# STUDIES OF THE RENIN ANGIOTENSIN SYSTEM IN THE HUMAN VASCULATURE

*by*

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# Declaration

The experimental design of the work presented in this thesis was that of the author and his supervisor, Professor John MC Connell. All experimental work was performed by the author with the exception of organ bath experiments in human internal mammary arteries. These were performed by Dr Mark C Petrie, as part of a collaborative project, using protocols devised by the author. Immunohistochemistry was performed by Mr Ian Downie and pathological specimens were prepared by Mr Niall Whyte, under supervision of the author and Dr George Lindop. ACE genotyping was performed by Dr Cathy Clarke and Miss Rosie Farmer, using standard methods. Assays for renin, aldosterone and angiotensin II were performed by Dr JJ Morton.

## Publications arising from this Thesis

The results presented in chapter 3 have been published as follows:

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Padmanabhan N, McGrath JC, Connell JMC. ACE-independent contraction to angiotensin I in human resistance arteries. Hypertension 1998; 32(4): PG 06. Presented at the European Council for Cardiovascular Research 1998.

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Petrie MC and Padmanabhan N, John E McDonald, Hillier C, Connell JMC, McMurray JJV. Angiotensin converting enzyme (ACE) and non ACE dependent angiotensin II generation in resistance arteries from patients with heart failure and coronary heart disease. Journal of the American College of Cardiology 2001; 37: 1056-1061. Presented at the 48th Annual Scientific Session of the American College of Cardiology 1999.

Padmanabhan N, Petrie MC, McDonald JE, Spiers AC, McMurray JJV, Connell JMC. Angiotensin II generation by non-ACE pathways in human resistance arteries. Journal of Human Hypertension 1999; 13: S3. Presented at the Annual Scientific Meeting of the British Hypertension Society 1999.

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# Summary

Pharmacological inhibition of the renin-angiotensin system (RAS) plays a crucial role in the treatment of cardiovascular and renal disease. In addition to systemic generation of angiotensin II (AII), experiments in animals and humans suggest that local, tissue-based, mechanisms are also important sources of the peptide. In particular, it has recently been suggested that a number of enzymes, other than angiotensin-converting enzyme (ACE), can generate AII within tissues. These alternative pathways may allow for escape from ACE-inhibition and thus be clinically significant.

The work presented in this thesis concentrates on the local generation of AII. Preliminary experiments using wire myography were performed in human resistance arteries from normal subjects, obtained by subcutaneous gluteal fat biopsy. In these vessels, AI stimulated a contractile response that was dependent on activation of the AII type 1 receptor (AT1R). Thus, conversion of AI to AII can occur within the vasculature. This conversion was resistant to inhibition of ACE with enalaprilat in human tissue. In contrast, AI responses in rabbit arteries were almost completely inhibited by enalaprilat. Further investigation demonstrated that the combination of enalaprilat and the chymase inhibitor, chymostatin (but neither agent alone), inhibited the response to AI in human resistance arteries. Thus, a dual pathway for AII generation exists in human arteries, probably mediated by ACE and chymase.

Since the significance of non-ACE AII generation may be greatest in patients taking ACE-inhibitors, further studies were conducted on resistance arteries from patients with chronic heart failure (CHF) who were receiving such medication, compared to patients with coronary heart disease (CHD). In patients with CHD a similar dual pathway to that observed in normal volunteers appeared to be present. However, in arteries from patients with CHF, the contribution of chymase to AII generation – as inferred from the effect of inhibiting ACE – appeared to be less. Thus, the activity of the enzymes responsible for AII generation may be modulated by either the syndrome, or its treatment.

In other studies on resistance arteries from patients with CHF and CHD, responses to AII, norepinephrine (NE), acetylcholine (ACh) and bradykinin (BK) were investigated. The response to AII appeared to be exaggerated in arteries from patents with CHF, but ACE and chymase inhibition had no effect in either CHF or CHD. Similarly, the responses to NE and ACh were unaffected by these inhibitors. There was, thus, no evidence that potentiation of counter-regulatory vasodilatory peptides accounted for the apparent inhibition of AI responses. The response to BK was, however potentiated by enalaprilat, consistent with previous studies.

In order to investigate whether non-ACE AII generation is limited to gluteal resistance arteries, or is more widespread, responses in human internal mammary arteries were also studied. The results of these experiments were consistent with those in smaller vessels. In addition, both internal mammary

arteries and samples of skin taken from the normal volunteers who had undergone gluteal biopsy, were used in an investigation of the localisation of ACE and chymase. Indirect immunohistochemistry was performed using monoclonal antibodies for ACE and chymase. Chymase was identified within mast cells in the adventitia of internal mammary arteries and small skin vessels. ACE was identified on the endothelial surface of skin capillaries and in the media of internal mammary arteries. Thus, ACE and chymase expression are spatially distinct and this may be reflected in a functional distinction.

Numerous studies have suggested that polymorphisms within genes encoding components of the RAS may associate with hypertension and cardiovascular disease. The ACE I/D polymorphism has acted as a paradigm for such investigations. However these studies have often been contradictory and their interpretation is limited by the failure to demonstrate a convincing intermediate phenotype, that explains in physiological terms, how polymorphic markers may influence disease predisposition. Since the D allele is associated with higher plasma and tissue ACE levels, I hypothesised that this might be reflected in greater local conversion of AI to AII in human blood vessels. An analysis of the response to AI, according to genotype, was therefore performed. This was limited by heterogeneity in the distribution of genotypes in experimental subjects, but did not support the hypothesis.

These studies convincingly demonstrate that AII generation in the human vasculature occurs by the action of ACE and an alternative enzyme –

probably chymase. However their interpretation is limited by a number of methodological issues. In particular, it has been suggested that these *in-vitro* studies exaggerate the importance of chymase because circulating AI is exposed to plasma and endothelial ACE, and is thus available to the enzyme. *In-vivo* experiments have consistently suggested that ACE is the predominant AII-generating enzyme. It is also possible that chymase does generate AII that acts locally within the vasculature, but not primarily as a vasoconstrictor. In this respect, AII is known to act in a paracrine fashion as a local growth promoter. However the experiments presented in this thesis have concentrated on the vasoconstricting action of angiotensin and have not addressed this issue.

These issues could be resolved in a number of ways. The development of a specific chymase inhibitor suitable for *in-vivo* use would facilitate studies of the pressor action of AI to determine whether chymase contributes significantly to AII generation in man. However, other *in-vitro* strategies may also be helpful. These include further functional studies, but also a molecular biological approach to investigate whether ACE and chymase expression in the vasculature are influenced by disease or treatment. Functional studies could utilise the known substrate specificities of ACE and chymase. Indeed, AI isopeptides have been synthesised that are converted by either ACE or chymase, but not both. These could be used to dissect out the relative contributions of ACE and chymase to AII generation. In addition perfusion myography could be used to investigate local AII generation in a more

physiological preparation. Molecular techniques might include rtPCR, in-situ hybridisation and immunohistochemistry to investigate the regulation of ACE and chymase expression. If confirmed, the existence of non-ACE AII generating pathways may have considerable therapeutic significance.

# Abbreviations

| | |
|---|---|
| Acetylcholine | ACh |
| Angiotensin Converting Enzyme | ACE |
| ACE inhibitor | ACEi |
| Adenosine Triphosphate | ATP |
| Angiotensin I | AI |
| Angiotensin II | AII |
| Angiotensin II type 1 Receptor | AT1R |
| Bradykinin | BK |
| Coronary Heart Disease | CHD |
| Chronic Heart Failure | CHF |
| Left Ventricular Ejection Fraction | LVEF |
| Norepinephrine | NE |
| Physiological Salt Solution | PSS |
| Potassium Kreb's Solution | KPSS |
| Renin Angiotensin System | RAS |

# Chapter 1

# Introduction and Literature Review

## 1.1 The Renin-Angiotensin System: Overview and Historical Perspective

The Renin-Angiotensin System (RAS) plays a fundamental role in the regulation of the cardiovascular system. Through the production of Angiotensin II (AII) and indirectly by AII-stimulated secretion of aldosterone, the RAS regulates vascular structure and tone, renal haemodynamics and electrolyte and fluid homeostasis. Inhibition of the RAS is effective in the treatment of hypertension, chronic heart failure and progressive renal disease. However, despite the proven effectiveness of such treatment, the mortality and morbidity from these conditions remains high and it is therefore likely that novel strategies to optimise blockade of the RAS (and similar neuroendocrine systems) will yield further clinical benefits. In this introductory review I shall take a historical overview of the RAS and discuss areas of controversy, in particular the existence of local renin-angiotensin systems, genetic factors influencing AII production and mechanisms of AII generation, concentrating, wherever possible, on data obtained from human studies.

### 1.1.1 History of the RAS

In 1898 Tigerstedt and Bergman described a landmark experiment in which an elevation in blood pressure in the rabbit was induced by injecting a crude extract from the kidney.[1] They named the hypothetical pressor substance renin. Although this is now regarded as a discovery of fundamental

importance, it was largely ignored for twenty years, until Volhard postulated that renin might be a humoral factor responsible for so-called "pale hypertension," now known as malignant hypertension.[2] Two separate groups then suggested that renin was not directly responsible for the increase in blood pressure, but instead generated a vasoactive substance from plasma. Page and colleagues, working in Cleveland, showed that renal extracts only induced vasoconstriction when incubated with plasma and subsequently identified the plasma product, which they named *angiotonin*.[3] Simultaneously Braun-Menendez, in Mendoza, came to the same conclusion by extracting venous blood from ischaemic kidneys, which contained a pressor agent. Purification of the pressor substance yielded a factor that, unlike renin, induced only a short-lived pressor response *in-vivo* and was thermostable. They named this substance *hypertensin*.[4] The two groups subsequently agreed that the two substances were identical and combined their names to give the name angiotensin.

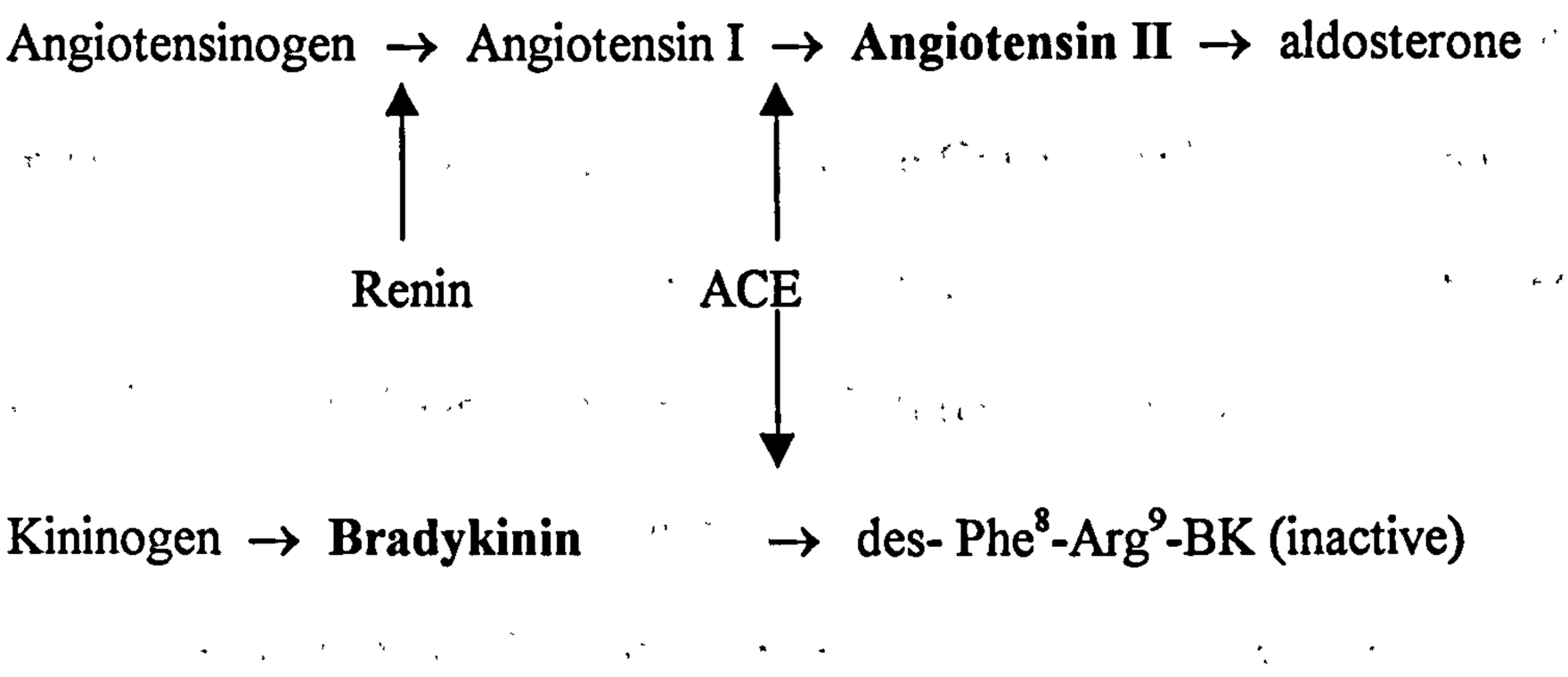
The existence of angiotensinogen was postulated by Page, who called it the "Renin Activator".[5] Plasma subjected to proteolytic digestion with pepsin was shown to liberate a pressor agent and by analogy it was suggested that renin was an enzyme which acted on a substrate - renin substrate (i.e. angiotensinogen), rather than being itself activated by a factor in plasma.[6] The existence of a further enzyme in the cascade was suggested by the work of Skeggs and colleagues who attempted to produce large quantities of angiotensin by incubating crude horse plasma angiotensinogen with porcine

renin. The constrictor factor was purified by countercurrent distribution with dialysis against distilled water and was shown to be a decapeptide. However, when dialysis was performed against 0.15M sodium chloride, a different form of angiotensin was identified. This is now known to be angiotensin II (AII), an octapeptide formed from the decapeptide precursor, angiotensin I by a chloride-sensitive dipeptidase.[7, 8] This enzyme was then identified and is known as angiotensin-converting enzyme (ACE).

Independently of the work on the generation of angiotensin, Erdos and co-workers were studying the metabolism of bradykinin (BK). Bradykinin is a vasodilator that was first identified in snake venom.[9] It is produced from its precursor, kininogen, by kallikrein (though other enzymes, such as trypsin, will also generate BK) and in the plasma is then rapidly degraded by removal of the C-terminal dipeptide, $Phe^{8}$-$Arg^{9}$. The enzyme responsible for the degradation of BK in plasma and kidney was identified and named kininase II (kininase I cleaves the terminal Arg only).[10] Purification of the enzyme and study of its substrate specificity subsequently showed that ACE and kininase II were identical.[11]

By the early 1970s the concept of the RAS as an endocrine system that generated AII by a series of enzymatic steps was firmly established. In addition it was known that the RAS interacted with the kinin system through the bifunctional actions of ACE to catalyse the conversion of AI to AII and to inactivate BK. This basic pathway for the generation of AII is summarised in Figure 1.1.

Figure 1.1: Basic pathway of the RAS




This diagram demonstrates the basic AII-generating pathways. In reality these pathways are considerably more complex than shown above. AII may be generated from angiotensinogen directly, without the intermediate step of AI formation. This is not mediated by ACE. Furthermore, ACE is not the only enzyme capable of releasing AII from AI. This issue is discussed further in section 1.4.3. Just as the generation of AII is more complex than was initially supposed, so is the degradation of this peptide. Several AII degradation products have been described and some of these are thought to be bioactive. This issue is discussed in section 1.1.5.

### 1.1.2 Renin Synthesis and Secretion

The major site of renin synthesis is the kidney. Renin is synthesised in specialised epitheloid cells of the juxtaglomerular apparatus (JGA) in the kidney. Ruyter first identified these cells in 1925 and Goormaghtigh then suggested that they were responsible for the secretion and activation of renin

(this subject has been extensively reviewed).[12] In man, renin is encoded by a single gene, located on chromosome 1q32.[13] It is synthesised as a 45kDa protein, pre-prorenin, from which the signal peptide is removed during transfer into the Golgi apparatus.[14] In the Golgi complex the prosegment is cleaved from some molecules to form active renin.[15] This is sequestered in granules, which have a number of features that suggest that they are lysosomal in nature. Mature granules, which are mainly comprised of active renin, are formed by the coalescence of protogranules, which are largely comprised of prorenin – suggesting that some inactive renin is cleaved within the granules. Export of renin is achieved by exocytosis of mature granules, though there is evidence that small immature granules containing prorenin are also exocytosed and as much as 90% of circulating renin is in this form. The fact that nephrectomy leads to a dramatic fall in plasma active renin suggests that the kidney is the principal site of active renin synthesis. This is not true of inactive renin, whose levels may be nearly normal after nephrectomy. Extra-renal sites of prorenin synthesis include the ovary and there are other local systems such as the vasculature and the central nervous system (see below). Whether prorenin is physiologically active is debatable. Prorenin plasma levels are reported to be elevated in a number of pathological states including diabetic nephropathy, but the significance of this is unclear and it may reflect a more general activation of the RAS.[16] Some studies, however, report a hypotensive, vasodilating, action of prorenin. This may be due to competition with renin for angiotensinogen or may reflect a genuine action of

the peptide itself.[17] In contrast, a recent study utilised rats transgenic for the human angiotensinogen gene to investigate whether prorenin was physiologically active.[18] Isolated rat hindquarters were perfused with human prorenin and/or renin and AI formation and mean arterial pressure were measured. There was no evidence that prorenin was activated to renin, nor of competition between the two peptides. Prorenin did not antagonise the effect of renin on blood pressure.

Renin is an aspartyl protease whose only known substrate is angiotensinogen, which it cleaves at a Leu-Leu bond to release the decapeptide, AI. In the conventional concept of the RAS as a circulating endocrine system plasma renin is thought to release AI from circulating angiotensinogen (which is almost exclusively derived from the liver). Since the concentration of plasma angiotensinogen is many times higher than the Michaelis constant for the conversion to AI by renin, it was generally accepted that renin is the rate-limiting step in the enzyme cascade.[19, 20] Recently, however, some studies have cast doubts on the kinetics of the renin-angiotensinogen reaction, putting the substrate concentration much closer to the $K_m$ of the enzyme and transgenic models suggest that the availability of angiotensinogen is also rate-limiting.[21] Thus, Kim et al showed that, in transgenic mice expressing a variable number of copies of the angiotensinogen gene, blood pressure correlated with both the number of copies of the gene and the plasma level of substrate.[22]

### 1.1.3 Regulation of renin secretion

The regulation of renin synthesis is complex and a detailed review of this subject is beyond the scope of this introduction (see review by Taugner and Hackenthal; [12]). Briefly, however, renin synthesis and secretion are regulated by a number of factors. These include renal nerves, hormonal factors, sodium status and pressure responses. Numerous studies suggest that β-adrenergic agents are able to stimulate renin secretion, probably via $\beta_1$ receptors activated by renal sympathetic nerves. The effect of α-adrenoreceptor stimulation is less certain. Stimulation of this receptor causes renal vasoconstriction and inhibition of renin release may therefore be secondary to induced haemodynamic changes. However some experiments have shown that stimulation of $\alpha_1$ receptors increases renin secretion – an effect that may be mediated by prostaglandins.

Renin synthesis is closely related to sodium status and is stimulated by sodium depletion. Whether sodium delivery to the JGA influences renin secretion is difficult to establish, but it appears that cells of the macula densa exert a tonic inhibitory effect by a local, as yet unidentified, signal. Autocrine regulation by nitric oxide (NO) and prostaglandins has been suggested.[23] Renin secretion is also responsive to changes in renal perfusion, with a marked rise as perfusion falls.[24] This is mediated in part by renal sympathetic nerves, with a significant contribution from the myogenic response of renal arterioles.[25] The set-point for this response is probably

very close to mean arterial pressure, suggesting that the baroreflex is an important physiological mediator of renin secretion.

The most important hormonal factor that influences renin release is AII. This has been shown to inhibit renin release as part of a negative feedback control mechanism. The effect of AII is mediated through its actions on renal haemodynamics and tubular function, but there is also a direct inhibitory effect on granulated cells.[26]

### 1.1.4 Generation of angiotensin II by ACE

The next step in the cascade is the generation of AII from AI. Initially this was also felt to occur in the plasma compartment. Vane and colleagues showed that a major site for AII generation was the pulmonary vascular bed.[27] ACE is an enzyme with a wide tissue distribution. It is present in plasma in a truncated form that lacks the C-terminal hydrophobic anchoring domain, implying that it is shed from the endothelial surface.[28] In the vasculature ACE is an ecto-enzyme present on endothelial cells of arteries and veins, and is present in large amounts in the lungs (because of their extensive vascular bed). Other sites rich in ACE are the brain, particularly in the choroid plexus, adjacent to the subfornical organ and the caudate nucleus, the kidney and the genital tract, especially the testis.[29] ACE has two distinct isoforms, germinal and somatic. Germinal ACE is derived from the same gene as the somatic form by differential splicing regulated by a tissue specific promoter.[30] Immunohistochemistry and in-situ hybridisation show that

germinal ACE is expressed uniquely in germinal cells.[31] Epithelial cells also contain large amounts of ACE. Thus immunohistochemical staining reveals large amounts of the enzyme in the epithelium of proximal tubules in the kidney.[28]

ACE is a chloride-sensitive C-terminal peptidase. Its primary structure suggests that it has two catalytic sites which can be shown to be independently active.[32] Both the active sites are able to hydrolyse AI and BK, but their $K_{cat}$ for these substrates and chloride sensitivity differ. Unlike renin, which shows great substrate specificity, ACE has a number of peptide substrates, limited by the presence of a penultimate proline residue. Alternative substrates for ACE include opioids, neurotensin, substance P and luteinizing hormone releasing hormone.[33, 34] These substrates are generally short and the rate of hydrolysis is related to the length, slowing considerably if the peptide has more than ten residues. Chloride dependence of the ACE-substrate reaction is variable; AI is chloride dependent, however BK is hydrolysed at much lower concentrations. The exact role of these reactions remains unknown, but clearly ACE has multiple functions beyond the generation of AI and inactivation of BK. ACE is not thought to be rate-limiting in the RAS.

### 1.1.5 Other derivatives of angiotensin I

The major product of the RAS is the octapeptide AII, although further cleavage of the peptide by a number of peptidases produces fragments that

may be active (Figure 1.2). Thus deletion of the C-terminal phenylalanine by a metalloendopeptidase produces Ang-(1-7) which is thought to have vasodilator and anti-proliferative properties. Ang-(1-7) is degraded by ACE and thus ACEi increase plasma levels of this peptide.[35] Other fragments of AII that may have physiological actions include angiotensin III (Ang III) and angiotensin IV (AIV). Ang III is the N-terminal aspartic acid deleted derivative of AII. Ang IV is produced from Ang III by the deletion of the N-terminal arginine. The peptidases responsible for the generation of Ang III and Ang IV include aminopeptidases A and B.[36] Ang III may also be generated independently of AII by initial cleavage of AI to Des-Asp$^{1}$ AI (mediated by aminopeptidase B), followed by removal of the terminal His-Leu dipeptide (by ACE). While Ang III is thought to exert its effects through binding to the AT1 receptor, this is not thought to be true of Ang IV and Ang-(1-7). Moreover the biological effects of these peptides are distinct from those of AII. Ang III acts predominantly in the brain where it is released in the paraventicular nucleus in response to water deprivation and induces a dipsogenic response.[37] Ang IV appears to act on a receptor found in the brain, kidneys, lungs, prostate gland and the gut. Its actions are not yet clear; in some vascular beds it mediates vasoconstriction, but in others causes vasodilation.[38]

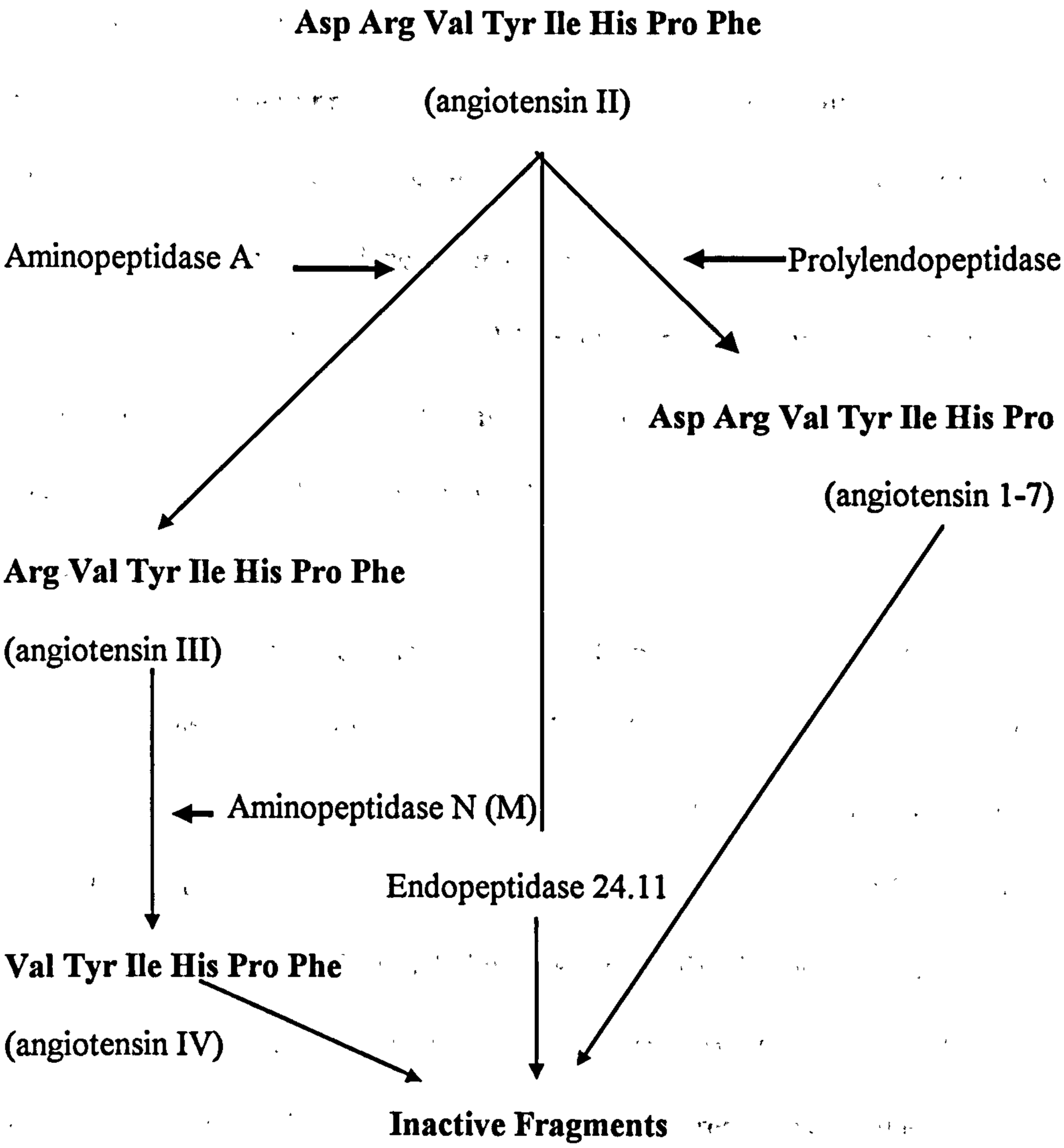
### 1.1.6 Angiotensin II receptors

AII acts at two principal receptors, though the existence of others has been postulated. Most of the known biological actions of AII are through binding to the type 1 receptor, AT1R. A second major receptor subtype, the AT2 receptor (AT2) has also been identified. The molecular biology and role of the two receptors has been comprehensively reviewed.[39, 40] Both the AT1R and AT2R are members of the family of receptors with seven transmembrane hydrophobic domains and are G-protein coupled. They are encoded by distinct genes and have only a 32% sequence homology. Pharmacologically they can be distinguished by their different binding affinities, AT1R showing a high affinity for the AII antagonist losartan, and AT2R for the compounds CGP42112A and PD 123177.

