinopril. Furthermore, the intra-individual variations in plasma angiotensin II over time are very large in this study and these changes cannot be due to the ACE genotype, which suggests non genotypic factors as discussed above are the main influences on plasma angiotensin II.

In addition to the neurohormonal data we failed to show any evidence of gradual deterioration in QT dispersion, or QTc dispersion. The data in the literature suggests that while high QT dispersion in a group does associate with increased mortality, especially sudden death in heart failure (Barr 1994) and post myocardial infarction (Higham 1995), there are large variations in individual data (Glancy 1995). In a small group like this particularly where a proportion (7/22) could not be assessed due to atrial fibrillation or left bundle branch block or bradycardia it is unlikely that any

specific conclusions can be made. No evidence was found that the QT dispersion is correlated with neurohormonal profiles, although there is now mounting evidence that aldosterone may influence myocardial collagen deposition (MacFadyen 1997), and that this may be reflected in QT dispersion (Bonnar 1999).

Summary points:

- Evidence in the literature suggests that neurohormonal reactivation, particularly of angiotensin II and aldosterone, associates with a poorer prognosis.
- In general it has been assumed that reactivation of angiotensin II and aldosterone are due to similar mechanisms, and should therefore occur together, and be persistent.
- The results presented show that reactivation of one of the two hormones occurs in about 10% of the population at any one time, but they can return to suppressed levels spontaneously, and do not appear to herald inevitable disease progress.
- Despite the small numbers involved the profiles of neurohormones appear to be different between the short and long acting ACE inhibitors. Despite this, the evidence in major trials suggest that captopril is effective in reducing mortality both in heart failure and post myocardial infarction. To date there is no randomised controlled mortality trial of captopril vs longer acting ACE inhibitor available.
- Monitoring neurohormones during routine therapy may be useful in targeting additional therapy.

CHAPTER 4:

ACE inhibitors in novel situations. The benefit of Lisinopril in hyperlipidaemia.

Introduction.

This chapter addresses an issue that has been extensively looked at in animals, but less so in humans. ACE inhibitors appear to reduce the structural damage inflicted by hypercholesterolaemia on the arterial wall, with animal data suggesting that the effect is in excess of reductions in blood pressure and may therefore represent an additional benefit of this family of drugs. This chapter reports the results of a study looking at the effect of lisinopril on endothelial function in a group of hyperlipidaemic humans.

Hypercholesterolaemia is a major risk factor for cardiovascular disease (Martin 1986), and lowering the cholesterol level has been shown to produce mortality benefits after myocardial infarction (Sacks 1996). Experimental work suggests that arterial responses to vasodilators are impaired prior to the development of frank atherosclerotic intimal lesions in hypercholesterolaemia (Vita 1990, Chowienczyk 1992, Reddy 1994). There is mounting evidence that ACE inhibitors may be beneficial in reducing atherosclerosis in cholesterol fed monkeys (Aberg 1990) and furthermore, they may also improve endothelial responses in animal models (Becker 1991). This suggests a potential interaction between the renin angiotensin system and endothelial dysfunction due to hyperlipidaemia, and also the development of atherosclerosis.

Brachial artery endothelial function has become a common tool to assess the effect of therapy on arterial function. Three main observations support its validity to study potential anti atherosclerotic therapies. Firstly, endothelial function in forearm resistance vessels correlates well with endothelial function in coronary arteries (Anderson 1995b, Vogel 1993). Secondly, risks that work synergistically to increase cardiovascular events also work synergistically to worsen endothelial function (Heitzer 1996). Finally, three therapies which reduce cardiovascular mortality in heart

failure; statins, ACE inhibitors, and spironolactone (Scandinavian Simvastatin Survival Group 1994, Garg 1995, Pitt 1999), also improve endothelial dysfunction (Treasure 1995, Nakamura 1994, Farquharson 1999).

Much of the evidence that the renin angiotensin system is involved in the atherosclerotic process comes from studies with ACE inhibitors. Firstly, they have been shown to reduce recurrent myocardial infarction in patients with left ventricular dysfunction (Rutherford 1994). Secondly, they improve endothelial dysfunction in heart failure patients (Nakamura 1994), in patients with ischaemic heart disease undergoing therapeutic angioplasty (Mancini 1996). The effects in diabetics have been variable, depending on the stimulus used and the type of diabetes. In type 1 insulin dependant diabetes, O'Driscoll et al (1997) showed an improvement in flow to acetylcholine, after only one month of therapy. However these results were not confirmed by either Mullen et al (1998) or McFarlane et al (1999) who both used flow mediated dilatation, and had longer treatment durations, suggesting type 1 diabetics may not respond in a similar way to ACE inhibitors. In type 2 diabetes O'Driscoll (1999) has again shown improvements in acetylcholine mediated dilatation however this is unconfirmed as yet by other studies in this model. Thirdly, in a series of studies in the Watanabe heritable hyperlipidaemic rabbit, captopril prevented development of atherosclerosis (Chobian 1990), in contrast to propranolol (Lichenstein 1989) and verapamil (Tilton 1985).

It is possible that these effects are mediated not by reductions in angiotensin II but by increases in bradykinin levels, as bradykinin is metabolised by ACE. However there is now increasing evidence to suggest that local angiotensin in the vessel wall is important in the pathogenesis of atherosclerosis.

Physiological concentrations of angiotensin II can promote the generation of superoxide radicals which degrade nitric oxide and thus reduce its activity (Griendling 1994, Rajagopalan 1996); furthermore oxidised LDL is more atherogenic (Parathasay 1992) and acetylated LDL will increase angiotensin converting enzyme expression on macrophages (Diet 1996). Hence ACE inhibitors could reduce local angiotensin II and therefore local production of superoxide radicals, increasing the bioavailability of nitric oxide.

Despite the compelling circumstantial evidence quoted above, there are no specific studies of the effect of manipulating the renin angiotensin axis in hypercholesterolaemic man. This chapter explores the possibility that patients with hypercholesterolaemia might benefit from treatment with an ACE inhibitor in conjunction with lipid lowering therapy. We wished to test the hypothesis that 6 months treatment with lisinopril would reduce endothelial dysfunction in hypercholesterolaemic patients. Six months therapy was chosen to correlate with animal studies where 4 or 6 months therapy has shown positive results (Aberg 1990, Becker 1991).

Methods:

Written informed consent was obtained from the subjects to each protocol. The protocol had previously been approved by the Tayside Committee for Medical Research Ethics and all investigations conformed with the principles outlined in the Declaration of Helsinki.

Population and patients:

Patients were recruited from the cardiovascular risk factor clinic. In total forty four patients were recruited with the intention of studying the effect of either lisinopril or placebo on brachial artery endothelial function.

The cardiovascular risk factor clinic has been in operation for over seven years, and looks after hyperlipidaemic patients identified by the general practitioner. Potential subjects were identified and invitations to take part were made by the clinic doctors, patients were then screened by the author and asked to participate. At the screening visit history, clinical examination, routine biochemistry, and ECG were taken. If patients agreed to participate, fasted lipid samples were taken at a subsequent visit prior to the study commencing. At the beginning of each study the patients general practitioner was informed of the study protocol and duration.

Of those patients recruited, familial hyperlipidaemia was clinically diagnosed in 12 according to the criteria of Durrington (1989) by a combination of lipid profile, and tendon xanthoma in patient or first degree relative. Patients had been stabilised on lipid lowering treatment for a minimum of six months, which remained constant throughout. Hypercholesterolaemia was defined for the study purposes as fasting total cholesterol greater than 5.8 mmol/L, despite treatment. Patients were excluded if they

had evidence of uncontrolled hypertension (diastolic blood pressure greater than 100mmHg), diabetes, or renal dysfunction (urea \geq 10 mmol/L; creatinine \geq 150 mmol/L). Baseline characteristics for the two groups after randomisation are found in table 4.1.

Table 4.1: Baseline haemodynamic and biochemical parameters of hyperlipidaemic patients studied (mean \pm SEM).

	Lisinopril group N=20	Placebo Group N=20	p value
Age (years)	49 \pm 1.9	48.5 \pm 2.05	0.80
Sex (M:F)	15/5 (75:25%)	14/6 (70:30%)	
Smoking History Never	11	11	
Ex Smoker	7	6	
Active smoker	2	3	
Cardiac History	5	7	
Familial/primary aetiology*	7/13	5/15	
Hypertensive on Rx	1	3	
Lipid lowering Rx HMG Co A (mean dose \pm SEM)	9 23 \pm 3mg	12 23 \pm 3mg	
Fibrate	9	6	
Concomitant medications: Aspirin	8	11	
Calcium channel antagonists	4	4	
Serum ACE activity	38.7 \pm 4.2	33.7 \pm 2.2	0.33
Systolic blood pressure (mmHg)	145 \pm 4	143 \pm 4	0.69
Diastolic blood pressure (mmHg)	84 \pm 2	83 \pm 2	0.8
Total cholesterol (mmol/L)**	6.43 \pm 0.3	6.64 \pm 0.3	0.63
HDL cholesterol (mmol/L)**	1.1 \pm 0.07	1.1 \pm 0.1	0.90
Triglycerides (mmol/L)†	2.2 \pm 0.3	2.38 \pm 0.6	0.84
LDL (mmol/L)**	4.33 \pm 0.34	4.58 \pm 0.35	0.54
Baseline Blood Flow (ml/min/100ml forearm)	3.41 \pm 0.86	2.99 \pm 0.56	0.45

for definition see Durrington

** to convert to mg/dl divide by 0.02586

† to convert to mg/dl divide by 0.0113

Study Design:

This study was of a double blind, randomised placebo controlled design. The author was not involved in the randomisation, which was done by a second investigator, who randomly assigned treatments at entry to the study, and controlled all medication

Subjects were randomised in a double blind manner to receive 6 months treatment of oral lisinopril or matched placebo (Zeneca Pharmaceuticals, Cheshire, UK), after baseline assessment of endothelial dysfunction.

The subjects underwent forearm plethysmography as described in Chapter 2, at baseline and following six months treatment with lisinopril or placebo. On both occasions subjects were studied at the same time each day and had abstained from food, caffeine, alcohol and cigarettes for at least four hours before the study, which was performed in an air conditioned room controlled between 23-24 °C.

A 27G needle was inserted into the brachial artery of the non dominant arm under local anaesthesia, and 0.9% saline was infused for 30 minutes prior to the establishment of baseline readings. Strain gauge measurements were taken at five minute intervals to establish three consecutive readings which were within 10%. The mean ratio of measurements from both arms at these time points was taken as the baseline ratio of forearm blood flow.

Following the establishment of baselines, acetylcholine 7.5, 15 and 30 µg/ml was infused, as the endothelial dependent vasodilator. Each dose was infused for 6 minutes

in total, with readings being taken over the last two minutes of each infusion period. Following the third dose, 0.9% saline was infused until the ratio between the blood flow in both arms had returned to baseline.

A second series of infusions were then started, with sodium nitroprusside was infused at 0.8, 1.6, and 3.2 $\mu\text{g/ml}$. An identical protocol was followed on each study day.

Statistical analysis: Comparisons between the biochemical parameters at baseline and follow up were performed using students t-test. For statistical analysis, the ratio of flow measurements from the infused arm over those from the control arm was used both of these measurements are accepted as standard ways of expressing forearm flow data (Benjamin 1995, Chin-Dusting 1999). The ratios of flow were analysed by a repeated measures analysis of variance, looking at the change in the ratio from baseline. Statistical significance was accepted for p values less than 0.05, and data is displayed as mean and standard error of the mean.

Results.

Forty four subjects were initially recruited to the study. Four subjects discontinued the study, two on each arm. Of the active patients who discontinued the study, the first tolerated the dose for five months before being given nicorandil for angina, this combination caused postural hypotension. The second patient had recurrence of endogenous depression and withdrew. One patient on the placebo limb moved out of the area with his job and the second suffered from headaches within a week of treatment and withdrew. The baseline characteristics of these patients were not significantly different from those who completed the study. One further patient who remained in the study had the dose reduced to 10 mg/day due to postural hypotension. Using the clinical criteria established by Durrington (1989) five of those patients in the placebo arm had familial hyperlipidaemia, as opposed to seven in the active group.

Baseline Parameters: At baseline all parameters were similar between the groups, as shown in Table 4.1 above. At follow up the active group showed a significant reduction in blood pressure against the placebo group (Table 4.2). If the fall between baseline and six months within the treatment group is compared we see a highly significant fall, systolic 145 ± 4 versus 128 ± 4 ($P < 0.001$), diastolic 84 ± 2 versus 74 ± 2 mmHg ($P < 0.001$). Serum potassium rose significantly in the active group, consistent with the effects of an ACE inhibitor, and serum circulating ACE activity also was markedly suppressed. Both the placebo and active group demonstrated a fall in baseline blood flow between the baseline study day flow and the follow up study day flow. Baseline blood flow was not statistically significantly different between the groups on each day, but the fall in the active group achieved significance ($p < 0.01$).

Table 4.2: Comparison of haemodynamic and biochemical parameters of randomised patients, at six months follow up.

	Lisinopril group N=20	Placebo Group N=20	p value
Weight (Kg)	80.7 ± 5.4	81.7 ± 2.8	0.86
Systolic blood pressure (mmHg)	128 ± 4	138 ± 3	<0.02
Diastolic blood pressure (mmHg)	74 ± 2	81 ± 2	<0.02
Total cholesterol (mmol/L)**	6.47 ± 0.4	6.5 ± 0.3	0.95
HDL cholesterol (mmol/L)**	1.19 ± 0.07	1.09 ± 0.08	0.3
Triglycerides (mmol/L)†	2.17 ± 0.3	2.40 ± 0.6	0.7
LDL (mmol/L)**	4.33 ± 0.49	4.38 ± 0.34	0.9
ACE activity	7.1 ± 1	34.0 ± 3	<0.001
Potassium (mmol/L)	4.42 ± 0.1	4.23 ± 0.05	<0.05
Baseline Blood Flow (ml/min/100ml forearm)	2.01 ± 0.15	2.5 ± 0.33	0.18

** to convert to mg/dl divide by 0.02586

† to convert to mg/dl divide by 0.0113

Blood Flow Responses:

Blood flow is expressed as the ratio of blood flow in the infused arm over that in the control arm. Vasodilation in the infused arm causes an increase in the ratio. Figure 4.1 shows the ratios at baseline and after six months treatment in the lisinopril group where there was a significant increase in blood flow responses to acetylcholine. Furthermore, in this group, we also demonstrated statistically significant increases in the responses to sodium nitroprusside (Figure 4.3). In contrast, in the placebo group there was evidence of a decline in the endothelial dependent responses to acetylcholine over time (Figure 4.2), with absolutely no change in the endothelial independent response to nitroprusside (Figure 4.4).

Figure 4.5 shows a dual regression analysis between change in systolic blood pressure over the six months and change in blood flow ratio in response to the top dose of acetylcholine (30 $\mu\text{g/ml}$). In the active group, delineated by the solid symbols, there is a significant correlation between change in blood pressure and the alteration in blood flow. In contrast, superimposed on those datapoints for comparison, are the results for the placebo group in open squares, which fail to show any trend.

Figure 4.1: Ratio of blood flow in the infused arm over the control arm (\pm SEM), during infusions of acetylcholine, at baseline and after 6 months treatment with lisinopril 20mg/day. $P < 0.02$ between baseline and post treatment.

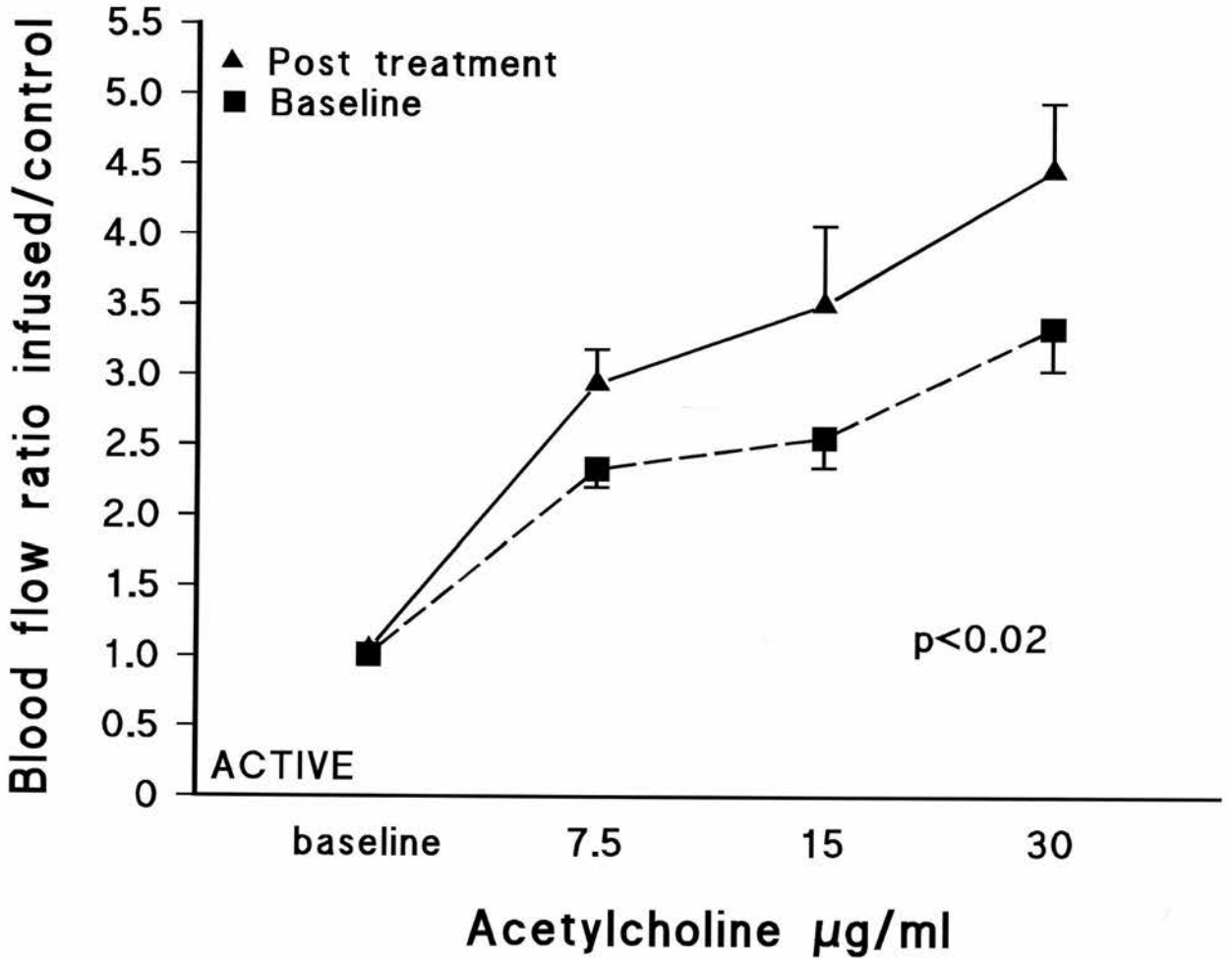


Figure 4.2: Ratio of blood flow in the infused arm over the control arm (\pm SEM), during infusions of acetylcholine, at baseline and after 6 months treatment with placebo.

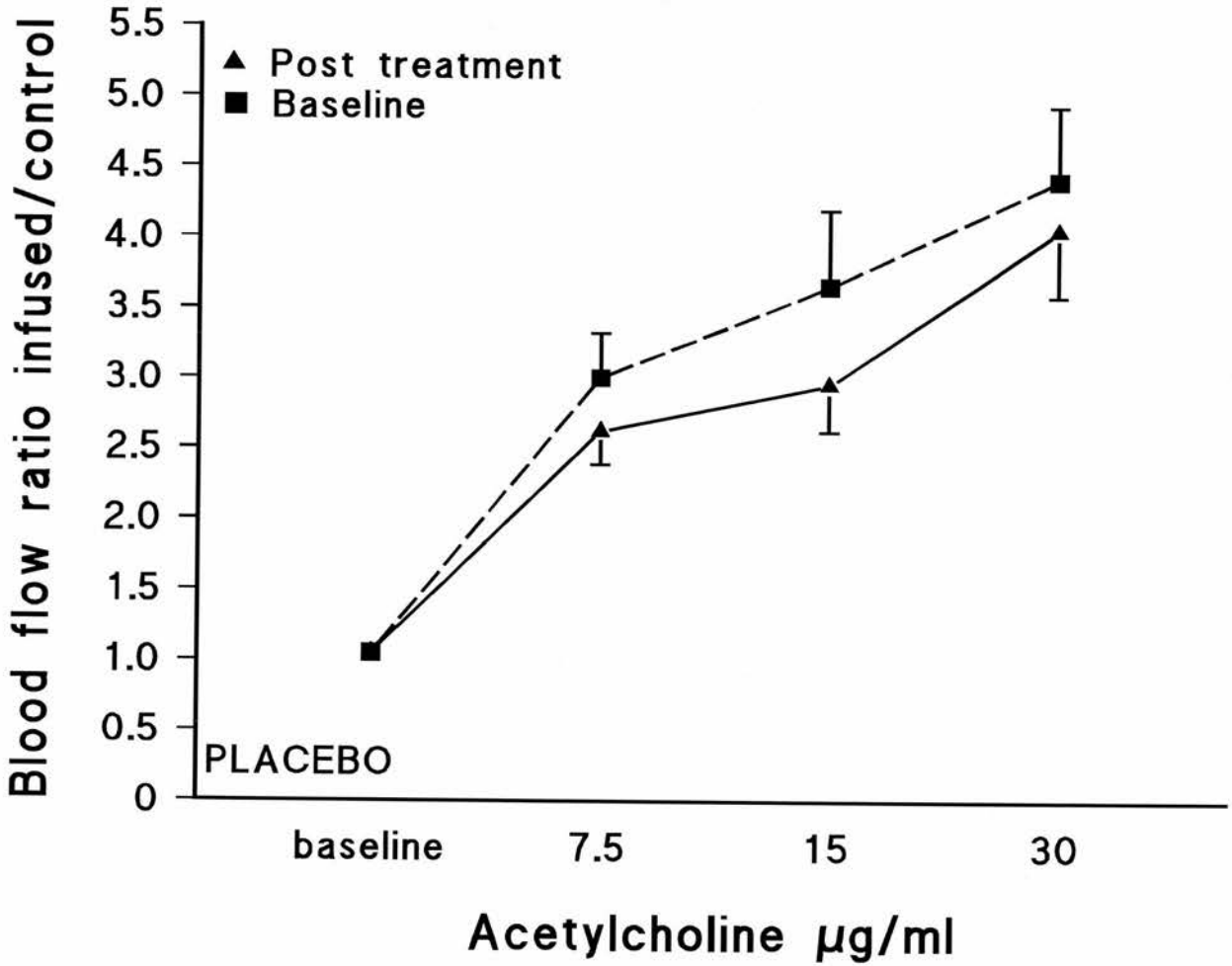


Figure 4.3: Ratio of blood flow in the infused arm over the control arm (\pm SEM), during infusions of sodium nitroprusside at baseline and after six months treatment with lisinopril. $P < 0.03$ between baseline and post treatment.