Binding of AII to AT1R is followed by the cleavage of phosphatidylinositol to diacylglycerol (DAG) and inositol-4,5-bisphosphate ($IP_2$). $IP_2$ and its metabolite, $IP_3$, initiate a rapid rise in intracellular $Ca^{2+}$, while DAG stimulates the activity of the membrane-associated kinase, protein kinase C. In turn, this phosphorylates serine and threonine residues on protein substrates and modifies their activity. Mitogen-activated protein kinases (MAP) are also stimulated and these may be responsible for activation of immediate early genes, such as c-*fos*, c-*jun* and c- *myc*. Other pathways activated by AII acting through AT1R include phospholipase $A_2$ and phospholipase D. Recently AII has been found to cause phosphorylation of janus kinases, JAK2 and TYK2, which in turn leads to tyrosine

phosphorylation and nuclear translocation of the $STAT_1$ and $STAT_2$ transcription proteins, thus providing a mechanism for the prolonged trophic effects of AII stimulation in a number of organs.

Figure 1.2: Metabolism of AII and related peptides



In contrast to the AT1R, very little is known about AT2R signal transduction. Activation of AT2R is not associated with a rise in intracellular $Ca^{2+}$, or the generation of phospholipid derived second messengers. While there is some evidence that the AT2R is associated with phosphotyrosine phosphatase activation, MAP inhibition and inhibition of T-type calcium channels, the biological effects of AT2 stimulation are unclear. The ability of AT2R stimulation to induce protein dephosphorylation suggests that it might counteract the growth promoting effects of AT1R. Consistent with this is the observation that over-expression of the AT2R in rat carotid arteries is associated with attenuated neointima formation.[41] There have also been suggestions that stimulation of the AT2R may result in apoptosis in vascular smooth muscle cells and in a rat phaeochromocytoma cell line.[42, 43] This is mediated through the dephosphorylation, and inactivation of specific "survival" proteins such as Bcl-2.

The differential actions of the AT1R and AT2R are mirrored in their tissue localisation and pattern of expression. Thus, AT1R is expressed in the myocardium, on vascular smooth muscle cells, fibroblasts, neuronal cells, in the adrenal cortex and in the kidney. Inhibition of the AT1R during pregnancy induces specific renal abnormalities in the foetus, characterised by papillary atrophy, tubular atrophy and vascular wall thickening.[44] This suggests that the AT1R is also required for normal renal development. AT2R is expressed in the adrenal medulla, ovary, brain and at high levels during foetal development. In the rat the AT2R is the predominant angiotensin

receptor in the aortic arch and pulmonary artery from day fifteen of gestation until fifteen days post-partum and is also expressed in the trachea. However, soon after birth, AT1R becomes the predominant receptor subtype in these tissues.[45] This suggests that, while the AT2R may play an important role in the development of the vasculature (though its precise role remains to be elucidated), regulation of the cardiovascular system in the adult is largely mediated by binding to the AT1R.

## 1.2 Local Renin Angiotensin Systems

The historical review of the biology of the RAS given above concentrates on its role as an endocrine system and suggests that AII is generated in plasma and acts as a circulating hormone. However, many lines of evidence suggest that there may be a parallel local RAS, distinct from, but related to, the systemic RAS. The possible role of locally generated angiotensin was first suggested by mechanistic studies of angiotensin metabolism. It was found that the rate of angiotensin metabolism was too high for generation in plasma by (plasma) renin to explain measured levels.[46] This was studied in some detail by Admiraal et al who infused [$^{125}$I]-labelled AI intravenously into hypertensive subjects at the time of renal vein sampling as an investigation for renovascular disease.[47] This allowed the sampling of venous blood to measure the extraction of AI across the forearm, kidney, leg and hepatomesenteric vascular beds. Placement of an

arterial catheter allowed the arterio-venous concentration gradient to be assessed. The extraction of labelled AI varied from 47% across the forearm to 96% across the kidney. However, in spite of the high degree of extraction, there was little or no difference in the plasma arteriovenous concentration gradient for AI, strongly suggesting that regional de novo production accounted for a large proportion of the generation of the peptide. From the blood transit time across the various vascular beds, and the known plasma renin level, the contribution of plasma renin to the generation of AI was estimated to be less than 20-30% across the kidney and at best 60% across the hepatomesenteric bed.

While the demonstration of local angiotensin generation is important it does not prove that there are tissue based renin-angiotensin systems, independent of the circulating system. Components of the RAS such as renin, angiotensinogen and ACE may be adsorbed from the bloodstream thus making local activity of the RAS dependent on delivery of precusors. For example, Loudon showed that after bilateral nephrectomy in rats, renin was detectable in the aortic wall for some hours, despite a profound and rapid fall in plasma levels, consistent with arterial wall uptake.[48] Thus, a clear distinction between circulating and tissue renin-angiotensin systems requires the demonstration of synthesis of components of the RAS in the tissues. Before molecular biological techniques to detect the presence of mRNA were available the existence of components of the RAS in tissues was inferred from the results of bioassays. This raised concerns that the activity measured was

not due to renin, but to other enzymes such as cathepsin G, which also release AII from its precursors, but at a more acid pH. However in recent years the presence of mRNA for all the components of the RAS has been demonstrated in many organs, including the heart, nervous system, kidney and the vasculature. In addition there is evidence that the expression of various components of tissue renin-angiotensin systems may be altered in various pathological states.

### 1.2.1 The Vascular Renin Angiotensin System

A. Evidence from physiological studies

The existence of a local vascular RAS is suggested by both *in-vivo* and *in-vitro* experiments studying pressor responses to angiotensin. Bund et al showed that porcine tetradecapeptide (TDP) renin substrate elicited a contractile response in isolated small human resistance arteries mounted in a wire myograph.[49] This contraction could be inhibited by a renin inhibitor, H261, suggesting that vascular wall renin was able to activate the precursor TDP. Interestingly this contraction could not be inhibited by captopril, but some inhibition was obtained using the serine protease inhibitor, aprotinin, and this was enhanced by combining aprotinin and captopril. Thus, it appeared that the vessel wall RAS included renin, ACE and a serine protease (or proteases) capable of generating AII from its precursors. The ability of locally generated AII to modulate the tone of resistance arteries was also studied in an elegant experiment by Henrion et al.[50] They perfused an

excised rat carotid artery (donor) in series with a smaller rat mesenteric resistance artery (recipient), thus allowing them to study whether vasoactive agents generated in the donor vessel influence the tone of the recipient. The vessels were perfused with Kreb's solution, mounted in separate chambers and immersed in a superfusate into which drugs could be added to each vessel individually. They found that AII was generated by the donor (despite the absence of any precursor in the perfusate) and that this constricted the recipient, an effect that was inhibited by adding cilazapril to the superfusate surrounding the donor artery. In addition incubation with both losartan and cilazapril caused the recipient artery to dilate. Their data suggest that AII was generated locally, in both the donor and recipient vessels, and was acting on the AT1 receptor. Furthermore the addition of a BK antagonist, HOE 140, suppressed the ACEi induced dilation of the recipient, suggesting that locally generated BK was able to influence vascular tone.

Local vascular conversion of AI to AII *in-vivo* has also been studied using venous occlusion plethysmography. Webb et al infused AI into the brachial artery of healthy volunteers and assessed the pressor response.[51] AI induced vasoconstriction could be inhibited by co-infusion with enalaprilat, suggesting that vascular ACE was responsible for converting AI to AII within the vessel. Interestingly, while infusion of enalaprilat alone had no effect on forearm vascular tone in sodium replete subjects, after sodium depletion, infusion of the ACEi increased resting forearm blood flow, suggesting that vascular AII generation may be influenced by sodium status. Further evidence

of regulation of the vascular RAS by sodium status was recently provided by Boddi et al who investigated AII production and AI degradation in the forearm and leg vascular beds in healthy subjects on a normal, low and high sodium diet.[52] Their methods were similar to those employed by Admiraal et al - using infusions of both [$^{125}$I]-labelled AI and unlabelled AI.[47] As in earlier work, they found that that both AI and AII were generated by the local vascular bed. Surprisingly, manipulation of the sodium status of the subjects revealed differential regulation of the circulating and vascular RAS. In sodium deplete subjects plasma renin activity increased but vascular AI and AII generation became undetectable, while in sodium replete subjects plasma renin activity fell and vascular AI and AII generation were significantly enhanced. The conclusion from this study, that sodium depletion is associated with reduced vascular ACE activity is obviously at variance with the results obtained by Webb and requires confirmation. Nonetheless, it used well-established methodology and measured vascular angiotensin generation more directly. If confirmed, this study does suggest that the vascular and systemic RAS are differentially regulated by sodium balance, which is clearly an important finding.

It is clear, from both in-vivo and in-vitro studies, that there is local vascular generation of AII and, in addition, there are tantalising hints that regulation of the vascular RAS is distinct from the circulating system. However, proof that the vascular RAS is differentially regulated at a genomic level requires the tools of molecular biology.

B. Evidence from molecular biology

Expression of renin

Whether renin is expressed in blood vessels has been hotly debated, with both positive and negative studies being reported.[53, 54] The persistence of renin-like activity in the vasculature after nephrectomy may indicate continuing synthesis outwith the kidney. However, other authors have suggested that when the pH is kept at 6.5, which is optimal for renin, activity rapidly declines, suggesting that other enzymes have been responsible for the generation of AII in bioassays.[55, 56] When renin mRNA has been detected in vascular tissue it has been at a low level. Thus, though Paul et al detected renin mRNA in human aorta and saphenous vein, the measured level in the latter (using a semi-quantitative competitive rtPCR reaction), was about 1% of that found in the kidney.[57] Since angiotensinogen and ACE mRNA are also expressed in aorta and saphenous vein, it is possible that all the components necessary for AII generation are present in vascular tissue, at least in large vessels. Whether this is true of resistance arteries is not known.

Expression of angiotensinogen

Although it is thought that the reaction between renin and angiotensinogen is the rate-limiting step in the RAS, surprisingly little is known about local production of this peptide. As discussed above, angiotensinogen mRNA is detectable in the human aorta and saphenous vein. Naftilan et al investigated angiotensinogen expression in the aorta of Wistar

and Wistar-Kyoto rats.[58] The aim of this study was to obtain information about both the level and spatial organisation of angiotensinogen expression and investigate the effect of sodium depletion on the former. Angiotensinogen mRNA, identified using northern blot analysis and localised by in-situ hybridisation, was located in the smooth muscle cell layer of the aorta, as well as in perivascular adipose tissue. On a normal sodium diet the expression of angiotensinogen mRNA was greater in the adipose tissue than in the aorta, but sodium depletion enhanced the latter and had no effect on the former. Shiota et al found that angiotensinogen gene expression was increased in the aorta and liver of two-kidney, one clip rats four weeks after clipping, but levels fell to normal after twelve weeks.[59] In the rat, at least, these two studies suggest that increased local angiotensinogen expression may contribute to the vascular abnormalities seen in hypertension. This hypothesis has been tested further in rats transgenic for the human angiotensinogen gene (which is not cleaved by rat renin).[60] In-situ hybridisation detected mRNA for human angiotensinogen in the smooth muscle cell layer of skeletal muscle resistance arterioles from the rat hindlimb, though precise cellular localisation was not possible. When the isolated hindlimb was perfused with active human renin, AI was detectable in the effluent in transgenic but not control rats. After cessation of the infusion plasma renin quickly became undetectable, but AI continued to be detected for 30 minutes. Renin infusion also stimulated AII production and induced vasoconstriction. These results suggest that

plasma renin can be taken up from the circulation and that this is able to generate AII by cleavage of locally synthesised angiotensinogen.

Expression of ACE

ACE can be detected in smooth muscle cells from the media of blood vessels, as well as in the endothelium.[61, 62] ACE has also been identified in the adventitia of the rat aorta. Given the evidence for vascular localisation of angiotensinogen, it is likely that all three layers of larger arteries may possess the components of a local RAS. Again, the location of components of a putative local RAS in resistance arteries is not yet known, though these vessels have a similar three-layer structure to conduit vessels.

Interest in ACE as a regulator of local AII generation has been stimulated by the identification of a polymorphism that associates with plasma and cellular ACE levels and which may be associated with cardiovascular disease (see section 1.3).[63, 64, 65] In addition, ACE expression is increased in a number of animal models of hypertension. Thus Miyazaki and co-workers have shown that ACE activity and gene expression is increased in the rat two-kidney, one clip model of hypertension and that this is associated with an increased contractile response of isolated arteries to AI.[66] This work was extended by Muller et al who used the same model to measure both aortic ACE gene expression, by an mRNA protection assay, and AII formation from infused AI in the isolated hindlimb.[67] AII generation was linear over a wide range of infused AI concentrations in both hypertensive and

control rats and did not show saturation, consistent with the known kinetics of the ACE/AI reaction. Nonetheless, AII generation and ACE gene expression were significantly higher in the hypertensive rats, suggesting that vascular ACE was able to regulate local AII formation. This finding may be of particular importance since it suggests that while ACE may not be rate limiting in the systemic RAS, it may act to regulate the final concentration of AII in tissues, especially in conditions where there is increased expression of the RAS.

The significance of increased local AII generation secondary to increased ACE expression may lie not only with regulation of vascular tone, but with long-term regulation of vascular structure (see section 1.4). Consistent with this hypothesis, Morishita et al were able to increase carotid artery ACE expression in the rat using a gene transfer approach and this was associated with an increase in wall to lumen ratio, an effect that could be abolished by an AT1R antagonist.[68]

Transgenic models

Transgenic models have also investigated the possibility that local expression of the RAS may be of pathophysiological significance. The model that has generated most interest is the Ren-2 transgenic rat.[69] This model, developed by Mullins and co-workers, expresses the mouse salivary gland Ren-2 (i.e. renin) gene and develops fulminant hypertension. While there is some debate about what the true plasma renin levels are in this model

(radioimmunoassay detects low plasma renin, but this may be rat renin and the possibility remains that mouse renin circulates in the plasma at high levels) there is little doubt that the Ren-2 gene is expressed in vascular tissue. This is associated with increased angiotensin generation in the isolated hindlimb, compared to control rats.[70] In addition, continuing angiotensin generation in the isolated hindlimb following bilateral nephrectomy suggests continued vascular renin gene expression. The low plasma renin level suggests that local angiotensin generation secondary to local over-expression of the transgene may be responsible for the phenotype.

Most of the evidence that there is a vascular RAS has come from animal studies, or from histological examination of large human vessels. A recent study, however, detected mRNA for all the components of the RAS, including renin, in (smaller) uterine spiral arteries, which undergo considerable remodelling during pregnancy.[71] It is therefore possible that components of the RAS are expressed widely in the human vasculature and that they are regulated independently from the circulating system. There is preliminary evidence to suggest that dysregulation of the vascular RAS could contribute to the pathogenesis of hypertension and may be responsible for some of the vascular and end-organ abnormalities seen in this condition.

### 1.2.2 The Cardiac Renin Angiotensin System

There is a large literature concerning the cardiac RAS. Several lines of evidence suggest that AII is generated within myocardial tissues. The heart

appears to be a net secretor of angiotensins and the tissue concentrations of AI and AII are too high to be explained by diffusion from the blood and distribution into the interstitium.[72] The site of cardiac angiotensin production has been studied in some detail. Using a modification of the Langendorff isolated perfused rat heart, de Lannoy et al studied the relative contributions of interstitial and intravascular AI production.[73] No AI production was observed when the heart was perfused with Tyrode's buffer alone, but AI was detectable in interstitial fluid during renin infusion and in greater quantities in both interstitial fluid and coronary venous effluent during combined renin and angiotensinogen infusion. However, though the appearance of AI in interstitial fluid could be explained by the renin-angiotensinogen reaction in that compartment, the concentration of AI measured in the coronary effluent was 4.6 times higher than could be explained by intravascular generation alone. This suggests that the majority of cardiac AI production occurs in the extravascular compartment. The authors thus proposed that AII is generated at two sites - the interstitium and the vascular endothelium. This study also suggested that cardiac AI generation was dependent on the delivery of renin, and possibly angiotensinogen, by plasma. This work was extended by van Kats et al, who measured cardiac AI and AII production in pigs using a radiolabelled tracer technique.[74] Again they found that most of the cardiac-derived AI and AII were synthesised locally. In addition, they investigated the effect of captopril treatment on cardiac AI and AII production and showed that, while plasma

AII was suppressed, tissue AII levels were unchanged. Thus, tissue AII production was maintained under conditions in which circulating AII production was inhibited.

These studies provide strong evidence that the heart is able to synthesise AI and AII locally. However, proof of the existence of an independent cardiac RAS requires the demonstration of local synthesis of angiotensinogen or renin. Bilateral nephrectomy has been shown to cause a fall in cardiac renin to low or undetectable levels in the rat and other species, casting doubt on this possibility.[75, 76] In contrast, other studies have shown that the heart does contain mRNA for renin, angiotensinogen and ACE.[77, 78, 79] Thus, gene expression of components of the RAS may contribute to increased local cardiac AII generation independent of the systemic RAS.

Activation of the cardiac RAS after myocardial infarction

Falkenhahn et al studied ACE distribution using immunohistochemistry in an experimental rat model of myocardial infarction and also in human post-mortem specimens.[80] In non-infarcted myocardium ACE was largely confined to endothelial cells. One day after myocardial infarction no ACE was identified in the zone of necrosis, but after three days began to appear within granulation tissue at the border of this zone. Intense ACE expression was then identified in the endothelium of sprouting capillaries and this continued as fibrosis developed. ACE was also identified in

macrophages and fibroblasts within the scar tissue. AII is known to promote hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts, inhibits matrix metalloproteinase type I and also stimulates cardiac fibroblasts to increase production of type I and type III collagen.[81, 82] Increased cardiac ACE expression may thus contribute to remodelling after myocardial infarction. Cardiac remodelling is also a feature of congestive heart failure (CHF) and may contribute to the progressive myocardial dysfunction seen in this condition. It is, therefore, no surprise that inhibition of the RAS is associated with marked mortality and morbidity benefits in established CHF and post myocardial infarction.[83, 84, 85, 86]

### 1.2.3 Other Organ Specific Renin Angiotensin Systems

The conventional view of the systemic RAS as an endocrine system places the kidney in a pivotal position since it is the major source of circulating renin and is also the site of action of AII and aldosterone in regulating sodium balance and extracellular fluid volume. In fact, the kidney is also a net secretor of AII, and studies assessing the total renal content of the peptide suggest that there is far more than can be explained by diffusion from the plasma compartment into the interstitium.[87] It is likely that AII is generated from both locally synthesised and systemically delivered angiotensinogen and acts in a paracrine manner within the kidney.[88, 89] Thus, angiotensinogen mRNA has been identified in the proximal tubule by a variety of techniques and ACE is found in large quantities at the proximal

tubule brush border; as well as on endothelial cells in the renal vasculature.[90, 91] The AT1R is also found on the luminal membrane in the proximal and distal tubule, as well as on mesangial cells and both afferent and efferent arterioles, and it is likely that AII is generated in the nephron, where it is also active.[92] Braam et al confirmed that AII is detectable within proximal tubule fluid in the nanomolar range and also showed that AII can be detected in microperfused tubules.[93] In perfused tubules the source of the AII cannot be glomerular filtrate, and this finding provides very strong evidence that AII is generated within the nephron. Whether this is as a result of the action of renin synthesised in the tubule is open to question: some authors report the detection of renin mRNA in small quantities within proximal tubule cells and Leyssac reported measurable renin within tubular fluid.[94] Since renin is not filtered at the glomerulus it is likely that there is some intra-tubular renin synthesis, albeit at a low level. The renal tubules thus appear to express all the components of the RAS.

AII generated within the tubule acts to promote tubular sodium absorption through binding to the AT1R. It is also likely that locally generated AII has trophic effects within the kidney, just as it does in the vasculature. Thus, AII stimulates growth of cultured mesangial and proximal tubular cells.[95] In addition to promoting cell growth AII also stimulates extracellular matrix deposition by these cells. The trophic effects of AII in the kidney may be mediated by stimulating the paracrine secretion of various cytokines such as TGF-β, PDGF and endothelin-I.[96, 97] In proximal

tubular cells AII induces transcription of type IV collagen, an effect that is blocked by a neutralising antibody to TGF-β, emphasising the importance of cytokine secretion in mediating the local trophic effects of the RAS.[98]

In addition to the examples of local AII synthesis and action presented above there is evidence for a local RAS in the brain, adrenal gland and reproductive tissue, but a review of these data is beyond the scope of this thesis. There is little doubt, however, that AII is generated locally in a variety of tissues and that this may be significant in a number of pathological states. It is also likely that the RAS has an extended role at a local level, as a growth promoter and to stimulate extracellular matrix remodelling. These effects are likely to be of pathophysiological significance and emphasise the potential benefits of pharmacological interruption of the RAS in conditions such as CHF and renal failure. Finally, I have alluded to recent evidence that polymorphisms of genes encoding the RAS may influence AII generation and so contribute to genetic susceptibility to cardiovascular disease. In the following section I shall briefly review the evidence for this hypothesis.

## 1.3 Polymorphisms of Genes within the Renin Angiotensin System

Most, if not all, genes are subject to minor variations in their sequences and are considered polymorphic if the frequency of the rarest allele is ≥ 1%. Some of these polymorphisms may influence the biological activity

of the gene product, for example the expression of a gene or the functional properties of the encoded protein. This is the intermediate phenotype. Possession of one, or many, of these variants may confer a genetic liability to develop a particular trait.

There are two basic strategies for identifying the genetic determinants of any given trait: candidate gene analysis and total genome searches.[99] The former tests for an association between a polymorphism in a candidate gene and a trait, aiming to show that the variant is more frequent in disease than in controls, or that individuals with a common polymorphism also share the same natural history. This approach relies upon our understanding of the intermediate phenotype and hence the choice of the candidate gene. It is also dependent on the choice of appropriate controls, requires a large candidate gene pool, and is limited in the presence of genetic heterogeneity between populations. In a total gene search strategy, a search is made for the cosegregation of marker loci with the trait in sample pedigrees (such as a family or amongst siblings). This approach is more laborious as it requires the identification of family members and, for this reason, most studies of the genetic basis of cardiovascular and renal disease have utilised a candidate gene approach. Given the importance of the RAS in cardiovascular homeostasis, genes encoding its components are obvious candidates.

### 1.3.1 Renin gene polymorphisms

Renin is thought to be the rate-limiting step in the RAS, making it an obvious candidate gene in hypertension and cardiovascular disease. Early studies in rats suggested that renin gene variation may contribute to the risk of hypertension. For example a restriction fragment length polymorphism (RFLP) within the renin gene, that was identified in the spontaneously hypertensive rat (SHR), has been shown to co-segregate with an increase in blood pressure in F2 progeny of the SHR and Wistar-Kyoto (WKY) cross.[100, 101] A number of RFLPs have been identified in the human renin gene, recognised by the restriction enzymes Taq1, HindIII, BgI I, BgI II and MboI.[102, 103] However most studies using these markers have failed to show any association with blood pressure.

### 1.3.2 The Angiotensinogen M235T Polymorphism

Transgenic strategies inducing over-expression of the angiotensinogen gene in mice suggest that this gene is associated with the regulation of blood pressure. In this model introduction of multiple copies of the angiotensinogen gene led to a dose-dependent increase in blood pressure.[22] Angiotensinogen is thus a strong candidate gene for hypertension. A number of polymorphic variants of this gene have been identified, and their association with hypertension investigated. One such variant of the angiotensinogen gene is the M235T polymorphism, which encodes for the substitution of a threonine for methionine at position 235. Interest in this polymorphism was

stimulated by a sibling-pair study from Salt Lake City and France, which suggested that the gene locus was associated with hypertension in men, particularly in severely affected sib-pairs.[104] Two variants of the gene, the M235T and the 174M were found to be associated with hypertension in this study and the former was found to be associated with higher angiotensinogen plasma levels. Other studies have also found that the M235T variant is associated with essential hypertension in both American and Japanese populations.[105, 106] The M235T polymorphism has also been found to be associated with coronary heart disease and, in addition, the T allele occurred at a higher frequency in women with pre-eclampsia.[107, 108]

As with the ACE I/D polymorphism, association studies involving the M235T polymorphism can be viewed with more confidence if an intermediate phenotype can be identified. One study investigated the role of the M235T polymorphism in determining the renal plasma flow response to AII infusion.[109] The hypothesis was that, since the T allele is associated with higher angiotensinogen levels, it might also be associated with greater intra-renal AII generation and a blunted plasma flow response to infused AII. This was observed and, in addition, there was an interesting interaction between the genotype and obesity, with obese patients exhibiting greater blunting of the response in TT homozygotes. The reason for this interaction is unclear but may reflect greater generation of AII in adipose tissue.

Further support for a physiological role of the M235T polymorphism derives from the demonstration that it may be associated with variation in

gene expression. A common variant in the proximal promoter of the angiotensinogen gene, characterised by the presence of adenine instead of guanine, has been identified.[110] This appears to be in close linkage disequilibrium with the M235T polymorphism and appears to influence transcription of the gene. The authors speculated that the T variant and its associated regulatory element were, in fact, the original version of the gene and had evolved in the sodium-deprived conditions of the African subcontinent. In this scenario hypertension, related to this polymorphism, develops upon a change in environment, such as an increase in sodium intake - a version of Neel's "thrifty gene hypothesis."[111]

In contrast to these studies there have also been a number of negative studies relating the angiotensinogen gene and hypertension. In a study of 223 untreated hypertensives and matched controls from the East-Anglian region of the UK there was no association between the M235T polymorphism and blood pressure.[112] Similarly a study of 508 patients with hypertension from Finland failed to demonstrate any association with this polymorphism.[113] Similarly negative results were also obtained in a German population.[114] Another study suggested that while the angiotensinogen gene itself was associated with hypertension, the M235T polymorphism was not.[115] Thus, it is possible that genetic variation within the angiotensinogen gene may be implicated in the pathophysiology of hypertension, but the role of specific polymorphisms is less clear.

### 1.3.3 The ACE I/D Polymorphism

While there are polymorphic markers in the genes encoding renin, angiotensinogen, ACE and the AT1R, most attention has been focused on the ACE gene since the discovery by Rigat of an insertion/deletion (I/D) polymorphism that accounts about half of the inter-individual variation in plasma ACE levels. This polymorphism is characterised by the presence or absence of a 287 base pair fragment within intron 16 of the ACE gene and is thus not part of the coding sequence.[63] Subsequent studies have confirmed a strong association between serum, cellular and cardiac ACE levels and this polymorphism.[64, 116] The mean plasma ACE level in Caucasian DD homozygotes is about twice the level found in II homozygotes, with an intermediate level in heterozygotes suggestive of co-dominant inheritance. It is suggested that the polymorphism is in strong linkage disequilibrium with a putative regulatory gene locus that controls ACE levels.[117]

<u>The ACE I/D polymorphism and the risk of ischaemic heart disease</u>

Interest in this polymorphism was first generated by the finding of an excess risk of myocardial infarction (MI) in male DD homozygotes.[65] Several other studies, in a variety of populations, have also suggested an association between the I/D polymorphism and coronary artery disease.[118, 119] In particular two studies have shown an association between possession of the D allele and a parental or grandparental history of MI.[120, 121] While these data were initially persuasive there have also been a number of negative

studies.[122] In particular, Lindpaintner analysed the relationship between the polymorphism and ischaemic heart disease in the large, prospectively followed, cohort of the Physicians Health Study and found no association between the ACE I/D polymorphism and the risk of ischaemic heart disease.[123] A subsequent meta-analysis gave a mean odds ratio for MI in DD homozygotes of 1.26, but a funnel plot of odds ratio against sample size suggested that publication bias may have confounded the result.[124] More recently the ISIS III database was interrogated to examine the association between the D allele and the risk of MI in 4629 patients and 5934 controls. No significant association was found between the DD genotype and the risk of MI (odds ratio 1.10, 95% CI 1.00-1.21).[125] In the same paper the results of an up-dated meta-analysis of the association between MI and the D allele were reported. When the results from ISIS were combined with those from the larger hypothesis-generating trials (involving 200 or more cases) the odds ratio was 1.02 (99% CI 0.95 – 1.11). The study was also able to assess the impact of the polymorphism on patient survival and found no association. The authors concluded that, while there may be an excess risk of MI (of approximately 10 – 15%) associated with the D allele, studies with more than 10,000 cases would be needed to provide a reliable answer.