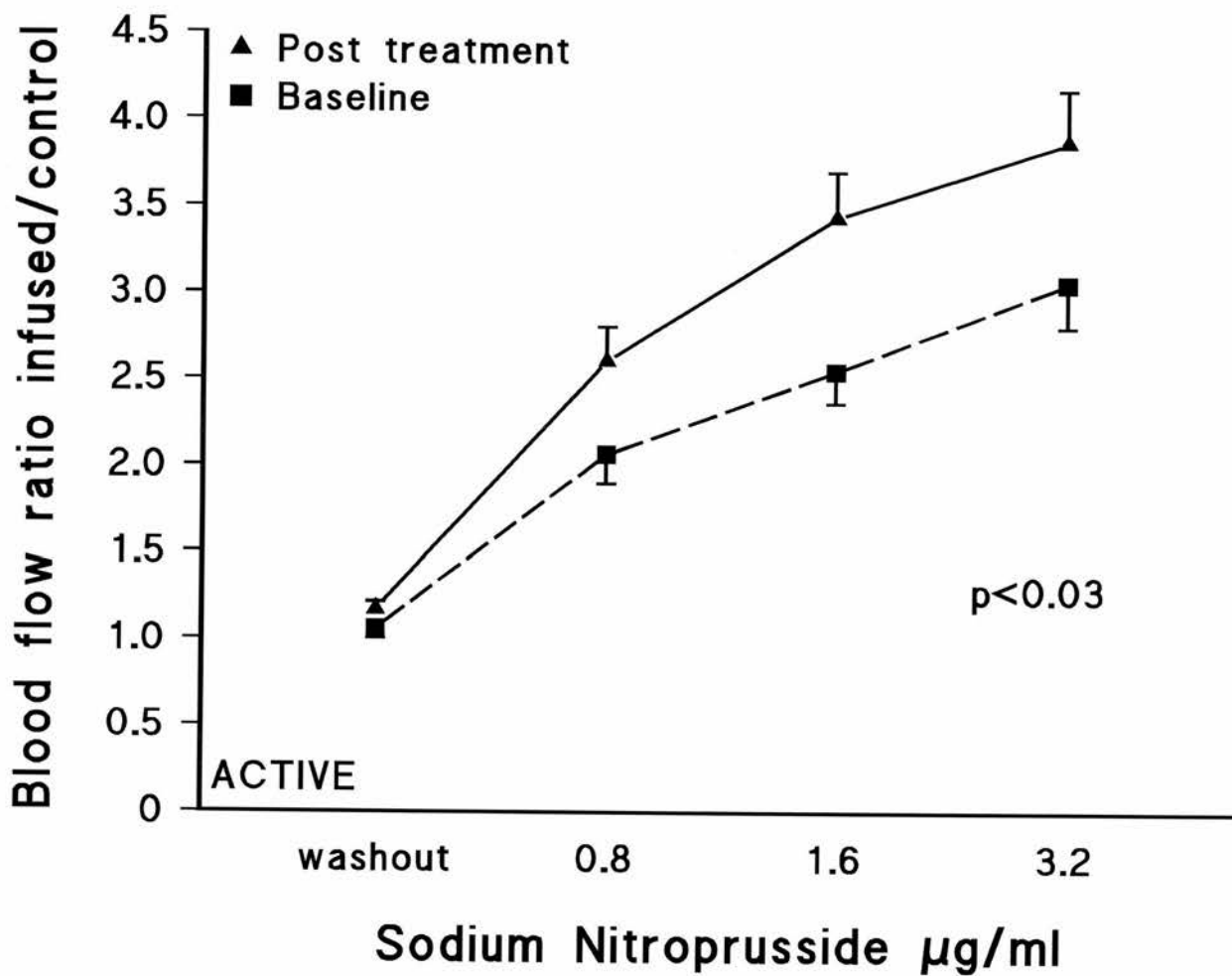


Figure 4.4: Ratio of blood flow in the infused arm over the control arm (\pm SEM), during infusions of sodium nitroprusside, at baseline and after six months treatment with placebo.

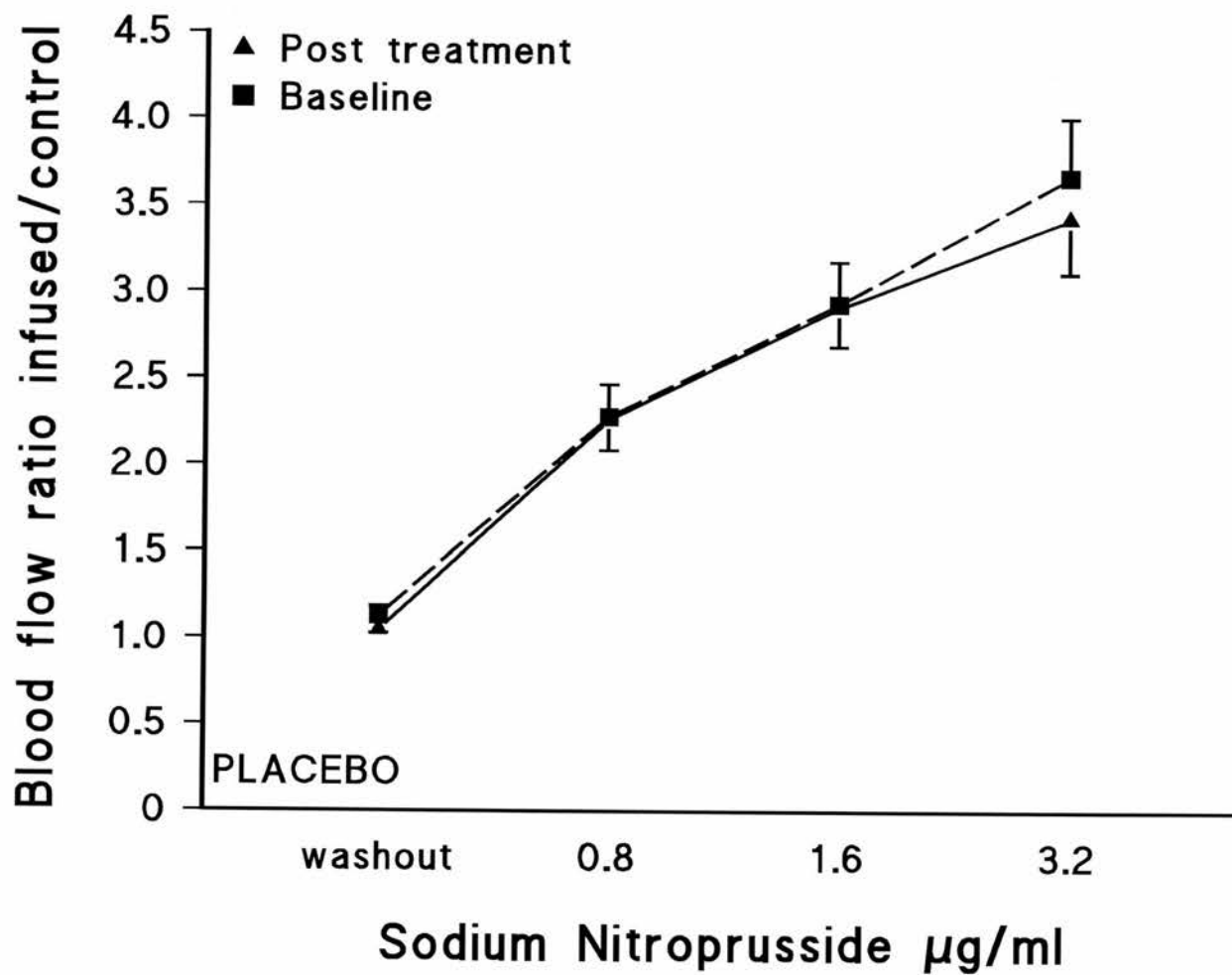
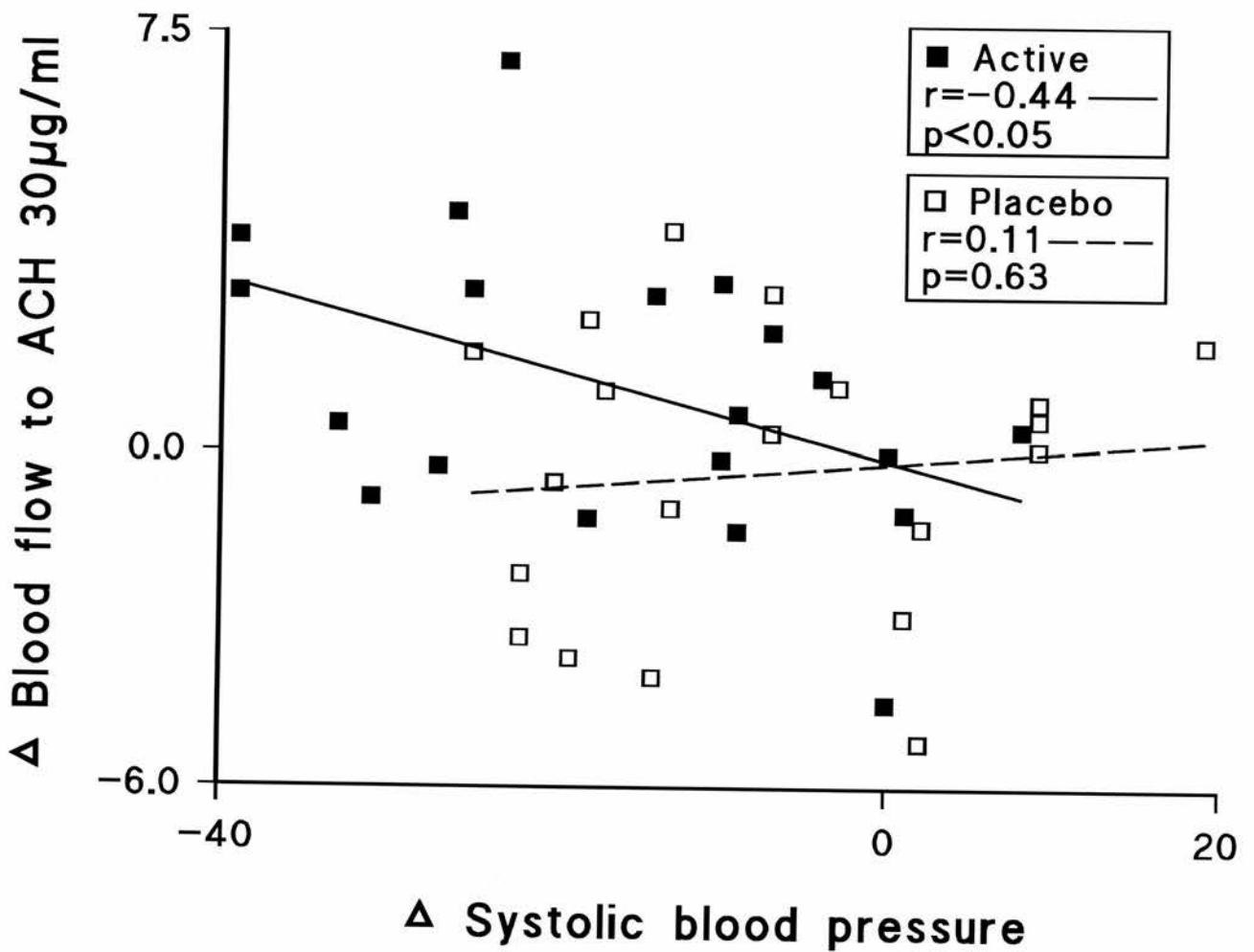


Figure 4.5: Regression analyses of the correlation between the change in blood pressure during the treatment phase, and the change in blood flow response to acetylcholine at the highest dose (30 μ g/ml) between baseline and post treatment. The placebo group are those with open squares, and those on active treatment in solid squares. The regression lines are solid for active treatment, and dashed for placebo.



DISCUSSION:

This chapter describes an investigation into the effects of an ACE inhibitor on arterial function in hypercholesterolaemic patients. We chose to use acetylcholine as the endothelium dependent vasodilator as it is known that, compared to controls, vasodilatation to this compound is reduced in hypercholesterolaemia (Chowiencyzk 1992). Nitroprusside was used as a standard endothelial independent vasodilator. The results demonstrate that over time endothelial function, as assessed by the vasodilatory response to acetylcholine, deteriorates in a group of hypercholesterolaemic patients, albeit non significantly, and that lisinopril 20 mg/day reverses that trend, causing a significant increase in responses both to endothelial dependent acetylcholine and endothelial independent nitroprusside. ACE inhibitors have been shown in animal models to reduce hypercholesterolaemic damage in animal models and we believe that this is the first demonstration of such an effect specifically in hypercholesterolaemic patients.

Baseline Data:

The baseline data were well matched between the groups. The observed changes in blood pressure, potassium and serum ACE activity suggested good compliance with the therapy. For a group of treated hypercholesterolaemics, the achieved total cholesterol and LDL may seem high, but the mean total cholesterol levels in the general population from which this cohort are derived is around 6.0 mmol/L. The evidence suggests that endothelial function is best in hyperlipidaemics with lower

levels of LDL although there appears to be little further improvement below 3.2 mmol/L (Sacks 1996). Baseline forearm blood flows were comparable between the groups, both before and after six months treatment. Although both values fell between baseline and follow up only the fall in the active group reached statistical significance.

The observed fall in blood flow after six months of lisinopril may initially appear surprising, but is consistent with the limited information available in the literature about the effects of chronic ACE inhibitor therapy. In a similar study in normotensive insulin resistant subjects, six months treatment with an ACE inhibitor also produced a reduction in baseline flow (Bijlstra 1995), but failed to improve evoked responses. In shorter term studies in other normotensive populations there is no evidence of a fall in baseline blood flow, suggesting that the effect may take some time to develop (O'Driscoll 1997) The mechanism of this remains obscure, in the longer term ACE inhibition appears to decrease both medium sized vessel diameter and capillary bed cross-sectional area (Wang 1990), however ACE inhibitors may increase compliance (Van Bortel 1995). Hence as blood flow is the product of driving pressure, and resistance, which reflects vessel compliance, our observed fall in blood flow may relate to the reduced driving pressure seen, and increased vessel compliance.

The above fall in blood flow complicates our results, but for several reasons this finding supports, rather than undermines our primary observation. Firstly, although blood flow fell between baseline and follow up in both the active and the placebo group, the response to acetylcholine actually diverged between the groups, with the

active group demonstrating an improvement in response and the placebo group showing a deterioration over time. Secondly, Benjamin et al (1995) observed that the lower the baseline flow the more difficult it is for an artery to vasodilate in response to a stimulus. In the present study, the active group had a lower baseline flow but actually increased their blood flow more in response to both vasodilators which is contrary to what would be expected non-specifically from the baseline flow changes. Thirdly, the fact that the active group demonstrates a correlation between the increase in blood flow and the fall in blood pressure supports our results, as had our changes been a chance finding it would be unlikely that we would find such a correlation with changes in blood pressure.

Potentially, the benefits observed in arterial function in this study could be largely the result of the reduction in blood pressure, especially since a correlation was observed between the reduction in blood pressure and the change in vasodilatation to acetylcholine (Figure 4.5). For several reasons it appears unlikely that the results are simply a blood pressure effect. Firstly, the TREND study (Mancini 1996), found that quinapril improved endothelial function in a group of patients, despite an unexpected increase in blood pressure in the treatment group. Secondly, in contrast to the available data on ACE inhibitors, calcium channel antagonists do not seem to improve endothelial function despite reducing blood pressure. In particular, in the animal model of familial hyperlipidaemia, (watanabe heritable hyperlipidaemic rabbit) ACE inhibitors prevented atherosclerosis (Chobian 1990, 1992) whereas propranolol (Lichenstein 1989), and verapamil (Tilton 1985) did not. Furthermore there has been some debate as to whether there is endothelial dysfunction in hypertensives

(Cockcroft 1994, Panza 1990) and whether anti-hypertensives actually improve endothelial responses in truly hypertensive groups (Panza 1993), hence making it unlikely that our small reduction in blood pressure in normotensives is the reason behind the benefit seen.

There are several possible mechanisms underlying our present results:

Firstly, ACE inhibitors both reduce angiotensin II production and prevent the degradation of endothelial bradykinin which releases NO. That ACE inhibitors potentiate the vasodilator effect of applied bradykinin is not under debate, due to the wealth of in vitro and in vivo supporting data, as kinase II which converts angiotensin I to angiotensin II is also responsible for the degradation of bradykinin (Horning 1997). The acute effect of bradykinin inhibition, using specific antagonists such as HOE-140, on acetylcholine mediated vasodilation has suggested that much of the acute benefit of ACE inhibitors is bradykinin mediated (Berkenboom 1995), however the proportion of acetylcholine induced dilation due to bradykinin release is not known (Zanzinger 1994). There are few studies looking at the long term effects of co-administration of ACE inhibitors and bradykinin antagonists, hence it is difficult to assess whether the effect on bradykinin is maintained in the longer term, or whether a new equilibrium is reached. In longer term studies angiotensin II antagonists and ACE inhibitors have been found to be equally effective in preventing endothelial dysfunction due to Cyclosporin A (Auch-Schwelk 1995). Furthermore, even in studies of only two weeks, Ramipril showed benefits on arterial structure following balloon denudation, which was only partially reversed by HOE-140 (Farhy 1993). We feel that it is unlikely that increased vasodilation in response to acetylcholine presented here is due to changes in the release or metabolism of bradykinin, particularly since

baseline blood flow fell, and one would expect that if there were chronically elevated levels of bradykinin overall then those in the active group should have higher baseline blood flow. It is possible that after 6 months therapy with ACE inhibitors bradykinin production is reduced, such that a new plateau is reached.

Acetylcholine is believed to release nitric oxide which mediates vasodilation. The TREND study (Mancini 1996) showed improvements in coronary endothelial responses following six months treatment with quinapril, in normotensive patients. It is particularly noteworthy that in the TREND study the main beneficial effect of quinapril on endothelial function occurred in those with a low density lipoprotein (LDL) fraction > 130 mg/dL (≥ 3.36 mmol/L) (Pitt 1997b). Similarly, the beneficial effect of quinapril, in the QUIET study, on coronary vessels was mainly seen in those with high LDL (Cashin Hempell 1997). These findings agree with the present study and suggests that ACE inhibitors are indeed beneficial in those with elevated lipids. In vitro work has also provided evidence to support this, since monocytes which are activated to become macrophages by acetylated LDL begin to express ACE (Diet 1996), and lipid laden macrophages in atherosclerotic plaques express ACE (Diet 1994), hence in theory circulating LDL can increase tissue levels of angiotensin II.

Angiotensin II has been shown to increase production of superoxide anions in vascular smooth muscle cells (Griendling 1994). If angiotensin II levels are elevated in atherosclerotic plaques, then there could potentially be an increase in superoxide anions released, reducing the bioavailability of nitric oxide due to increased degradation.

We have also demonstrated that lisinopril increased the response to nitroprusside which is believed to be an endothelial independent vasodilator. This is not the first demonstration of improvement in nitroprusside responses, with a variety of beneficial therapies. Jeserich et al (1994) also showed improvements in nitroprusside responses following long term ACE inhibition in heart failure patients. Similarly Gilligan et al (1994b), showed that post-menopausal women improved responses to both acetylcholine and nitroprusside with oestrogen treatment. Indeed, in the literature several reports show that the responses to nitroprusside in hypercholesterolaemia are subnormal (Creager 1992, Casino 1994), although statistical significance is only rarely reported (Creager 1990, Gilligan 1994d). In diabetics who are also known to have accelerated atherosclerosis, it has been shown that blockade of nitric oxide synthesis causes less vasoconstriction than in normals, and that the response to applied nitroprusside was markedly reduced in the diabetics compared to normal controls, suggesting that in this group there is abnormalities of nitric oxide metabolism (Calver 1992). Animal evidence suggests that the more severe the arterial damage, the more likely that endothelial independent responses are also reduced (Verbeuren 1986). Potentially, in severely damaged arteries, beneficial therapies might first improve acetylcholine responses, and later improve nitroprusside responses.