The ACE I/D polymorphism and left ventricular hypertrophy

Following the initial suggestion of an association with the risk of MI, further studies have been performed to investigate the contribution of the I/D

polymorphism to a variety of other conditions in which over expression of the RAS may play a role. Thus the relationship between the I/D polymorphism and left ventricular hypertrophy (LVH), hypertension and progressive renal disease has also been studied. There is now an extensive literature on the possible role of polymorphisms within the RAS in renal disease and this has been comprehensively reviewed.[126]

Several studies have suggested that the D allele may associate with LVH, or other conditions that are due to abnormal cardiac remodelling. Thus Schunkert et al found that the D allele was over-represented in subjects, drawn from population of 711 women and 717 men, found to have LVH by electrocardiographic criteria.[127] The association was particularly strong in normotensive men, who would otherwise be considered at low risk for LVH, but the relationship was not present in women. Other conditions found to be associated with the I/D allele include ischaemic and idiopathic cardiomyopathy and hypertrophic cardiomyopathy (HCM), especially in families with HCM and a history of sudden death.[128, 129] Although other studies, particularly from Japan, have also found an association between LVH and the ACE I/D polymorphism, there have been a number of negative studies as well. Kupari et al used the more sensitive technique of echocardiography to investigate the role of the I/D polymorphism in determining LVH and found no association.[130] This was confirmed by Lindpaintner, using the cohort from the Framingham Study.[131]

Although there have been many studies of the role of the I/D polymorphism in cardiovascular regulation there is, as yet no definite conclusion. There are many reasons for this. Many studies have been small and have lacked statistical power. Genetic heterogeneity (i.e. different genetic associations in different populations) may have confounded the results, the high frequency of the D allele limits its genetic informativeness and there is an inherent weakness in association studies - which are limited, for example, by the choice of an appropriate control population and by the fact that they take a "snap-shot" of a dynamic situation. Greatest doubt has been cast on the association between the D allele and MI, as discussed above.

Twin studies are an attractive alternative to association studies, though recruitment is obviously harder. Only one twin study has been published investigating the role of the I/D polymorphism and cardiovascular structure. Busjahn et al studied cardiac dimensions in 91 monozygotic (MZ) and 41 dizygotic (DZ) twins.[132] There was a significant relationship between both serum ACE activity and possession of the D allele and posterior cardiac wall thickness. However study of the within-pair differences in wall thickness between MZ, concordant DZ and discordant DZ twins suggested a major contribution of other genes in determining cardiac structure.

## The ACE I/D polymorphism and the regulation of blood pressure

There are a number of reasons to consider ACE to be a candidate gene for hypertension. In a cross between stroke-prone spontaneously

hypertensive rats (SHR/SP) and WKY rats, a major locus determining blood pressure was identified on chromosome 10.[133] This region is homologous to chromosome 17q in man – on which the ACE gene is located. Studies in other strains of hypertensive rats also suggested that ACE may be an important locus in determining blood pressure.[134] However, serum ACE activity does not correlate with blood pressure and there have been a number of studies that did not suggest the ACE gene is a determinant of hypertension.[135] Thus, in both Finnish and Japanese populations, and in a French sib-pair analysis, no association was found between the ACE gene and hypertension.[136, 137, 138]

Despite this some studies have suggested that the D allele of the ACE gene may contribute to the development of hypertension in specific populations, such as African-Americans.[139] More recently O'Donnell et al used a cohort of 3095 participants in the Framingham Heart Study to study the association between the ACE gene and hypertension.[140] This study used both the ACE I/D locus and a microsatellite associated with the human growth hormone (hGH) gene – which is tightly linked to ACE – as markers in a linkage analysis on 1044 sibling pairs from 484 families. Logistic and linear regression analyses were also performed to investigate the risk of hypertension associated with the ACE I/D polymorphism in the whole cohort. The odds ratio for hypertension among men who were homozygous at the D allele was 1.59 (95% CI, 1.13 to 2.23), but there was no such association in women. In the linkage analysis a weak association was found between the I/D

locus and the hGH microsatellite and diastolic, but not systolic, blood pressure in men, but not women. While such studies may suggest that the ACE locus is an important determinant of blood pressure they do not exclude the possibility of an association with other genes in linkage disequilibrium with ACE. Julier et al performed a sib-pair analysis in 357 French and UK families to investigate further the linkage between the homologous region on human chromosome 17 and blood pressure.[141] They found evidence that two closely linked markers, D17S183 and D17S934, were linked with hypertension, but these were located 18 centi-Morgans proximal to the ACE locus. Thus, while chromosome 17q may well contain an important blood-pressure determining locus, it is probably not ACE.

Further doubt on the role of the ACE gene in blood pressure regulation has been suggested by transgenic models. Krege et al found that mice heterozygous for an insertional mutation that inactivates the ACE gene had a lower blood pressure than controls.[142] However a subsequent study from the same group found that, in marked contrast to animals over-expressing the angiotensinogen gene, over-expression of the ACE gene had no effect on blood pressure, despite markedly higher serum ACE levels.[143] Thus, the weight of evidence is against any significant role of the ACE gene in determining blood pressure, or the risk of developing hypertension.

Intermediate phenotypes for the ACE I/D polymorphism

Although the D allele is a major determinant of serum ACE levels it has been more difficult to identify an intermediate phenotype (i.e. a physiological effect) for the polymorphism that might explain its association with cardiovascular disease. Ueda studied the pressor response to AI infusion in normotensive men homozygous for the I and D alleles. Venous levels of AII during AI infusion were higher in DD subjects than in II subjects and the dose required to raise the blood pressure by 20 mmHg ($PD_{20}$) was significantly lower.[144] This response was enhanced in sodium depleted subjects, suggesting that the polymorphism may be more important in conditions when the RAS is activated. Another study assessed the effect of the I/D genotype on the contractile properties of isolated internal mammary artery rings *in-vitro*.[145] Contractile responses to AI were expressed as the % of the maximum response to AII and the difference in the areas between the AI and AII curves was taken to represent vascular AI-AII conversion (thus greater conversion would be associated with a smaller difference in area). Although the area between the curves did fall from II through ID to DD homozygotes, this was not significant. However, there did appear to be greater AI-AII conversion associated with the DD and ID genotypes taken together, compared with the II genotype. There was also a significantly lower sensitivity to the endothelium-dependent vasodilator methacholine in patients with the D allele, suggesting that stimulated release of nitric oxide (NO) was lower. By contrast, the increase in phenylephrine stimulated contraction in

the presence of the NO synthase inhibitor L-NMMA, was greater, suggesting higher basal NO release in those possessing the D allele. Again these results were analysed in terms of the presence or absence of the D allele. Another study investigated forearm vasodilation induced by acetylcholine (ACh) in hypertensive and normotensive individuals.[146] ACh induced less vasodilation in hypertensive DD homozygotes, but only when compared with ID and II subjects taken together. Though these results are initially persuasive it is debatable whether analysis based on combining ID and DD genotypes is appropriate, given that the alléles are co-dominant. A further criticism of these studies is their failure to take into account the possibility of non-ACE AII generation, an issue that will be discussed at great length throughout this thesis. When phenotypic variation, related to the I/D polymorphism, is studied in terms of the response to AI, the presence of alternative pathways for AII generation may be an important confounding factor.

In contrast to the above studies that suggest that the I/D polymorphism is associated with an intermediate phenotype of increased pressor response to AI, Lachurie et al did not find that either the blood pressure response, or the achieved AII concentration, to AI infusion was different between II and DD subjects.[147] This negative result was replicated by Chadwick et al who, using a very similar protocol to Ueda, were unable show a greater pressor response in DD homozygotes.[148] Unequivocal demonstration of an intermediate phenotype for the ACE I/D

polymorphism is thus awaited. On present evidence it is not likely that this polymorphism is a useful marker for cardiovascular risk.

### 1.3.4 The AT1R A1166C Polymorphism

The A1166C polymorphism and blood pressure

Since AII acts principally on this receptor, it is an obvious candidate gene for cardiovascular disease. The A1166C polymorphism of the AT1R gene has been identified, and an association was reported between the C allele and hypertension in a small population of 206 patients.[149] However, subsequent studies have been contradictory. One case-control study showed that the frequency of the C allele was increased in a group of 108 hypertensive patients with a strong family history and early onset of disease.[150] However this association was not confirmed in another, larger, study.[151] A further study failed to show any influence of this polymorphism on blood pressure within a group of patients without hypertension.[152]

The A1166C polymorphism and the risk of ischaemic heart disease

The role of polymorphisms within the AT1R in determining cardiovascular risk has also been extensively investigated. Following the demonstration of a putative association between the ACE I/D polymorphism and MI, a similar analysis was performed for the A1166C polymorphism.[153] Although the frequency of the C allele was not increased in patients with MI, there appeared to be an interaction with the ACE DD

genotype, such that the odds ratio for MI in DD homozygotes was increased in patients also carrying the C allele. This result was not replicated in a similar study from Norway, though there was a suggestion that the frequency of the C allele was increased in patients with MI who were thought to have been at low risk (low body mass index and apolipoprotein B levels).[154] Since these early studies there have been a variety of others considering the effect of this polymorphism on cardiovascular risk and progressive renal disease but no consistent association has been found. Other studies have concentrated on the role of the A1166C polymorphism in determining cardiovascular structure, and these are described in section 1.3.3.

Intermediate phenotypes for the A1166C polymorphism

As suggested above demonstration of an intermediate phenotype is crucial for a polymorphism to be considered to be physiologically significant. Such data for the A1166C polymorphism has recently been provided by Miller at al.[155] In this study healthy, sodium replete, men and women were chosen to form two groups characterised by the presence or absence of the C allele i.e. AA or AC/CC. Renal haemodynamic function was assessed using inulin and para-aminohippurate clearance at baseline and after administration of losartan or AII by infusion. Baseline glomerular filtration rate (GFR), renal plasma flow and renal blood flow were lower in the AC/CC group but there was no difference in mean arterial blood pressure (MAP). Losartan induced an increase in GFR and a fall in MAP in AC/CC, but not AA, subjects, with

no effect on renal blood, or plasma, flow. Although there was a trend to a fall in aldosterone after losartan in AA subjects, it was significant only in AC/CC subjects. Response to AII infusion also varied according to genotype. Thus AII induced a fall in GFR only in AC/CC subjects. These results show a clear difference in renal haemodynamics associated with this polymorphism. Since MAP was equal in the two groups the reduced renal plasma, and blood, flow associated with the C allele reflected increased renal vascular resistance. Given the differential effects of losartan in the two groups it is likely that this was due to increased intra-renal AII activity in those possessing the C allele. However, while initially persuasive, this was a small study and has yet to be replicated.

Another study investigated the relationship between this polymorphism and vascular reactivity in human internal mammary arteries.[156] This showed that the potentiation of phenylephrine-mediated contraction of isolated rings induced by AII was greater in subjects possessing the C allele. Again, this result has to be viewed with caution, since there was no association between the polymorphism and AII-induced contraction alone. Further studies are required to define an intermediate phenotype of the A1166C polymorphism and, as yet, there is no really convincing evidence that this marker truly associates with either cardiovascular disease or hypertension.

### 1.3.5 Polymorphisms of the RAS and Vascular Structure

It is possible that genetic variation within the RAS, by influencing AII generation or action directly at a cellular level, or indirectly through the control of blood pressure, may be associated with variation in cardiovascular structure. High-resolution ultrasound probes can visualise the carotid, femoral and radial arteries and allow wall thickness to be measured in man *in-vivo*. In addition ultrasound can be used to measure arterial pulse wave velocity, which is an indirect estimate of vessel distensibility, or compliance, and can visualise the boundary between the intimal and medial layers. Low arterial compliance, which may be due to abnormal vascular structure, has been associated with cerebrovascular disease.[157] Thus, changes in vascular structure and compliance may act as markers for cardiovascular and cerebrovascular risk.

It has been hypothesised that the importance of polymorphisms in the RAS is that, even if they are not directly involved in the pathogenesis of hypertension, they modify the response of the cardiovascular system to elevated blood pressure. Numerous studies have therefore studied the relationship between genetic variation in the RAS, hypertension and vascular structure.

Castellano et al studied carotid artery intima-media thickness (IMT) in 199 members of a randomly selected population from the Vobarno region in Italy, who were genotyped for the ACE I/D polymorphism.[158] A multivariate analysis showed that carotid IMT was significantly higher in DD

subjects than in ID or II subjects. The study was, however, confounded by the fact that a higher proportion of II homozygotes was on antihypertensive therapy than in the other two groups. A similar finding has been reported in a group of patients with type II diabetics - though this study did not find an association with femoral artery IMT and again analysed for possession versus absence of the D allele.[159]

Several similar studies have investigated the effect of the A1166C polymorphism of the AT1R on vascular structure. Benetos et al studied the relationship between possession of the C allele and pulse wave velocity (hence arterial compliance) in normotensive and hypertensive patients. The C allele was found to be associated with higher pulse wave velocity and lower arterial compliance.[160] A further study investigated the interaction between this polymorphism and treatment of hypertension with a calcium channel blocker, nitrendipine, and the ACE inhibitor perindopril.[161] Treatment with perindopril reduced pulse wave velocity (increased compliance) to a greater extent in carriers of the C allele than in AA subjects, whereas treatment with nitrendipine had the opposite effect. Thus a genetic marker in the RAS influenced vascular structure and appeared to determine the response to treatment. However, although this work is provocative, it has not been replicated. Castellano et al failed to show a relationship between the A1166C polymorphism and either carotid IMT or blood pressure in a similar study.[162]

In conclusion, there is considerable evidence that genetic variation in the RAS may influence cardiovascular structure and risk. However the strength of the associations remain uncertain and it is likely that their importance (and particularly that of the ACE I/D polymorphism) has been over-stated. The existence of numerous contradictory studies engenders scepticism, which is compounded by lack of evidence of an intermediate phenotype to link genotype and phenotype. In addition, mechanisms by which polymorphisms in non-coding regions of these genes can influence their expression have not been described. Authors have consistently suggested that the ACE I/D and AT1R A1166C polymorphisms, for example, may be in linkage disequilibrium with regulatory elements, but these have yet to be identified or characterised at a molecular level. It is equally possible that they are also in linkage disequilibrium with other major loci. Further work is clearly required to determine whether specific polymorphisms, or combinations of polymorphisms, will be useful in quantifying cardiovascular risk or guiding treatment. Future studies will need to consider other candidate genes, both within the RAS and distinct from it. Other studies are already being performed which do not depend on the knowledge of a candidate gene but instead use the tools of population genetics to identify useful markers. Finally, the possibility of gene-gene and gene-environment interactions adds another layer of complexity to an already confusing subject.

## 1.4 Alternative Angiotensin II Generating Pathways

In recent years the "classical view" of the RAS has had to be modified to incorporate the likelihood of local AII generation and has been expanded to include the study of the genetic variation in its expression. Furthermore, alternative enzymatic pathways for AII generation have recently been discovered.

Inhibition of the Renin-Angiotensin System (RAS) is a cornerstone of the treatment of cardiovascular and renal disease. ACEi reduce symptoms, slow the progression and improve mortality in chronic heart failure (CHF) and their use is now virtually obligatory in that syndrome.[163] Similarly, they slow the progression of chronic renal failure (CRF), an effect that cannot be explained simply by their anti-hypertensive action.[164] However, despite their manifest benefits, mortality in patients with CHF treated with ACEi remains high, and their use slows, but does not stop the progression of CRF. One explanation for the limited effectiveness of ACEi in these situations is that other neurohumoral systems are activated, and that these are not susceptible to inhibition of the RAS. For example, the sympathoadrenergic system is activated in CHF – hence the benefit of β-blockers in this syndrome - and the endothelin system is activated in glomerulosclerosis.[165, 166] Alternatively the effect of ACEi may be limited by incomplete inhibition of the RAS. Strategies that maximise the suppression of the RAS may be associated with greater clinical benefits. This would be consistent by the results of the

ELITE I study in which treatment with an AT1R antagonist in patients with CHF was associated with a greater reduction in mortality than conventional treatment with captopril.[167] Clearly these viewpoints are not mutually exclusive. In this section I shall describe the evidence that ACEi fail to adequately suppress the RAS and go on to suggest possible explanations for this.

### 1.4.1 Clinical Evidence of Incomplete Suppression of the RAS by ACE Inhibitors

ACEi block the final step in the generation of AII. Their use is therefore associated with an acute fall in the plasma concentration of AII and this is usually regarded as their predominant mode of action. However studies of the long term use of ACEi do not show a consistent fall in AII. In one study, while plasma AII levels fell acutely after oral administration of enalapril, at 24 hrs they had risen significantly, despite continuing low plasma converting enzyme levels.[168] After six months of treatment AII levels, measured 12-16 hrs post dose, were not significantly different in patients receiving enalapril compared to placebo. Despite this, blood pressures in the active drug group were reduced. Similarly, in the CONSENSUS trial, in which patients with severe (NYHA class IV) CHF were randomised to enalapril vs. placebo, long term suppression of ACE was established, but AII was only partially suppressed.[169] In another small study of patients with CHF, left ventricular ejection fractions (LVEF) were measured before and at a

mean of 42 months after administration of an ACEi.[170] At the end of this period plasma AII concentrations measured in the patients treated with ACEi were higher than those measured in age and sex matched patients without CHF, not on ACEi. In the majority of these patients LVEF had fallen during this period, indicating progression of CHF despite treatment.

Physical exercise is known to be associated with renin release and activation of the RAS. Accordingly, in a further study of the ability of ACEi to reduce AII levels, the effect of captopril on the exercise-induced rise in AII was studied in normal human subjects.[171] After 3 days of treatment with captopril 50 mg thrice daily the baseline, pre-exercise, plasma AII was significantly reduced. However, the subsequent rise in AII during exercise was not significantly suppressed in the ACEi treated group compared to placebo.

### 1.4.2 Mechanisms by which AII may overcome ACE Inhibition

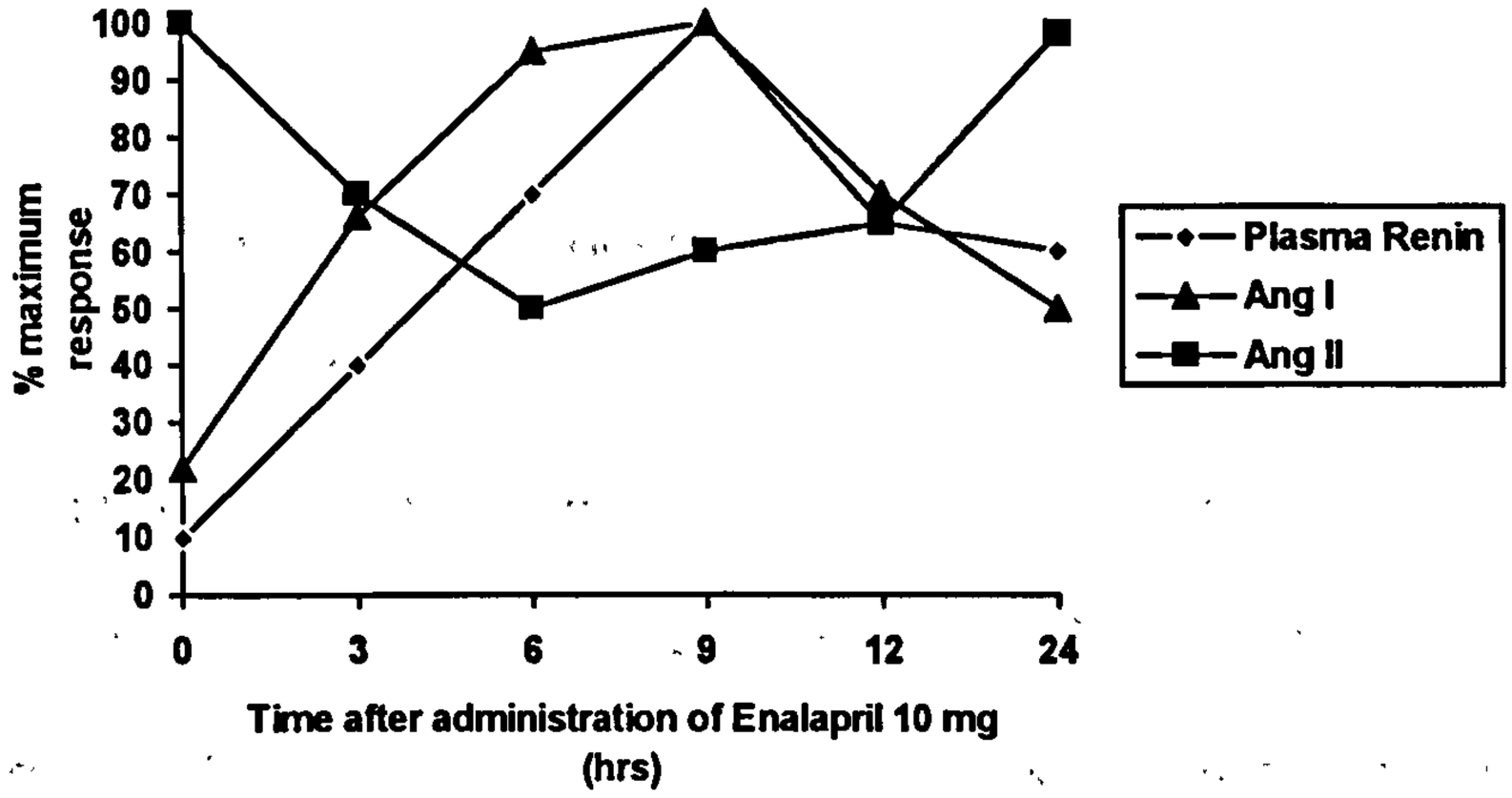
There are two main mechanisms by which AII may be able to escape the effects of ACEi: The first relates to the kinetics of the enzymes contributing to AII generation, together with the pharmacokinetics of ACEi. The second mechanism relates to the existence of a number of alternative enzymes able to catalyse the conversion of AI to AII and thus bypass ACE.

Although the existence of ACEi has focused attention on this enzyme, the conversion of angiotensinogen to AI by renin is thought to be the rate limiting step in the RAS. Treatment with an ACEi is associated with an

increase in renin, and in turn, AI levels, by interruption of the negative feedback loop. Thus, towards the end of a dosing interval with an ACEi, AII will be generated by a "mass action" effect through residual ACE activity and will tend to return to its baseline concentration. This is demonstrated in Figure 1.3, which shows the time course of plasma renin, AI and AII after administration of enalapril in humans. Similarly, Juillerat et al found that plasma AII levels returned to normal less than 24 hrs after administration of enalapril or benazepril, despite the fact that ACE was still suppressed - continuing production of AII being driven by the persistently high plasma renin and AI levels.[172] These data may explain the flat dose response curve of ACE inhibitors.[173].

It follows from this argument that combined ACE and AT1R inhibition may have additive effects. Thus administration of an ACE inhibitor might blunt the rise in AII that occurs when the feedback loop is interrupted by blockade of the AT1R and similarly blockade of the receptor might blunt the effect of rising AII levels driven by the high AI level that results from inhibition of ACE. Azizi et al tested this hypothesis by examining the effects of 10 or 20 mg of enalapril and 10 mg enalapril + 50 mg losartan on blood pressure and active renin in normotensive sodium depleted volunteers.[174] The combination caused a greater fall in blood pressure than 10 mg enalapril alone and a much more prolonged fall than was achieved by doubling that dose.

Figure 1.3: Time course of plasma renin, AI and AII following administration of enalapril (data adapted from Azizi et al)[174]




The potential benefits of combined ACE and AT1R blockade in heart failure have been investigated in a pig rapid pacing model which exhibits progressive changes in left ventricular function and neurohormonal activation, similar to those which occur in clinical CHF.[175] Treatment of the pigs with benazeprilat ameliorated the increase in left ventricular end-diastolic diameter and reduced plasma noradrenaline to a greater extent than treatment with the AT1R antagonist, valsartan. However, the combination was more effective than either drug alone. In a similar study of humans with CHF, Baruch et al studied the effect of addition of valsartan or placebo to standard ACEi therapy.[176] This was associated with an improvement in both haemodynamic parameters, such as pulmonary capillary wedge pressure and pulmonary artery diastolic pressure, and the neurohormonal profile as assessed by plasma aldosterone and noradrenaline. The authors also

measured plasma AII levels at baseline and after administration of valsartan. At baseline AII was within the normally reported range in all patients, despite treatment with an ACEi, and it did not rise after administration of valsartan – exactly as predicted above.

### 1.4.3 Non-ACE pathways for AII generation

The octapeptide AII is formed by cleavage of the C-terminal dipeptide, His-Leu, from the precursor decapeptide AI. ACE is conventionally thought be largely responsible for this conversion in human tissues. ACE has low requirements for substrate specificity: requiring the presence of a terminal COOH group and the absence of proline in the penultimate position in the peptide. It therefore catalyses the cleavage of a number of peptides, including bradykinin and enkephalins (see above). Just as the actions of ACE are not specific to the generation of AII, so the cleavage of AI may be mediated by a number of other enzymes. Moreover, *in-vitro* other enzymes are able to generate AII by cleavage of AI and directly from angiotensinogen. Thus, AII formation as a result of the action of trypsin, chymotrypsin, tonin, cathepsin G and kallikrein has been described.[177] Kallikrein appears to be bifunctional depending on the local pH conditions. At low pH it favours the generation of AII directly from angiotensinogen, while at higher pH it will generate kinins, thus contributing to the so-called "kinin-tensin" system.[178] This may have significance in ischaemic tissue, in which the pH is low. This hypothesis was tested in nephrectomized dogs,

after occlusion of the left anterior descending artery. AII generation, assayed in blood extracted from the coronary sinus, was inhibited by aprotinin (trasylol) but not captopril. This suggests that a serine protease effective at the low pH induced by ischaemia may generate AII. That the dogs had undergone bilateral nephrectomy strongly suggests that the reaction was independent of renin and the protease responsible has subsequently been identified as kallikrein.[179] The physiological role of these reactions in humans is not known. Human urinary kallikrein has been shown to generate AII from both angiotensinogen and AI *in-vitro*.[180] However, the amount of AII liberated directly from angiotensinogen was considerably lower than that from AI. Nonetheless these data suggest that the kinin-tensin system may also be physiologically relevant in humans.

Further evidence of the role of serine proteases as putative AII generating enzymes follows from experiments in a number of other animal models. Thus, Okunishi et al demonstrated that an ACEi reduced, but did not abolish, the contraction of vascular strips from the mesenteric and pulmonary arteries of dogs and monkeys to AI.[181] However, addition of chymostatin, a serine protease inhibitor of chymase and chymotrypsin, abolished the contraction. Inhibition of AII generation by vascular extracts followed the same pattern. This non-ACE, chymostatin-sensitive component of Ang II generation has been termed "CAGE."