If we were observing a reduction in superoxide particles, we would expect lisinopril to augment not only the blood flow responses to acetylcholine, which is believed to release nitric oxide, but also those to sodium nitroprusside, as we have observed. It is also possible that the beneficial arterial responses which we observed are due to the ACE inhibitor being an anti-oxidant itself and reducing the degradation of nitric oxide.

Lisinopril does not possess a sulph-hydryl group in contrast to captopril (Godfrey 1994) but both have been associated with intrinsic anti-oxidant activity (Mira 1993). Alternatively, the local reduction in angiotensin II may reduce superoxide activity. Both hypercholesterolaemia (Ohara 1993) and angiotensin II (Griendling 1994, Rajagopalan 1996) at physiological concentrations can stimulate superoxide anion production, which could reduce the half life of available nitric oxide. In this way ACE inhibitors should amplify NO bioactivity as seen here. The theory that enhanced NO degradation is a feature in atherosclerosis is supported by animal work which shows that atherosclerotic vessels appear to produce more rather than less nitric oxide, despite endothelial dependent vasodilatation being severely blunted (Minor 1990). Interestingly, in hyperlipidaemia, treatment with antioxidants has been shown in animals to improve endothelial function (Simon 1993), and in man to improve arterial function (Anderson 1995a) and structure (Azen 1996). ACE inhibitors could therefore act beneficially on NO degradation by preventing superoxide radical production, preventing LDL oxidation either directly, or by reductions in local angiotensin II. If so, this could be the mechanism underlying our observation that lisinopril improves the arterial responses to both acetylcholine and nitroprusside.

Summary points:

- Six months treatment with lisinopril caused an improvement in arterial responses to acetylcholine, and sodium nitroprusside in patients with hyperlipidaemia.
- The mechanism behind this improvement was not addressed by this protocol, but may be due to alterations in the bio-availability of nitric oxide. This might be due to either decreased degradation of bradykinin, or a fall in the rate of degradation of nitric oxide.
- There is growing evidence that anti-oxidants improve arterial response, as do ACE inhibitors in a variety of animal models.
- Evidence in the literature suggests that ACE activity is promoted in atherosclerotic plaques and angiotensin II can promote superoxide radical formation, and that this may be important in the premature destruction of nitric oxide in the vascular intima.

CHAPTER 5:

The effect of ACE inhibition on plasma nitrite and nitrate in hypercholesterolaemia, a comparison with normal controls.

In the previous chapter it was shown that six month therapy with lisinopril caused improved endothelial function in a group of hyperlipidaemic patients. The hypothesis proposed was that angiotensin II stimulates superoxide radical formation via cellular components within the vessel wall, and that atheromatous arteries express more angiotensin converting enzyme, and hence more free radicals which reduced the bio-availability of endothelially derived nitric oxide. Therefore, with lisinopril administration the resulting fall in angiotensin II caused an improvement in the balance of nitric oxide and free radicals, and hence improved flow both to endogenous and applied nitric oxide. This chapter goes on to address the question of whether or not there is evidence of altered nitric oxide synthesis in hyperlipidaemics, and the effect of lisinopril on nitric oxide production.

Introduction:

Endothelial dysfunction in hyperlipidaemia is believed to relate to alterations in the synthesis or metabolism of nitric oxide. In the literature, comparing hyperlipidaemics to normals, L-Arginine both orally and intra-arterially has been shown to improve endothelial dysfunction, suggesting a defect in nitric oxide synthesis (Creager 1992, Clarkson 1996). However this has not been uniformly confirmed by other studies, for example Casino et al (1994) applied L-Arginine intra-arterially, and improved blood flow response to acetylcholine in normals, but not in hyperlipidaemic patients. Hence the defect in arterial responses in hyperlipidaemia may be in metabolism rather than production of nitric oxide in the vascular intima. In theory if the defect is due to inadequate production, then overall nitric oxide levels, and those of the breakdown

products should be reduced. On the other hand cardiovascular risk factors could increase free radicals which are believed normally to inactivate NO. In that situation, NO breakdown products might be unaltered or even increased.

Plasma nitrate and nitrite are breakdown products of nitric oxide (Leone 1994), and their levels have been used to assess whether reduced NO bio-activity is due to reduced production, or increased breakdown. Furthermore, plasma nitrite and nitrate have been used to assess the effect of drugs on nitric oxide release, with a variety of therapies (Nakashima 1996, Cincinelli 1998).

In the hyperlipidaemia population, plasma nitrite and nitrate results have been equivocal. Tanaka et al (1997) studied inpatients who were suspected of coronary artery disease and compared these to normal controls. The study showed that basal nitrite/nitrate expressed together as NO_x correlated negatively with total cholesterol and LDL in the patients, and NO_x was significantly lower than in normal controls. Another study from the same group (Nakashima 1996), showed that simvastatin could increase basal NO_x pre treatment from 38µmol/L to 57µmol/L post treatment in a group of hyperlipidaemic patients. Mean cholesterol pre-treatment was 7.2mmol/L. Even more interestingly, the increase in plasma NO_x correlated not with the reduction in total cholesterol but with the increase in HDL.

In contrast two studies have suggested that hypercholesterolaemia will cause higher levels of nitrite excretion. Ferlito et al (1997) looked only at female subjects with acute and chronic coronary disease, and risk factors for coronary disease. They found that while hypertension appeared to increase the level of excreted nitrite, the factor that increased excretion most was hypercholesterolaemia. Takahashi et al (1992)

looked at both men and women and found correlations between nitrate and total cholesterol in women, with multiple regression analysis nitrate correlated positively with total cholesterol, triglycerides, and blood pressure. These results led the authors to suggest that endothelium derived NO is increased in the atherosclerotic process.

A separate but related question is whether treatments which improve endothelial dysfunction do so by increasing NO production, or by reducing NO breakdown. As demonstrated in the previous chapter ACE inhibitors improve endothelial function in hyperlipidaemia, and have also been shown to improve endothelial function in insulin dependent diabetes (O'Driscoll 1997), heart failure patients (Nakamura 1994), and in coronary heart disease patients with higher lipid levels (Pitt 1997b, Cashin-Hempell 1997). Hence ACE inhibitors improve endothelial function, and indeed mortality in the larger trials, but there is no direct evidence that they affect nitric oxide levels.

Nitric oxide has an extremely short half life and is rapidly degraded to several ions within the vessel wall (Leone 1995). The commonest of these are nitrite (NO_2^-) and nitrate (NO_3^-), with nitrate being the more stable. Neither of these ions are easy to measure, due to easy contamination, and also because blood samples have to undergo immediate centrifugation to avoid degradation of nitrite to nitrate. It is also important that nitrite and nitrate are measured simultaneously (Leone 1995) and there are a variety of factors that could influence the results.

Fasting status markedly affects the plasma levels, and there is large inter-individual/intra-individual variation depending on substances ingested prior to sampling. Wang et al (1997) found that four days of a low nitrite/nitrate diet were

required before stable measurements could be taken. Furthermore, diurnal variation occurs in non fasted subjects (Tanaka 1997), levels are affected in trained athletes (Poveda 1997), and following infarcts (Akiyama 1998). It is therefore essential that appropriate fasting protocols are used to produce reliable results.

In hyperlipidaemia, whether the defect occurs in synthesis or breakdown of nitric oxide is unknown, and in the previous chapter it was discussed that the benefit derived from lisinopril may relate to reduction in the formation of superoxide radicals causing improved survival of tonic and stimulated nitric oxide. We would expect therefore that those patients on placebo would exhibit the highest levels of nitrites/nitrates, with a reduction in the groups on lisinopril due to improved bioavailability, and lower levels still in the control group.

With this rationale we decided to assess the production of nitric oxide in the hypercholesterolaemic volunteers pre and post the cessation of their trial medication to see whether or not there was an alteration in overall nitric oxide metabolism.

Materials and methods.

Written informed consent was obtained from the subjects to each protocol. All protocols were previously approved by the Tayside Committee for Medical Research Ethics and all investigations conformed with the principles outlined in the Declaration of Helsinki.

Subject recruitment:

Hyperlipidaemic Patients:

Patients were recruited from the cardiovascular risk factor clinic as discussed in the previous chapter. Within the group of forty four who initially volunteered to assist with the forearm plethysmography study, twenty agreed to take part in this study looking at nitric oxide production on and off trial medication. For patient characteristics of the full forty subjects see previous chapter.

Patients were excluded if they had evidence of uncontrolled hypertension (diastolic blood pressure greater than 100mmHg), diabetes, renal dysfunction (urea \geq 10 mmol/L; creatinine \geq 150 mmol/L). This study was also double blind randomised as the code was only unblinded after the last subject had completed the fasts. The characteristics of the two hyperlipidaemic groups and the normal controls are seen in Table 5.1.

Control population.

Eleven normal volunteers (50 ± 3 years) were identified via advertisements in the local press, and in the university. Volunteers were taking no medication, and had no

previous illnesses. Furthermore, clinical examination, baseline biochemistry, haematology, and 12 lead electrocardiogram were all normal. Subjects were excluded if the total cholesterol was greater than 5.5mmol/L. Due to the prevailing local genetics several people were screened for every eligible subject, so it was not felt appropriate to aim for lower values. Overall baseline bloods and haemodynamics are given in Table 5.1. Subjects attended the department on two occasions separated by eight weeks, having fasted overnight from 7.00pm as in the protocol below.

Protocol:

Patients and volunteers were fasted overnight, with fasting starting at 7.00pm, and attending the department at 10.00am the next day. Subjects were allowed to drink only Milli Q+ water (Millipore, Bedford MA), and limited to 1L water. Smoking was also prohibited, during the fast. Cannulae were inserted into antecubital fossa vein at 10am and first samples were taken at 11.am for nitrite, nitrate, and lipid profiles with further sampling at 12.00pm and 1.00pm for nitrite and nitrate only. Nitrate/nitrite samples were collected immediately onto ice, and spun at 3500rpm for 15 minutes, in a refrigerated centrifuge, at 4°C. Lipid profiles were collected into Vacutainer tubes, and processed the same day using automated analysers. Samples for nitrate/nitrite were collected into needles and syringes and thereafter into lithium heparin tubes all sampling equipment was rinsed three times with Milli-Q+ water.

Statistical analysis:

Baseline data were compared between groups using single ANOVA (Table 5.2). There was little variation in nitrite/nitrate levels between the three timepoints 15, 17, and 18 hours so, after discussion with the biostatisticians, it was decided to analyse

the data as weighted areas under the curve. These are equal to the area under the curve divided by the time intervals, to give numerical values consistent with the primary data for ease of comprehension. These results were then compared using single ANOVA.

Statistical significance was accepted for p values <0.05 . Results are expressed as mean \pm SEM

Table 5.1: Baseline characteristics of the three groups prior to stopping the randomised tablets, compared to the normal controls.

	Hypercholesterolaemic subjects		Normal cholesterol
	Lisinopril	Placebo	
	Mean ± SEM	Mean ± SEM	Mean ± SEM
Age (years)	51.5 ± 2.77	49.7 ± 2.33	50 ± 3.3
Sex (M:F)	8:3	6:3	7:4
Systolic blood pressure (mmHg)	125±5	136±3	133 ± 6
Diastolic blood pressure (mmHg)	77±3	82±2	76 ± 4
Total cholesterol (mmol/L)	5.9±0.27	6.1±0.29	5.13 ± 0.17*
HDL (mmol/L)	1.1±0.09	1.2±0.1	1.4 ± 0.13
Trigs (mmol/L)	2.24±0.4	1.9±0.6	1.4 ± 0.23
Lipid lowering Rx:			
Statin therapy	6 (55%)	4 (44%)	
Fibrate therapy	4 (36%)	2 (22%)	
Other therapy	1 (19%)	3 (33%)	
HRT	1	1	1
Aspirin	3	4	
Ca -channel antagonists	2	3	
Vitamin E	1	1	
Active Smokers	2	1	1

** p<0.01 vs hyperlipidaemic groups

Results:

Baseline data:

The three groups were well matched at baseline (Table 5.1). The normal controls had significantly lower levels of total cholesterol, and low density lipoprotein cholesterol, but other cholesterol fractions were comparable. Blood pressure was lower in the lisinopril group than in either of the other groups, although this failed to reach statistical significance ($P=0.08$ active vs placebo). The cholesterol and fractions were stable over the eight weeks between study days, stopping the lisinopril having no effect on lipid parameters (Table 5.2).

Nitrite and Nitrate data:

Figures 5.1 and 5.2 demonstrate that there were no significant differences overall in plasma nitrite, or plasma nitrates either over time, or between the groups.

Figure 5.1 shows the data for nitrate and nitrite for the three groups, for both visits as weighted areas under the curve. The mean value for each hyperlipidaemic group pre and post is seen at the side of the individual data points. The normal control groups are also shown for comparison although they took no medication between their two visits. Using ANOVA for the AUC values there was no significant differences seen between any group.

Figure 5.2 shows each individuals data on and off randomised medication for nitrate, for both hyperlipidaemic groups, to demonstrates that there was no trend in either direction between the visits.

Figure 5.3 is a regression analysis between total areas under the curve for nitrite and nitrate and shows that there is reasonable correlation between these two ions but that

a lot of the nitrite values are grouped around 0.6 $\mu\text{mol/L}$ which may represent in vitro breakdown of this ion.

In view of the studies in the literature looking at cholesterol and nitrate data we also decided to assess whether there was a correlation between the levels of these ions and any of the lipid fractions. As we had failed to show any effect of the study drug on either nitrite or nitrate it was felt to be appropriate to pool the data from both study days, for each group.

We could not show any correlation between cholesterol and either of the ions in the hyperlipidaemic groups. Figure 5.4 panels 1 and 2 show that while there was no correlation for the whole population, there was a significant if loose correlation between nitrate and both LDL and total cholesterol in the normal control group only. The r values for the other groups, and other lipid fractions are given in Table 5.3.

Table 5.2: Lipid profiles for all three groups, on both study days. Note that the hyperlipidaemic patients discontinued either lisinopril or placebo between visit 1 and 2 whereas the normal controls were on no medication.

	Hypercholesterolaemic subjects				Normal subjects	
	Lisinopril		Placebo		Visit 1	Visit 2
	On trial- Visit 1	Off trial- Visit 2	On trial- Visit 1	Off trial- Visit 2		
N=	11	11	9	9	11	6
Total cholesterol (mmol/L)	5.9±0.3	6.0±0.4	6.1±0.3	6.1±0.3	5.13 ± 0.2**	5.15±0.2*
HDL (mmol/L)	1.1±0.09	1.2±1.9	1.2±0.1	1.2±0.1	1.4 ± 0.13	1.7±0.1
Trigs (mmol/L)	2.24±0.4	2.14±0.5	1.9±0.6	1.7±0.33	1.4 ± 0.44	1.1±0.13
LDL (mmol/L)	3.8±0.27	3.9±2.7	4.15±0.31	4.11±0.32	3.1±0.20*	2.9±0.3*

**p<0.01 vs hyperlipidaemic groups

* p<0.05 vs hyperlipidaemic groups

Table 5.3: Correlation co-efficient values between lipid fractions in the three groups, and calculated plasma nitrate as a weighted AUC.

	Hypercholesterolaemic subjects				Normal control	
	Lisinopril		Placebo			
Total cholesterol	r=0.05	NS	r=-0.18	NS	r=0.58	p<0.02
LDL cholesterol	r=0.03	NS	r=-0.05	NS	r=0.51	p<0.05
HDL	r= 0.09	NS	r=-0.24	NS	r=-0.06	NS
Triglycerides	r=0.04	NS	r=-0.11	NS	r=-0.07	NS

Figure 5.1: Plasma nitrate (panel 1) and nitrite (panel 2) weighted areas under the curve for individual subjects in the three groups, hyperlipidaemic subjects on lisinopril, and placebo, and normal controls. Mean and SEM is seen beside the data.

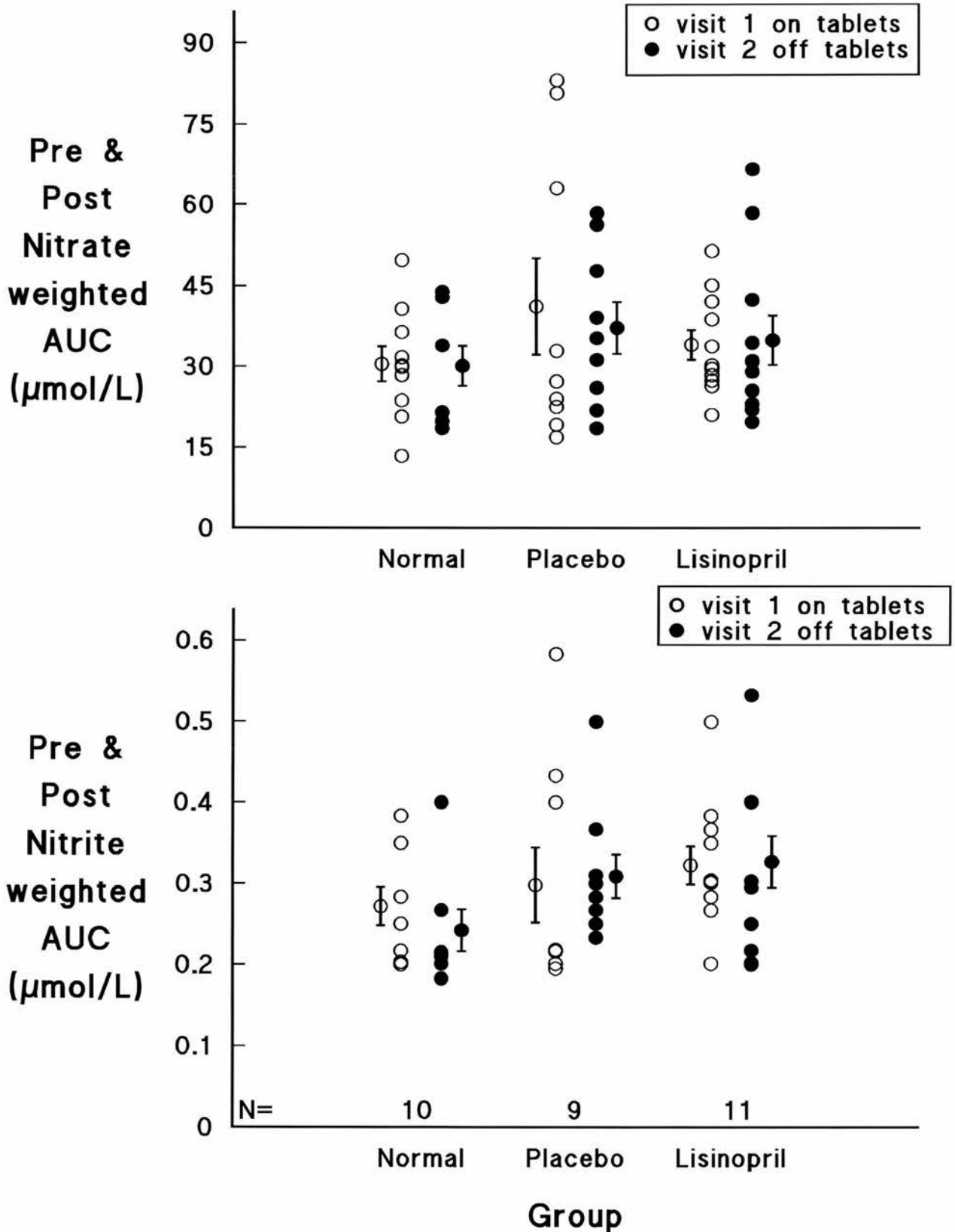


Figure 5.2: Plasma nitrate as a weighted area under the curve showing the two hyperlipidaemic groups, panel 1-placebo, panel 2-lisinopril, and the effects stopping treatment on each individual. Mean and SEM is seen beside the individual data.

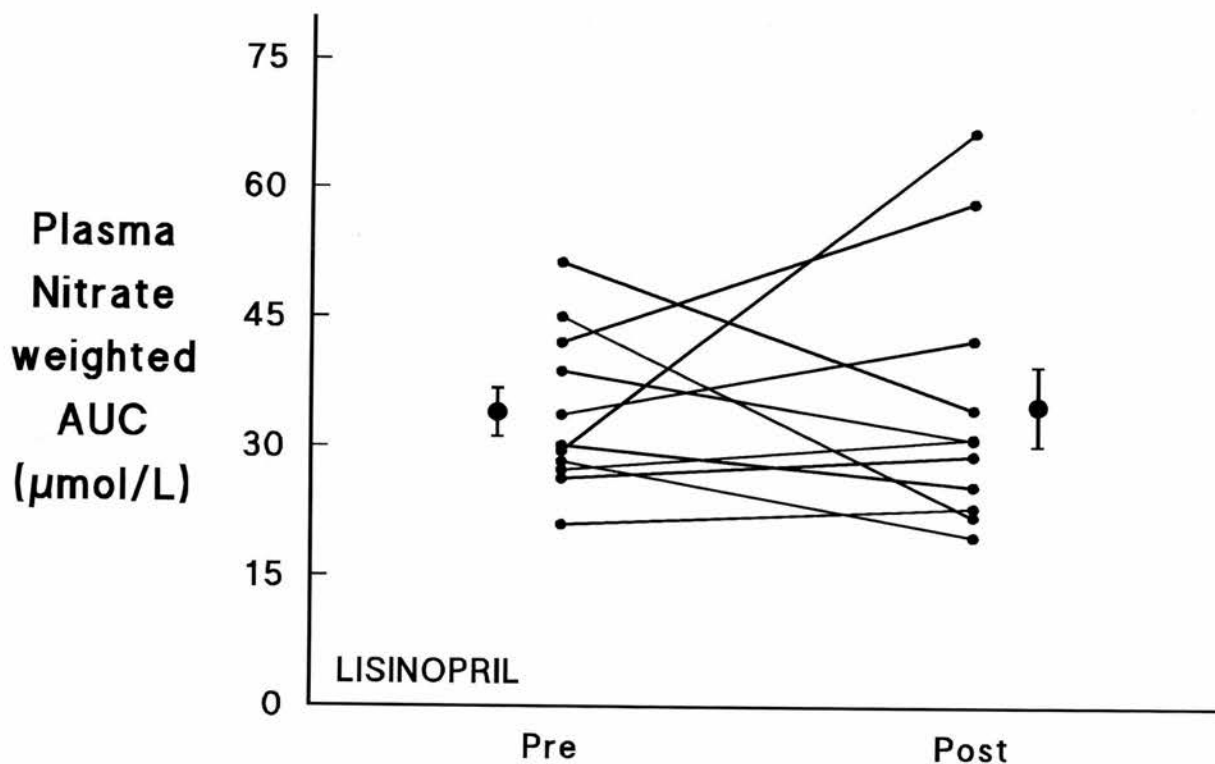
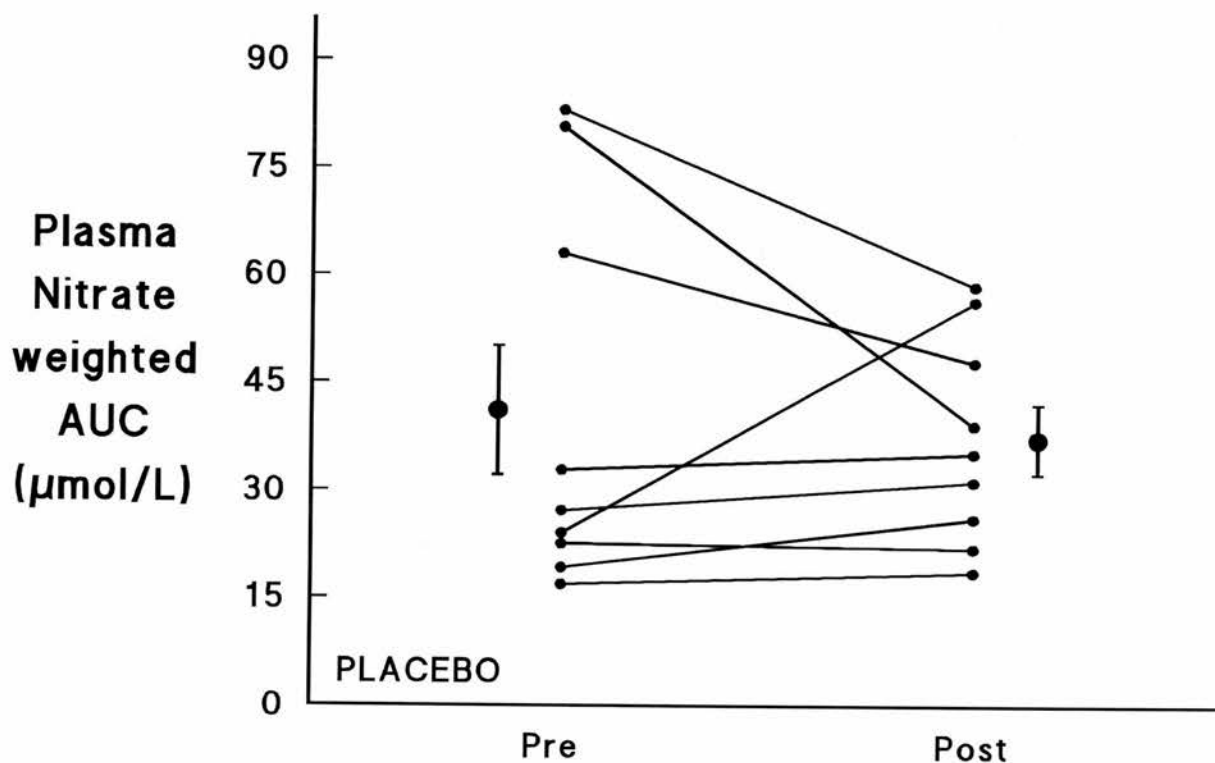


Figure 5.3: Correlation between areas under the curve for plasma nitrite and nitrate in all subjects at both visits.

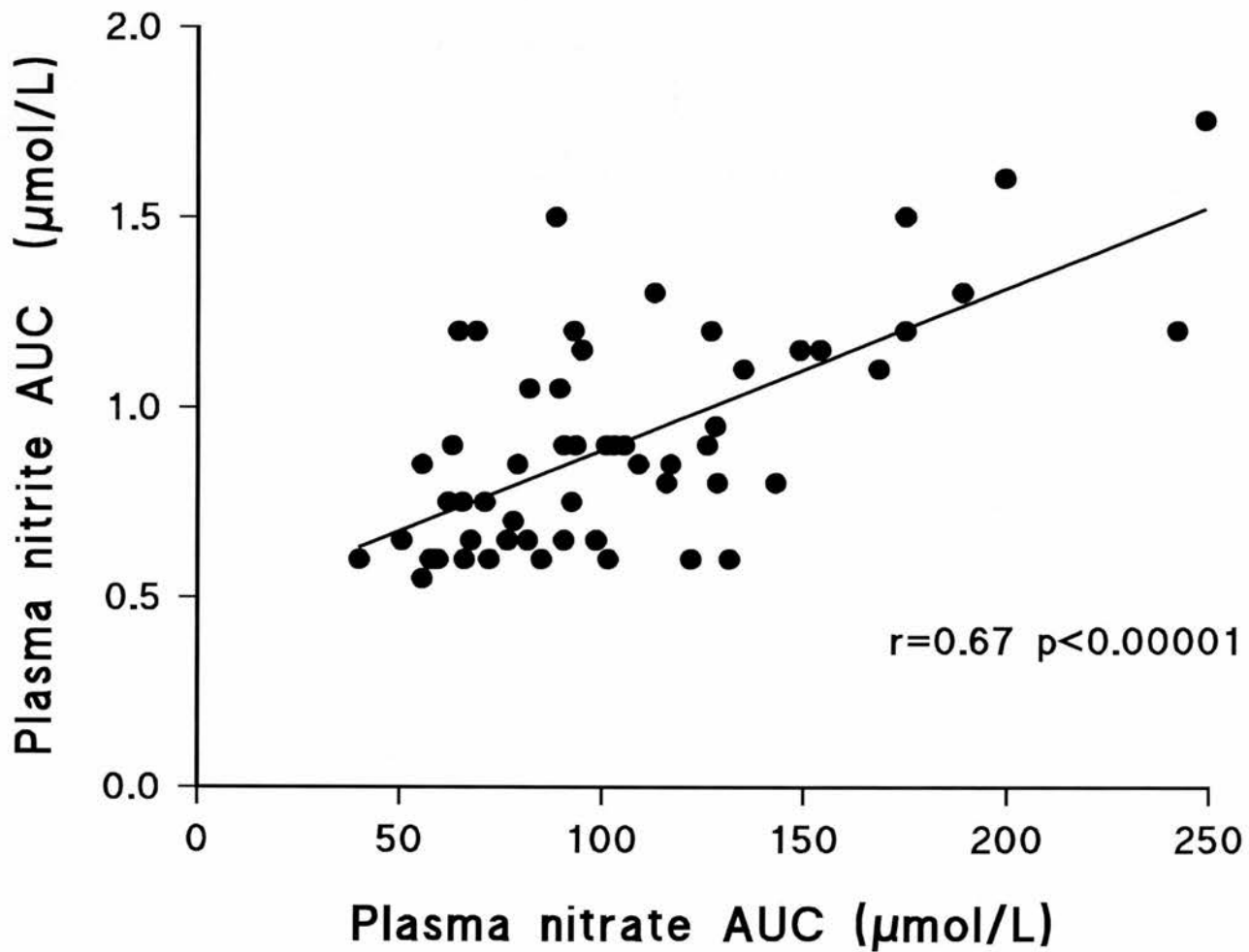
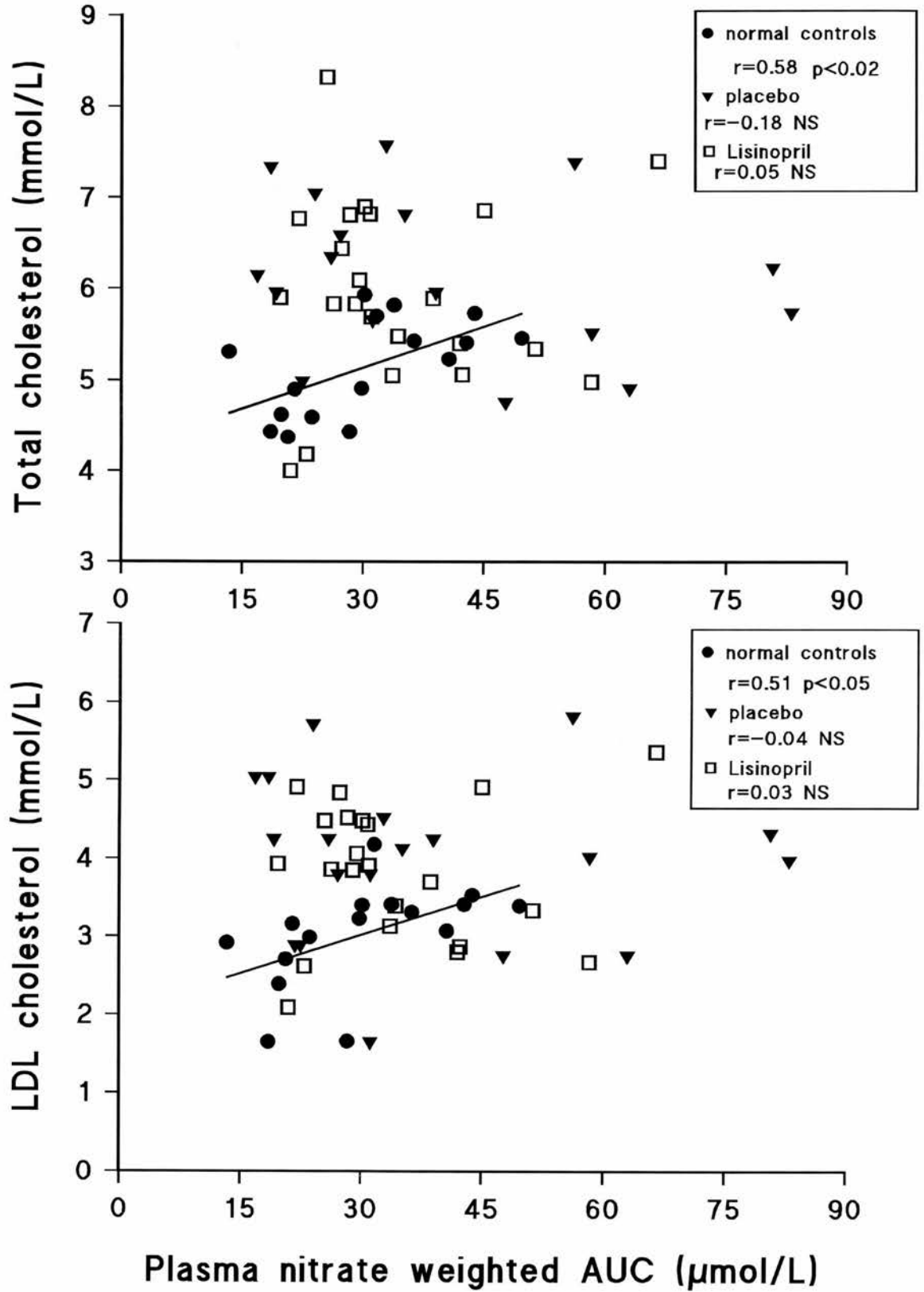


Figure 5.4: Correlation in the three groups between total cholesterol and LDL cholesterol and nitrate weighted AUC. There is a significant correlation only for the normal control subjects.



Discussion:

This study was a substudy of the previous chapter that was able to benefit from the fact the hyperlipidaemic subjects were already randomised to either placebo or active lisinopril tablets. Only half of the patients agreed to take part, and due to the double blind randomisation, there were eleven in the active arm and nine in the placebo arm. The control volunteers were taken from the general population and, as the mean cholesterol from the local population is 6.00mmol/L, it required several people to be screened for one subject to be eligible, particularly in the older age group, hence there were only eleven control subjects, six of whom agreed to repeat the fast after eight weeks although there was no difference between the two time points.

Baseline data:

Despite the relatively small numbers the baseline data was well matched between the groups except total and LDL cholesterol. There was some difference between the medication taken by the two groups with more of the lisinopril patients on HMG Co A reductase therapy than the placebo group. Furthermore, some of the hyperlipidamic patients were also taking cardioactive medications, particularly aspirin and calcium channel antagonists, whose effect on nitrate/nitrite levels are unknown. One patient in each group was taking HRT, which is believed acutely to affect plasma nitrite and nitrate (Cincinelli 1998), although the effects of longer term administration are unknown.

Nitrate/Nitrite data.

We did not demonstrate a significant difference in plasma levels of nitrate or nitrite between the three groups, which is disappointing. The large inter-individual variability seen in the figures show that a larger study would be required to definitively exclude any effect.

In humans it has been noted that plasma nitrite and nitrate are variable between individuals and also dependant on dietary intake, which may explain the marked variation in levels in the literature. Poveda et al (1997) in young healthy trained volunteers showed nitrite levels of $4.9\mu\text{mol/L}$, compared to $1.9\mu\text{mol/L}$ for untrained controls, which is six times our nitrite levels, although they do not appear to have fasted their subjects. Our basal levels agree with those found in both NIDDM and normal controls for nitrate (Catalano 1997), and are comparable to Ferlito et al (1997) in group of coronary artery disease patients.

Ferlito (1997) and Takahashi (1992) both noted an association with high total cholesterol and high nitrate production in their subjects, as we found in the normal controls. This is however in marked contrast to the work of Nakashima et al (1996), who found lower levels of nitrate/nitrite (NO_x) in hyperlipidaemic patients ($38 \pm 17\mu\text{mol/L}$) and found that levels were increased by treatment with Simvastatin for four weeks ($44 \pm 28 \mu\text{mol/L}$). They further showed that the percentage increase in NO_x showed a positive correlation with HDL change. This suggests that therapy with HMG Co reductase therapy may affect nitrite/nitrate levels, but not directly related to

the total cholesterol or LDL. The study by Nakashima (1996) does however have several flaws, not least the absence of a normo-cholesterolaemic control group. They also studied a heterogeneous population which drew together 26 patients with co-morbidity varying from hypertension, to arrhythmias and valvular heart disease whose effects on nitrate metabolism is unknown. Furthermore the fasting was stated to be “overnight” which may not be adequate to provide accurate basal nitrite and nitrate levels especially in those patients with co-morbidity.

The failure to demonstrate beneficial effects of ACE inhibitors on excretion of nitrite and nitrates is somewhat surprising, especially given the results from the previous chapter. Furthermore, as ACE inhibitors are known to prolong the activity of bradykinin, which is known to increase endothelial release of nitric oxide (Horning 1997), it would be expected that nitrites/nitrates should increase on treatment, even in the absence of alterations in superoxide radicals due to reduction of angiotensin II. It is possible that after five months treatment with an ACE inhibitor the effects on bradykinin have achieved a plateau, where although there is no excess production of nitric oxide that produced has a greater effect, hence the results presented in the previous chapter which showed lisinopril was beneficial in hyperlipidaemia

HMG Co A reductase inhibitors were unevenly balanced between the groups, and evidence from the literature suggests that this family may alter nitrite/nitrate metabolism.

Simvastatin and pravastatin are well known to improve mortality post infarct (Sacks 1996, Scandinavian Simvastatin Survival study 1994), and also to improve endothelial function in hypercholesterolaemic patients (Treasure 1995, Egashira 1994).

Furthermore, in rats applied statin appears to up regulate vascular endothelial Nitric Oxide Synthase (eNOS) (Endres 1998), and in cell culture statins have been shown to inhibit the suppression of eNOS by oxidised LDL (Hernandez-Perera 1998). They have also been shown to affect the levels of nitrite and nitrate in plasma, within the limitations of that study as discussed above (Nakashima 1996). Within our data set we tried to look at the population of patients on statin therapy against those on fibrate therapy and found that there was no significant differences in the mean levels of nitrate, 39 ± 4.4 vs 34 ± 3.3 $\mu\text{mol/L}$ respectively. We also went on to look at correlations between nitrate levels and cholesterol differentiating between the two types of therapy, and found no significant correlation in either drug type, although the numbers are small.

There is interest in the literature in the specific effects of statin therapy on endothelial function, arterial structure, and on nitric oxide synthase levels, as some of their effects appear to be in excess of, or unrelated to their effects on cholesterol lowering (Byington 1995). In cell culture HMG CoA reductase inhibitors appear to antagonise the suppression of nitric oxide synthase by oxidised LDL (Hernandez-Perera 1998), and they can also reduce smooth muscle cell proliferation (Bellosa 1998). Furthermore pravastatin has direct vasodilatory effects in isolated aortic rings, that is not associated with alterations in cholesterol (Kaesemeyer 1999), but appears to be due to alterations in nitric oxide release. It may be that those individuals on statin therapy have several influences on the production and catabolism of nitric oxide and this has affected these results.

It is somewhat surprising that lisinopril as an ACE inhibitor did not alter whole body nitrate/nitrite excretion, since ACE inhibitors are known to potentiate bradykinin in the human vasculature, which is believed to cause nitric oxide dependent vasodilation. On the other hand, the increased NO bio-activity seen with ACE inhibitors may relate more to withdrawal of AII induced superoxide radicals which inactivate NO. If this were the case then nitrate/nitrite levels would be unchanged, or even lower. Clearly our neutral result could be due to a combination of these two opposing forces.

Other potential reasons for our unexpected results may be the ubiquity of nitric oxide and the huge potential for other confounding factors, even with the stability of the diet and medication in this group. Nitric oxide synthase is used not only in vasomotor tone, but also in platelet function, in macrophage metabolism (Akiyama 1998), in myocardial cell death (deBelder 1993) and also as a paracrine messenger in many systems (Kato 1992, Corbett 1993, Costa 1993). It is likely that the total body nitrate will be the sum of all these various aspects, and that alterations in the vascular wall that cause benefits in endothelial function as seen in the previous chapter produce only tiny effects on the total body nitrate and nitrite. In this case our sample size may not have been adequate to demonstrate this. Indeed, it has been shown that even the application of acetylcholine, which is believed to release nitric oxide in the forearm bed does not alter the nitrate and nitrite in the venous effluent plasma, which might mean that other factors are more important in the overall production of plasma nitrate/nitrite (Butler 1998).

The other potential cause for the disparity in the expected effect and the result may relate to the difficulty in measuring these ions. The assay used here is believed to be one of the most reliable and sensitive available, and has the advantage of allowing simultaneous assessment for both nitrate and nitrite. However some of the contradictions in the literature may relate to the large variation in the methods of assay used, and the techniques for fasting and the use of concomitant medication. This study attempted to minimise these effects by using a specific assay, and strict fasting protocol.

Summary Points:

- The previous chapter suggested that nitric oxide bio-availability may be improved in hyperlipidaemics on lisinopril. This chapter went on to look at the whole body excretion of nitrate and nitrite as the breakdown products of nitric oxide, before and after cessation of the trial medication.
- Previous studies in hyperlipidaemia have given variable results on the effect of cholesterol levels on nitrate/nitrite excretion.
- The results of this chapter show that while there was some evidence that the normal control population had lower levels of nitrite and nitrate, this was not statistically significant, and there was no evidence that treatment with an ACE inhibitor had any effect on the levels of nitrate, or nitrite in the patients.
- These results may reflect the many factors that influence whole body nitrate and nitrite, including fasting state, and medication. Furthermore, the work in the literature suggests that statin therapy may affect nitrate and nitrite levels, and a proportion of the subjects studied were taking these drugs which could have influenced the results.
- While this chapter has not shown evidence of an effect of lisinopril on whole body nitrate excretion the sample size may not have been adequate to avoid a type II error.

CHAPTER 6:

Ovarian hormones in man: their effects on resting vascular tone, angiotensin converting enzyme activity and angiotensin II induced vasoconstriction.

This chapter looks at a potential interaction between female ovarian hormones and the renin angiotensin system. Specifically it looks at whether or not the acute vasodilation seen when oestrogens are administered in human models is due to an interaction between these hormones and vascular ACE activity, or the vasoconstriction seen with angiotensin II.

Introduction:

Pre-menopausal women have a lower prevalence of atherosclerotic vascular disease (Stevenson 1994), which is believed to relate to endogenous ovarian hormones, in particular oestrogens. Hormone replacement therapy reduces cardiovascular events although not stroke in the post-menopausal population (Stampfer 1991), and only part of this benefit is mediated by alterations in lipids profiles (Stevenson 1994).

Oestrogens appear to also have specific effects on the vascular tree, causing vasodilation, and increasing responses to applied vasodilators. Local and systemic oestrogens cause vasodilation within 60 minutes (Magness 1989) and resistance vessel blood flow varies with the menstrual cycle in premenopausal women (Bartelink 1990). Benefits of oestradiol have also been seen acutely in clinical situations, Rosano et al (1993) showed that in women, with known ischaemic heart disease, exercise tolerance is increased within 40 minutes of sub-lingual oestradiol, which they presumed to be secondary to increased myocardial blood flow via coronary vasodilation.