### 1.4.4 Role of chymase in AII generation

Interest in non-ACE AII generation in human tissues, although known to occur through the action of serine proteases *in-vitro*, was further stimulated by Urata et al who studied AII generation in membrane preparations from normal and failing human hearts.[182] Normal hearts were those that were unsuitable donors for cardiac transplantation and failing hearts were those removed from recipients. Membrane fractions were prepared from left ventricular tissue and AII formation was examined by incubation with [$^{125}$I]-labelled AI. Surprisingly captopril was able to reduce AII formation by only 11% in normal and cardiomyopathic hearts. In contrast the serine protease inhibitor, SBTI (soybean trypsin inhibitor), reduced cardiac AII formation by 80%. This observation strongly suggests that a dual pathway for AII generation exists in human myocardial tissue *in-vitro*, one limb mediated by ACE and the other by a serine protease. In a further study Urata et al isolated the AII generating serine protease from cardiac membranes using high salt extraction, gel filtration and HPLC chromatography.[183] From the amino-acid sequence the enzyme was identified as a member of the chymase family and was named human heart chymase (HHC). Subsequent biochemical characterisation showed that HHC has an optimum pH for AII generation of 7.5-9.0, is very specific for the AI-AII conversion, and has a very high catalytic rate (some 20 times higher than ACE).[184] The gene encoding HHC was then cloned and a cDNA identified.[185]

### 1.4.5 Biochemistry and molecular biology of chymase

Chymases are enzymes generally associated with mast cells, in which they are contained in secretory granules. Unlike digestive proteases, chymases are not stored as proenzymes, but as the active form. Analysis of the N-terminal sequence and comparison with the cDNA suggests that the proenzyme is cleaved and activated at an early stage of vesicle maturation. Both heparin and histamine at low pH inhibit the conversion of the proenzyme to the active form.[186] Chymases are serine proteases that catalyse the hydrolysis at the C-terminal of peptides, usually after an aromatic amino acid such as Phe, Tyr and Trp. In rodents they exist as a number of isoforms and glycoforms, encoded by different genes. Thus, mice appear to have five chymases (designated mouse mast cell proteases, MMCP), MMCP 1-5.[187] Rats have RMCP 1-5.[188] Humans appear to have only one gene for chymase and isolates of the enzyme from human heart, skin and tonsil have identical amino acid sequences, although there may be multiple glycoforms.[189] McEuen et al recently identified two major distinct forms of human chymase, B and C, that differed in their affinity for heparin and eluted at different concentration of NaCl on heparin-agarose chromatography.[190] A further peak, chymase A, did not bind to the column and was considered to be a contaminant, complexed to proteoglycans. These chymases had similar molecular weights and were both recognised by the same antibody, but had different tissue distributions. Chymase B was the predominant form identified in the skin and heart, but chymase C was

predominant in the lung. The authors were unable to show that these two chymases were products of different genes, but their distinct biochemical properties suggest that they may be functionally different.

Human chymase is unusual in having a high degree of substrate specificity. Initially it was thought that AI, from which it cleaves the Phe8-His9 dipeptide to liberate AII, was the only substrate. In contrast RMCP 2 cleaves the Tyr4-Ile5 bond in AI and hence acts as an angiotensinase. Recent phylogenetic analysis of chymases suggests that there are, in fact, two distinct groups, α and β.[191] α-chymases include HHC, dog chymase and RMCP 3. These enzymes cleave AI to AII but are unable to hydrolyse AII further because the Tyr4-Ile5 bond is resistant to their action. β-chymases include RMCP 1 and 2 and MMCP 1, 2 and 3 and these act as angiotensinases. Three-dimensional molecular modelling suggests that the different substrate specificity resides in the electrostatic properties of the substrate binding sites.[192]

The different specificities of the various chymases may be the reason that there are marked species differences in AII forming pathways. Animal experiments have not uniformly identified non-ACE AII generation, or CAGE. There is clearly a species difference between humans and rodents. The contractile response to AI in rat and rabbit vascular strips is completely blocked by captopril and unaffected by chymostatin, whereas in human vascular strips captopril has little effect.[193] Balcells et al studied AII generation in membranes prepared from the left ventricles of humans, dogs,

mice and rats.[194] Membranes were extracted in either high or low detergent (HD or LD), with the expectation that ACE, being membrane-bound *in-vivo* would localise to HD preparations and chymase to LD. AII forming activity could be identified in both HD and LD fractions and was greatest in the human heart compared to animal hearts. This difference was attributable to chymase dependent AII generation in the LD fraction, which was much greater than ACE dependent generation in the HD fraction. Akasu et al studied species and organ differences in AII generation using a similar method.[195] AII generating capacity was greatest in extracts of lung tissue and in all species (dog, rabbit, hamster, pig and marmoset), except humans, this was predominantly due to ACE. In humans chymase like activity predominated over ACE in the lung, heart and aorta. Chymase activity was significant in all species except the rabbit, in which very little non-ACE AII generation was identified.

### 1.4.6 Tissue distribution of chymase

Functional and ultrastructural studies suggest that ACE and chymase are spatially disssociated. In a study of cat femoral arteries the response to AI was incompletely inhibited by captopril.[196] Endothelium removal had a similar effect to captopril, but there was no additional inhibition when endothelium-denuded arteries were incubated with the ACEi. Chymostatin induced further inhibition in these vessels suggesting that the location of ACE is primarily the endothelium, with further AII generation occurring within the

vessel wall, probably catalysed by chymase. A similar situation pertains in dog arteries, where non-ACE AII generation is predominantly adventitial.[197]

Urata used immunohistochemistry and in-situ hybridisation to study the location of human chymase. The levels of expression were also examined using western blotting.[198] In the heart chymase was identified within secretory granules of mast cells and in the interstitium, associated with the extracellular matrix. Some endothelial cells also contained granules. Chymase activity was greater in the ventricles compared to the atria. Chymase could also be detected in coronary artery, aorta, kidney and spleen, though at very low levels in the latter two organs. In a subsequent study chymase was detected at high levels in the alimentary tract, especially the oesophagus and stomach, the tonsils and the colon.[199] Moderate levels were detected in cardiac ventricles and the lung, with low levels in atria and aorta. There was some discrepancy between the chymase expression as detected by western blotting and activity determined by bioassay, with skin, coronary artery and renal cortex all showing high levels of enzymatic activity, but lower levels of expression. The reason for this was not clear, but did not appear to be due to the activity of cathepsin G because the enzymatic activity was aprotinin-insensitive.

### 1.4.7 AII generation by chymase in the heart

The studies by Urata and his group suggest that the majority of AII generation in the human heart is mediated by chymase, located largely in the

interstitium and in mast cells. The potential importance of this was emphasised by a study by Shiota et al who studied chymase gene expression in the heart of the cardiomyopathic hamster.[200] Chymase gene expression was found to be increased in the early stages of the condition, concurrent with the development of fibrosis, while ACE expression was unchanged. However, whether chymase is the pre-dominant AII generating enzyme in the heart has been challenged in a number of studies which highlight the importance of experimental methodology in this field.

### 1.4.7 Importance of experimental methodology in assessing the role of chymase

The major criticism of *in-vitro* studies of AII generation is that homogenisation of cardiac tissue may release intracellular and bound extracellular chymase, presenting AI to an enzyme that would not normally be accessible to it. Thus, the apparent predominance of chymase mediated AII generation might reflect the technique used to assay for it. Zisman et al studied AII generation across the intact heart and in solubilised left ventricular membranes obtained from hearts removed from the recipients of transplants.[201] In intact hearts $^{125}$I-AI was infused into the left main coronary artery at the time of coronary catheterization, with sampling from the coronary sinus and femoral vein to allow calculation of AII generation. After 6 minutes of AI infusion a simultaneous infusion of enalaprilat was commenced in order to examine the effect of an ACE inhibitor on AII

generation. In a separate study a membrane fraction from left ventricular tissue was solubilised in a buffer containing 0.6% Triton-X100 and extensively dialysed against 0.01M Hepes to remove residual ACE inhibitor. AII generation by the membrane preparation was then measured in the presence and absence of enalaprilat. In marked contrast to the results of Urata, AII generation across the myocardial bed was reduced by 89% in the presence of enalaprilat and by 85% in the homogenized tissue. A similar experiment in dogs was performed by Balcells et al.[202] They showed that AII generation across the intact myocardial bed was inhibited by 60% in the presence of captopril, but by only 6% in homogenised tissue. Further doubt has been cast on the ability of chymase to generate AII *in-vivo* by the work of Kokkonen et al who showed that the activity of the enzyme in homogenised myocardial tissue was almost fully inhibited in the presence of interstitial fluid.[203] Since chymase is located mainly in the interstitium, this experiment suggests that the contribution of chymase to AII generation may be less that was previously supposed.

The results of these studies may be explained by the detergent content of the medium in which AII generation was assayed, as suggested in the study by Balcells et al.[194] Thus, solubilisation of heart tissue with a high detergent concentration, as in the method applied by Zisman, may result in the loss of chymase from the sample and retention of ACE. Wolny et al studied this directly and found that when the method of Zisman et al was applied there was large fall in AII generation due to the loss of chymase activity from the

sample.[204] Although this experiment suggests that chymase is, indeed, responsible for the majority of AII formation in homogenised heart tissue, it does not explain the dominant role of ACE when conversion across the myocardial bed is studied. Urata showed that chymase is not present in the circulation and thus it is likely that intravascular AII formation is mediated by ACE, probably located on the endothelial surface.[197]

### 1.4.8 Non-ACE AII generation in the vasculature

The role of chymase in angiotensin II generation in the vasculature has also been studied in both humans and animals. As previously discussed non-ACE angiotensin II generation has been shown in large arteries from the cat and dog.[195, 196] Mangiapane et al studied the pressor effect of AI and Pro$^{11}$, D-Ala$^{12}$-AI (SUB) on isolated superior mesenteric arteries from the dog.[205] SUB is an AI analogue that is resistant to ACE, but is cleaved by chymase to release AII.[206] Both AI and SUB were able to contract canine arteries. Responses to AI were inhibited by captopril, but this could be enhanced by combining captopril with chymostatin. The response to SUB was not reduced by captopril but was inhibited by chymostatin. Thus, there appeared to be both ACE and non-ACE AII generation in these vessels. SUB was also used to investigate ACE-independent conversion of AI in an intact vascular bed – the isolated cat hindlimb preparation.[207] Again SUB was able to induce vasoconstriction, which was subject to inhibition by an AT1R antagonist, candesartan, but not by captopril..

Takai et al were able to extract chymase from human gastroepiploic arteries and the same group studied the contractile response to AI in these vessels.[208, 209] Lisinopril reduced the contraction to 100nM AI by 30%, but addition of chymostatin increased this to 96%. Similarly the generation of AII in homogenised gastroepiploic arteries was reduced by 92% in the presence of chymostatin, but by only 14% in the presence of lisinopril. The natural protease inhibitor α-antitrypsin also reduced AII generation in homogenised vascular tissue, however, it had very little effect on AII generation by vascular slices, suggesting that, in intact tissue, chymase is less susceptible to natural protease inhibitors than previously supposed. Wolny et al studied the contraction of isolated coronary arteries taken from hearts removed at the time of transplantation (4 patients with idiopathic cardiomyopathy and 4 with ischaemic heart disease).[204] Their results were very similar, in that there was little effect of the ACEi cilazapril alone, but significant inhibition could be achieved with chymostatin and this could be enhanced by combining the two. Voors et al studied the contractile response to both AI and the ACE dependent analogue [$Pro^{10}$]-AI in human internal mammary arteries.[210] Here captopril slightly inhibited the response to AI, shifting the dose-response curve to the right, but the ACEi nearly abolished the response to $Pro^{10}$-AI. Chymostatin also inhibited the response to AI, with a similar shift in the dose-response curve. The combination of chymostatin and captopril caused much greater inhibition of the response to AI, shifting the dose-response curve and also reducing the maximum

response. Veins also contract to AI and AII. Borland et al studied human saphenous veins and found a similar pattern of response and inhibition to that seen in arteries.[211] Thus, the response to AI could not be inhibited by quinalprilat, but was very significantly inhibited by quinalprilat and soybean trypsin inhibitor (SBTI, which is known to inhibit chymase) together.

In addition to mediating pressor responses, AII generated by chymase may exert a trophic role in the vasculature. Chymase is induced by balloon injury to the dog carotid artery and monkeys fed a high cholesterol diet display marked activation of the enzyme in the atherosclerotic aorta.[212, 213] Shiota et al studied the response of the canine carotid artery to balloon injury and showed, not only that chymase was induced in the injured region, but that pre-treatment with the mast cell stabiliser, tranilast, very significantly reduced the intimal hyperplasia that occurred as a consequence.[214] The same group also provided evidence that chymase-induced AII generation was involved in the response by showing that the AT1R antagonist, candesartan, also reduced intimal hyperplasia after balloon injury.[215]

### 1.4.10 Non-ACE AII generation in other tissues

Chymase expression and non-ACE AII generation have been studied in a number of tissues other than the heart and blood vessels. There are high levels of chymase in the pineal and pituitary glands but whether it is responsible for AII generation in the brain is, however, not known.[216]

Chymase may be an important AII-generating enzyme in the renal and urogenital systems. AI causes contraction of human detrusor muscle and this can be inhibited by SBTI.[217] This response of detrusor muscle is inhibited only slightly by enalaprilat, but addition of the specific chymase inhibitor, CH5450 (a peptide inhibitor designed to inhibit chymase) causes additional inhibition.[218] Again, the functional significance of AII generation in the bladder is not known.

Rat glomeruli convert AI to AII and this can be reduced by 80% in the presence of chymostatin.[219] The role of non-ACE AII generation in the intact canine kidney was studied by infusion of AI and [$Pro^{11}$ D-$Ala^{12}$]-AI into the renal artery while arterial blood pressure and renal blood flow were measured.[220] Both peptides elicited a rise in blood pressure and reduced renal blood flow, though AI was effective at lower doses. Using an intravital needle probe the ability of these peptides to influence glomerular haemodynamics, through vasoconstriction of the efferent and afferent arterioles, was also studied. Again both induced a contractile response, but that induced by AI was greater. Interestingly, the sensitivity of the afferent and efferent arterioles to AI was similar, though previous studies have suggested that postglomerular vessels are more sensitive to AII than preglomerular vessels. The intrarenal AII concentration, before and after injection of peptide into the renal artery, was also measured directly by needle biopsy from the cortex. Both AI and [$Pro^{11}$ D-$Ala^{12}$]-AI increased renal cortical AII.

Renal AII generation is difficult to study *in-vivo* in humans. An indirect method is to compare the effect of ACEi, renin inhibition and AT1R blockade on renal plasma flow.[221] Although ACEi might be expected to increase renal plasma flow to a greater degree than renin inhibition, due to kinin-mediated vasodilation in addition to reduction of AII generation, they are substantially less effective. AT1R blockade is as effective as renin inhibition, suggesting that there is a renin-dependent, ACE-independent, AII generating pathway in the human kidney.

These studies demonstrate that non-ACE pathways exist in intact tissue, and furthermore, that they are physiologically significant in an organ that is a major target for AII and is fundamentally involved in cardiovascular regulation.

### 1.4.11 Functions of chymase outwith the RAS

Although it has been suggested that the only substrate for chymase is AI, recent evidence suggests that there are others, and that generation of AII is not the only function of chymase. The endothelin system has become a major focus of cardiovascular research since its discovery by Yanagisawa in 1988.[222] Endothelin 1 (ET-1) is a 21 amino acid peptide that is the most potent vasoconstrictor yet identified and is the major form produced by vascular endothelial cells and in the heart. It is derived from a precursor, big-ET1, by the action of endothelin converting enzyme (ECE) and has a major role in regulating vascular resistance. The system also includes endothelins 2

and 3, derived from their own precursors and encoded by separate genes (big ET-2 and big ET-3). Endothelin levels are elevated in heart failure and renal failure, suggesting that the endothelin system is part of the neurohormonal activation that occurs in these conditions.[223] Recent work suggests that human, but not rat, chymase (RMCP 1), is able to cleave the $Tyr^{31}$-$Gly^{32}$ bond within the big endothelin peptides.[224] This generates a novel set of 31 amino acid endothelin peptides, ET 1(1,31), ET 2(1,31) and ET 3(1,31). Preliminary studies indicate that these peptides are able to constrict rat trachea, and that this cannot be blocked by phosphoramidon (which blocks ECE), suggesting that further cleavage to the 21 amino acid derivative is not required.[225] Further studies have shown that these novel endothelin peptides can also constrict porcine coronary artery and induce an increase in intracellular calcium in cultured human vascular smooth muscle cells.[226] This latter action appears to be mediated through the $ET_A$ receptor, since it is blocked by the selective antagonist BQ123. The role of chymase as an endothelin converting enzyme and the biological actions of these novel endothelins clearly requires further study.

Since chymase is expressed by mast cells it might be expected to play a role in inflammatory responses. Injection of human chymase into the skin of guinea pigs induces an increase in microvascular permeability which can be blocked by SBTI.[227] This is associated with local accumulation of neutrophils and eosinophils.[228] In addition to facilitating the recruitment of inflammatory cells, chymase activates the potent pro-inflammatory cytokine

IL-1β.[229] Other important actions of chymase include the activation of procollagenase (matrix metalloproteinase 1), suggesting that it may play a role in tissue remodelling.[230] Mast cells accumulate at the shoulder region of coronary artery plaques and, in autopsy specimens, the number of degranulated mast cells at the site of plaque rupture relative to unruptured plaques or normal coronary artery intima, is increased.[231, 232] In another study mast cells associated with coronary artery plaques were shown to contain significant quantities of the inflammatory mediator TNF-α.[233] A recent study showed that chymase dependent AII generation in homogenised human internal mammary arteries correlated with total and LDL cholesterol.[234] Thus it is possible to speculate that mast cells may contribute to atherogenesis and, by releasing chymase, histamine and TNF-α, contribute to the inflammatory reaction that may precede plaque rupture. In addition to the preliminary evidence that links chymase to atherosclerosis there have also been suggestions that this enzyme may also induce apoptosis in cardiomyocytes. Hara et al studied the effect of co-culture of rat neonatal cardiomyocytes with mast cell granules and observed apoptosis that could be prevented by pre-treatment with SBTI.[235] Using a specific neutralising antibody, the specific chymase responsible for apoptosis was identified as RMCP-1.

In summary it is becoming clear that AII may be generated by non-ACE pathways in the heart and the vasculature, in addition to a number of other tissues. It is also clear that the enzyme largely responsible for bypassing

ACE in humans is chymase. However there are still many questions left to be answered. In particular, the physiological role of chymase is uncertain and its regulation unknown. Still less is known about the contribution of chymase to the pathophysiology of conditions such as heart failure and hypertension, in which the RAS is fundamentally important, although the existence of non-ACE pathways may be an important therapeutic consideration. There are currently no specific inhibitors of chymase available for use in humans and so *in-vivo* experiments cannot be performed. Furthermore, while non-ACE AII generation has been demonstrated in human large and medium sized arteries, similar studies have not been performed in resistance arteries, which, as discussed below, play an important role in cardiovascular regulation.

## 1.5 Resistance Arteries: Physiology and Pathophysiology

### 1.5.1 Small arteries are resistance vessels

As the work in this thesis concerns the regulation of AII generation in human resistance arteries, the structure and function of these vessels will be considered further. The physiology of small arteries and their role in regulating peripheral resistance in normal and pathological states has been comprehensively reviewed by Mulvany and Aalkjaer.[236] The importance of these vessels lies in their ability to regulate the distribution of blood to peripheral organs, through variation of their diameter and hence resistance to

flow. The vessels that contribute substantially to peripheral resistance are known as resistance arteries. The precise definition of resistance arteries is unclear: formerly it was thought that the major site of peripheral resistance lay at the level of arterioles (those vessels with no more than one complete layer of smooth muscle cells). However studies of the pressure drop across a variety of vascular beds in several animal species suggest that up to 50% of the peripheral resistance lies proximal to vessels with diameters of 100 μm (Figure 1.4). Although studies of this nature have not been made in the human vasculature it is assumed that the properties of human blood vessels are similar to those observed in animals. Mulvany and Aalkjaer suggested that small arteries with diameters of less than 500 μm which contribute substantially to the peripheral resistance be considered as resistance arteries.[236] This definition takes into account the functional studies mentioned above, together with previous histological definitions.

Small arteries control resting peripheral resistance through haemodynamic properties related to their structure and their tone. *In-vivo* small arteries have pronounced active tone which may be modulated by two major mechanisms:

<u>Local or intrinsic control</u>

Peripheral resistance may be modulated by the action of tissue metabolites and autocrine factors and responses to physical factors such as flow, wall stretch and pressure. Thus arteries constrict in response to increased intravascular pressure and this can be shown to relate to both

longitudinal stretch and internal pressure.[237] While both responses are calcium dependent, the stretch response appears to be endothelium-independent but the pressure induced response is abolished by removal of the endothelium.[238] Flow-mediated vascular responses are a function of shear stress, which is the force per unit area acting in the direction of blood flow at the endothelial surface. It is a function of both the vessel radius and the blood viscosity. Flow-mediated vasodilation has been shown to occur in both conduit and resistance arteries, and is endothelium-dependent in most preparations thus far studied.[239, 240, 241]

Extrinsic neurohumoral regulation

Resistance arteries receive external stimuli from autonomic nerves and circulating endocrine factors. Neural control of vascular tone is effected by a plexus located within the adventitia. Axons run along the adventitia-media junction but, in the systemic circulation, do not penetrate the media. Three classes of nerves have been identified: vasoconstrictor sympathetic nerves, vasodilator sympathetic and parasympathetic nerves and peripheral small sensory nerves. The predominant vasoconstrictor neurotransmitter is norepinephrine (NE), which is released by stimulation of sympathetic nerves. Co-transmission of NE and ATP has been identified, with the latter mediating rapid excitatory junctional potentials which summate to produce early depolarization via $P_2$ (purine) receptors. Slow prolonged depolarization is due to NE acting mainly on the $\alpha_1$ receptor.[242] Vasodilator autonomic nerves appear to act via ACh release and antidromic stimulation of

nociceptive sensory nerves is known to induce vasodilation through the release of CGRP and Substance P.[243] A number of circulating hormones may also regulate vascular tone. These include catecholamines, principally adrenaline and NE which mediate vasoconstriction via $\alpha_1$ (and possibly $\alpha_2$) receptors and vasodilation via $\beta_2$ receptors. Other endocrine vasoconstrictors include AII, endothelin and vasopressin. Vasodilation may be mediated through BK, ANP and Purines.[244]

Figure 1.4: Pressure drop through hamster cheek pouch circulation

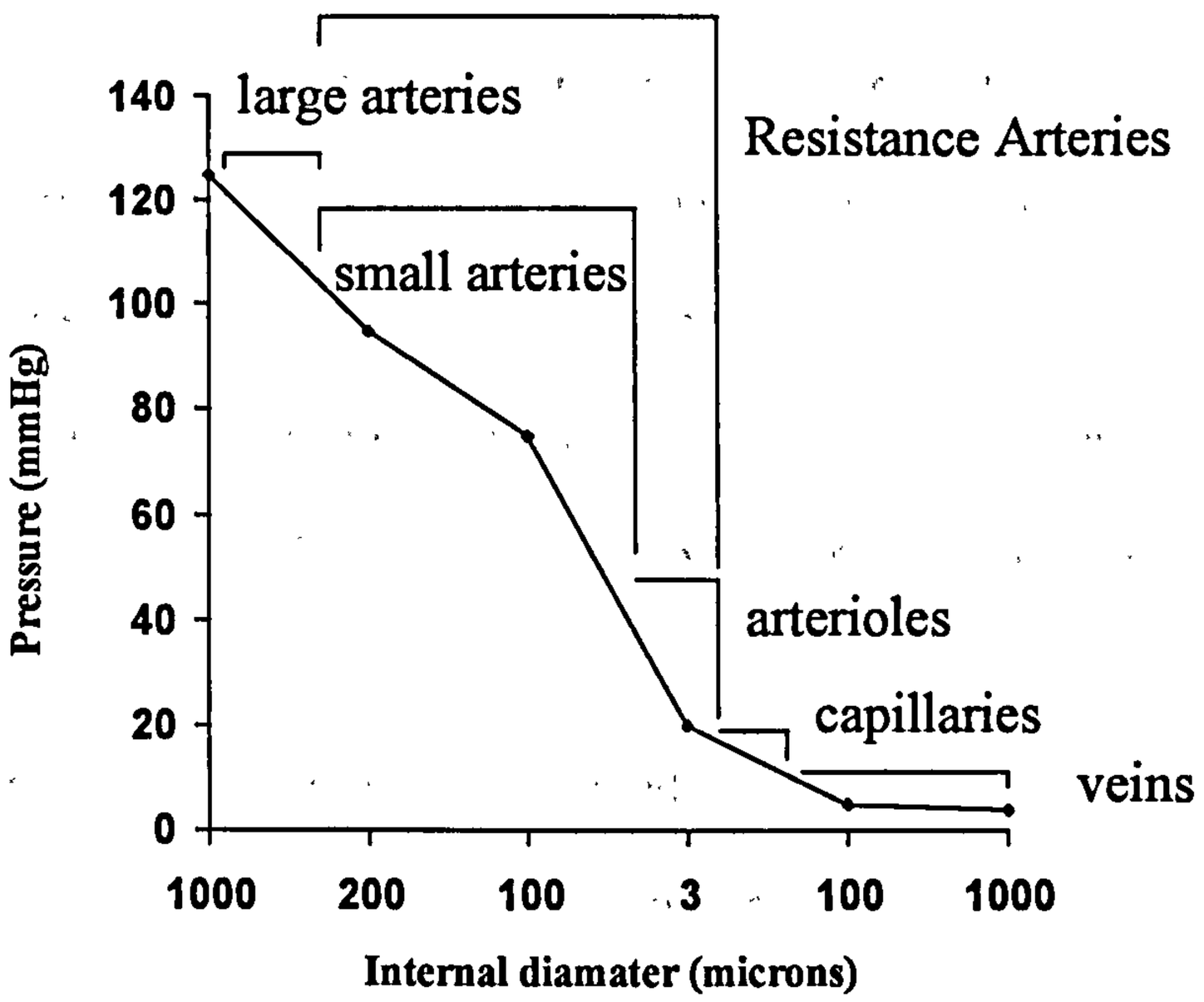



Fig 1.4 demonstrates that the majority of the fall in pressure is at the level of small arteries and arterioles and to significant degree occurs within vessels of > 100 μm diameter. From Davis et al.[245]

### 1.5.2 Regulation of resistance artery function by AII

AII regulates resistance artery structure and function by a number of distinct, but inter-related, mechanisms. AII causes vasoconstriction directly through its effects on vascular smooth muscle cells. This is mediated by the AT1 receptor through the $IP_3$ pathway and an increase in intracellular $Ca^{2+}$.[246, 247] In addition to a direct effect on smooth muscle cells AII may also increase vascular tone indirectly through facilitation of sympathetic stimulation of the vessel. Thus AII potentiates sympathetic neurotransmission in peripheral nerves by increasing norepinephrine release and reducing its re-uptake at nerve endings.[248, 249] Indeed, there is evidence for an interaction between local noradrenergic neurotransmission and locally generated AII, with AII release stimulated by the β adrenergic receptor.[250] The evidence cited in the section on the local RAS suggests that resistance arteries are capable of generating AII through the action of ACE on locally generated AI and that this acts as a paracrine agent to influence vascular tone.

A third mechanism by which AII may modulate vascular tone is through its influence on the endothelium and, in particular, on nitric oxide synthesis. AII is known to induce a counter-regulatory increase in NO synthesis which offsets AII mediated vasoconstriction.[251] Recently Ackermann et al showed that the contractile response to AI in rat vessels was reduced by direct nitric oxide donors such as SIN-1, suggesting that while AII induces NO synthesis, NO may have the opposite effect on local AII generation through ACE.[252] A further mechanism through which AII and

NO may interact arises through the action of vascular superoxide anion ($\bullet O_2^-$). This free-radical can reduce bioavailable NO and impair endothelium-dependent relaxation. AII induces $\bullet O_2^-$ production in human internal mammary arteries through activation of the AT1R.[253] Other studies have shown that AII infusion may induce endothelial dysfunction and an increase in $\bullet O_2^-$ production.[254] Thus AII stimulated NO synthesis may be offset by the scavenging action of superoxide.

In addition to short term effects on vascular tone, AII may also have chronic effects on vascular structure. When infused into rats at a dose below the threshold for acute vasoconstriction, AII raises blood pressure progressively over the course of a few days.[255] This is associated with increases in media thickness, media/lumen ratio and media cross-sectional area in the mesenteric vascular bed. When rats were treated with hydralazine to prevent the increase in blood pressure, similar changes in the mesenteric vessels were still observed, suggesting that the effect of AII was blood pressure-independent.[256] Thus, AII may have a long-term trophic effect on resistance arteries. This trophic effect of AII on artery structure may also be mediated through an interaction with other vascular regulators. There is evidence that the local vascular RAS and NO systems interact in the long-term regulation of vascular structure. Takemoto et al showed that vascular remodelling and myocardial hypertrophy could be induced in rats by treatment with L-NAME which inhibits endothelial nitric oxide synthase.[257] This was associated with an increase in myocardial and aortic ACE activity and both

the vascular changes and the increase in ACE activity could be attenuated by treatment with the ACEi, temocapril.

The ability of resistance arteries to respond to vasoactive agents and neural stimuli enables acute regulation of peripheral resistance and allows regulation of blood flow to individual organs. However, of equal importance is the long term regulation of vascular structure. The best evidence that the structure and function of resistance arteries is altered in a pathological state comes from studies in hypertension.