In vitro and in vivo, oestrogens have been suggested to cause vasodilation by multiple mechanisms, i.e. via nitric oxide (Gilligan 1994c, Kawano 1997) via calcium channel blocking type mechanisms (Belfort 1996), via prostaglandins (Belfort 1995) or via

endothelial independent mechanisms (Bardett Faucett 1999). However recently there has been a suggestion that oestrogens may interact with the renin angiotensin system. In chronic dosing studies, hormone replacement has been shown to reduce angiotensin converting enzyme activity in both primates and man (Brosnihan 1997, Proudler 1995, 1996). Proudler et al (1995) found that hormone replacement therapy, as a combination of oestrogen and progesterone, causes a reduction in serum ACE activity in post-menopausal women, and followed by observing that serially administered oestrogens and progesterone can reduce serum ACE in this population (Proudler 1996). They do, however, note that the preparation of HRT appears to be important in producing this effect. Mabe et al (1992) in male subjects, suggested that female hormones may act at a later stage in the renin angiotensin system, by reducing the pressor responses to angiotensin II, an effect that has also been shown in women (Magness 1994).

Men are at greater risk of atherosclerotic vascular disease than women. Previous clinical studies in males using high dose oral oestrogens have shown a higher mortality in the treatment group, largely due to thromboembolic events, hence they were stopped early (The Coronary Drug Project Research Group 1973). This observation may relate to recent work which suggests that oestrogens cause activation of inflammatory markers, such as C-reactive protein (Ridker 1999), and hence increased thrombotic events. However there is good evidence to suggest that administered oestrogens may have beneficial effects in the male vasculature. Oestrogen receptors and aromatase enzyme are present on human male and female coronary endothelial cells (Diano 1999), and the data presented by Diano et al (1999) suggests that

diseased arteries have greater immuno-staining for aromatase enzyme than non diseased vessels.

Recent clinical studies have shown that intra-venous oestrogen administration improves coronary blood flow to acetylcholine (Blumenthal 1997) and cold pressor test (Reis 1998) within 15 minutes of administration. These are supported by long term studies in men, where endothelial response in male to female transsexuals approach those of females after prolonged treatment with fairly high dose ovarian hormones. This effect was not due to alterations in cholesterol and fractions, which were not significantly changed by treatment (McCrohon 1997).

We therefore chose to study normal male volunteers, because their oestrogen and progesterone hormone status could be more easily manipulated, without any bias from endogenous cyclical hormones. Furthermore, the above evidence led us to believe that oestrogens are likely to cause acute vascular effects in human males, and we could therefore study vascular ACE activity and its importance in vasodilation due to oestrogen.

A major problem in assessing ACE activity is that a large amount of ACE is bound to the luminal surface of the endothelial cell. To study this "vascular ACE" we used a technique used previously by Davidson et al (1996a) which compares vasoconstriction in response to angiotensin I (which requires conversion via ACE to angiotensin II prior to biological activity), to vasoconstriction in response to applied angiotensin II. This model therefore assesses both the effect of oestrogen on the activity of vascular ACE and on vasoconstriction to angiotensin II.

This study was designed to see whether ovarian hormones cause vasodilation in males, and whether in doing so they alter either vascular ACE, or angiotensin II responses. In addition we looked at the effect of acute dosing with oestradiol and medroxyprogesterone on serum lipids, as Bagatell et al (1994) has shown high density lipoprotein levels are sensitive to hormonal change in males, and lipids levels are known to affect vascular responses (Anderson 1995a).

Methods:

Healthy, non-obese, male volunteers, were recruited by word of mouth and advertising. Eight subjects were used, age range 20-35 years. At the beginning of each study the subject's general practitioner was informed of the study protocol and duration. All subjects had normal clinical history and examination, 12-lead electrocardiogram, and haematological and biochemical profiles. Subjects had taken no medications for at least one month before the study. The subjects had minimal risk factors for vascular disease, 4 subjects smoked between 5-10 cigarettes/day, no subject was hypertensive, hyperlipidaemic, or diabetic, or had a family history of vascular disease.

The study was of a double blind randomised crossover design. Randomisation was done by a third party prior to the start of the study. Order of medication was randomly assigned and all trial medication was boxed as sets of three treatments which were numbered and allocated sequentially to subjects. Medication was taken as two doses, the first twelve hours before and the second two hours before the study. All subjects took 2 mg oestradiol valerate per dose (Climaval, Sandoz), 20 mg medroxyprogesterone acetate per dose (Provera, Upjohn), or one placebo tablet per dose. In addition, except to take the medication, all patients had fasted for a minimum of 12hrs, and had abstained from caffeine, alcohol and cigarettes for at least eight hours before the study, which was performed in an air conditioned room controlled between 23-24°C. Blood was taken for fasted total cholesterol, high density lipoproteins (HDL), triglycerides (Trigs), and circulating ACE activity; further serum

samples were collected for oestradiol, progesterone, and testosterone, on arrival in the department.

On each study day the subject underwent forearm plethysmography as described in Chapter 2.

A 27G needle was inserted into the brachial artery of the non dominant arm under local anaesthesia, and 0.9% saline was infused for 30 minutes prior to the establishment of baseline readings. Strain gauge measurements were taken at five minute intervals to establish three consecutive readings which were within 10%. The mean ratio of measurements from both arms at these time points was taken as the baseline ratio of forearm blood flow.

Angiotensin I and II were supplied by Calbiochem/Novobiochem Nottingham, England, and were the same batch for all studies. Peptides were made into solution within 45 minutes of the study starting. The peptides were dissolved in 0.9% saline, and the infusion rate was kept constant at 1ml/min throughout the study period (Grasby 310 syringe pump, Grasby Medical). Discussion with the company established that the peptides were stable for up to 24hr after reconstitution.

Following the establishment of baselines, angiotensin I was infused at three concentrations, 12 pmol/min, 24 pmol/min, and 48 pmol/min which were expected to produce 25%, 35% and 50% reduction in blood flow under normal conditions. Each dose was infused for 7 minutes in total, with readings being taken over the last two minutes of each infusion period. Following the third dose, 0.9% Saline was infused until the ratio between the blood flow in both arms had returned to baseline. A second series of infusions were then started, with angiotensin II, which was infused at 4 pmol/min, 8 pmol/min, and 16 pmol/min; again these concentrations were expected to

produce 25%, 35%, and 50% reductions in flow. An identical protocol was followed on each study day.

Statistical analysis: Comparisons between the biochemical parameters were performed using students t-test. For statistical analysis, the ratio of flow measurements from the infused arm over those from the control arm was used, as was the percentage reduction in absolute flow from baseline, both of these measurements are accepted as standard ways of expressing forearm flow data (Benjamin 1995, Chindusting 1999). They have the advantage of having a contemporaneous internal control, and allowing adjustment for alterations in baseline blood flow. The reductions in flow to angiotensin I and II were compared on each study day by calculating the area under the curve, and comparing these using paired students t-tests (Matthews 1990). All results are expressed as mean \pm SEM, and statistical significance was accepted for p values <0.05 .

Results:

There was no subjective, or objective evidence of side effects of hormone treatment.

Biochemical parameters:

Serum lipids and ACE concentration: Table 6.1 shows the figures for the results of treatment on circulating ACE activity, and total cholesterol, including HDL and triglycerides. We failed to demonstrate a statistically significant difference between the two treatments or between either treatment and placebo.

Serum Hormone levels: Table 6.1 shows the levels of oestradiol, progesterone and testosterone measured in the samples taken prior to each blood flow study. These samples were therefore taken two hours following the second dose of treatment, and twelve hours after the first dose. Testosterone levels were unaffected by the short term treatment. Oestradiol levels rose in all volunteers, with an increase in the means from 76.8 ± 14 on the placebo day, to 163.6 ± 22 pmol/L ($P < 0.0001$) this is comparable to the menstrual phase in pre-menopausal females (Bartelink 1990). Progesterone was also measured, but medroxy-progesterone does not cross react with the assay hence no variation was seen.

Blood Flow Results:

Baseline: Oral oestradiol produced significant baseline vasodilation against placebo in the subjects, see Table 6.1 and Figure 6.1, with no significant vasodilation to progesterone compared to placebo.

Vasoconstrictors: Figure 6.3 shows the absolute blood flow on the three treatment days in response to the vasoconstrictors. In figure 6.2 blood flow is expressed as the ratio between the flow in the infused arm against the control un-infused arm. A fall in this ratio corresponds to vasoconstriction. There were no significant differences noted between the treatments, except at baseline. These figures demonstrate the advantage of using the internal contemporaneous control of the contra-lateral arm to allow for alteration in baseline flow.

Table 6.1: Biochemical parameters, and baseline forearm blood flow following treatment with oestradiol or medroxyprogesterone against placebo.

	<u>Treatment</u>		
	Placebo	Oestradiol 4mg	Medroxyprogesterone 40mg
n=8			
Serum ACE activity in units/L (mean ± SEM)	42.8±8	38.2 ±5	48 ± 7
	<i>Serum hormones (mean± SEM)</i>		
Oestradiol [pmol/L]	77±5	164±8** p<0.0001 vs. placebo	76 ± 6
Progesterone [nmol/L]	1.8 ±0.1	1.6 ±0.1	1.8 ± 0.1
Testosterone [nmol/L]	28 ± 0.7	26 ± 0.5	26 ± 0.9
	<i>Serum lipids in mmol/L(mean ± SEM)</i>		
Cholesterol	3.8 ± 1.1	3.9 ± 0.8	3.8 ± 0.8
HDL	1.13 ± 0.3	1.19 ± 0.3	1.17 ± 0.3
Triglycerides	0.94 ± 0.6	0.99 ± 0.8	0.93 ± 0.3
	<i>Forearm blood flow ml/100ml/min</i>		
Baseline blood flow (mean ± SEM)	1.5 ± 0.1	2.0 ± 0.2* p<0.03 vs. placebo	1.6 ± 0.2

Figure 6.1: Baseline blood flow with placebo, and oral oestradiol in all subjects, with mean \pm SEM annotated at the

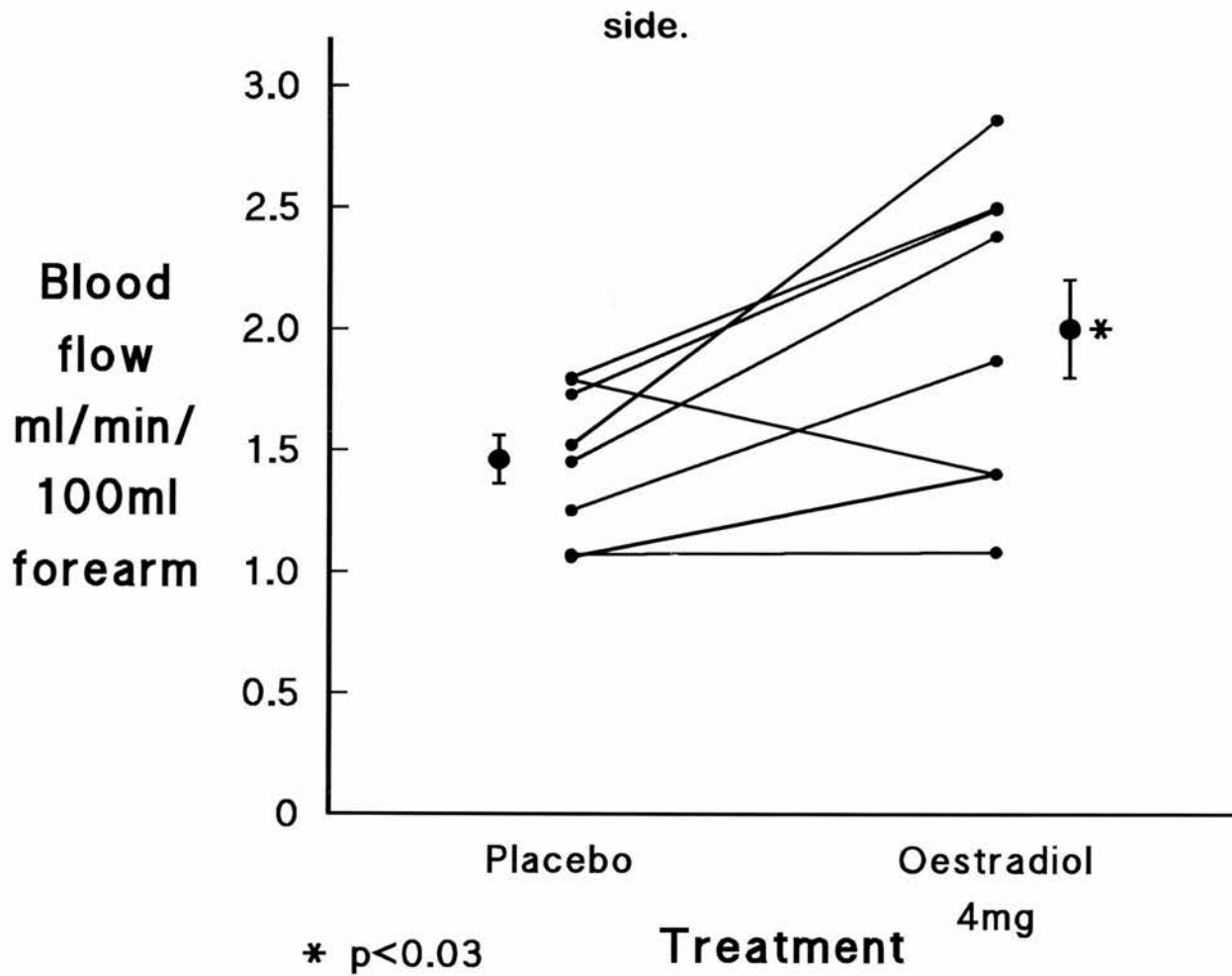


Figure 6.2: Ratio of blood flow in infused arm/control arm during infusions of angiotensin I and II, with oral oestradiol, medroxy-progesterone and placebo. (mean \pm SEM)

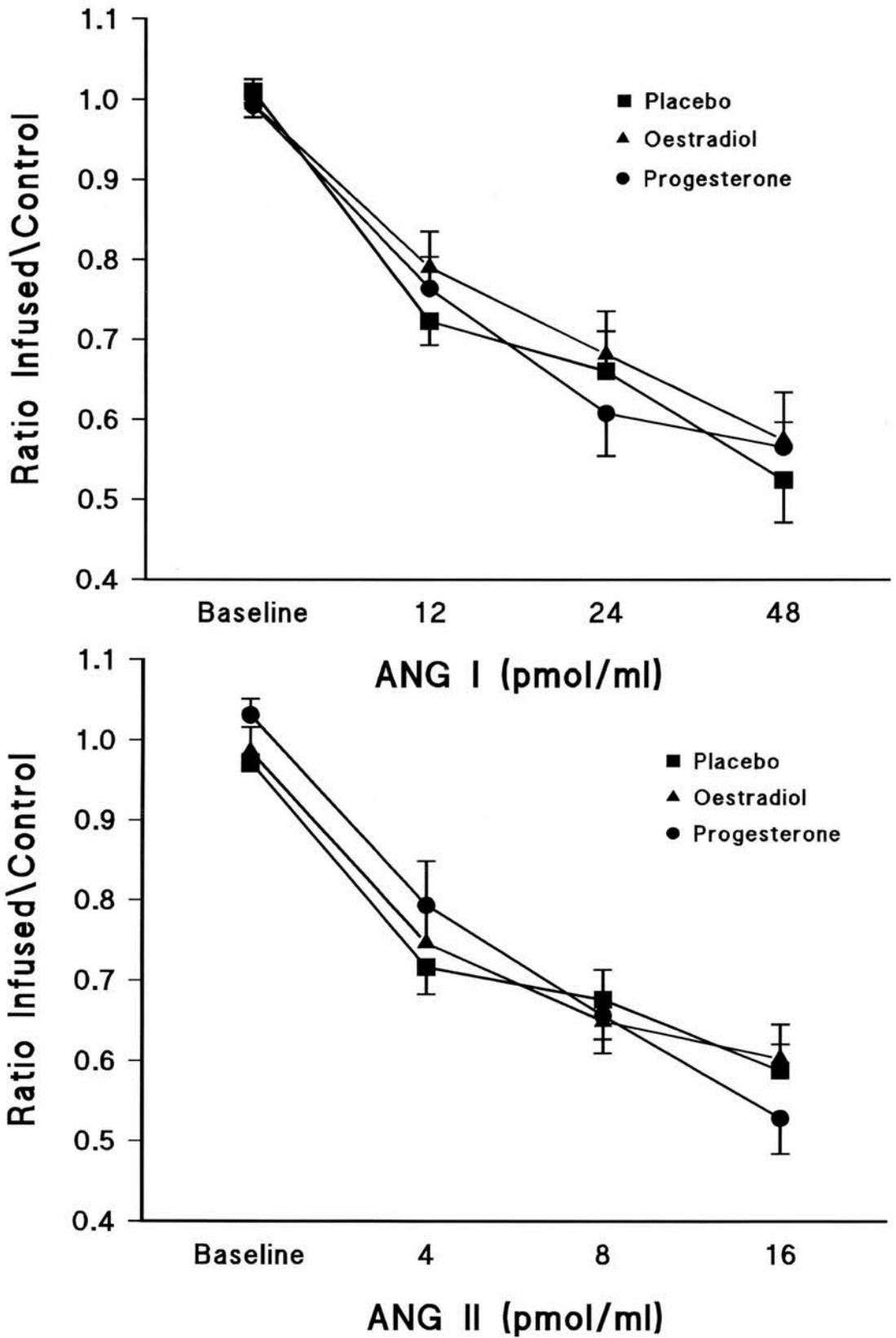
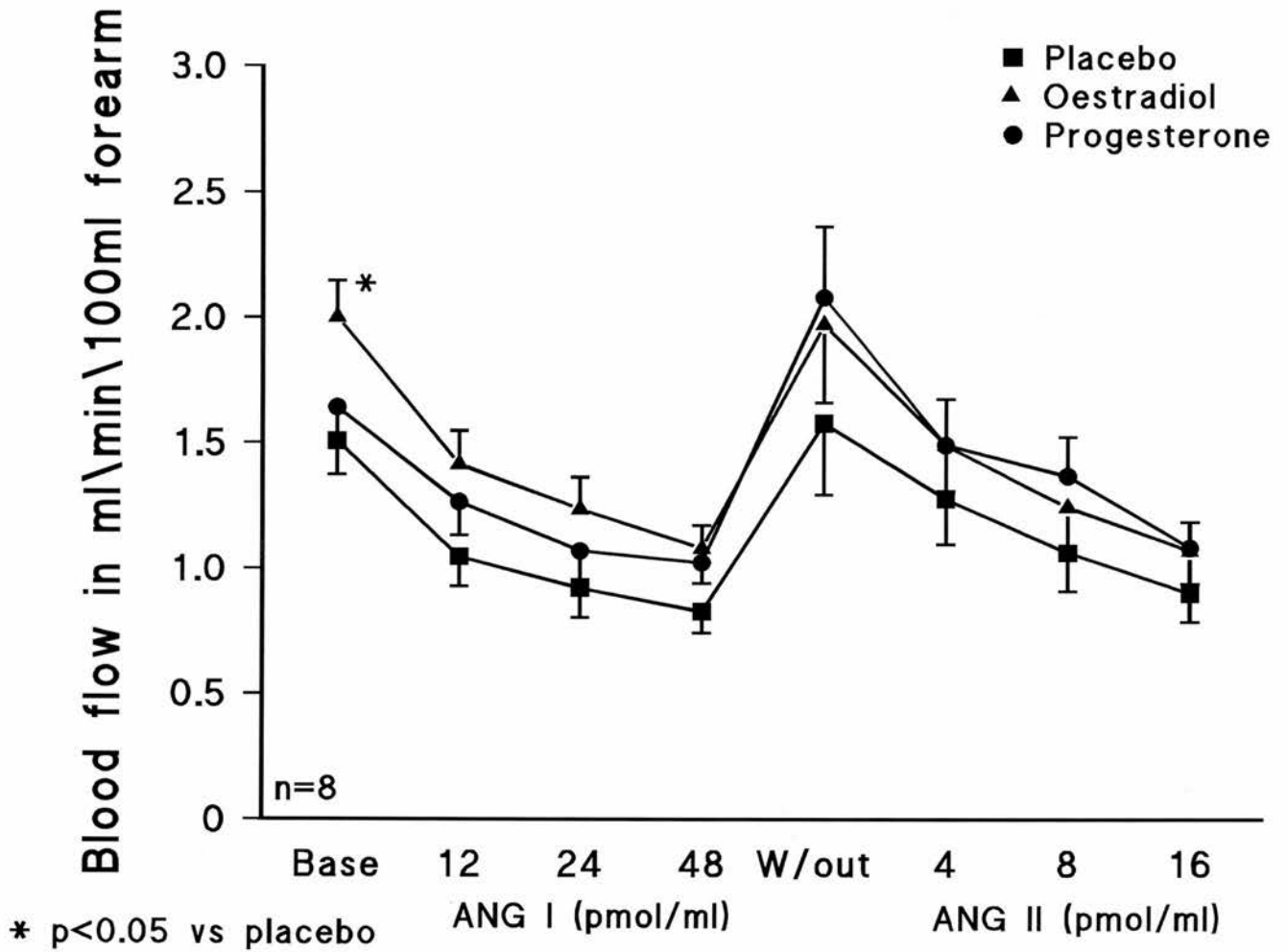


FIGURE 6.3: Absolute blood flow in ml/100ml forearm/minute in the subjects on oestradiol, medroxyprogesterone, and placebo, and the effects of angiotensin I and II induced vasoconstriction.



Discussion.

This study has shown that in normal male volunteers, short term dosing with female ovarian hormones causes vasodilation in the subjects, which is consistent with the effects that have been noted in animals (Magness 1989, Davis 1992), women (Hilliard 1992, Pines 1991) and in vitro (Belfort 1995, 1996). We found that neither oestradiol or medroxyprogesterone had any effect on ACE activity, assessed either as plasma ACE or as vascular ACE activity. Similarly we found no effect of oestradiol, or medroxyprogesterone on the vasoconstrictor response to angiotensin II. This evidence suggests that the acute effects of oestradiol in causing vasodilation in males are not due to alterations in the vascular response to either angiotensin I or angiotensin II.

There has been great interest in the mechanism behind the acute vasodilatory effect of oestradiol, and its ability to potentiate the effects of vasodilators in animals (Williams 1990, 1992) women (Gilligan 1994a, 1994b, Kawano 1997), and more recently in men (Blumenthal 1997, Reis 1998). Interest has been heightened by the demonstration that sublingual oestrogen has an anti-anginal effect, if given acutely (Rosano 1993). Early in vitro work suggested that the effect might be endothelial independent, as denudation of the arterial endothelium did not affect the relaxation induced by oestradiol, and might relate to calcium channel antagonism (Jiang 1991). However there is increasing in vivo evidence to suggest that oestrogens have direct vascular effects (Pines 1991, Williams 1992).

Our data does not support the belief that in men acute vasodilatory effects are due to decreased activity of the ACE enzyme, either serum ACE or endothelial ACE. This model however does not give any data on longer term effects. Proudler et al showed

evidence of reduction in ACE activity in post-menopausal women after six months treatment (Proudler 1995), and reductions in serum ACE has been shown in cynomolgus monkeys after eight months (Brosnihan 1997), but this is the first study to look at acute effects of oestrogens in human males.

In vitro data using rat tissues, has suggested that an observed reduction in ACE activity may reflect alterations in mRNA concentration, and hence reductions in protein synthesis leading to a fall in ACE concentration (Gallagher 1999). This may be why we have failed to demonstrate a fall in ACE activity using short oestrogen dosage, in contrast to the longer term studies in the literature. Proudler et al (1995) demonstrated effects on ACE in women and there is little in the literature to suggest that the actual ACE protein has a different profile between the sexes. For example, in the major post infarct trials (Latini 1995) or the major heart failure trials (Garg 1995), direct ACE inhibition had similar beneficial effects on men and women.

Oestradiol may have several mechanisms of action, as the results in vitro are conflicting. However, in clinical studies there is mounting evidence that they enhance nitric oxide release, or alter metabolism. Acute transdermal oestrogen has been shown to increase the production of nitric oxide metabolites in women (Kawano 1997, Cincinelli 1998). Furthermore, in post-menopausal women, co-infusion of oestradiol augments the increases in arterial flow in response to acetylcholine (Gilligan 1994a), and this effect can be blocked by the addition of a nitric oxide synthase inhibitor (Gilligan 1994c). It seems likely that at least part of the acute vasodilation is due to alterations in nitric oxide release or metabolism, because oestrogens have been shown to improve nitric oxide mediated flows in both men and women, suggesting at least potentiation of nitric oxide (Reis 1998, Gilligan 1994a).

The use of young male subjects limits the scope of our results, however we have shown that even in this group oestrogens have relatively acute effects, and that these acute effects are not due to alterations in ACE activity either in plasma or on the vascular endothelium. Our results differ from those of Mabe et al (1992), who showed that progesterone, but not oestrogens in young men could increase the dose of angiotensin II required to cause a 20mmHg increase in diastolic blood pressure. They used a combined protocol with six days of norethisterone following on from six days of mestranol rather than oestradiol, and the study days for the progestogen may have still been influenced by the effects of the prior oestrogen, as there was no washout phase. We have not shown any effect of progesterone on angiotensin II induced vasoconstriction in our model.

The present study looked at the vasoconstrictor responses to angiotensin I against those of angiotensin II to examine vascular ACE activity in vivo in man. Substances that block angiotensin converting enzyme selectively alter angiotensin I responses, and this technique has previously been used to show that C-type natriuretic peptide is an endogenous inhibitor of vascular ACE (Davidson 1996a). It is possible that we did inhibit the ACE enzyme, but failed to observe the effect because other pathways converting angiotensin I to angiotensin II were operative. However enalaprilat, a specific ACE inhibitor infused into the forearm almost completely abolishes vasoconstriction to angiotensin I (Benjamin 1989) suggesting that alternative conversion pathways are not significant in the forearm in normal males.

Another limitation of this study is the relatively small sample size. A previous study from our group with similar subject numbers have demonstrated vascular ACE inhibition by C-type natriuretic peptide (Davidson 1996a). In the present study, from

available statistical tables the number of subjects had 65% power to show a 30% fall in vasoconstriction to angiotensin I, i.e. from -48% to -32%, at 0.05 significance (Machin 1987). Davidson et al (1996a) showed that C-type natriuretic peptide caused a 60% fall in the vasoconstriction to angiotensin I with the similar model. Therefore although the sample size was small we do not feel that we have failed to detect important inhibition of vascular ACE.

Hormones and Lipids: As the main end points in this study are negative, it is important to confirm that subject compliance was adequate. All eight subjects demonstrated 60-100% increases in serum oestradiol on the oestradiol treatment day, which suggest that the subjects were very compliant with treatment. Unfortunately, no assay is available to determine medroxyprogesterone levels and so we were unable to check compliance on the medroxyprogesterone limb of the study. As the treatments were double blinded it is unlikely that the subjects would have selectively taken the oestrogen, and not the progesterone.

While we demonstrated no effect on serum lipids, Bagatell et al (1994) inhibited endogenous oestrogen production in a group of males for four weeks and found that the plasma HDL level fell when circulating endogenous oestradiol was pharmacologically reduced. Our study was a short term study which is probably why we failed to demonstrate changes in plasma lipids. In genetic males taking long term high dose oestrogens, HDL levels approach those of females, showing that over the longer term ovarian hormones have lipid effects in males (New 1997). This evidence supports the belief that circulating oestrogens do influence the production of cholesterol and lipid fractions in genetic males.

Summary Points:

- Studies in post menopausal women have suggested that HRT can affect circulating ACE activity and this may be one of the mechanisms behind the acute vasodilatory response to oestrogen. Males have been shown to have improved coronary vasodilation to endothelial stimuli with acute intravenous oestrogen.
- The aim of this study was to see if oestradiol or medroxy-progesterone cause acute vasodilation in men, and if so does this relate to effects on either circulating or vascular ACE activity or angiotensin II induced vasoconstriction.
- The results showed that acute dosing with oestrogens causes baseline vasodilation in normal male volunteers, in contrast to acute medroxyprogesterone administration.
- Ovarian hormones had no effect on lipids or lipid fractions in this population.
- Neither oral oestradiol, or medroxy progesterone had an effect on circulating angiotensin converting enzyme activity, or vascular ACE activity as assessed by the differential vasoconstrictor response to angiotensin I and angiotensin II.
- The results presented suggest that the acute vasodilatory response to administered oestrogen is not dependent on an interaction with the renin angiotensin system. Evidence in the literature suggests that it may be due to alterations in nitric oxide synthesis.

CHAPTER 7:

Interaction between the renin angiotensin system and nitric oxide. Does nitric oxide inhibition influence renin release?

Introduction:

So far the previous chapters have looked mainly at the effects of blocking renin angiotensin activation via ACE inhibition in a variety of clinical situations. In chapter five I was unable to identify a direct link between the release of nitric oxide and angiotensin reduction via ACE inhibition. This chapter looks at the reverse angle, i.e. to see if inhibition of nitric oxide has a direct effect on renin release, as the first step in the renin angiotensin cascade, suggesting that the two hormone systems are inter-related.

There are several potential reasons that nitric oxide could affect renin release. At a very basic level it is believed that nitric oxide is responsible for the maintenance of basal vascular tone, and peripheral resistance, which would feedback via the sympathetic nervous system to alter renin release (Stamler 1994).

A second potential method might be the direct use of nitric oxide as a signalling molecule in the renal parenchyma. There has recently been much experimental evidence to suggest that nitric oxide is important in the control of the secretion of a variety of hormones, including pituitary secretion of growth hormone (Kato 1992), insulin from pancreatic islet cells (Corbett 1993), and hypothalamic secretion of CRF (Costa 1993). This evidence would suggest that nitric oxide may be important as a signalling molecule in these situations, possibly working in a paracrine fashion.

Isoforms of nitric oxide synthetase have been found in the macula densa cells around the renal tubules (Mundel 1992), i.e. the same cells that are believed to control the secretion of renin from the juxtaglomerular cells (Hackenthal 1990). Nitric oxide synthetase has also been localised in a variety of other sites within the kidney

including vascular and tubular elements (Reid 1995a). Furthermore, activation of nitric oxide synthetase in the macula densa appears to occur under conditions which would be expected to increase renin secretion, e.g. frusemide stimulation (Reid 1995b). In whole animal experiments, mainly in rats, nitric oxide antagonists can be shown to inhibit the release of renin in response to frusemide, which is believed to be a macula densa stimulus (Itoh 1985). This suggests a permissive role for nitric oxide in renin release due to frusemide (Reid 1995b, Beierwaltes 1995, Johnson 1994).

However considering pressure dependant renin release the results are conflicting. Sigmon et al (1992) found that the inhibition of renin by nitric oxide antagonists was due to their effects on renal perfusion pressure, and that if this was controlled, the inhibition of renin could be reversed. They showed that if renal perfusion pressure was maintained by an aortic balloon, and sympathetic stimulus controlled by β blockers, then nitric oxide blockade by L-NAME actually increased renin secretion. However, more recently Reid et al (1995b), using a rabbit model, have demonstrated that the effect is not pressure dependant, but is in contrast, a direct effect of nitric oxide inhibition, as renin was not suppressed by an equipressor dose of phenylephrine.

The above evidence suggested that there might be a direct and specific role for nitric oxide in the control of the release of renin from the kidney. We therefore investigated the effect of nitric oxide blockade on renin secretion in response to a small intravenous bolus of frusemide. We also extended our study to examine whether any observed effect on renin release was due to its pressor effect per se, or whether it was a specific and direct effect of nitric oxide independent of pressure as suggested by Reid et al (1995b).

Materials and methods:

Ten healthy, non-obese male volunteers were recruited by word of mouth and advertising. At the beginning of each study the patients general practitioner was informed of the study protocol and duration. All had normal clinical history and examination, 12-lead electrocardiogram, echocardiogram and haematological and biochemical profiles. Subjects had taken no medications for at least one month before the study. The subjects had minimal risk factors for vascular disease, 4 subjects smoked between 5-10 cigarettes/day, no subject was hypertensive, hyperlipidaemic, or diabetic, or had a family history of vascular disease.

Volunteers were studied at the same time of day on two occasions in a randomised, double blind, cross-over design. Subjects lay supine for one hour prior to the start of the study to establish baseline haemodynamics, and renin levels. Baseline readings were taken ten minutes before (-10mins) and at the start of the infusion (Time 0).

After the rest period in a double blind fashion they received either a front loaded infusion of 4mg/kg of N^G-monomethyl L-Arginine (OXONON BioAnalysis, Emeryville, California) dissolved in 25mls N Saline, over 50 mins or volume matched infusion of 0.9% N Saline. At the start of the infusion, a priming bolus of either volume matched placebo or N^G-monomethyl L-Arginine (L-NMMA) 4mg/kg in 25mls 0.9% N saline was given over two minutes. Treatment was randomly assigned by a second investigator before the start of the experiment. Fourteen minutes into the infusion, an iv injection of frusemide (Antigen Pharmaceuticals, Roscrea, Ireland) 5mg iv was given to stimulate renin release.

Measurements:

Haemodynamic: Blood pressure, as mean arterial pressure, systolic and diastolic pressure, and heart rate was measured by semi-automatic sphygmomanometer (Vital Signs Monitor, Critikon, Tampa, FL, USA,) and taken as the mean of three readings at each time point. Readings were taken at -10, 0, 5, 10, 15, 20, 30, 40, and 50 minutes.

Renin: Renin samples were taken at -10, 0, 5, 10, 20, 30, 40, and 50 mins. See chapter 2 for collection and analysis protocols.

Data analysis:

Comparisons were made between active and placebo treatments by analysis of variance (ANOVA). Where the overall ANOVA was significant, multifactorial analysis of variance was used to determine differences at individual time points. A P value of less than 0.05 was considered significant and results are expressed as means \pm SEM.

PROTOCOL 2:

Five of the initial ten subjects underwent an identical protocol, using phenylephrine (Tayside Pharmaceuticals, Dundee) to mimic the pressor effects of L-NMMA to assess the effect this might have on renin release. This protocol was not blinded.

Subjects again rested supine for one hour prior to baseline haemodynamic and renin levels. Phenylephrine was commenced at 0.5ug/kg/min, as an infusion, with a 25ml

bolus of N Saline at the start of the infusion. Frusemide 5mg iv was administered as a bolus 14 mins into the infusion as in protocol 1. Phenylephrine dose was decided on following discussion with Dr H Elliot (University of Glasgow), and produced the appropriate haemodynamic changes in four volunteers, the fifth had the dose titrated up to 0.75ug/kg/min after four minutes to achieve an adequate haemodynamic response. Haemodynamic and renin measurements were collected at the same intervals as above.

Results:

PROTOCOL 1:

Haemodynamic: The doses of L-NMMA used caused a significant pressor effect prior to the bolus of frusemide, with an average increase at 10 mins of 7.5mmHg ($P<0.05$) in mean arterial pressure (MAP) (Figure 7.1 panel 1). Similarly, diastolic pressure at that point increased by an average 6.8mmHg ($P<0.01$), and there was a small but non significant rise in systolic pressure of 1mmHg (Figure 7.1 panel 2). Heart rate was suppressed on average by 7.2 beats per minute ($P< 0.05$) (Figure 7.1 panel 3). The bolus of frusemide increased the blood pressure in both the active and treatment days, but had no effect on heart rate.

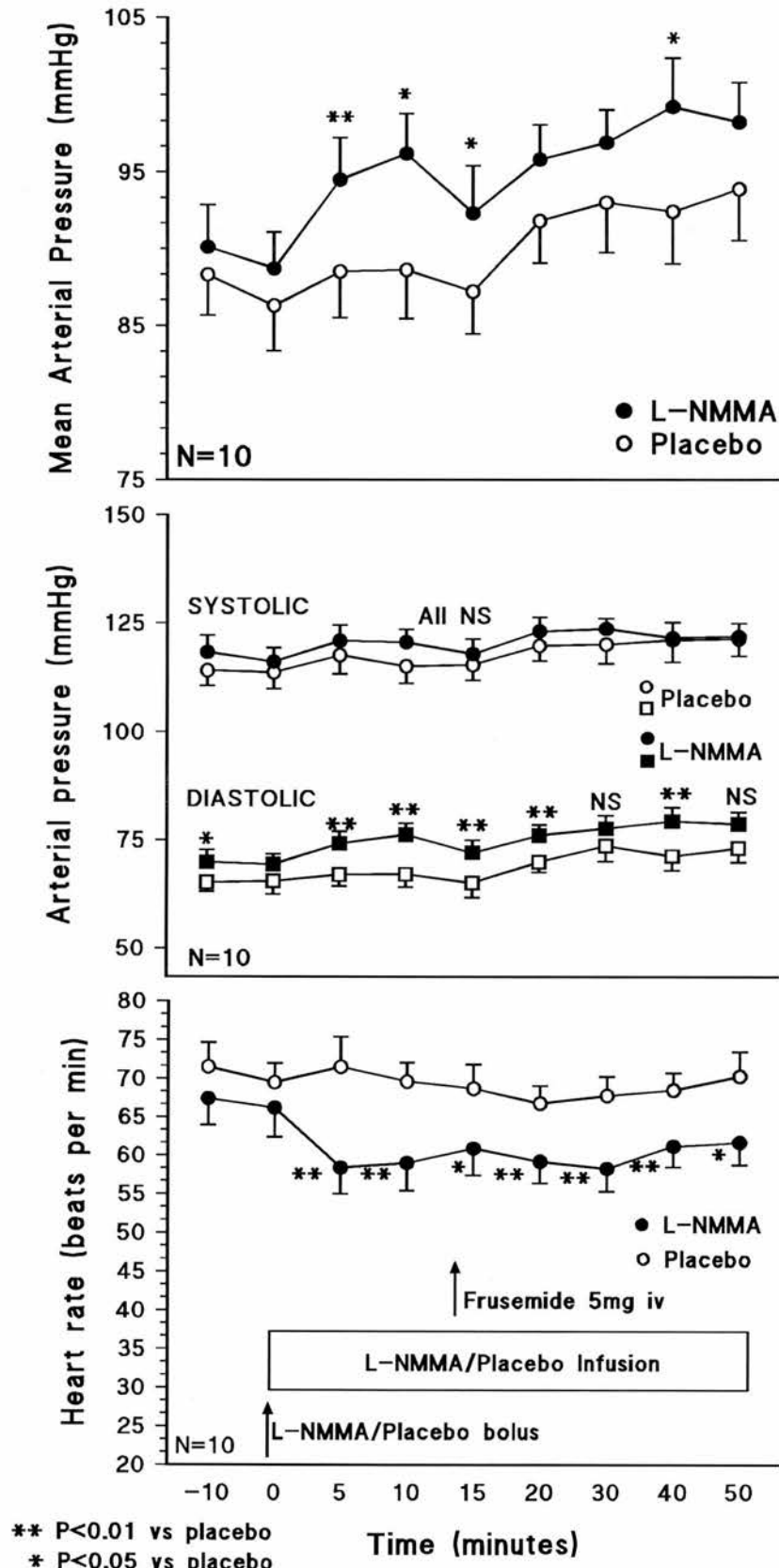
Renin: Baseline renin was unaffected by the infusion of L-NMMA, but there was a significant suppression of the renin response to frusemide ($p<0.01$) at all timepoints after the frusemide bolus (Figure 7.2).

PROTOCOL 2: (5 volunteers only)

Haemodynamic: The phenylephrine doses were aimed to mimic the pressor effects noted with the L-NMMA infusion. A similar reduction in heart rate and increase in mean arterial pressure were noted. Heart rate became significantly lower than placebo at the final two time points (Figure 7.3).

Renin: The renin response to frusemide was completely suppressed to the level noted during the L-NMMA infusion. This difference between placebo and phenylephrine reached significance at all timepoints (Figure 7.4).

Figure 7.1: The haemodynamic effects of nitric oxide synthesis inhibition with L-NMMA in normal controls, compared with volume matched placebo, note frusemide injection at 14 minutes.



** P<0.01 vs placebo
* P<0.05 vs placebo

Figure 7.2: Plasma renin activity during nitric oxide synthesis inhibition with L-NMMA, and following an injection of frusemide to stimulate renin.

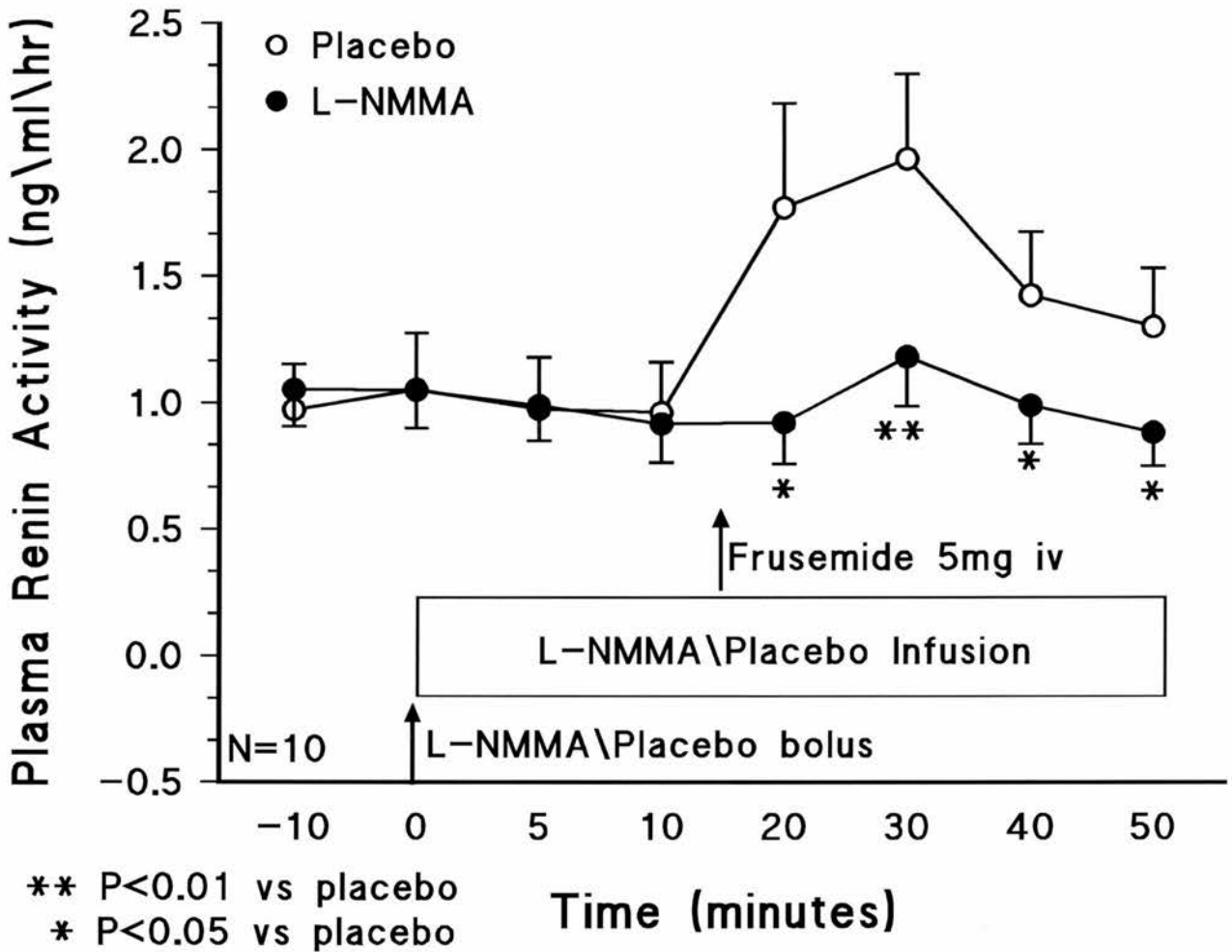


Figure 7.3: Haemodynamic effects, in five volunteers, of an infusion of phenylephrine, compared to those due to L-NMMA, and placebo.