### 1.5.3 Abnormalities of resistance artery structure and function in hypertension

Established essential hypertension is associated with increased peripheral resistance.[258] While there is evidence that an increased response to vasoactive agents may be partly responsible for this - as suggested by an enhanced pressor response to infused agonists - the observation that peripheral resistance is increased under conditions when the vasculature is completely relaxed (e.g. during reactive hyperaemia) suggests that essential hypertension is associated with structural changes in small arteries.[259, 260] These structural changes may take several forms; increased resistance could be due a reduction in the internal diameter, an increase in length, or rarefaction (reduction in the number and therefore cross-sectional area), of peripheral vessels. Moreover while abnormal haemodynamic responses have been shown in some studies, others have not replicated these findings; indeed

increased vascular reactivity in hypertension per se does not imply a causal relationship, since it may be related to basal tone (contractility being related to the degree of stretch imposed on the vessel), which is itself influenced by blood pressure.

These problems have led to the development of in-vitro techniques which allow the study of defined isolated vessels under standardised conditions. Perfusion and wire myography (see methods) allow the study of resistance arteries under defined transmural pressures, allowing quantitative structural and functional comparisons to be made. These techniques have been utilised in a series of landmark studies by Mulvany's group in Aarhus and Heagerty in Manchester. Thus, Aalkjaer et al showed that essential hypertension is associated with a 29% increase in media to lumen ratio in resistance arteries from human subjects.[261] This was associated with an increased maximum contractile response to NE, vasopressin and AII, with no change in sensitivity to any of these agents. When corrected for the increased media thickness the force production per unit of smooth muscle (active media stress) was found to be unchanged compared to normotensive controls, suggesting that the increased pressor response was due to altered vascular structure and not to abnormal smooth muscle cell function; if anything calcium sensitivity (tested by the response to standard NE activation in the presence of varying calcium concentrations) was reduced in vessels from hypertensive subjects. These findings have been confirmed using pressure myography and in addition defective endothelium-dependent vasodilation to

ACh was identified.[262] The arteries studied were dissected from subcutaneous gluteal fat, taken under local anaesthesia. Although small arteries supplying skin may not be the best model for the human vasculature, their accessibility has led to this technique being widely adopted.

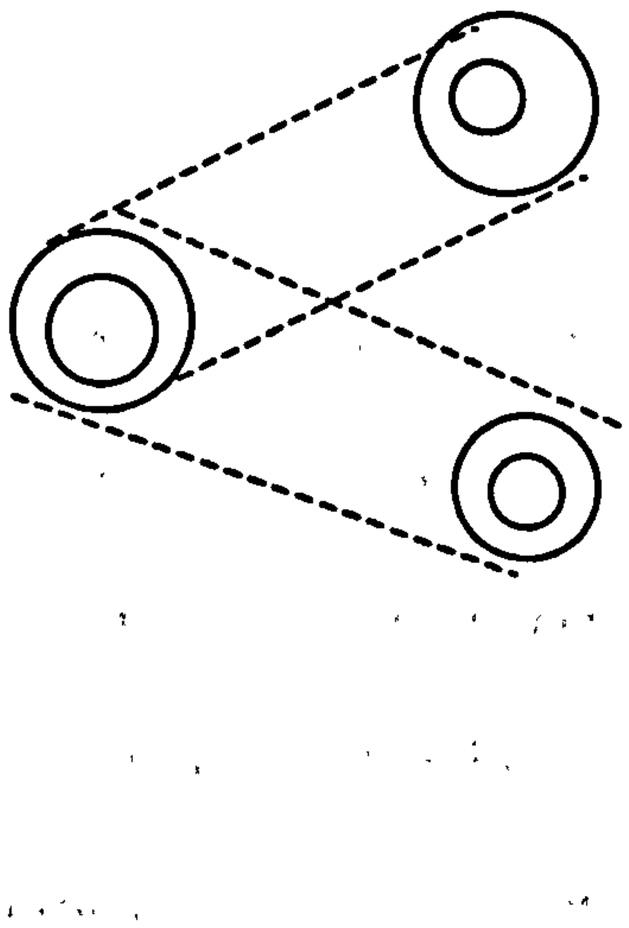
Structural changes in peripheral resistance arteries arise from a combination of two processes: eutrophic remodelling and hypertrophic remodelling.[263] This is illustrated in Figure 1.5. In eutrophic remodelling there is rearrangement of existing material in the media around a smaller lumen, resulting in a greater media-lumen ratio and reduced external diameter. Hypertrophic remodelling involves a thickening of the media resulting in a reduced luminal diameter, but leaving the external diameter unchanged. These processes can co-exist, leading to the development of "remodelling" and "growth" indices to try to quantify their relative contributions.[264]

It is thought that eutrophic remodelling predominates in mild essential hypertensives, but hypertrophic remodelling predominates in models of severe hypertension, such as 1-kidney, 1-clip Goldblatt rats, and in secondary hypertension.[260, 265] Thus, in one study the morphology of resistance arteries from patients with secondary hypertension (primary hyperaldosteronism, phaeochromocytoma and renovascular hypertension) and essential hypertension were compared to normotensive controls.[266] Media-lumen ratio was increased in all hypertensive patients, but media cross-sectional area (consistent with growth rather than remodelling) was increased only in patients with renovascular hypertension, with a smaller and non-

significant growth effect observed in primary hyperaldosteronism. The authors speculated that this was due to the activation of the RAS that accompanies renovascular disease.

Indirect evidence that the RAS may be involved in vascular hypertrophy and remodelling, independent of any effect on blood pressure, may also be inferred from studies of the effects on ACEi on small artery structure. Thus, in a double blind randomised trial of treatment with perindopril or atenolol in subjects with essential hypertension, a reduction in media-lumen ratio and an increase in lumen diameter were observed only with the ACEi, despite a greater fall in blood pressure obtained with the β-blocker.[267] Similarly Schiffrin showed that treatment with cilazapril was associated with a reduction in the media-lumen ratio of subcutaneous resistance arteries from patients with essential hypertension while atenolol had no effect.[268]

Figure 1.5: Hypertrophic versus eutrophic remodelling of resistance arteries




Hypothetical cross-sections of vessels undergoing growth and remodelling. Growth is associated with a smaller lumen, but the external diameter of the vessel is unchanged. Remodelling is associated with rearrangement of existing material around a smaller lumen, and thus both internal and the external diameters are reduced.

Although the evidence cited above suggests that hypertension is associated with changes in resistance artery structure and an increased peripheral resistance, this does not necessarily imply a causal relation. An increase in media-lumen ratio, by normalising media stress, may be an adaptive response to raised intravascular pressure. Thus, it is interesting to note that in the vessels studied by Aalkjaer the degree of blood pressure elevation correlated with the increase in media-lumen ratio. In addition the greater efficacy in normalising vascular structure observed with ACEi than with other anti-hypertensives suggests that neurohumoral mechanisms, and in particular the RAS, may influence vascular structure independent of blood pressure. Mulvany suggested that, while structural changes in the vasculature certainly increase peripheral resistance, the level of blood pressure may be set elsewhere in the cardiovascular system.[236]

### 1.5.4 Abnormalities of resistance artery structure and function in chronic heart failure

Chronic heart failure is associated with neurohormonal activation at an early stage of the disease. Poor myocardial performance and falling cardiac output result in activation of the sympathetic nervous system and a rise in plasma catecholamines.[269, 270] Also, a fall in renal perfusion pressure results in sodium retention, probably mediated by the action of AII and aldosterone at the distal tubule. Stimulation of renal sympathetic nerves also promotes sodium retention. Thus, patients with early cardiac failure are

unable to excrete sodium when given a high salt diet, despite normal renal haemodynamics.[271] Sodium excretion is normalised by enalapril, emphasising the role of the RAS. High circulating levels of NE and AII (as well as vasopressin, epinephrine, dopamine and endothelin-I) increase peripheral resistance through vasoconstriction, and volume expansion occurs secondary to sodium retention.

In addition there is thought to be endothelial dysfunction in CHF. Thus, vasodilation mediated by ACh in the forearm resistance bed, as assessed by venous occlusion plethysmography, and flow-mediated dilation of conduit arteries (which is endothelium dependent) are reduced.[272, 273] These changes, though initially adaptive, overload the failing myocardium and contribute to the progression of the syndrome.

Although it is widely accepted that peripheral resistance is increased in heart failure there have been very few studies looking directly at the structure and function of resistance arteries from patients with CHF. Angus et al studied resistance arteries from 6 patients with symptomatic CHF and compared them with healthy controls.[274] Vessels were obtained from gluteal fat, following the method of Aalkjaer et al. Maximum responses to NE, AI and AII were significantly reduced in vessels from patients with CHF, with no change in agonist sensitivity (as assessed by EC50, the concentration for half maximum response). In addition, responses to ACh were reduced (in only 2/6 vessels studied was any response obtained), but relaxation with an endothelium-independent agonist, sodium nitroprusside, was normal. In one

vessel a rightward shift in the concentration-response curve to AI was seen in the presence of enalaprilat. No structural data was obtained in this small study and it is possible that the poor response to ACh was due to damage to the endothelium at the time of dissection or mounting of the vessels. More recently, Stephens et al studied 27 patients with heart failure, who were a subset of those recruited for the Acute Infarction Ramipril Study (AIRE), and compared them with 10 healthy controls.[85, 275] As part of the AIRE study protocol patients with clinically evident heart failure were randomly assigned to treatment with the ACEi, ramipril, from 3-10 days post myocardial infarction. The degree of heart failure was mild (Left ventricular ejection fraction 41 ± 4%) and patients with severe (NYHA grade IV) heart failure were excluded from the study. Resistance arteries were obtained from gluteal fat biopsy and arterial wall morphology, together with the responses to AII, NE, ACh and electric field stimulation, studied using wire myography. There were no differences in wall morphology (media-lumen ration and cross sectional area) between control or either patient group, though lumen diameter tended to be higher and media-lumen ratio lower in arteries from patients treated with ramipril. In contrast to the previous study, responses to NE and AII in the control and placebo treated CHF group were the same, implying that there was no difference in the responses to vasoconstrictor agonists as a result of heart failure. However, response to AII and NE were significantly enhanced in patients treated with ramipril. The authors suggested that this may represent an interaction between AII and NE, possibly

at the level of the AT1R. Again, in contrast to previous findings, there was no difference in the response to ACh between groups, except at high concentrations where there was an apparent reduction in the response in patients treated with ramipril.

Studies of human resistance arteries in other pathological states have been limited. Aalkjaer et al studied vessels obtained from gluteal biopsy in 20 patients with renal failure requiring dialysis.[276] The patients were heterogeneous in terms of aetiology of renal failure and 2 had received a renal transplant previously. The patients were subdivided into 3 groups according to whether they were currently hypertensive and receiving anti-hypertensive medication, previously hypertensive but not currently receiving medication, or had never been hypertensive. No differences in vascular morphology were observed between any patient group and controls, though there was a correlation between blood pressure and media-lumen ratio. Similarly, no differences were observed in the response to NE, AII or activation by potassium depolarization. These results are surprising given the vasculopathic state and levels of blood pressure commonly associated with uraemia. However it was an early study and more subtle alterations in morphology and endothelial function may be detected in future.

## 1.6 Summary

There is considerable evidence that abnormalities in peripheral resistance vessels contribute to the pathophysiology of hypertension and CHF. The RAS is clearly implicated in the control of vascular structure and tone and the greater ability of ACEi to normalise the structural abnormalities seen in hypertension argue for a role for this neurohormonal system in their aetiology. However, little is known about the regulation of the RAS in resistance arteries. In this thesis I shall concentrate on three major issues:

1 The existence of a local RAS in human resistance arteries

There is little direct evidence that an active local RAS exists in human resistance arteries, though the evidence cited in section 1.2 would suggest that this is very likely.

2 Influence of RAS genotype on phenotype of resistance arteries

Although genetic variation within the RAS can be shown to influence its systemic activity, no similar intermediate phenotype has been shown in the resistance bed.

3 Non-ACE AII generation in resistance arteries

There is evidence that AII generation may occur through non-ACE pathways in the heart and large arteries, but this has not been adequately demonstrated in resistance vessels.

The work presented in this thesis is therefore a study of the actions, mechanism of generation and regulation of AII in resistance arteries from human subjects.

# Chapter 2

# Materials and Methods

## 2.1 Study of Resistance Arteries using Wire Myography:

### Introduction

Contractile responses of human resistance arteries were investigated in two separate studies. The first was in healthy volunteers and New Zealand white rabbits, the second was in patients with chronic heart failure and coronary heart disease.

The initial aim of the project in healthy volunteers was to investigate the role of the ACE I/D polymorphism in determining the response of human resistance arteries to AI. Thus, after initial genotyping, only subjects who were homozygous at either the I or D alleles of the ACE gene I/D polymorphism were invited to participate in the study. However, the protocol was subsequently modified because preliminary data suggested that the role of the I/D polymorphism might be less than had been expected, due to the possibility of non-ACE AII generation. It was then decided that selection according to genotype was unnecessary. This obviously had the additional benefit of speeding recruitment. Owing to difficulties in recruiting healthy volunteers willing to undergo gluteal biopsy abdominal wall fat biopsies were obtained from healthy patients undergoing hernia repair. Responses obtained from healthy human subjects were compared with those from a control species, the New Zealand White rabbit. This species was chosen because tissue was readily available in the laboratory and because it has been used as a

model of CHF, in which the possibility of non-ACE AII generation is important.

The results of the study of AI mediated responses in healthy volunteers stimulated a further study in patients with CHF, treated with ACEi, compared to patients with CHD who were ACE-naïve. This study was performed in the light of the results obtained from experiments on resistance arteries from normal volunteers and was carried out in collaboration with Professor JJV McMurray and Dr MC Petrie. Dr Petrie recruited experimental subjects and carried out gluteal biopsy and echocardiography. I dissected resistance arteries, performed all myography and analysed the data.

Stated here are details of the methods common to all myography studies, namely materials, artery preparation, myography normalisation procedures and statistical analysis. Also included are details of preliminary experiments which were performed prior to the main studies. Details of patient selection and specific experimental protocols are included in the relevant chapters.

### 2.1.1 Materials

Human AI, AII, bradykinin, noradrenaline and acetylcholine were purchased from Sigma (Sigma-Aldrich, UK). Chymostatin was purchased from Bachem (Safron-Walden, UK) and enalaprilat was a gift from Merck, Sharp and Dohme Ltd. CH5450 was a gift from Ferring Research,

(Southampton, UK). All myography experiments were performed on a Mulvany-Halpern four channel myograph (JP Trading, Aarhus, Denmark).

### 2.1.2 Human cutaneous resistance artery preparation

In most patients studied, a subcutaneous gluteal fat biopsy was performed under local anaesthesia with 1% lignocaine. Following the method of Aalkjaer et al an ellipse of skin 1.5cm x 0.5 cm was excised and adherent fat dissected free to a depth of approximately 1.5cm.[276] This was placed immediately to cold 0.9% NaCl solution and then transferred to cold Kreb's solution (composition in mM: NaCl 118.4, KCL 4.7, $MgSO_4.H_2O$ 1.2, $KH_2PO_4$ 1.2. Na $HCO_3$ 24.9, $CaCl_2$ 2.5, glucose 11.1, EDTA 0.023 which gives a pH of 7.4 when gassed with a 5% $CO_2$ / 95% $O_2$ mixture).

Subcutaneous fat biopsies were also obtained from the abdominal wall of male patients undergoing routine open hernia repair. This method of obtaining resistance arteries from human subjects was described by Aalkjaer et al, who found no difference in the properties of vessels from the two sources when mounted in a wire myograph.[276] These samples were of skin and adherent fat taken from the initial skin incision by sharp dissection. Diathermy was not used prior to obtaining the biopsy. The biopsy was immediately transferred into cold 0.9% NaCl solution and then to Kreb's solution.

Resistance arteries were dissected free of fat using watchmaker's forceps and spring scissors, under a Zeiss dissecting microscope. Care was taken to avoid handling the vessels directly to minimise traumatic damage.

This was achieved by handling the vessel using a side branch that was not subsequently mounted in the myograph, or by handling adherent connective tissue. Once free, vessels were placed in fresh cold Kreb's solution and stored overnight at 4°C, after first extruding any residual blood from the lumen by gentle manipulation with forceps. Approximately 24 hrs after the biopsy the vessels were divided into segments approximately 2mm in length. The aim was to obtain 4 resistance arteries and usually these were segments from one longer vessel. Resistance arteries were then mounted on two 40μm diameter stainless steel wires in a four channel Mulvany-Halpern myograph. In this set of experiments the responses of cutaneous resistance arteries from a control species, in this case, the rabbit, were also studied. Details of the rabbit cutaneous artery preparation are given below.

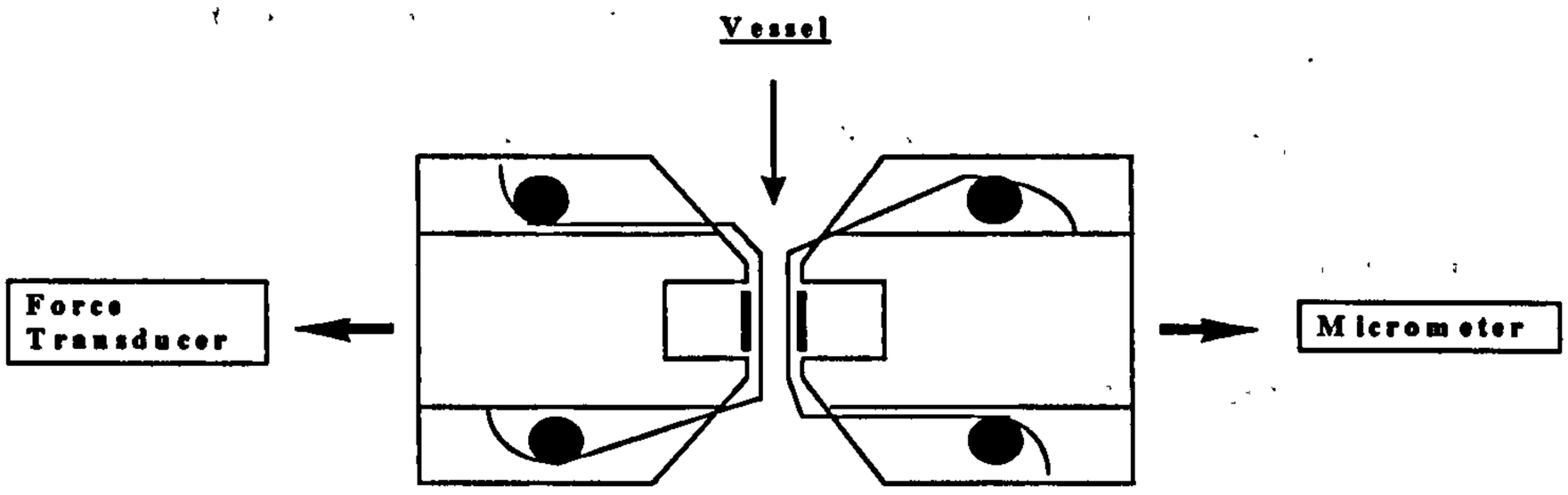
### 2.1.3 Rabbit cutaneous resistance artery preparation

After sacrifice a flap of skin from the area overlying the gluteal muscles was removed. On the deep surface of the skin flap the cutaneous vascular network could be seen, with arteries and veins running parallel to each other. An artery of suitable size was selected and dissected free from overlying connective tissue using the same instruments as described above. During dissection the surface of the skin flap was kept moist by applying Kreb's solution. Vessel preparation was as described above.

### 2.1.4 The Mulvany-Halpern Myograph

The myograph was first described by Mulvany and Halpern in 1977.[277] This device allows the study of vessels with diameters in the range of 100-1000μm. Parameters that can be determined with this device include isometric responses to agonists, internal diameter normalised for transmural pressure and simple morphological measures, such as wall thickness and adventitia, media and intima thickness. In this technique segments of artery, approximately 2mm in length. are mounted as a ring preparation on two 40 μm diameter stainless steel wires, using a no-touch technique as far as possible. A schematic diagram of the myograph is shown below in Figure 2.1.

Figure 2.1 The Mulvany-Halpern Myograph (not to scale)




The force transducer was connected to a specially designed myo-interface and thus to a chart recorder, allowing the force across the vessel wall to be recorded. The preparation was bathed in Kreb's solution which could be extracted using a suction device. The myograph was kept at 37° C during the experiments by an in-built heater and thermostat.

The experiments described in this section were all performed in a four-channel myograph. This consisted of a base-unit on which were mounted four myograph blocks, and thus allowed the study of four arteries simultaneously. Once each vessel had been obtained it was cannulated either in a Petri dish or by threading it onto a wire already fixed in the myograph. The second wire was then passed down the lumen taking care to avoid touching the endothelial surface. Both wires were secured under screws such that, as the screw was tightened, tension was applied to the wire, ensuring that the wires were taut and that slippage could not occur as the vessel contracted, or was stretched in the normalisation procedure. Any part of the vessel protruding from the jaw of the myograph was cut away and if it extended into the jaw a longitudinal incision was made to the vessel wall, to ensure that this segment was not able to contract. Using a micometer eyepiece that had previously been calibrated using a graticule, the length of the vessel was then measured to the inner edge of the jaw. Using the micometer the heads of the myograph were then moved together until the wires were just touching and the micometer reading at that point, $x_0$, was recorded. This was done either under direct vision using the microscope, or could be determined from the chart recorder, which gave a sudden negative reading at that point, as the force across the vessel wall fell to zero.

### 2.1.5 Normalisation

The reasons for following a normalisation procedure are threefold. Firstly, it is obvious that the size of an elastic structure, such as a blood vessel, is influenced by the transmural pressure, and that this needs to be defined. Secondly, the active response of the vessel is dependent on the degree of stretch it is under. Thirdly, the sensitivity of the vessel to pharmacological stimulation is also influenced by stretch. For this reason the size of the vessel is first determined at a given transmural pressure and then set to the pressure at which contraction is optimal. Following the original work from Mulvany's group the internal circumference of the vessel was determined for a transmural pressure of 100 mmHg ($IC_{100}$ = 13.3 kPa), with the vessel relaxed. The size of the vessel that was optimal for contraction was the $IC_1 = 0.9 \times IC_{100}$. This was determined by Mulvany's early work on rat mesenteric arteries, and it was assumed throughout these myography studies that the properties of human arteries are similar.[277]

Normalisation was performed by exploring the passive internal circumference - tension relationship of the artery. Each artery was distended stepwise, using the micrometer, and the tension developed across the wall was measured using the chart recorder. The Laplace equation relates effective internal pressure, wall tension and internal circumference. Thus:

$$P_i = \text{Wall tension} / (\text{internal circumference} / 2 \bullet \pi)$$

Where $P_i$ is the effective pressure

The internal circumference was calculated from the micrometer reading (subtracting the reading taken when the wires were just touching, $x_0$, for each successive stretch) and the known diameter of the wires. Wall tension was calculated from the force measured across the wall, divided by twice the segment length (which had been measured using the micrometer eyepiece). The force was measured from the displacement of the pen on the chart recorder, which had previously been calibrated against a force of known magnitude. Thus for each distension a micrometer reading and chart reading were taken. In practice these were entered into a computer, or programmable calculator, able to calculate the actual values from the readings, given the relevant calibration factors. The stepwise distension was continued at one minute intervals, to allow for "stress relaxation" (with the force recording being taken at the end of each interval), until the effective pressure exceeded 100 mmHg. At that point the computer fitted an exponential curve to the internal circumference - pressure data and determined the point corresponding to 100 mmHg. This gave the $IC_{100}$, as described above. From this the $IC_1$ could be calculated and the computer was able to interpolate the equivalent micrometer reading. The vessel was then set to that reading. The internal diameters equivalent to the $IC_{100}$ and $IC_1$, $L_{100}$ and $L_1$ respectively were calculated from the equation $L = IC / \pi$. The aim was to study arteries in the range of $L_1 = 250$ -350 μm, but they were not discarded unless $L_1$ was greater than 500μm (using Mulvany's definition of resistance arteries [236]).

### 2.1.6 Experimental protocol

In preliminary experiments normalisation was followed by activation of each artery through exposure to KPSS, on three occasions, followed by one exposure to KPSS and norepinephrine (NE) 10 μM together (NEK), with the intention of examining the maximum contractile capability of the vessel. ACh was added on the plateau achieved with NEK in order to stimulate endothelium-dependent vasodilation. This protocol was discontinued because there was concern that failure to achieve significant vasodilation in vessels pre-contracted with NEK might reflect the unphysiological nature of the stimulation, and not damaged endothelium. Thus the protocol was modified to include activation with KPSS twice and NE 10 μM once. After a plateau contraction had been attained with NE (defined as a stable contraction for at least 2 minutes), ACh 3μM was added to the bath. Vessels that were unable to contact to either KPSS or NE, or that showed no relaxation to ACh (and were therefore considered to have no functionally intact endothelium), were discarded.

### 2.1.7 Experimental protocols: human vessels

Preliminary experiments were performed in order to establish that AI was able to elicit a contraction in human subcutaneous resistance arteries, and to compare this response with that elicited by AII. In addition, the effect of inhibition of the AI response with the ACE inhibitor, captopril, and the AT1R antagonist, losartan, were investigated. In the light of these experiments the

protocol was modified to investigate the effect of the ACE inhibitor, enalaprilat and a chymase inhibitor, CH5450, on the responses to AI. CH5450 is a peptide designed to inhibit chymase specifically.[278] Concentrations used were enalaprilat, 1μM and CH5450, 10μM. Since a review of the literature suggested that the action of chymase is commonly defined in terms of inhibition by bacterial product, chymostatin, a more formal study was then designed to investigate the response to AI in the presence of enalaprilat, chymostatin and their combination in arteries from normal patients. Details of the experimental protocol are given in chapter 3. Experimental protocols in patients with CHF and CHD are shown in chapter 5.

### 2.1.8 Experimental protocol: rabbit vessels

Rabbit vessels underwent a similar standard activation to human vessels. Dose-response curves to AI were constructed over the same concentration range as for human vessels. Preliminary experiments suggested very marked inhibition in the presence of enalaprilat. The effect of chymase inhibition was studied using CH5450. Since AII generation has been described by serine proteases, such as cathepsin G and kallikrein, which are inhibited by trasylol, the effect of this inhibitor was also studied in rabbit vessels. The full protocol is given in section 3.2.

### 2.1.9 Data Presentation and Statistical analysis

Contractile responses were expressed as an increase in active effective pressure (P, mmHg), calculated as an increase in isometric tension (T) above resting divided by the normalised internal radius. Because there was great variability in the magnitude of the responses of the arteries to AI and AII, responses to these agonists were expressed as the % of the response to a standard vasoconstrictor. For this, I chose the response to the second exposure to KPSS at plateau.

Where possible, agonist potency in the presence or absence of inhibitor was expressed in terms of the EC50 values, this being the concentration required to produce 50% of the maximum response. The EC50 was derived from fits of each separate curve (calculated directly by interpolation in Microsoft Excel spreadsheets, using the "FORECAST" function). To facilitate analysis, these values were also expressed as the PD2 - which is the negative logarithm of the corresponding EC50 and is a whole number. Maximum responses were taken directly from the curves.

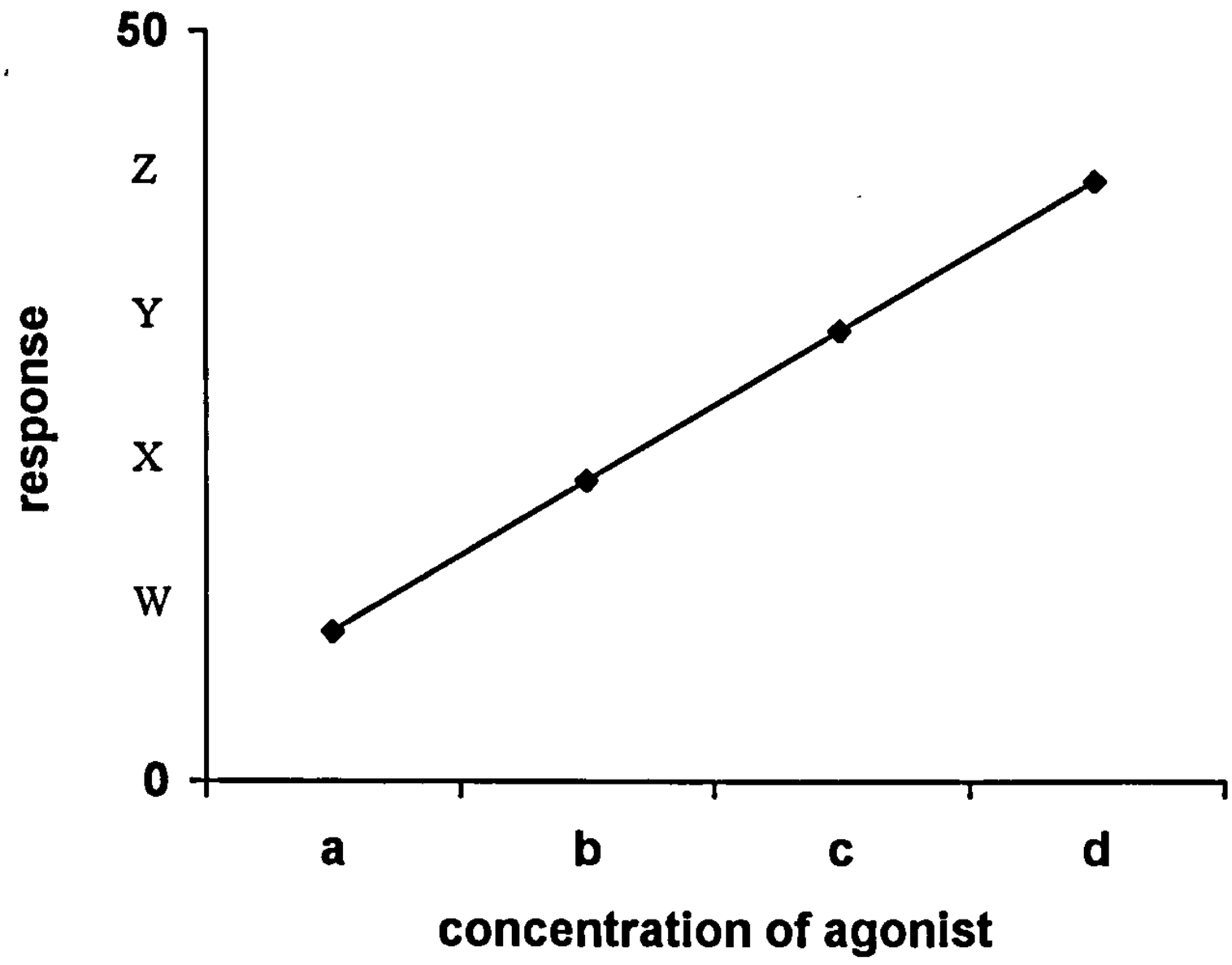
Since some responses did not achieve a clear maximum even at the highest concentration of agonist, EC values could not be calculated for all curves. Thus, in an initial analysis, the area under the curve was calculated for each concentration-response curve (using the response normalised to KPSS) by a simple trapezoidal rule. This gave a dimensionless numerical value expressing the contractile response. Calculation of the AUC is given in schematic form in figure 2.2.

Responses to AI for each inhibitor were then compared as a group with control responses. An Anderson-Darling test for normality was made on responses as expressed as AUC. This determined whether parametric or non-parametric statistics were used. Thus, for responses to AI in normal human volunteers, where a matched control was not available for all curves, a Mann Whitney U test was employed. AUC was also calculated for responses to BK and, because a matched control was available for all experimental curves, a Wilcoxon Rank Sum test was employed. Analyses were performed using SPSS or Minitab software. Analysis of data obtained from patients with CHF or CHD was performed in the same way, for the sake of consistency. Where data appeared to be described by a normal distribution, paired or unpaired t-tests were performed, as appropriate.

Re-consideration of the analysis of angiotensin dose-response curves in the absence of a clear maximum response led to an analysis of variance (ANOVA) procedure being employed. This was employed to compare EC50 and maximum responses where there was a clear maximum. Where no clear maximum could be identified, the threshold concentration - defined as the concentration at which a response was first observed - was noted for each individual dose-response curve. This followed the suggestion of Voors et al, who faced similar problem in analysing the response of human internal mammary arteries to angiotensin I.[210] Threshold concentrations were then compared by ANOVA using GraphPad Prism software (GraphPad Inc). An appropriate correction for multiple comparisons (Dunnett's or Bonferroni -

depending on whether each experimental curve was compared only to control or to other experimental curves as well) was made and a p value of less than 0.05 (after correction) was considered significant. In the text the results of both methods of analysis are described and any discrepancies are discussed.

Figure 2.2: Calculation of AUC




AUC was calculated using a simple trapezoidal rule. Based on the schematic above the equations would be:

$$W \times (b-a) + \{[0.5 \times (b-a)] \times (X-W)\} + \{X \times (c-b) + [0.5 \times (c-b) \times (Y-X)]\} \ldots \text{etc}$$

## 2.2 Experiments with human internal mammary arteries in an organ bath

Details of patient selection, vessel preparation and experimental protocols are given in chapter 6. Statistical analysis was based on the methods employed in studies of human resistance arteries. Thus, the responses to AI in IMAs were expressed as the percent contraction to that elicited by 80mM KCl in that vessel. Analysis was similar to that performed for resistance arteries. Thus the EC50 and maximum response were used to compare curves by ANOVA. For the sake of consistency with previous analyses AUC was also calculated using the same simple rule as for resistance arteries. Mean AUC values were compared as a group for each experimental curve compared to control, using a Mann Whitney U test.

## 2.3 Identification of ACE and Chymase in human internal mammary arteries: Studies using immunohistochemistry

This study was performed in collaboration with Dr George Lindop in the Department of Pathology, Western Infirmary. Vessel preparation was performed by Mr Niall Whyte. Immunohistochemistry (IHC) was performed by Mr Iain Downie, using established methodology within the Pathology Department.

Patient details are given in chapter 7. Vessel preparation and standard operating protocols for immunohistochemistry are stated in the appendix to this chapter.

## 2.4 Appendix to Methods

Included in this appendix are details of standard methods that were being employed in the laboratory and which were used in the preparation of this thesis.

### 2.4.1 Genotyping for the ACE gene I/D Polymorphism

A sample of whole blood was drawn from each patient into a standard 5ml K-EDTA bottle. This was stored in a -20°C freezer. DNA was extracted using a modified standard procedure.[279] Genotyping for the ACE I/D polymorphism was then done in two batches – these samples being included in protocols being run as a routine in the laboratory by Dr Cathy Clark. The first batch (patients 1-20) was analysed using a 2-primer PCR; the second (patients 21-47) utilised an updated 3-primer PCR. Both protocols are given below.

A) <u>2-Primer PCR for the ACE I/D Polymorphism</u>

The I/D polymorphism is located in intron 16 of the human ACE gene. The D allele arises from the absence of a 287 base pair sequence from this intron. Initial genotyping was performed using a modification of the 2-primer method developed by Rigat.[280, 281] This gives a 190 base pair (bp) fragment in the absence of the insertion (D allele) and a 490 bp fragment in the presence of the insertion (I allele). DNA was incubated with the following reaction mixture:

Commercial PCR buffer without $MgCl_2$ (Promega Ltd, Southampton UK)

$MgCl_2$ 3.0mmol/l

dATP, dCTP, dGTP, dTTP (Promega) 0.5mmol/l each

DMSO 5%

*Taq* DNA Polymerase 1U (Promega)

Primer 1: 5' CAGGAGACCACTCCCATCCTTTCT 3'

Primer 2: 5' GATGTGGCCATCCACATTCGTCAGAT 3'

Cycling conditions were as follows:

Initial denaturation 94°C for 3 min

30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2min

Final extension 72°C for 2 min

The PCR product was visualised after electrophoresis on 1.5% agarose gels with ethidium bromide staining. The results were subsequently checked using a third primer to avoid mistyping of ID for DD (see below).[281]

B) <u>3-Primer PCR for the ACE I/D Polymorphism</u>

This method utilised a "nested" PCR primer situated within the insertion sequence of the I allele. Genomic DNA (50ng) from each subject was added to a well of a microtitre plate and dried down at 60°C for 25 minutes. Reaction mix (25µl/well) was then added containing:

Tris HCl (pH 9.0) 10 mmol/l

KCl 50 mmol/l

0.1% triton X-100

$MgCl_2$ 1.5mmol/l

dATP, dCTP, dGTP, dTTP (Promega Ltd, Southampton UK)

100μmol/l each

DMSO 5%

*Taq* DNA Polymerase 1U (Promega Ltd)

Primer 1: 5'CCCATCCCTTCTCCCATTTCTC3'

Primer 2: 5'GGTTTCACCGTTTTAGCCGGGA3' 10pmol/l each

Primer 3: 5'CCATGCCCATAACAGGTCTTCA3'

Cycling conditions were as follows:

Initial denaturation 94°C for 3 min

30 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 1min 30sec

Final extension 72°C for 5 min

PCR products were visualised on a pre-stained 7.5% acrylamide-bisacrylamide (19:1) gel using diagonal gel electrophoresis (MADGE; MadgeBio Ltd, Grantham, Lincolnshire UK). The banding patterns of the 3 possible genotypes were: DD 210 base pair (bp) fragment; II 498 and 264 bp fragments; ID 498, 264, 210 bp fragments.

### 2.4.2 Assays for Renin, AII, Aldosterone and ACE

In the study patients with CHF and CHD blood was drawn for estimation of plasma AII, renin and aldosterone. These assays were performed by Dr JJ Morton, using established laboratory methodology. AII and renin were estimated using "in-house" radioimmunoassays.[282, 283] Aldosterone was estimated using a commercially available radioimmunoassay (DPC, Los Angeles, USA). ACE was estimated using established techniques in the biochemistry laboratory at the Western Infirmary.

### 2.4.3 Immunohistochemistry

A) Materials

Murine anti-human mast cell chymase monoclonal antibody was obtained from Chemicon International Ltd (Harrow, UK). A mouse anti-human ACE monoclonal antibody was a gift from Dr F Alhenc-Gelas (Paris). Secondary antibodies (human anti-mouse monoclonal antibody) were from DAKO Ltd. Alkaline phosphatase and peroxidase substrate kits were obtained from Vector Labs (UK).

B) Human internal mammary artery and skin biopsy preparation

Vessels were transferred to the pathology laboratory in cold 0.9% NaCl solution. They were then divided and one portion was embedded in OCT compound and then placed into liquid nitrogen. This was then wrapped in aluminium foil and stored at -70°C until required. The other portion was

formalin-fixed and embedded in paraffin. Skin biopsies were prepared in the same way.

C) Standard Operating Procedure

Indirect immunohistochemistry was employed. Formaldehyde-fixed specimens were sectioned on a microtome and mounted on glass slides. Frozen sections were prepared on a cryostat at -20°C and transferred to glass slides. Primary monoclonal antibodies were diluted in 1% bovine serum albumin (BSA). Thereafter the procedure was as follows:

1. Frozen sections were air-dryed after sectioning on the cryostat
2. Sections were then acetone-fixed for 10 minutes and allowed to air-dry
3. Cover sections in 1% BSA in TBS/Tween for 10 minutes at room temperature
4. Serum drained and excess wiped off
5. Cover sections with primary antibody diluted in 1% BSA and incubated at room temperature for 1 hour
6. Slide rinsed in TBS/Tween and washed in 2 changes of TBS/Tween for 5 minutes each
7. Slide covered in secondary antibody, appropriately diluted in 1% BSA at room temperature for 30 minutes
8. Repeat step 7
9. Appropriate substrate solution (alkaline phosphatase or peroxidase)

then applied and visualised according to manufacturer's instructions

10 Counter-stain with haematoxylin

11 Slides dehydrated in alcohols and xylene, then mounted.

In preparation of this thesis the original slides were photographed using a digital camera. Colour balance and contrast of the resulting images were adjusted in Adobe Photoshop.

# Chapter 3

# Studies of resistance arteries in normal Subjects and New Zealand White Rabbits: effect of ACE and chymase inhibition

## 3.1 Introduction

In section 1.4 I discussed the possibility of non-ACE AII generation in human tissue and suggested that this may reflect the activity of the enzyme chymase. Urata has demonstrated chymase-dependent non-ACE AII generation in the human myocardium.[182] A number of studies have investigated the possibility of chymase-dependent AII generation in the vasculature, but these studies have concentrated on large and medium-sized arteries and veins. Thus, chymase plays an important role in AII generation in human gastroepiploic, coronary and internal mammary arteries.[204, 208, 210] However, there have been no studies investigating the role of non-ACE AII generation in human resistance arteries.

The potential importance of the structure and function of resistance arteries in cardiovascular disease has been discussed in section 1.5. In particular hypertension is associated with remodelling within the vascular tree – but whether this is cause or effect is not known.[259, 261] Once established, however, the increased vascular resistance that results from this process may contribute to the maintenance of hypertension and its outcome. Since the RAS is a major therapeutic target in cardiovascular disease, the possibility of non-ACE AII generation in resistance arteries is clearly important.

Small artery structure and function have been extensively studied in animal models. However, as alluded to in section 1.4, there are thought to be

major differences in the mechanisms of AII generation between species.[192] This has implications for study design, particularly if interventions affecting the RAS are being contemplated. The aim of the studies presented in this chapter was therefore to investigate the possibility of non-ACE AII generation in human resistance arteries and compare these responses with those observed in a commonly used animal model, the rabbit.

## 3.2 Studies in Human Volunteers

### 3.2.1 Volunteer and patient selection

Non-smoking healthy male volunteers were identified by advertisement. Interested volunteers were invited to attend the Research Unit at the Western Infirmary, Glasgow, where they completed a basic health questionnaire. Volunteers with a history of cardiovascular disease, hypertension, diabetes or renal impairment were excluded from the study. All subjects gave written informed consent to participate in the study, which was approved by the Hospital Ethics Committee. Venous blood was drawn for measurement of electrolytes, random glucose and cholesterol. An additional sample of whole blood was drawn and placed into a freezer at –20°C for subsequent DNA extraction and genotyping for polymorphisms within the RAS. After 10 minutes rest, blood pressure was recorded in the right arm, sitting, with a standard mercury sphygmomanometer (Accoson UK). A

gluteal fat biopsy was then performed according to the method described in chapter 2.

Additionally some volunteers were healthy men who were admitted to a surgical ward for repair of inguinal hernia. Only men with no history of cardiovascular disease, diabetes, hypertension or hyperlipidaemia were included in the study. Blood pressure was measured and blood drawn as described above. These patients signed an informed consent form distinct from that described above. In these patients, a fat biopsy was taken from the edge of the initial incision at the time of hernia repair. Diathermy was not used prior to biopsy.

### 3.2.2 Experimental protocols

Resistance arteries were dissected, mounted in a wire myograph and normalised as described in section 2.1. They than underwent a standard activation to assess their contractile properties. Following this resistance arteries were incubated for 30 min in either Kreb's solution alone (control, vessel 1) or with enalaprilat 1μM (vessel 2), chymostatin 10μM (vessel 3) or both enalaprilat 1μM and chymostatin 10μM (vessel 4), respectively. Chymostatin replaced CH5450 as the chymase inhibitor because a review of the literature suggested that chymase activity is generally defined by its susceptibility to inhibition with this product ("CAGE," see introduction). A cumulative concentration response curve (CRC) was then performed to AI from 0.01nM to 3μM in log molar increments. Arteries were exposed to each

had been reached, whichever was sooner, since it was observed that tachyphylaxis developed even during a single dose-response curve. Owing to tachyphylaxis, vessels could not be exposed to AI more than once, precluding further concentration-response curves to this peptide, or to angiotensin II (AII).

In a subset of vessels responses to bradykinin (BK) were investigated. Following the dose response curve to AI, baseline was re-established by washing with PSS. Vessel 2 was incubated for 30 mins in the presence of Enalaprilat 1μM, vessel 1 remaining the control. Pre-contraction was then established with NE 10μM and once a stable plateau had been reached vessels were exposed to BK 0.01nM to 3μM. A summary of the experimental protocol is given in table 3.1

Table 3.1 Experimental protocol for resistance arteries from normal human volunteers

| | Vessel | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| **Standard Activation** | **KPSS**<br>**KPSS**<br>**NE 10μM**<br>**ACh $3\times10^{-6}$μM** | | | |
| **Incubation**<br>**30 mins** | **Control**<br>**Vehicle** | **Enalaprilat**<br>**1μM** | **Chymostatin**<br>**10μM** | **Enalaprilat**<br>**+**<br>**Chymostatin** |
| **CRC to AI** | **AI (0.01nM, 0.03nM, 0.1nM, 0.3nM etc … to 3μM)** | | | |
| | **Wash to baseline** | | | |
| **Incubate**<br>**30 mins** | **Control**<br>**Vehicle** | **Enalaprilat**<br>**1μM** | | |
| **Pre-constrict** | **NE 10μM** | | | |
| **CRC to BK** | **BK (0.1nM, 1nM, 10nM etc… to 3μM)** | | | |

### 3.2.3 Volunteer characteristics

In total gluteal biopsies were obtained from 29 normal volunteers. Fat biopsies from the anterior abdominal wall at the time of hernia repair were obtained from a further 9 patients. Subcutaneous arteries were not obtained from all biopsies, and several were discarded according to the criteria described in section 2.1 (F). Thus, the vessels studied came from 13 gluteal biopsies and 6 abdominal wall biopsies. The clinical characteristics of the volunteers who underwent gluteal biopsy, and from whom at least one vessel was studied, are shown in table 3.2. Similar data is shown for patients from whom an abdominal wall fat biopsy was obtained.

As can be seen from the tables all subjects were normotensive and had normal renal function, cholesterol and haemoglobin. None were diabetic. Although the age of those patients from whom abdominal wall biopsies were taken tended to be higher, there were no significant differences in the clinical characteristics of the subjects in each group.

### 3.2.4 Characteristics of arteries studied

Concentration response curves to AI alone (control) were performed in 15 arteries from separate patients. In early experiments the response to AI in the presence of losartan was also investigated (n=3). Subsequent experiments examined the response to AI in the presence of enalaprilat (1μM) in 12 arteries, chymostatin (10μM) in 6 arteries and the combination of chymostatin and enalaprilat (1μM, 10μM) in 7 arteries. In other experiments the response

to AI in the presence of CH5450 (10μM) or trasylol (100U/ml) was investigated in 8 and 3 vessels, respectively. The sizes of the vessels in each group are given in Table 3.3. Also given in Table 3.3 are the responses of the vessels in each group to the second exposure to KPSS, NE and ACh. There were no significant differences between control and experimental groups in terms of vessel diameter, contractile ability to either NE or KPSS, or endothelial-dependent vasodilatation to ACh.

Table 3.2 Clinical characteristics of patients studied

| Patients who underwent gluteal biopsy (n=13)<br>All values mean ± SD | | | | | |
|---|---|---|---|---|---|
| Age (years) | BP (mmHg) | Creatinine (μmol/l) | Cholesterol (mmol/l) | Glucose (mmol/l) | Haemoglobin (g/dl) |
| 30.15 (8.29) | 128.5/78.6 (14.0/10.7) | 91.17 (8.88) | 4.68 (0.42) | 4.79 (0.57) | 14.58 (1.10) |

| Patients who underwent abdominal wall biopsy (n=6)<br>All values mean ± SD | | | | | |
|---|---|---|---|---|---|
| Age (years) | BP (mmHg) | Creatinine (μmol/l) | Cholesterol (mmol/l) | Glucose (mmol/l) | Haemoglobin (g/dl) |
| 39.00 (17.13) | 133.0/76.0 (6.42/12.33) | 86.00 (5.15) | 4.75 (1.26) | 4.60 (0.55) | 14.80 (1.48) |

Table 3.3 Characteristics of human arteries

| | AI Control | AI +E | AI +C | AI+E +C | AI + CH5450 | AI + trasylol |
|---|---|---|---|---|---|---|
| Number of vessels | 15 | 12 | 7 | 6 | 8 | 3 |
| $L_1$ (diameter μm) | 331.16 (35.17) | 307.51 (28.58) | 305.70 (20.58) | 337.42 (27.27) | 326.06 (95.49) | 325.39 (97.11) |
| Contraction to KPSS (kPa) | 21.61 (7.42) | 21.62 (6.60) | 22.42 (5.71) | 22.83 (5.20) | 22.00 (13.04) | 28.48 (11.69) |
| Contraction to NE (kPa) | 22.72 (7.50) | 26.64 (3.86) | 26.82 (4.99) | 28.54 (6.78) | 28.63 (7.94) | 23.08 (15.21) |
| % Relaxation to ACh | 84.23 (8.72) | 80.95 (10.93) | 84.09 (8.74) | 83.32 (13.84) | 77.43 (22.76) | 80.24 (21.78) |

Table 3.3. Where $L_1$, is the internal diameter for optimal contraction, determined as normalised internal diameter, $L_{100} \times 0.9$. Also shown are the response to the second exposure to KPSS, the response to NE (10 μM) and the response to ACh (3μM). All values are mean (SD).

### 3.2.5 Responses of human resistance arteries to AI: The effect of inhibition of ACE and chymase

Arteries constricted to KPSS and NE and were able to maintain contraction for several minutes. The response to the second exposure to KPSS was larger than the first, and the response to NE was generally larger still. ACh induced a near-maximal vasodilation in most vessels. The responses to KPSS and NE given in table 3.3 are the absolute increases in effective pressure above baseline (kPa) and the response to ACh is the reduction in effective pressure as a % of the pre-contraction (thus full relaxation is equivalent to 100%). The data presented below represents only vessels that were able to constrict in response to both KPSS and NE and relax on exposure to ACh.

In the absence of any inhibitors, AI induced a concentration-dependent contractile response in human resistance arteries with a threshold (defined as the concentration at which the first response was detected) of [mean ± (SD)] 4.04 (4.09) $x10^{-9}$M and a maximum at 0.1 μM. The response followed a stereotypical pattern, with marked tachyphylaxis developing during the dose-response curve. This determined how the CRC was performed. Thus, the dosing interval was 4 minutes initially but once the threshold was reached the next dose was added when the peak response at that concentration had been achieved. A consistent observation was the inability of the artery to maintain contraction at high concentrations of AI, giving the dose-response curve a

characteristic "inverted U" shape. A representative CRC to AI in the absence of any inhibitors is shown in Figure 3.1.

Effect of losartan on responses to AI

In preliminary experiments the dependence of the contractile response to AI on activation of the AT1R, and, by inference, on conversion to AII was studied. Arteries were incubated with the AT1R antagonist losartan 1μM for 30 minutes before a CRC to AI was performed. A representative trace is shown in Figure 3.2. It can be seen from this figure that losartan almost completely inhibited the response to AI. This experiment was performed in 3 arteries.

Effect of ACE and chymase inhibition on responses to AI

The effect of co-incubation with enalaprilat, chymostatin, chymostatin + enalaprilat, CH5450 and trasylol are shown in figures 3.3-3.8. In these graphs, responses to AI are expressed as the % of the contraction observed with the second exposure to KPSS in that vessel. This was done in order to try to control for the variability in the responses to AI that was observed. Neither enalaprilat, chymostatin, nor CH5450 inhibited the response to AI. Due to the small number of vessels studied it is difficult to draw firm conclusions about the effect of trasylol, but inspection of the CRC revealed little evidence of inhibition with this agent. The concentrations of AI required for 50% (EC50) of maximum contraction in the presence of enalaprilat,

chymostatin and CH5450 are shown in table 3.4. Also shown in table 3.4 are the threshold concentrations and maximum responses to AI in the presence of these agents.

In contrast to the lack of inhibition of AI responses in the presence of ACEi or chymase inhibitors alone, the combination of enalaprilat and chymostatin had a very marked inhibitory effect. The CRC was shifted to the right, with a threshold response of 1.55 (1.15) $x10^{-7}$M, some 40-fold higher than the control response. The degree of inhibition was so marked that the maximum response was not achieved within the range of concentrations used. Thus neither the EC50 nor the maximum could be calculated for this response. In order to compare the response to AI in the presence or absence of inhibitors the AUC was calculated as described in methods. AUC values are shown in table 3.5. There was no significant difference between the AUC for AI alone compared to AI in the presence of enalaprilat ($p=0.48$), chymostatin ($p=0.063$) or CH5450 ($p=0.12$) when these values were compared using a Mann Whitney U test. However the AUC in the presence of both enalaprilat and chymostatin was significantly lower than control ($p=0.028$). Thus only the combination of enalapril and chymostatin significantly inhibited the response to AI.

In a subsequent analysis the threshold concentrations for AI in the presence of each inhibitor were compared to control using one-way analysis of variance (ANOVA) with a Bonferroni correction for multiple comparisons. When analysed in this way, similar results were obtained i.e. enalaprilat, chymostatin and CH5450 alone did not significantly increase the threshold to

AI ($p>0.05$ by ANOVA), but the combination of enalaprilat and chymostatin did increase the threshold to AI ($p<0.01$).