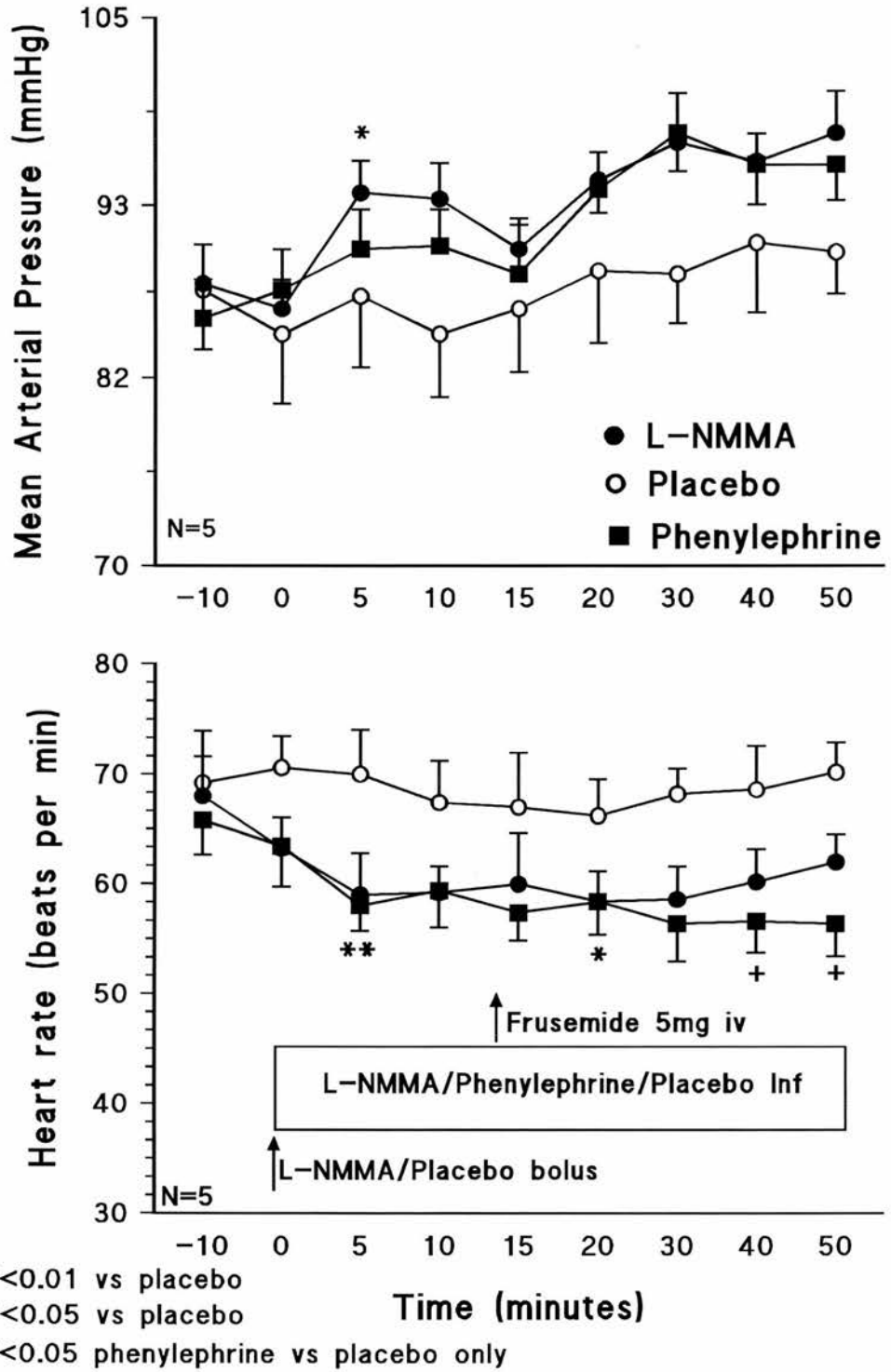
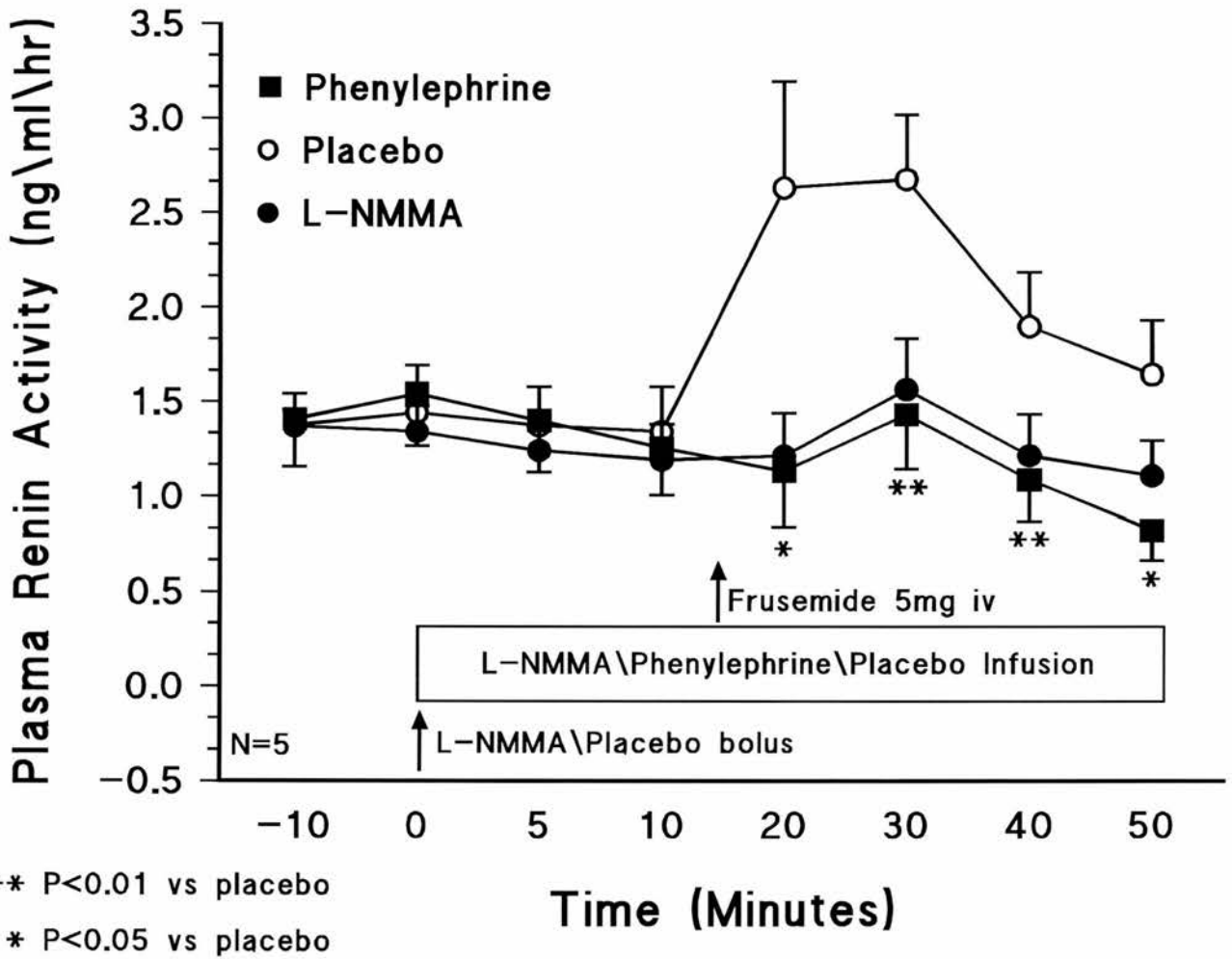


Figure 7.4: Plasma renin activity in five volunteers, during infusions of L-NMMA, and phenylephrine, compared to placebo, and following an injection of frusemide to stimulate renin.



Discussion:

Haemodynamics:

In this study L-NMMA produced the expected haemodynamic effects associated with nitric oxide inhibition. We chose to use a dose of L-NMMA which was between the second and third doses given by Stamler et al (1994), in their dose ranging study, and the alterations that we produced in MAP and diastolic pressure are comparable. Equally, Haynes et al (1993) gave a bolus of 3mg/kg over 5 minutes to a group of normal subjects, and found a 10% rise in mean arterial pressure, with a 19% reduction in heart rate, at ten minutes into the infusions: our respective changes from baseline were similar at 8.5% and 12.7%. We used a front loaded infusion, with the initial 4mg/kg bolus in order to ensure adequate inhibition of nitric oxide at 14 minutes, when the frusemide was given to stimulate renin. Previous work had shown that the haemodynamic effects of an L-NMMA bolus take fifteen minutes to reach peak effect (Haynes 1993), and we felt therefore that the combination of a bolus with an infusion, would give the best overall profile of nitric oxide inhibition over the whole timecourse of the study.

It is interesting to notice that following the injection of frusemide, the MAP and diastolic pressure rose on both the active and placebo days. This effect has been observed previously, in both normals (Johnston 1983, 1984) and in patients with high left atrial pressures (Lal 1969). It has been shown that frusemide invokes an initial venodilation, which causes a fall in blood pressure, followed by a subsequent rise in MAP, which is believed to be due to an increase in systemic vascular resistance. Some authors have argued that this late pressor effect in normals is due to activation of the

renin angiotensin system, as a linear response was shown between Δ plasma renin activity and Δ blood pressure (Johnston 1984). Furthermore, the elevation in blood pressure could be blocked by captopril (Johnston 1983).

In heart failure patients the haemodynamic responses are more variable. Although a reduction in wedge pressure is universally seen, the effects on systemic pressure are equivocal, as are the acute effects on the renin angiotensin system, although with chronic dosing renin-angiotensin system activation is accepted (Raftery 1994).

Our results differ slightly from those of Johnston et al (1983) as we found a dissociation between renin and blood pressure. We found that blood pressure increased following frusemide on the L-NMMA and phenylephrine days despite no concomitant rise in plasma renin. This does not however eliminate the possibility that angiotensin II is mechanistic in the rise in pressure via other pathways, as we have not looked at either angiotensin II or aldosterone. The beneficial effects of frusemide in acute pulmonary oedema are often clinically apparent prior to the actual diuresis, and may be due to the observed direct effects on venous capacitance, and pulmonary capillary wedge pressure.

Renin:

In this protocol our volunteers were salt replete, and hence our baseline renin levels were very low at around 1ng/ml/hr in all subjects. We failed to observe any suppression of baseline renin by L-NMMA despite animal studies in the literature where L-NAME reduced baseline renin within fifteen minutes in both rats (Sigmon 1992), and rabbits (Reid 1995b). In man, nitric oxide inhibition was shown to significantly reduce baseline renin only after 15 minutes (Chiu 1996). Our

measurements ended at 10 minutes, as we applied frusemide to stimulate renin at fourteen minutes, it is possible if we had continued the observation period we might have shown a fall in baseline renin, although given the low baseline levels this would have been technically difficult.

L-NMMA did suppress the renin response to frusemide, but this did not appear to be a specific effect, because phenylephrine, in equipressor doses, had an identical effect in inhibiting the renin response to frusemide.

This demonstration of the effects of nitric oxide synthesis inhibition on stimulated renin release in man agrees with much of the published animal data, as in animal models frusemide induced renin release is inhibited by nitric oxide synthesis inhibition (Reid 1995b, Beierwaltes 1995). It is however, difficult to reconcile our data with that of Reid et al (1995b), who performed essentially this experiment in rabbits. They did not find that phenylephrine inhibited frusemide induced renin release, despite producing similar haemodynamic effects to the nitric oxide inhibition. In their study both L-NAME and phenylephrine significantly reduced the baseline secretion of renin, showing the importance of pressure in control of renin secretion in the upright animal. Our study raises the distinct possibility that there are species specific differences in the effect of NO on renin release. Another factor that also requires consideration, is the large difference in dosage of frusemide between the studies. On a mg/kg basis Reid et al (1995b) gave an equivalent of 2mg/kg bolus in contrast to our 0.07mg/kg, nearly thirty times the dose. Our frusemide dose was chosen to allow effective renin secretion without the desire to void (McMurray 1989).

Considering our results further, the ready conclusion is that the inhibition of renin by nitric oxide inhibition is pressure dependent, and an indirect effect. Haemodynamic

factors are well known to be important in renin release (Scholtz 1993): below a certain level of renal perfusion pressure, renin secretion appears to double for every 2-3mmHg reduction (Hackenthal 1990). It is this pressure dependence, which complicates any attempt to unravel the role of nitric oxide in renin release in the whole animal since nitric oxide donors reduce perfusion pressure, and nitric oxide inhibitors increase perfusion pressure.

The literature contains a variety of conflicting results on the effect of either nitric oxide inhibition, or stimulation on renin release in animal models. The issue is further clouded by a variety of mechanisms used to stimulate renin release.

Frusemide is a common experimental stimulus for renin secretion, and several studies have shown that nitric oxide inhibition reduces both renin release (Reid 1995b, Beierwaltes 1995,) and renin gene (Schrickler 1995) expression.

However, experiments have been less consistent if perfusion pressure is used as the stimulus for renin release. Studies have tended to show that haemodynamic factors are more important than changes in nitric oxide levels in determining renin release (Sigmon 1992), which is consistent with our results. In rats, Sigmon et al (1992), showed that if all control mechanisms of renin secretion are blocked by a combination of stable renin perfusion pressure, ureteric obstruction, and propranolol, then L-NAME actually stimulates renin release. If however renal perfusion pressure was not controlled then L-NAME reduces renin secretion, emphasising the importance of pressure in control of renin secretion. Similarly, in dogs, Persson et al (1993) found that systemic nitric oxide inhibition had the greatest effect on inhibiting renin release at the lowest levels of perfusion pressure.

To try to decide whether nitric oxide is inhibitory, or stimulatory on renin release, one can look at isolated cell culture of the juxtaglomerular cells (JG cells), which should be devoid of pressure effects. However rather than clarifying the issue this seems to further confuse, as in pure isolated JG cell culture, nitric oxide application using sodium nitroprusside initially produces an inhibition of renin release within one hour (Greenberg 1995), followed by a stimulation over twenty hours (Schricker 1993a).

In a more complex model, where endothelial cells are co-cultured with the JG cells, the presence of endothelial cells suppresses renin release (Kurtz 1991, Schricker 1993b). Endothelial cells release a variety of mediators in addition to nitric oxide, and by a series of experiments Kurtz et al (1991) demonstrated that the presence of endothelial cells in the culture has different effects on renin secretion depending on the chemical applied to stimulate the JG cells. This raises the possibility that endothelial cells can secrete several mediators that have opposing effects on renin.

In an elegant study by He et al (1995), using isolated perfused juxtaglomerular apparatus, they showed that if L-arginine was applied directly to the macula densa via the tubular lumen to increase local production of NO, then renin release was stimulated, but if L-arginine or sodium nitroprusside were applied to the whole preparation, then it was found to be inhibitory on renin. They conclude that nitric oxide originates from several sources within the kidney, and may exert different effects.

Therefore the data from isolated cells suggests that the macula densa does indeed use nitric oxide as a signal for renin release, and the isolated cell data suggests that nitric

oxide is stimulatory on renin release. In models with more than one cell type the nitric oxide appears to have different effects depending on the other cells present.

When it comes to applying this information to a whole animal model or in man further complexities arise. For example, nitric oxide inhibitors cause alterations in renal blood flow which affect renin release (Gardes 1992,1994). Equally nitric oxide is believed to suppress baroreceptor activity and hence the sympathetic nervous system (Matsuda 1995), and nitric oxide inhibition increases the sensitivity of tubuloglomerular feedback (Thorup 1993) all of which will combine to control the ultimate renin response.

Using an isolated perfused kidney model, with falling perfusion pressure used as the stimulus for renin release, we again find conflicting results. For example, Sholtz and Kurtz (Sholtz 1993) demonstrate that renin secretion directly parallels renal perfusate flow, and that stimulation of renin by NO donors is pressure dependent, and is limited at normal perfusion pressure, but marked at artificially low perfusion pressure. This led them to suggest that the control of renin secretion has two components: a stimulatory effect of nitric oxide on renin release that is suppressed by higher perfusion pressures by baroreceptor function.

The difficulties of investigating renin and nitric oxide inhibition in the whole animal model is underscored by the variety of results seen and also the stimulus to renin used. For example, in the rat Beierwaltes et al (1995) found that NO inhibition had no effect on renin in response to alterations in perfusion pressure, findings supported by others

(Chiu 1995). In a similar study in rats, using chronic L-NAME administration, Knoblich et al (1996) found that renin was suppressed by L-NAME treatment at all perfusion pressures but specifically at lower pressures. However Johnson et al (1994) found that L-NAME suppressed renin at high perfusion pressure, but at hypotensive levels renin was actually stimulated despite the blockade of NO synthesis, which lead them to suggest that pressure is more important than nitric oxide inhibition per se. Finally, Sigmon et al (1992) using the rat model showed that with control of both renal perfusion pressure, and sympathetic nervous system, L-NAME could actually enhance renin secretion, a finding supported by other studies in dogs (Schnackenberg 1997).

One potential explanation for this complex series of results is that nitric oxide inhibition affects not only the cells of the macula densa in the preparation, but also renal blood flow and mean arterial pressure, particularly in systemic application studies, causing baroreceptor activation, and alterations in tubuloglomerular feedback. In our study we have shown that a small renin stimulus can be inhibited by the application of a nitric oxide inhibitor, which increased arterial pressure and via baroreceptor reflex reduced heart rate. A similar pressure increase using a non specific pressor agent also abolished the renin response, in an indirect way. The nitric oxide inhibitor may have caused its effect via several mechanisms, but unravelling the mechanisms behind this is not ethically possible in man, since it would be unethical to inhibit tubuloglomerular feedback via bilateral ureteric obstruction (Sigmon 1992), or block the sympathetic nervous system using renal denervation. Hence in man, pinpointing the absolute mechanism may be impossible, and is beyond the information

available in this chapter. Nevertheless, it is important to address this question in man in vivo, since species differences in renin are well recognised.

One final point to address is the effect of phenylephrine on renin release, as it is an α -1 adrenoceptor agonist which could also affect renin release. In dogs, α receptor blockade reduces renin release (Blair 1985), suggesting the phenylephrine should if anything stimulate renin release. In man, phenylephrine had no effect on renin secretion due to controlled hypotension, despite epinephrine causing increases in renin (Zayas 1993), and situations where phenylephrine is applied directly to the renal arteries, stimulation of renin, not inhibition, is seen suggesting that the suppression seen here was purely a pressure effect (Blair 1985, Naess 1991).

We have demonstrated in man that nitric oxide inhibition has direct effects on renin release, although the mechanisms behind this are not delineated in this study. The importance of the interaction between nitric oxide and renin release warrants further study, as this may be a direct interaction between the two systems. The data above suggest that a major part of the reduction by nitric oxide inhibition is due to indirect stimulation of the baroreceptor inhibition of renin.

Summary Points:

- Nitric oxide inhibition using L-NMMA causes a rise in systemic pressure, and a fall in heart rate, which reflects the importance of nitric oxide in the maintenance of vascular tone.
- L-NMMA infusion in normal salt replete volunteers did not cause a reduction in basal renin levels.
- Renin release stimulated by frusemide bolus was inhibited by the L-NMMA infusion, to a similar extent to the inhibition seen with an equipressor dose of phenylephrine.
- A study in human volunteers is limited as to the mechanistic information that is available as physiological control mechanisms such as the renal sympathetic nerves and tubuloglomerular feedback have to be left intact.
- A great deal of further work will be required to unravel whether nitric oxide has direct effects on components of the renin angiotensin system, or indirect effect via haemodynamics. The literature suggests that renin release is affected by nitric oxide at multiple sites within the normal physiological control mechanisms.

CHAPTER 8:

Discussion of results, limitations of studies, and suggestions for further research stimulated by this thesis.

The preceding series of investigations have addressed the issues of the adequacy of neurohormonal suppression by ACE inhibitors, as used in heart failure, and whether ACE inhibitors are of use in other clinical situations such as hyperlipidaemia. Furthermore, we looked at the renin angiotensin aldosterone axis, and its relationship with other hormonal systems, to see if there is evidence of direct interactions between these physiological systems.