Figure 3.1 Concentration Response Curve to AI in human resistance arteries – representative traces

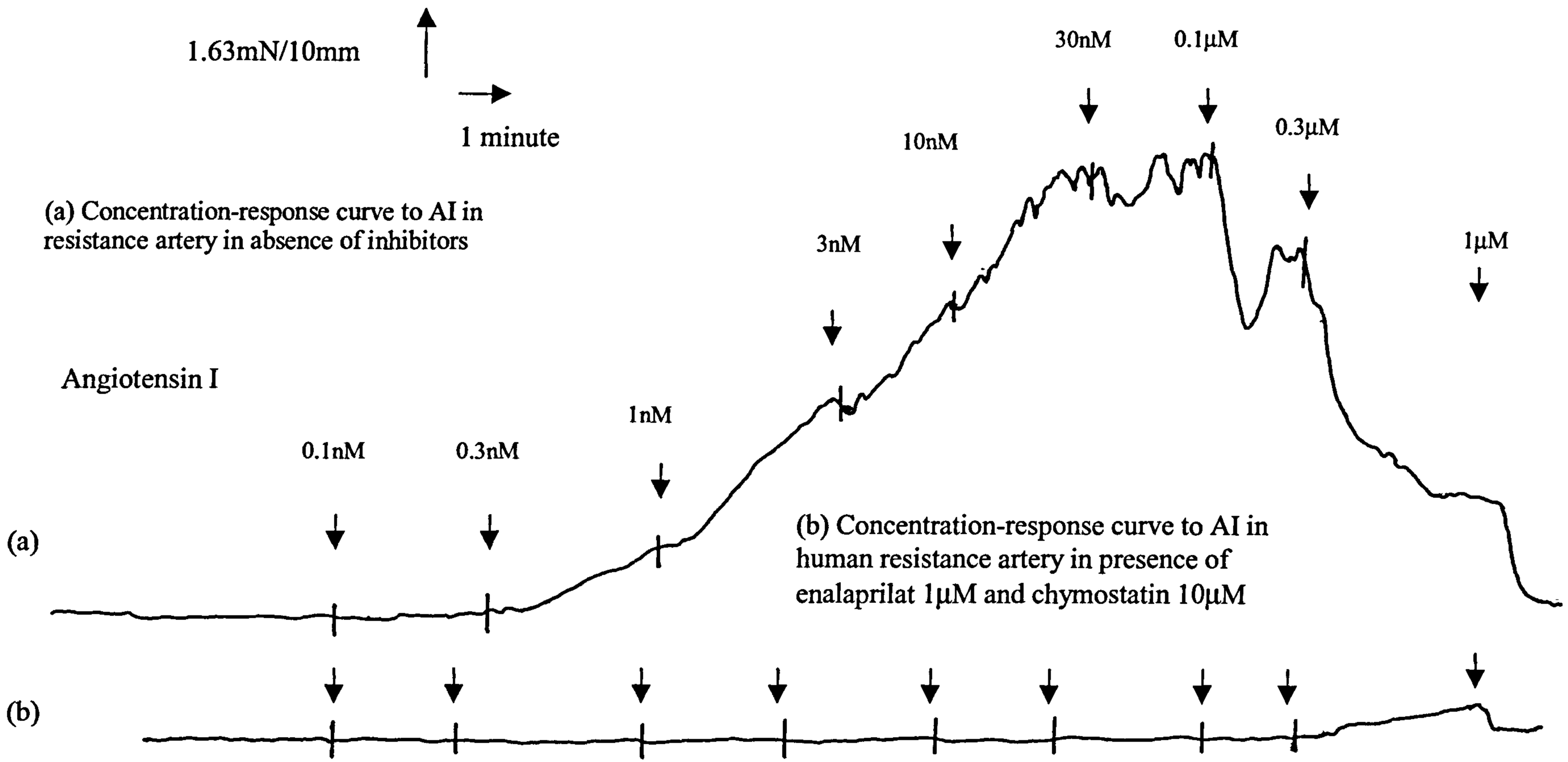


Figure 3.2 Concentration Response Curve to AI: Effect of co-incubation with losartan 1μM

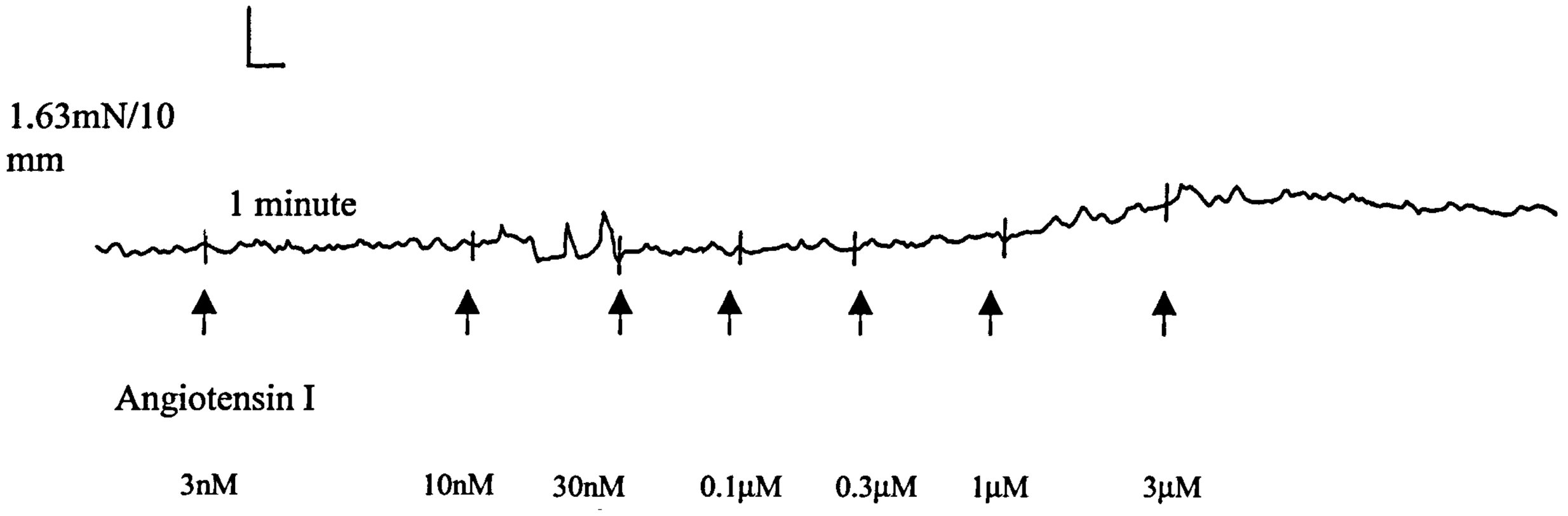


Figure 3.3: Concentration-Response Curve to AI: control

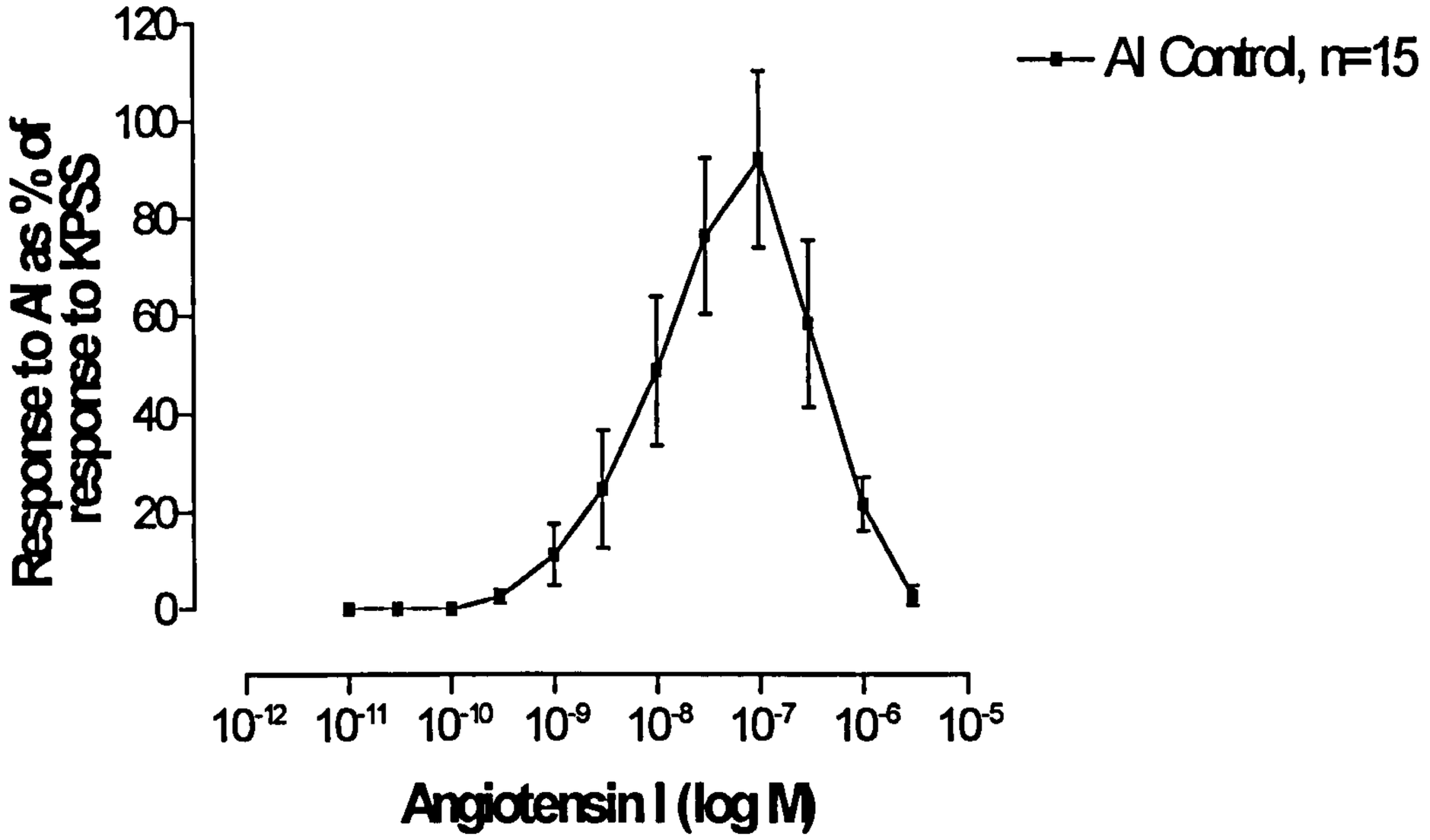



Figure 3.3 shows the cumulative concentration-response curve to AI in the absence of any inhibitors.

Figure 3.4: Concentration-Response Curve to AI: effect of enalaprilat 1μM

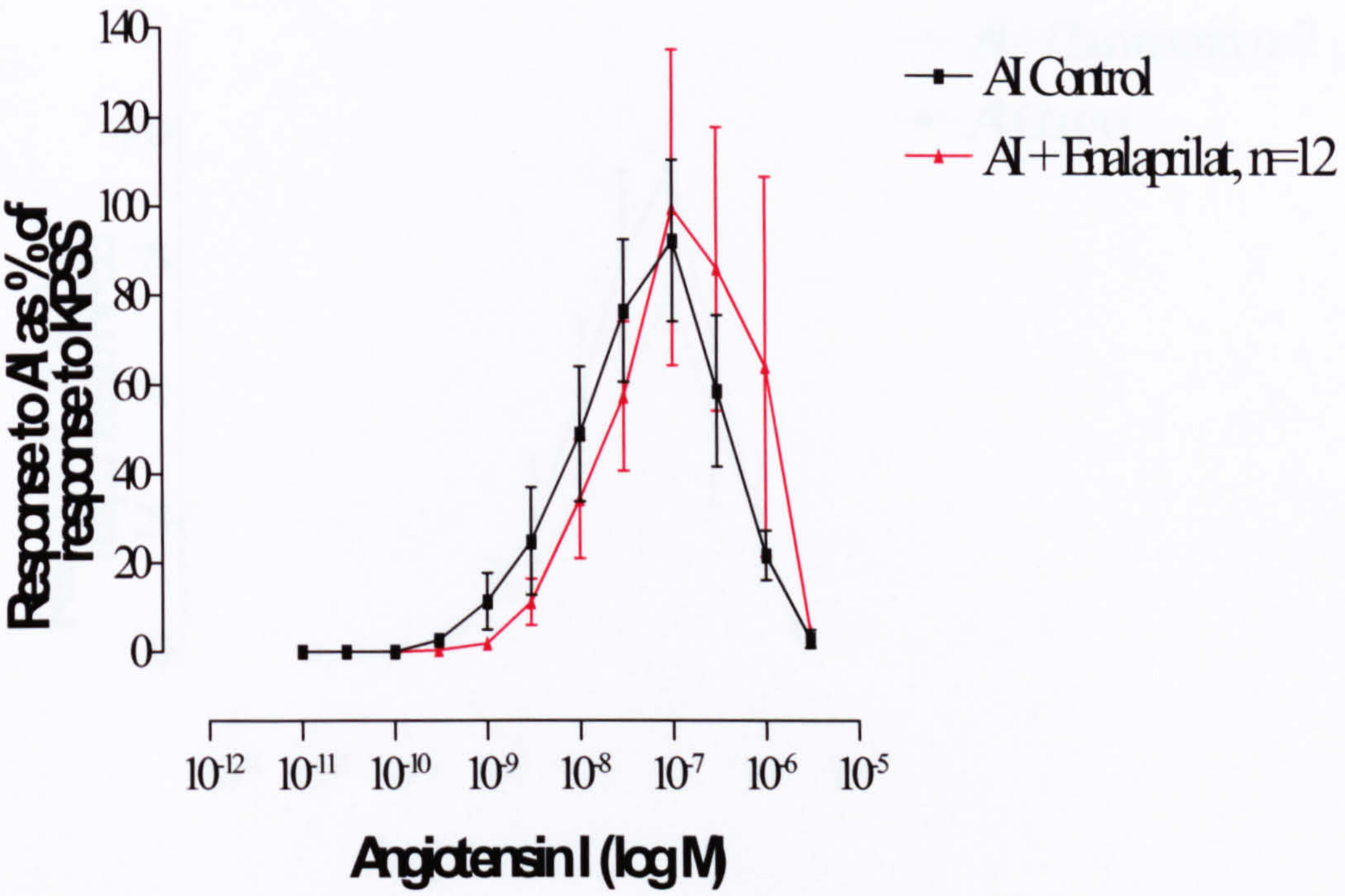



Figure 3.4 shows the cumulative concentration-response curve to AI after incubation with enalaprilat for 30 minutes.

Figure 3.5: Concentration-Response Curve to AI: effect of chymostatin 10μM

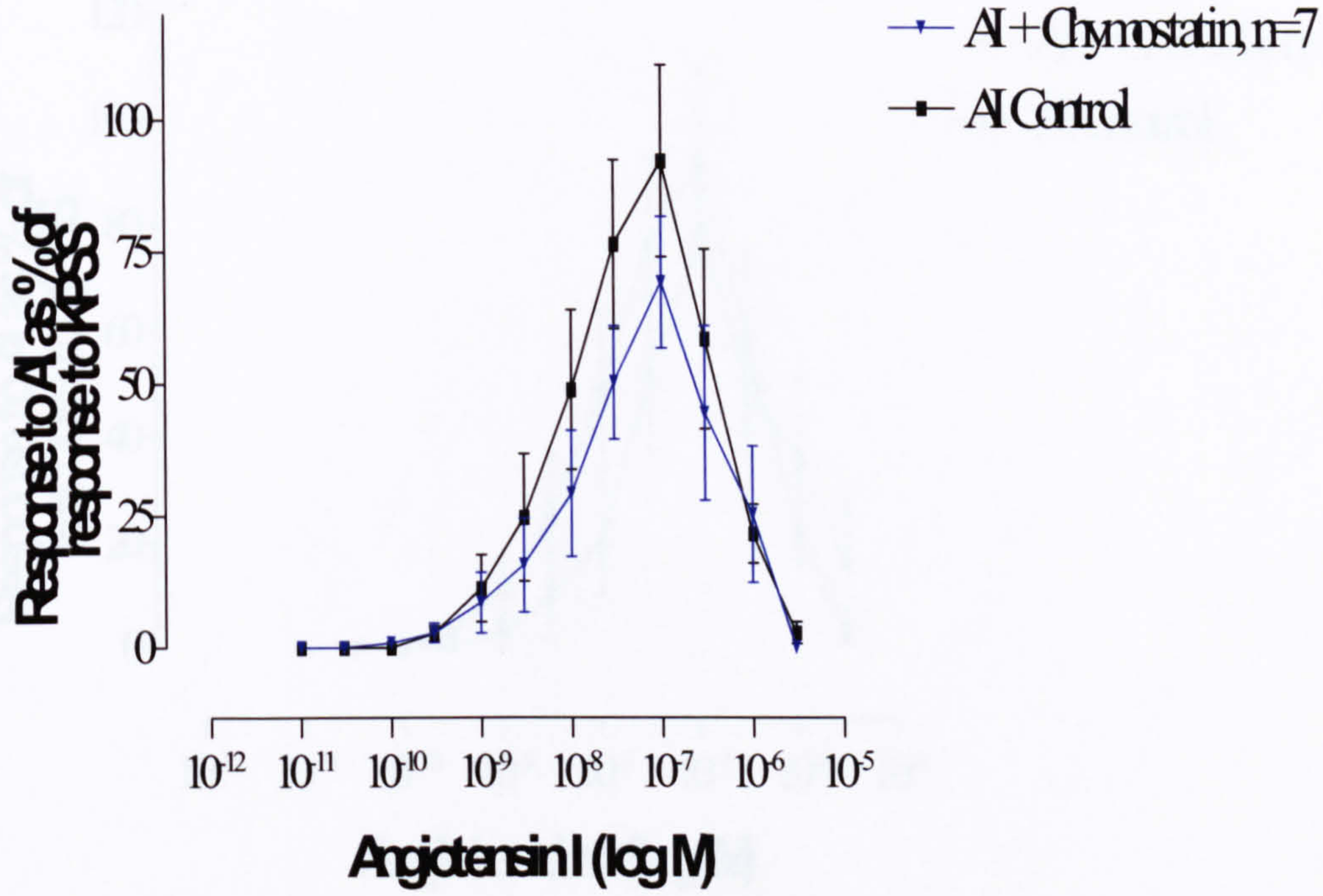



Figure 3.5 shows the cumulative concentration-response curve to AI after incubation with chymostatin for 30 minutes.

Figure 3.6: Concentration-Response Curve to AI: effect of CH5450 10μM

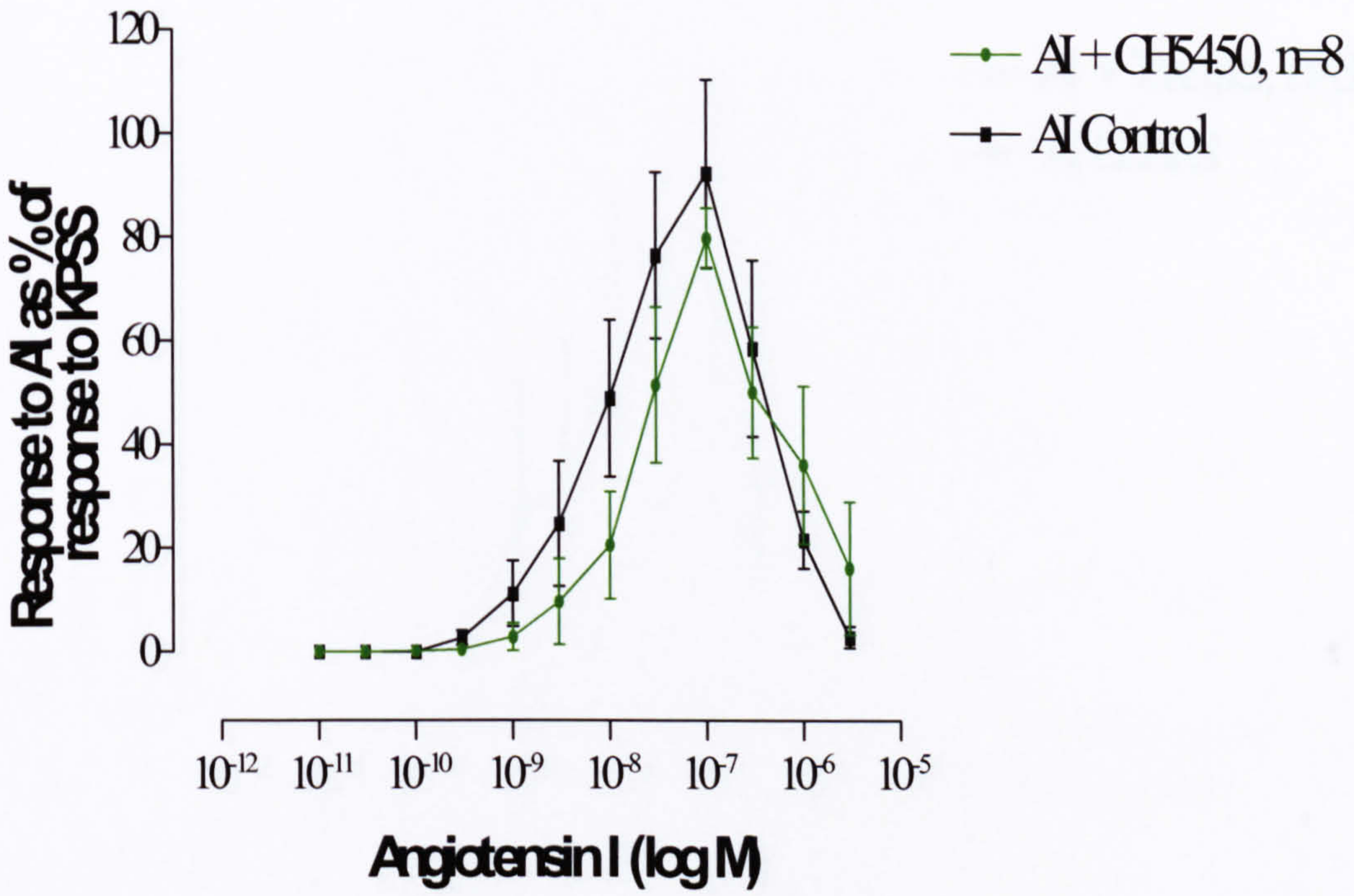



Figure 3.6 shows the cumulative concentration-response curve to AI after incubation with CH5450 for 30 minutes.

Figure 3.7: Concentration-Response Curve to AI: effect of trasylol 100U/ml

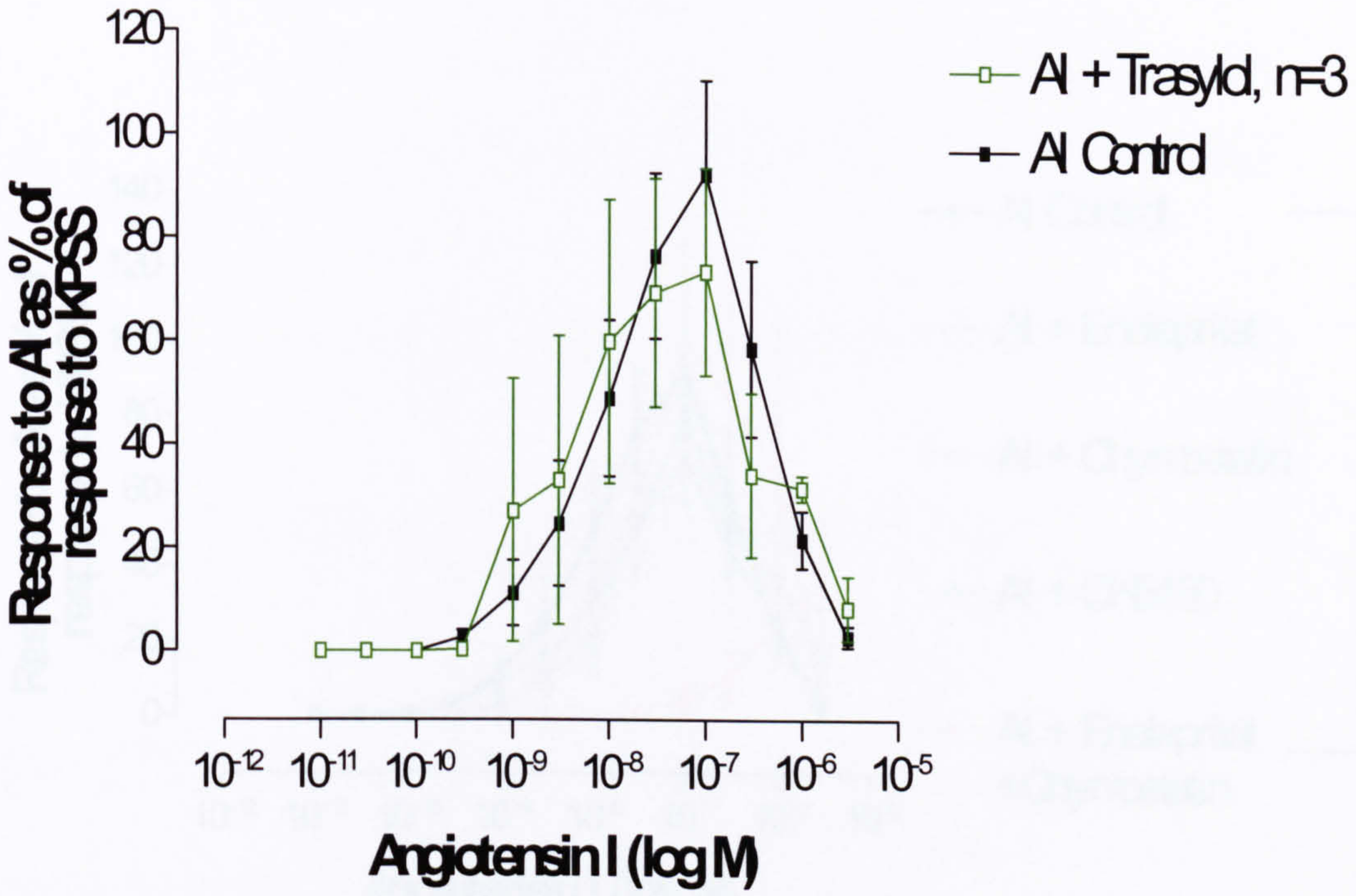



Figure 3.7 shows the cumulative concentration-response curve to AI after incubation with trasylol for 30 minutes.

Figure 3.8: Concentration-Response Curve to AI: comparative effects of inhibitors

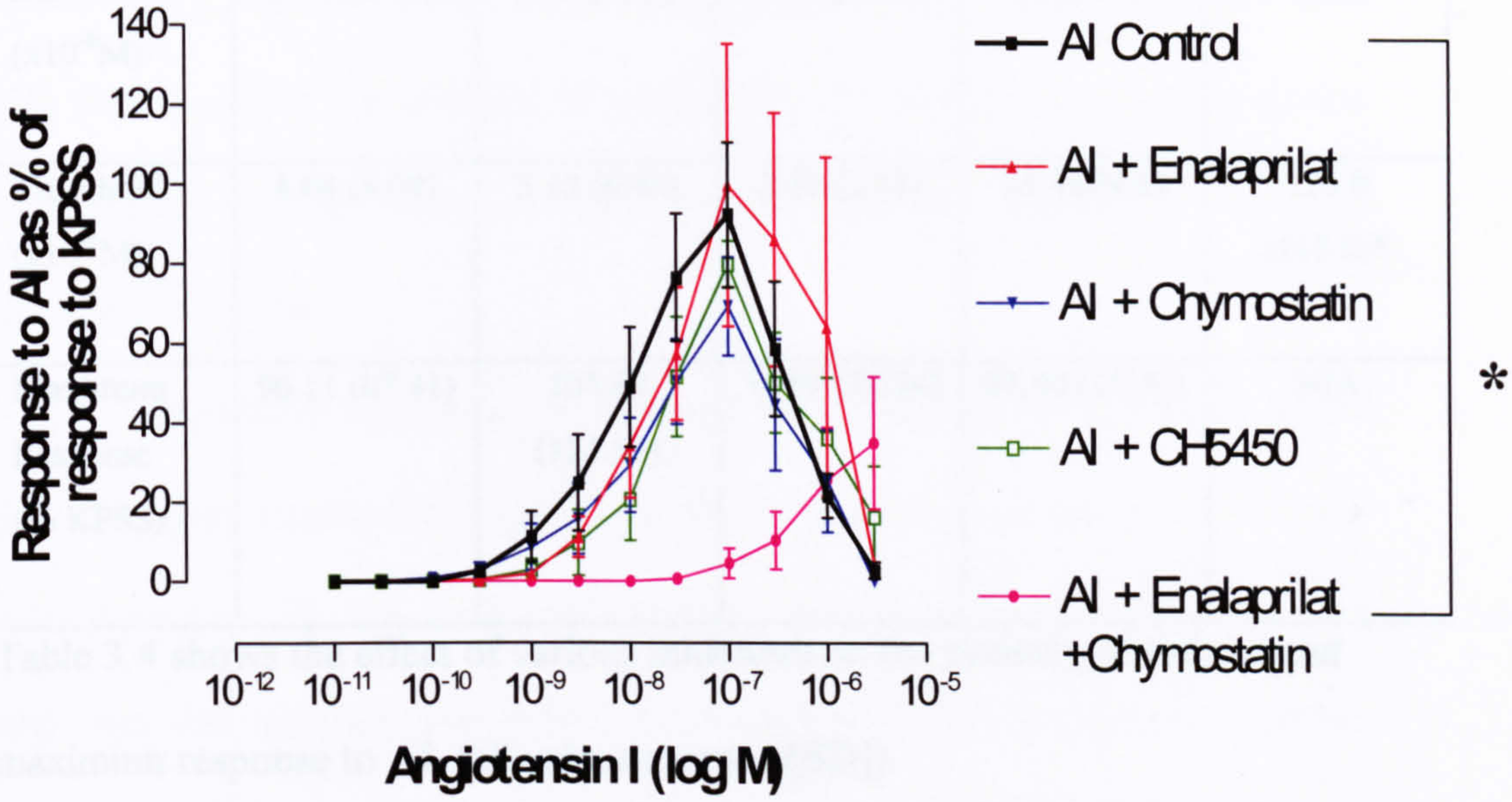



Figure 3.8 shows the cumulative concentration-response curve to AI in the presence of inhibitors compared to control.