Monitoring neurohormonal profiles in heart failure patients:

The study in chapter one looked at the actual profile of neurohormonal suppression in a group of heart failure patients which has not previously been addressed in this length of follow-up. ACE inhibitors are now first line treatment and have accepted mortality benefits in heart failure (Clinical Quality Improvement Network Investigators 1996). However there are several recent studies that have demonstrated that additional therapy with ACE inhibitors may improve upon the mortality and morbidity reductions. For example The CIBIS II study (1999) demonstrated that blockade of beta adrenoreceptors produces additional benefit in heart failure. More recently the RALES study (Pitt 1999) has shown that additional therapy with spironolactone causes mortality and morbidity reductions in subjects stabilised on ACE inhibitors. This suggests that receptor blockade for both noradrenaline, and aldosterone is beneficial in heart failure. The RALES data suggests that there is not blanket suppression of aldosterone by ACE inhibition, as was seen in chapter three. One potential limitation of the data in chapter three is the relatively small numbers of people followed over the period. Despite this, the incidence at any one time of inadequate suppression of either aldosterone or angiotensin II, as defined by our

criteria was 10% of the population, which given the size of the world population on ACE inhibitors is a significant deficit.

The ATLAS trial aimed to address the issue of appropriate doses of lisinopril in heart failure, looking at 5mg versus 35mg/day. While the mortality end-points showed that there was no statistically significant difference, higher lisinopril dosage resulted in fewer hospitalisations and the combined endpoint of all cause mortality, and cardiovascular morbidity was significantly better if higher doses were given (Hobbs 1998). Our data suggests that this may not relate to adequacy of hormonal suppression as some subjects on only small doses of ACE inhibitors had good suppression of both hormones, whereas some individuals with much higher doses of ACE inhibitors showed evidence of escape, although the majority of the patients took either 10 or 20mg of lisinopril or enalapril per day. The fact that benefit can be derived from a variety of doses is shown in the post infarct population (Latini 1995).

We found that those patients on captopril had more frequent episodes of escape, but the numbers were small. Nicholls et al (1982) showed that there was a significant rise in angiotensin II in subjects on captopril between one hour post dose, and six and a half hours post dose, which suggests that the shorter duration of action may be a factor in our results. In contrast they found that overall levels of angiotensin II and aldosterone taken one hour post dosing were stable over a four to six months treatment period. However they used 150mg doses given three times daily, which is a higher dose than is usually administered in present clinical practice. These results were not, however, confirmed by a similar studies in hypertensives, where aldosterone concentration increased from 38pg/ml to 164pg/ml (Lijnen 1982) over a 1 year period.

The data presented did not demonstrate an overall mean increase in any of the neurohormones, and we do not therefore feel that there is an inexorable rise in neurohormones with time in heart failure patients. Our results may differ to those in hypertensive patients, as they have a different disease process. Other studies in hypertensives by the same group using captopril appear to support a rise in aldosterone but with persistent improvement in blood pressure (Staessen 1981) and suppression of angiotensin II. This may reflect the reduction in ACE suppression seen with captopril in longer term dosing, or a reactive rise in aldosterone due to other factors such as magnesium or potassium (Rodriguez 1986).

The literature does not show a linear relationship between ACE inhibitor dose, or indeed plasma ACE levels and neurohormonal levels (Swedberg 1990). In one of the few studies looking at dose ranging and neurohormonal levels by Nussberger et al (1994), plasma ACE was dose dependantly suppressed by 5, 10, or 15mg of quinapril, with concomitant reductions in angiotensin II of 42%, 59%, and 68%. However while there was an effect of dose, the actual levels of ACE activity were markedly suppressed at the lowest dose (90% reduction), but the range of results was much greater. Pouleur et al (1993) showed that there appears to be individuals whose hormones are suppressed on Enalapril 20mg/day, and those whose angiotensin II and noradrenaline remain elevated, and it is those subjects with persistent elevation who appear to have deterioration in LV function. The above discussion was limited to the effects on resting neurohormones, exercise studies demonstrate that despite adequate ACE inhibition with captopril, exercise causes rises in both angiotensin II and aldosterone (Aldigier 1993).

There are limitations of the study outlined in chapter three, primarily the small numbers of patients on captopril as opposed to either enalapril and lisinopril. Furthermore, the fact that most of these individuals either died or were converted to longer acting ACE inhibitors reduces the information that can be obtained. Similarly we did not count tablets, and therefore have very limited knowledge regarding compliance. The overall excellent suppression of ACE activity in the long acting ACE group suggests that compliance was good, however this parameter is not available for those on captopril, as the captopril-enzyme complex appears to dissociate in vitro and therefore the results do not reflect the in vivo situation (Unger 1981). Furthermore, the captopril takers in general have more frequent dosing, and hence are more likely to have poor compliance.

Overall the data do not suggest that reactivation of either angiotensin II or aldosterone is a widespread phenomenon, however the RALES study showed additional benefit from suppression of aldosterone (Pitt 1999), and we await the publication of the ongoing studies looking at additional therapy with angiotensin II receptor antagonists.

One of the most recent epidemiological study to be released is The HOPE study (2000a), which was presented at the European Society of Cardiology meeting in the autumn of 1999. This was a large primary prevention study in high risk groups. The HOPE study looked at 9,297 high risk patients for cardiovascular disease without known left ventricular dysfunction. Subjects eligible were diabetics with one other risk factor, or patients with previous coronary artery disease, stroke or peripheral vascular disease. The results demonstrated a significant reduction in cardiovascular mortality

and morbidity due to ramipril, despite limited reductions in blood pressure. This suggests that ACE inhibitors could be useful in a high risk population in reducing atherosclerotic progression, and preventing ischaemic events and death. It is interesting that 65% of the trial population had elevated cholesterol. From the results shown in chapter four and the subgroup analyses of the TREND (Pitt 1997b) and the QUIET (Cashin-Hempell 1997) data, these patients may be found to have an excess benefit from ramipril.

Angiotensin converting enzyme inhibitors in hyperlipidaemia:

Angiotensin converting enzyme inhibitors are now first line in the treatment of heart failure, and are already established in hypertension. There is mounting evidence that they have beneficial effects at tissue level, the mechanisms of which are yet to be established. The epidemiological data shows mortality benefit for these drugs in heart failure (Garg 1995), post infarct (Latini 1995), and in diabetes. In particular diabetics appear to benefit both by reduction in blood pressure, but also because the renal function is preserved in those individuals taking ACE inhibitors. This was investigated by the MICRO-HOPE study (Gerstein 1996) which demonstrated reduction in cardiovascular mortality and overt nephropathy in those treated with ramipril (The HOPE study investigators 2000b). As has been discussed above, this group of drugs have benefits far in excess of blood pressure reduction. In chapter four several potential local mechanisms were discussed, and given the results presented it was felt that the reduction in local production of angiotensin II improves the bio-availability of nitric oxide, and hence arterial function.

Chapter four showed for the first time in man that lisinopril can improve endothelial function in hyperlipidaemia, this has previously only been shown in animals, and represents a significant move forward. In the TREND and the QUIET trials benefit of quinapril was particularly obvious in those cohorts with higher levels of LDL cholesterol (Pitt 1997b, Cashin-Hemphell 1997). It is possible that ACE inhibitors favorably influence plaque dynamics to improve vasodilator/vasoconstrictor balance, hence reducing angiographic progression in the higher LDL sub-groups. They may also influence oxidation of LDL which makes it more atherogenic (Godfrey 1994, Mira 1993). This is borne out by the major mortality trials such as the SOLVD (1991) and SAVE trials (Rutherford 1994) which suggest that ACE inhibitors reduce the incidence of myocardial infarction in the treated population.

A similar protocol to ours has been performed both in subjects with type I and II diabetes using acetylcholine (O'Driscoll 1997, 1999) and shown similar results, suggesting benefits within the vasculature in high risk patients. This result has not been confirmed in other studies using flow mediated dilatation in type 1 diabetics (Mullen 1998, McFarlane 1999) rather than acetylcholine. One further study in type I diabetes does suggest the benefit of ACE inhibition in flow mediated dilatation (Arcaro 1999) but this study used only one week treatment and showed beneficial effects on endothelial independent responses in addition. Furthermore their patient group had evidence of complications in the form of microalbuminuria, a subgroup who are known to benefit from primary ACE inhibition (The HOPE study investigators 2000b). Some of this controversy may arise from the different experimental stimuli

that have been applied in diabetes. Biljstra (1995) failed to show a benefit in insulin resistant subjects to methacholine in contrast to O'Driscoll in NIDDM (1999) using acetylcholine. This may reflect the fact that methacholine may not stimulate the release of nitric oxide to cause vasodilation (Chowiencyzk 1993), and indeed acetylcholine-induced vasodilatation only partly reflects nitric oxide release (Newby 1996). The differences noted may be explained by the variation in the ability of the stimuli used to release nitric oxide as opposed to other mediators such as prostaglandins, or bradykinin.

Vasodilation is gauged in most experimental protocols by assessing brachial artery flow, and is dependent on nitric oxide release from the endothelial cells. We have suggested that our results represent an increase in the bioavailability of nitric oxide, due to reduction in angiotensin II which promotes the production of superoxide radicals causing degradation of endothelial nitric oxide.

There are limitations to the study as in all clinical trials. It was decided at the beginning of the study that we could accept that historical controls had proven the impairment of endothelial function by hyperlipidaemia, (Chowiencyzk 1994, Creager 1990, Gilligan 1994d) hence a control group of normo-cholesterolaemics were not recruited. A control group would have allowed us to assess the effect of an ACE inhibitor on the nitroprusside response in normals, as there is some discussion in the literature as to whether nitroprusside is less effective in hypercholesterolaemics. Creager et al (1990) showed that patients with higher cholesterol have significantly reduced dilatation to nitroprusside confirmed by Gilligan (1994d), but Creager et al then failed to confirm a significant difference in endothelial independent responses in later studies (Creager 1992). A control group of patients without elevated cholesterol

would have allowed us to check that the effect of lisinopril on nitroprusside was specific to the hypercholesterolaemics, rather than a non specific effect. In hindsight a control group would have been of great value, but as discussed in the recruitment section for chapter five, might have been difficult to find appropriately matched for age, sex, and forearm volume. A control group was used in the subsequent study, chapter five, as there is limited and controversial data on the effects of cholesterol levels on nitrate/nitrate, hence no historical controls exist in the literature.

A further limitation was the change in baseline blood flow between the two study days in the active group. This could potentially confound our results but as discussed in the chapter, we feel that this actually underlines the positive results. Throughout this thesis the results of the forearm blood flow studies have been presented as ratios of infused arm to control arm, which is the best way to present the data to avoid the problems of variable baselines (Benjamin 1995, Chin-Dusting 1999).

Other potential ways of representing the data are percentage change from baseline, or forearm vascular resistance. Both of these methods are going to emphasise errors due to changes in baseline blood flow whereas ratios allow for minor alterations in study conditions within the study, and variation in forearm volumes between subjects. Ratios provide a more consistent way of presenting the data. The potential problems of using absolute flow data, without the contemporaneous, and intrinsic control of the contra-lateral arm, are demonstrated by the absolute flow data for the study discussed in chapter six seen in figure 6.3. This figure shows the blood flow for the infused arm only in the study looking at the effect of oestrogen, and progesterone on vasoconstriction to angiotensin I and angiotensin II. In this study we expected that the oestrogen would cause vasodilation, and this gives the visual impression that the

effect of the angiotensin I and II are reduced. However the ratios of flow are actually unchanged as seen in figure 6.2 which has eliminated a potential error due to the alterations in baseline if only absolute flows were addressed.

Similarly if the data is expressed as forearm vascular resistance, there is not only the potential for alterations in baseline, but also FVR is a calculated variable derived from an equation which reflects laminar flow in a non-distensible system, rather than pulsatile flow in a distensible system, which could introduce further errors.

Chapter four established that lisinopril improves both endothelial dependent and independent responses in a hyperlipidaemic population. There is mounting evidence that these drugs are of use in primary prevention in a high risk population. The results seen here might suggest that they could be useful in primary prevention in hyperlipidaemia, and we await appropriately directed large scale studies to answer this question.

Angiotensin converting enzyme inhibitors and nitric oxide:

From chapter 4 looking at the effects of lisinopril on arterial function the next study addressed the possibility that this effect was indeed due to increases in available nitric oxide. Chapter 5 discusses a study looking at the production of nitrite and nitrate in hyperlipidaemics as an assessment of nitric oxide release in this group. We failed to demonstrate an effect of either hyperlipidaemia, or administration of ACE inhibitors on nitric oxide production.

This was disappointing as our hypothesis was based both on extensive circumstantial evidence in the literature, and also that other beneficial therapies cause increases in

nitrate release (Cincinelli 1998). However on the basis of plasma nitrite and nitrate sampled after an eighteen hour fast, there was no significant change in nitric oxide levels in response to lisinopril. Our hypothesis was that patients with hyperlipidaemia will produce more nitric oxide, as atherosclerotic vessels have an imbalance of vasoconstrictors and vasodilators, and atheroma causes increases in inducible nitric oxide synthase (Behr 1999, Buttery 1996), and hence increased nitric oxides (Minor 1990). We did show that in comparison to the normal group both the hyperlipidaemic groups had slightly higher levels of nitrite/nitrate, but these were in the order of 15-20%, and would require larger patient numbers to achieve a significant result. The other point was that in the control group we demonstrated a linear relationship between cholesterol and nitrate, and it may be that there would have been a demonstrable difference between the normal controls and the hyperlipidaemic placebo group if a tighter criteria had been applied to total cholesterol for inclusion into the normal control group. The results in chapter five do not however exclude an effect of ACE inhibition on superoxide formation, and free radical degradation of nitric oxide, as while there was no specific alteration in the overall production, it may be that the available nitric oxide was more effective.

The other limitation in the protocol was that the subjects had taken five months treatment with either lisinopril, or placebo on the first study day, and were then studied again about four weeks after cessation of the medication. It is possible that the failure of the ACE inhibitor to affect nitrite/nitrate relates to structural changes in the artery which persisted after the cessation of the medication. If one looks at short term studies of ACE inhibition, there is evidence that these drugs do improve nitric oxide

metabolism, as vasodilation in response to quinaprilat appears to be nitric oxide dependent (Haefeli 1997). Indeed given that ACE inhibitors are accepted to potentiate bradykinin (Benjamin 1989) it is surprising that there is not a difference in nitric oxide in those subjects on lisinopril.

However, the results failed to demonstrate a significant change in nitric oxide levels on cessation of lisinopril in the group of hyperlipidaemics, this may be due to the small sample size or to the use of concomitant medication. Further studies could attempt to address the issue using larger groups and stopping all other medication.

Oestrogen, progesterone and angiotensin converting enzyme activity.

Hormone Replacement Therapy (HRT) in post menopausal females has been shown to result in a 50% reduction in cardiovascular mortality (Stampfer 1991), only 30-50% of which is attributable to alterations in lipids (Newnham 1993).

There are several suggested interactions between female ovarian hormones and the renin angiotensin system and we specifically studied the effect of oestrogens and progesterone on ACE activity, and vasoconstrictor response to angiotensin II. Neither of these end points appeared to be important in the vasodilatory response to oestrogens.

The limitation of this study is the group to whom it is directly applicable. Although we have shown that normal males appear to have oestrogen receptors, and the literature suggests that these receptors are important in coronary artery dynamics (Blumenthal

1997, Reis 1998), the epidemiological studies have not suggested any benefit of estrogen in men (The Coronary Drug Project 1973) which is in sharp contrast to the benefits seen in women. Recent studies have highlighted that oestrogen replacement acutely causes alterations in inflammatory markers, and this may account for the deleterious effects seen in males in the longer term (Ridker 1999).

It is difficult to say that the model is of no value, because it is well known that oestrogens do have effects in males, and the protocol was a clean way to study the effects of these hormones on angiotensin converting enzyme in the brachial artery. There is no evidence in the literature to suggest that there is any sex difference between males and female regarding the ACE protein molecule, indeed many of the studies looking at the effect of ACE inhibitors on heart failure have recruited both males and female equally, and there was no evidence that there was a difference in the effectiveness of ACE inhibitors in women (SOLVD 1991, The HOPE study 2000a). Furthermore, evidence in the literature with long term oestrogen does suggest that male vasculature can approach the responses of genetically female arteries with time (McCrohan 1997) suggesting that a longer term study might have produced different results. This is particularly pertinent as the HRT study which did show an effect on ACE enzyme in women looked at six months treatment (Proudler 1995). Given the literature evidence that longer term oestrogens are detrimental in males it was not felt appropriate to study a longer term dosage, had the volunteers been prepared to take a longer course.

The results in chapter 6 suggests that the acute vasodilatory effects of oestrogens are not dependent on inhibition of ACE activity. Evidence from the literature has suggested a variety of other possible mechanism behind the acute vasodilation seen. In

the longer term, the beneficial effects of HRT may however be due to changes in ACE activity. An interesting further study would be to look at both circulating ACE and vascular ACE, as assessed by the model used here, with long term HRT in post menopausal women.

Renin release and nitric oxide inhibition:

The above studies attempted to identify whether manipulation of the renin angiotensin system by ACE inhibitors, or ovarian hormones could be shown to affect arterial function, and nitric oxide metabolism. Chapter 7 looked at whether manipulations of nitric oxide could be shown to directly affect renin release.

The evidence suggests circumstantially that nitric oxide is an important signalling molecule in a variety of systems, and the evidence presented within chapter seven is consistent with the available data that renin release is influenced by nitric oxide.

Nitric oxide inhibition was found to reduce the frusemide induced renin release, and this is the first demonstration of this effect in humans. As such this is an important study as it was more physiological than many of the animal studies where the renal artery pressure is controlled via aortic snare (Knoblich 1996), or the renal sympathetic nerves interrupted (Naess 1993). These sorts of intervention are unethical in human volunteers, so any study looking at the effects of nitric oxide inhibition will be hampered by the physiological consequences of alterations in haemodynamics. Any manipulation of the nitric oxide system will influence prevailing pressure, and

therefore the organism will attempt to maintain homeostasis, by alterations in heart rate and renin release.

It is difficult to envisage a study in normal volunteers that could remove the effects of the physiological control of renin release. One criticism of the study presented in chapter 7 is that we did not look at other vasoactive agents in addition to L-NMMA, however again this would have affected the pressure rise in response to L-NMMA and would therefore not be an isolated stimulus. Despite this, the concomitant administration of Verapamil, or other non NO dependent vasodilator would have produced very interesting results, and is a further study that should be provoked by this investigation. The reflex control of blood pressure is likely to hamper unravelling the true interaction of nitric oxide and renin release in man, as the literature suggests that the pressure dependence of renin release is of paramount importance.

Renin release is now exciting more interest with the development of renin antagonists which may become clinically useful in heart failure, hypertension, and other high renin states. Frusemide is one of the very potent stimuli to renin release, and even the tiny dose (5mg) used in our study provoked a two fold rise in renin release. ACE inhibitors and diuretics are standard therapy in heart failure, and renin rises with both these agents. The reactive rise in renin may contribute to the escape of angiotensin II and aldosterone. The results above are valid in a physiological situation, and suggest that there are several mechanisms involved in renin secretion and its control. The importance in physiology of this hormone cannot be underestimated, and the fact that there are at least three main controls for its production underline this point. Furthermore we found in chapter 3 that renin levels correlated both with angiotensin

II and aldosterone in those subjects on captopril, which suggests that renin is an important determinant in reactivation of these hormones.

Whether nitric oxide is the molecule used as the signal between the macula densa, and the juxtaglomerular cells is not known at present, but it is likely that nitric oxide will be identified as a paracrine stimulus in a variety of hormonal systems, not least in the release of renin. Establishing this in the human model may pose serious problems because of the reflex control of blood pressure, and renal perfusion.

SUMMARY AND SUGGESTIONS FOR FURTHER WORK:

- We have shown that ACE inhibitors at stable dosage in heart failure do not produce blanket suppression of neurohormones. Furthermore that longer acting ACE inhibitors appear to produce better suppression than captopril, but the numbers are small and this deserves further study, especially with the still widespread use of captopril. Much useful information could be obtained on this issue with a study over a similar period looking at groups randomised to either captopril or longer acting ACE inhibitors, and assessing neurohormonal levels over the period with no alteration in therapy. The study discussed here however represents true clinical practice, and as such is of clinical relevance.
- We have shown for the first time in man that ACE inhibitors have beneficial effects on both endothelial dependent and endothelially independent vasodilation, in hyperlipidaemia. This is a useful point as these drugs may be of increased benefit in hypertensives with high cholesterol, and this may reflect the effects seen on ischaemic endpoints in the major heart failure trials such as SOLVD and SAVE. They have been found to reduce mortality in primary prevention situations, which would reduce the risk of myocardial infarction in high risk groups. Further studies should go on to look at primary prevention with ACE inhibitors in high risk groups of hyperlipidaemics. This is particularly interesting in the recent light of the HOPE study, discussed above.

- We have shown that the administration of an ACE inhibitor to hyperlipidaemic patients does not appear to affect the levels of plasma nitrate/nitrite, although there may be differences seen in a larger group of patients, or those patients not taking a HMG Co A inhibitor for cholesterol lowering. More studies should look at groups of untreated hyperlipidaemics, with standardised fasting protocols, and in normal patients on and off ACE inhibitors.
- It was demonstrated in males that acute administration of oral oestradiol causes vasodilation, but that this effect is not due to alterations in angiotensin converting enzyme activity. Further studies should address the effects of acute dosing and long term treatment on vascular ACE in women, as the studies in the literature suggest that the effects of HRT on ACE activity occur with six months therapy.
- The investigation into the dependence of renin secretion on nitric oxide release in the kidney suggested that there are several important mechanisms involved, and that in human experiments mechanistic data may be difficult to establish. Nitric oxide inhibition does reduce the release of renin in response to frusemide, and further studies should look at whether this effect is maintained in the face of a vasodilator in addition to L-NMMA to maintain the blood pressure at the baseline level.

*“The outcome of any serious research can only be
to make two questions grow where only one grew before.”*

-Thorsten Veblen (1857-1929)

The Place of Science in Modern Civilization

CHAPTER 9:

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