* $p<0.01$ comparing the threshold for combination with that of control

Table 3.4: Potency of AI in human arteries

| | Control<br>n=15 | Enalaprilat<br>n=12 | Chymostatin<br>n=7 | CH 5450<br>n=8 | Combination<br>n=6 |
|---|---|---|---|---|---|
| EC 50<br>($x10^{-8}M$) | 2.14 (1.97) | 3.43 (2.10) | 2.09 (1.59) | 4.36(3.06) | N/A |
| Threshold<br>($x10^{-9}M$) | 4.04 (4.09) | 5.13 (4.40) | 2.89 (3.41) | 18.4 (34.4) | 155.0<br>(115.0)* |
| Maximum<br>Response<br>(% KPSS) | 96.11 (67.41) | 104.60<br>(121.50) | 72.00 (32.24) | 83.96 (15.33) | N/A |

Table 3.4 shows the effect of various inhibitors on the potency, threshold and maximum response to AI. (all values means ±[SD])

* $p<0.01$ combination vs. control by ANOVA

Table 3.5: AUC values for AI: effect of inhibitors

| | Control | Enalaprilat | Chymostatin | CH 5450 | Combination |
|---|---|---|---|---|---|
| AUC<br>(mean ± SD) | 163.12<br>(128.07) | 169.82<br>(215.43) | 113.78<br>(73.22) | 111.44<br>(53.98) | 29.15<br>(73.22)* |

Table 3.5 shows the mean AUC values of AI curves

* $p=0.028$ vs control

### 3.2.6 Response of human resistance arteries to bradykinin: Effect of Enalaprilat

These experiments were performed in a subset of vessels, which had already been exposed to AI. In human resistance arteries pre-constricted with NE 10μM, and in the absence of enalaprilat, BK induced a dose-dependent vasodilation. The threshold was 0.1nM and the maximum response approached 100%.

Cumulative concentration-response curves to BK are shown in figure 3.9. In these graphs the response to BK is expressed as the % of the pre-constriction to NE. For each experimental vessel there was a matched control and data was normally distributed, allowing the use of parametric statistics. Enalaprilat did not influence the maximum response to BK ($p=0.32$ compared to control, by paired t-test). However there was a significant shift in the dose response curve to the left. Thus the EC50 for BK in the presence of enalaprilat was 4.46 (6.79) $x10^{-8}M$ compared with 3.33 (6.02) $x10^{-7}M$ in control vessels ($p=0.0026$, by paired t-test). There were no significant differences in the sizes of arteries studied in each group, or their responses to NE. Table 3.6 summarises these data.

Figure 3.9: Response of human resistance arteries to bradykinin: effect of enalaprilat 1μM

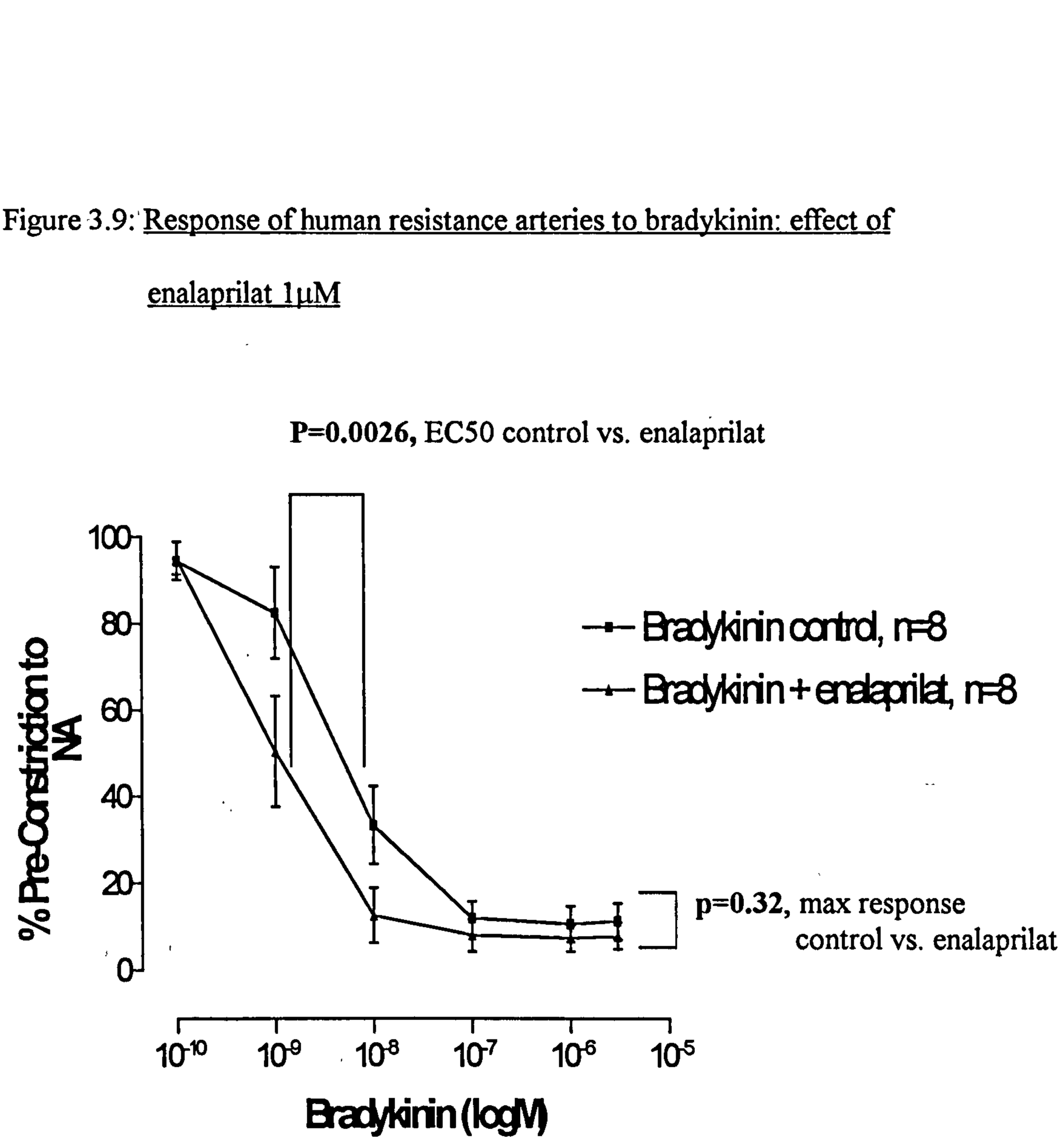



Figure 3.9 shows the cumulative concentration-response curve to BK in untreated arteries and those pre-treated with enalaprilat 1μM. The EC50 for BK in the presence of enalaprilat was significantly lower than for control, but the maximum response was unchanged.

Table 3.6: Potency of BK in human resistance arteries

| | Control | Enalaprilat |
|---|---|---|
| $L_1$ (diameter, μm, mean ±SD) | 316.78 (50.71) | 306.41 (68.84) |
| Contraction to NE (kPa, mean ± SD) | 26.74 (9.48) | 28.22 (5.72) |
| Maximum response to BK (% pre-constriction, mean ± SD) | 94.53 (12.40) | 95.18 (10.68) |
| EC 50 ($x10^{-7}M$) | **3.33 (6.02)** | **0.45 (0.68)*** |

Table 3.6 shows the sizes of arteries exposed to BK. These arteries are a sub-set of those described in table 3 2 under the headings AI control and AI + E. There were no differences in the responses to NE, KPSS or ACh during the standard activation. Data presented in this table shows no difference in the sizes of this sub-set of arteries, or in their response to NE (pre-constriction for BK CRC).

* p=0.0026 vs. control by paired t-test

## 3.3 Studies in New Zealand White Rabbits

### 3.3.1 Animals studied

The animals used in these experiments were sham operated controls for a rabbit coronary artery ligation model of heart failure and had normal left ventricular function as determined by echocardiogram. Experiments were carried out in male rabbits weighing 3.0-3.5 kg. Animals were sacrificed by an overdose of pentobarbitone into the ear vein. Biochemical data equivalent to human data is not available for these animals.

### 3.3.2 Experimental protocols

The protocol was essentially the same for rabbit cutaneous arteries, the difference lying in the inhibitors used. Rabbit arteries were incubated with either enalaprilat 1μM, trasylol 100U/ml or CH5450 10μM. Trasylol is a serine protease inhibitor, to which chymase is not susceptible. The combination of enalaprilat and CH5450 was not used because the inhibition achieved with the former was so marked (see results). Rabbit vessels did not appear to relax to bradykinin. The experimental protocol used is summarised in table 3.7

Table 3.7: Experimental protocol for resistance arteries from New Zealand white rabbits

| | Vessel | | | |
|---|---|---|---|---|
| | **1** | **2** | **3** | **4** |
| **Standard Activation** | **KPSS**<br>**KPSS**<br>**NE 10μM**<br>**ACh $3\times10^{-6}$μM** | | | |
| **Incubation**<br>**30 mins** | **Control**<br>**Vehicle** | **Enalaprilat**<br>**1μM** | **CH5450**<br>**10μM** | **Trasylol**<br>**100U/ml** |
| **CRC to AI** | **AI (0.01nM, 0.03nM, 0.1nM, 0.3nM etc … to 3μM)** | | | |

### 3.3.3 Characteristics of Rabbit Arteries Studied

Dissection of rabbit cutaneous resistance arteries was considerably easier than that of human vessels and it was possible to obtain vessels within the desired range of normalised internal diameters. Arteries larger than $L_1$ = 350μm were discarded and thus the mean sizes of rabbit vessels was smaller than their human equivalents. Rabbit arteries were subjected to the same "warm-up" as human arteries. They contracted when activated with KPSS and NE and exhibited a vasodilatory response to ACh. Mean internal diameters of the arteries studied and responses to KPSS, NE and ACh are given in table 3.8. It was a consistent finding that rabbit arteries exhibited a greater contractile response to KPSS and to NE, and a smaller vasodilatory response to ACh.

Table 3.8: Characteristics of rabbit arteries

| | AI<br>Control | AI +<br>Enalaprilat | AI +<br>CH5450 | AI+<br>Trasylol |
|---|---|---|---|---|
| Number of vessels | 9 | 8 | 6 | 6 |
| $L_0$ (diameter μm) | 298.58<br>(30.05) | 293.62<br>(33.60) | 265.90<br>(19.29) | 279.94<br>(23.53) |
| Contraction to KPSS (kPa) | 32.12<br>(4.01) | 36.10<br>(5.41) | 30.90<br>(6.18) | 34.78<br>(2.28) |
| Contraction to Norepinephrine (kPa) | 30.04<br>(6.82) | 36.09<br>(9.13) | 30.12<br>(8.29) | 34.31<br>(4.46) |
| % Relaxation to Acetylcholine | 64.46<br>(11.73) | 51.73<br>(7.30) | 64.22<br>(13.49) | 62.02<br>(11.80) |

Table 3.8 shows normalised internal diameters of vessels in each group and responses to KPSS and NE, together with % relaxation to ACh in vessels pre-contracted with NE as part of the "warm-up." There were no significant differences between the sizes of the arteries studied in each group, or in their response to any of these agonists.

### 3.3.4 Responses of rabbit resistance arteries to AI: The effect of inhibition of ACE and chymase

AI stimulated a dose-dependent contraction in rabbit cutaneous resistance arteries. In the absence of inhibitors, the threshold response was at 8.33 (8.80) $x10^{-9}$M and the maximum response was at 0.3μM. Thus, rabbit cutaneous arteries were slightly less sensitive to AI than were human arteries. As in human resistance arteries there was marked tachyphylaxis which followed a similar pattern. Concentration-response curves were therefore performed in the same way, with addition of the next concentration of agonist at 4 minutes, or whenever the peak response had developed, whichever was sooner.

<u>Effect of ACE and chymase inhibition on responses to AI</u>

The maximum response and EC50 to AI in the presence of trasylol were not different from control. CH5450, an inhibitor of chymase appeared to reduce the maximum response slightly ($p<0.01$ by ANOVA) but had no effect on the EC50. However, enalaprilat at the same concentration as employed in human vessels almost completely abolished the response, shifting the curve markedly to the right. The threshold in the presence of enalaprilat was 1.61 (1.48) $x10^{-6}$M and no maximum response was observed within the concentration range utilised in this set of experiments. Accordingly AUC was calculated and used to compare the responses. The AUC (mean ± SD) calculated for AI in the presence of enalaprilat was 21.43 (22.89) compared to

210.00 (55.60) for control (p=0.012; Wilcoxon Rank Sum Test). Neither CH5450 (p=0.075) nor trasylol (p=0.116) exerted any significant inhibitory effect.

The threshold concentrations for AI in the presence of the inhibitors were also compared to control in a one-way ANOVA with Bonferroni correction for multiple comparisons. The result of this was consistent with the comparison of AUC values – revealing significant inhibition only in the presence of enalaprilat ($p<0.001$). Threshold concentrations for all curves, together with EC50 and maximum responses for AI control, CH5450 and trasylol are shown in table 3.9.

Figure 3.10: Concentration-Response Curve to AI in the absence of inhibitors

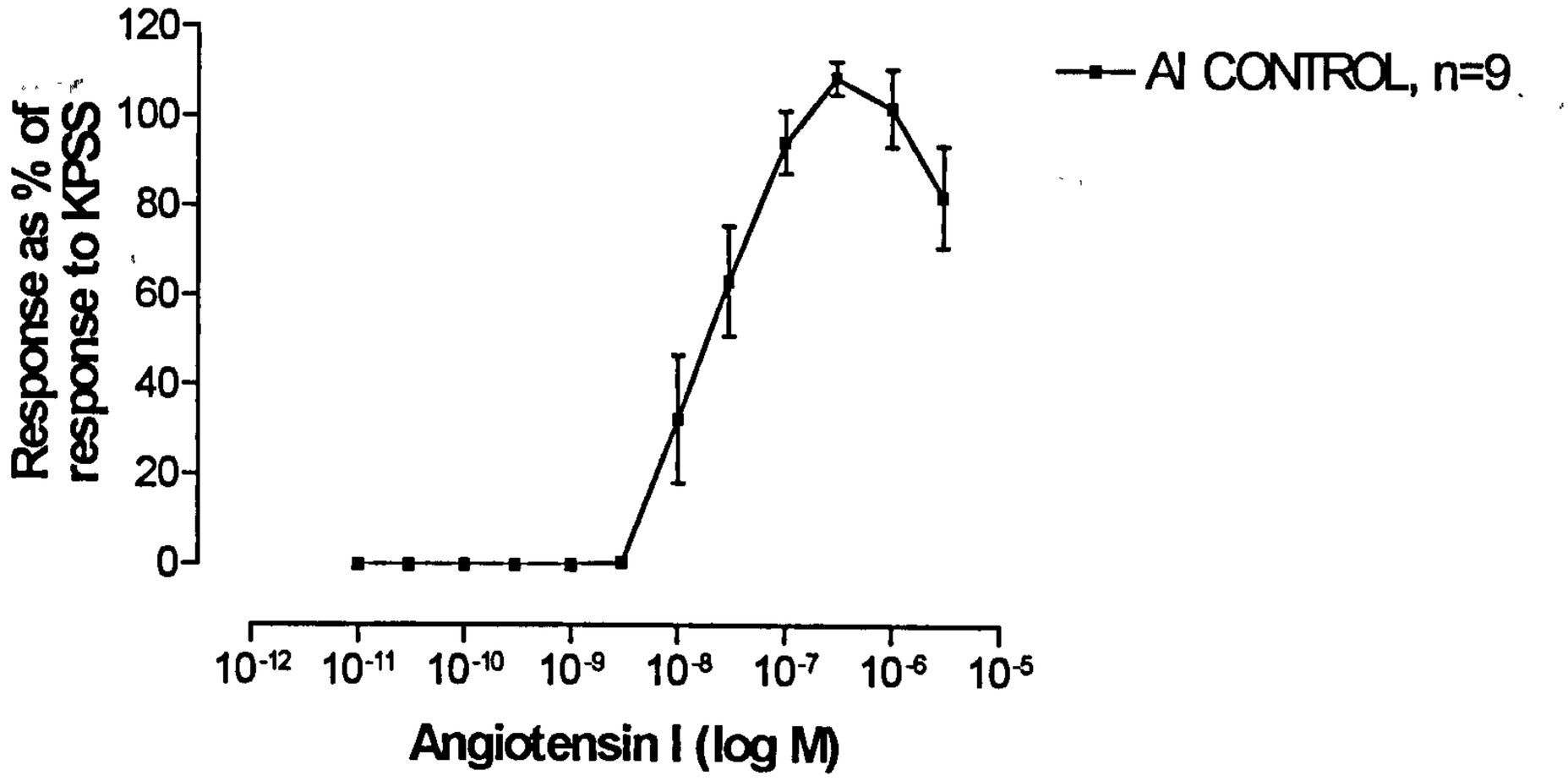



Figure 3.10 shows the cumulative concentration-response curve to AI in the absence of any inhibitors.

Figure 3.11: Concentration-Response Curve to AI: effect of enalaprilat 1μM

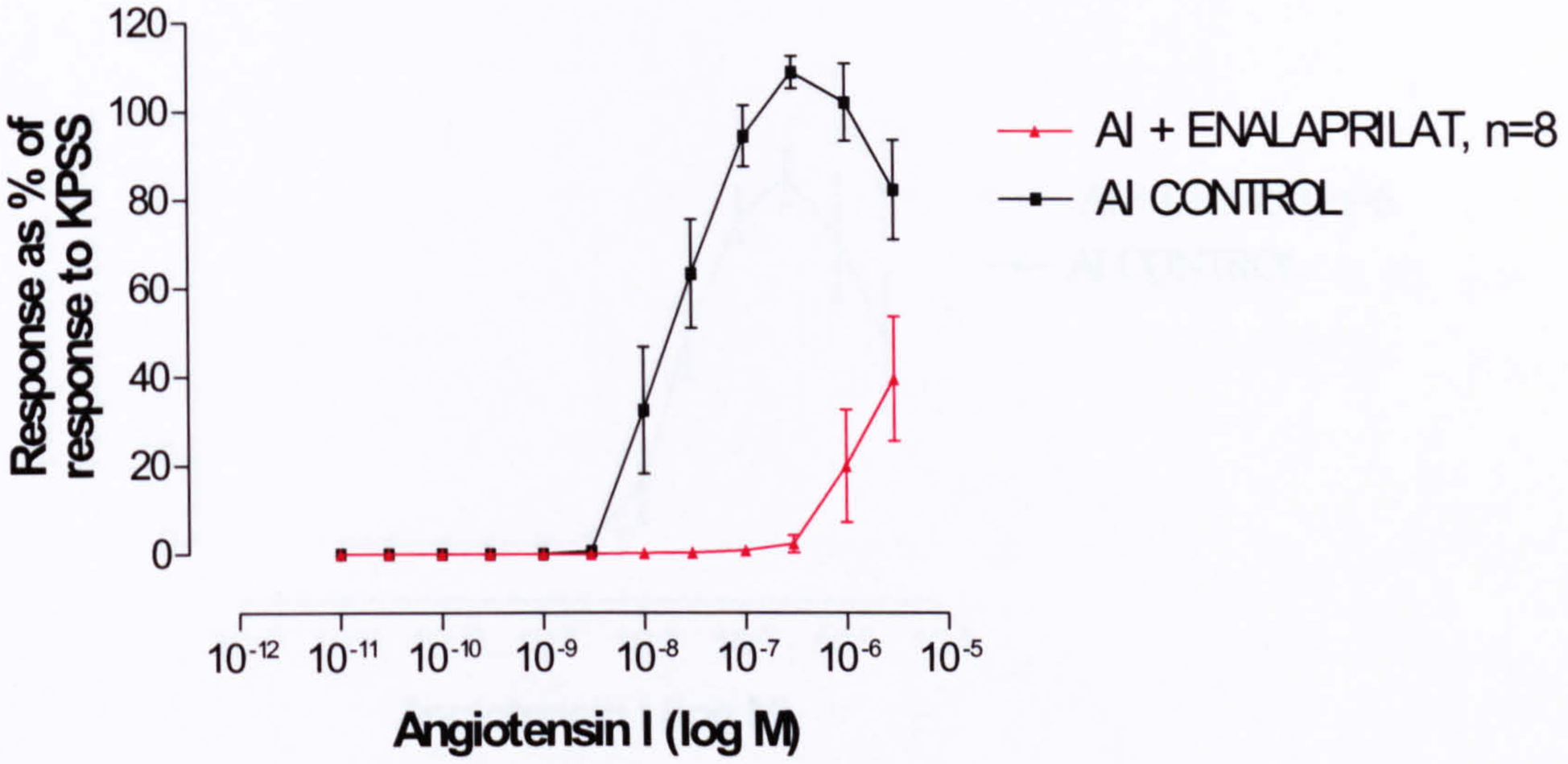



Figure 3.11 shows the cumulative concentration-response curve to AI after incubation with enalaprilat. The threshold was significantly increased ($p<0.001$) demonstrating marked inhibition by enalaprilat

Figure 3.12: Concentration-Response Curve to AI: effect of CH5450 10μM

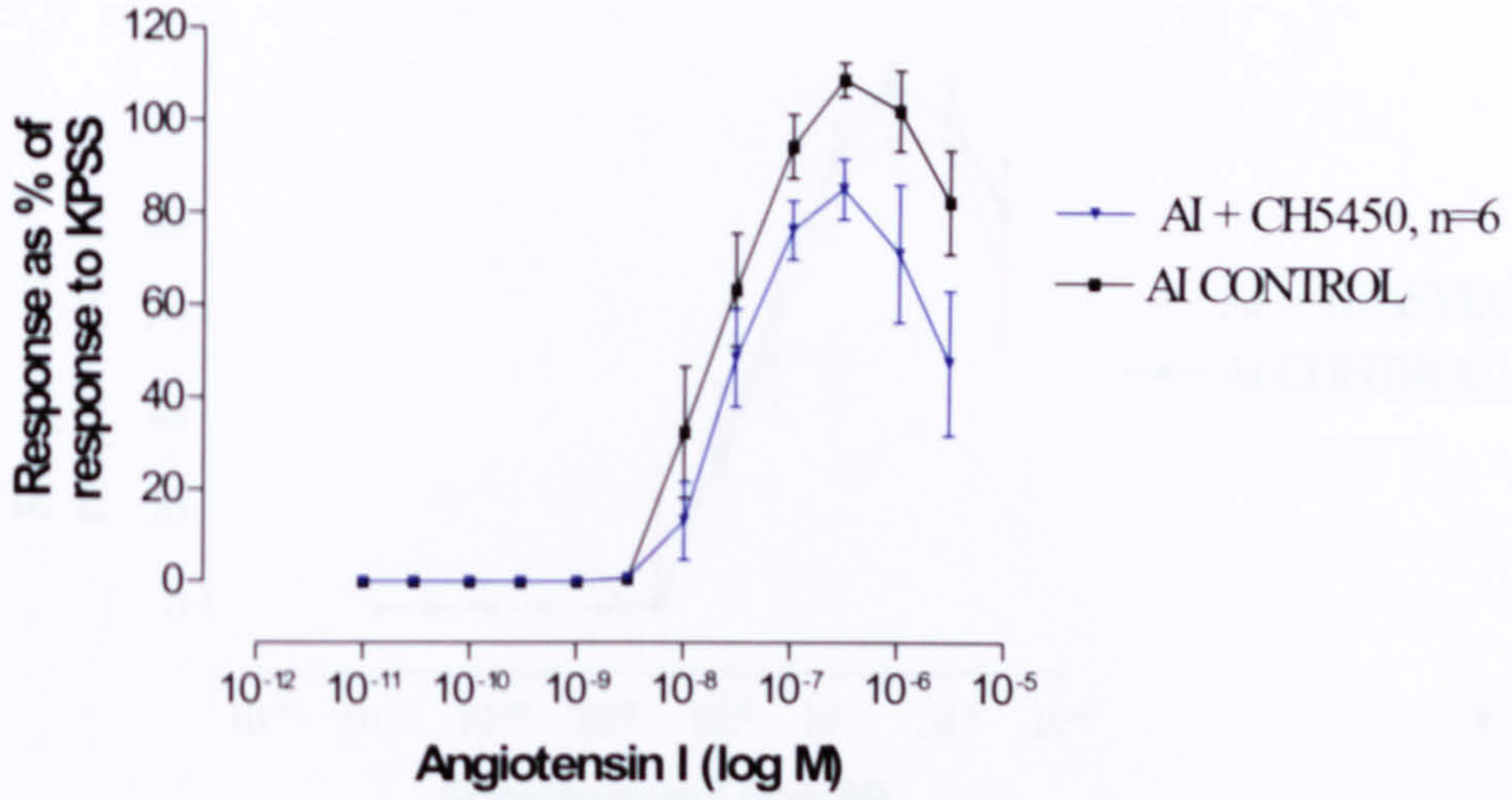



Figure 3.12 shows the cumulative concentration-response curve to AI after incubation with CH5450. The maximum response is reduced in the presence of CH5450 ($p<0.01$).

Figure 3.13: Concentration-Response Curve to AI: effect of trasylol 100U/ml

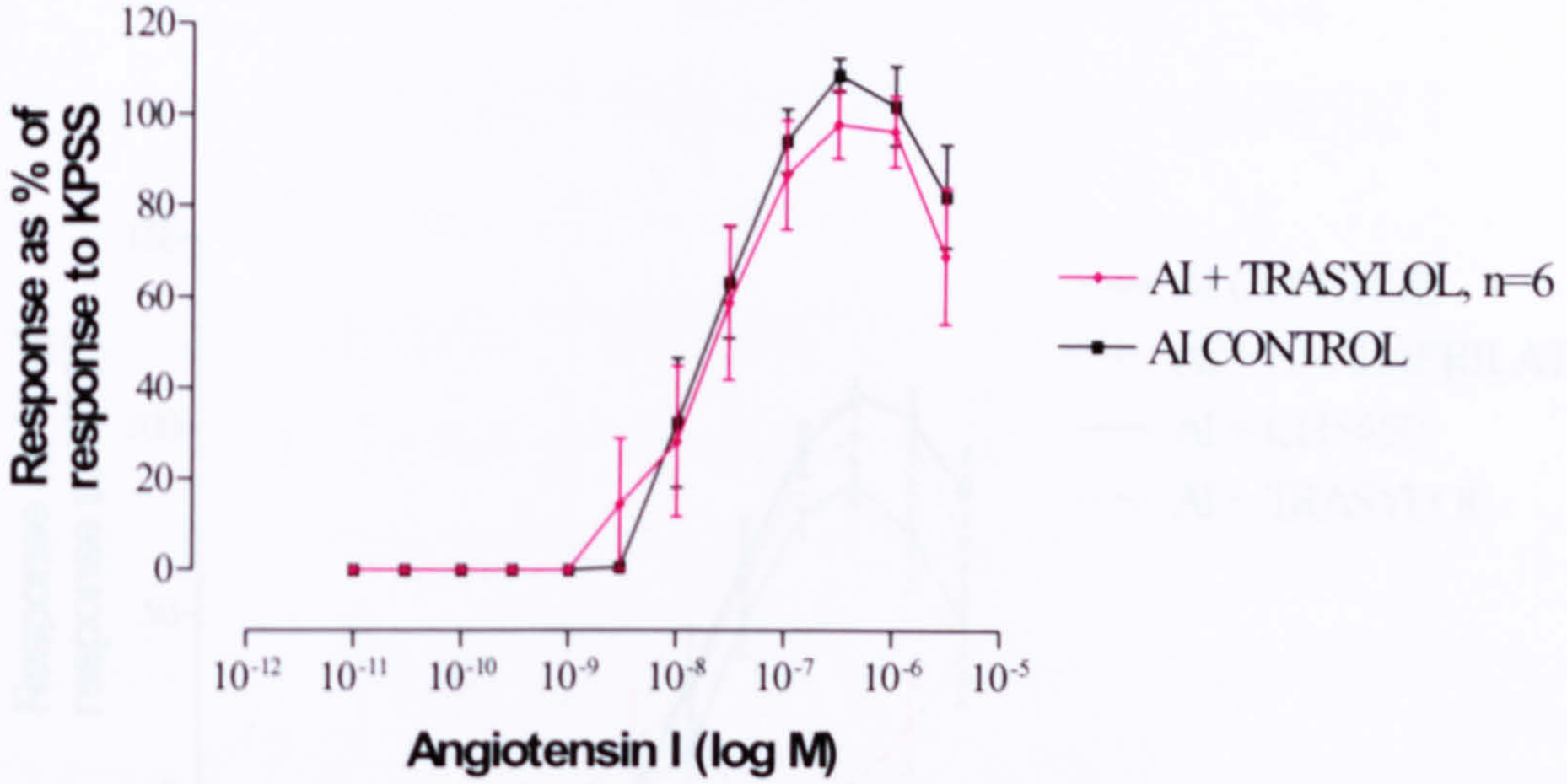



Figure 3.13 shows the cumulative concentration-response curve to AI after incubation with trasylol.

Figure 3.14: Concentration-Response Curve to AI: comparative effect of inhibitors

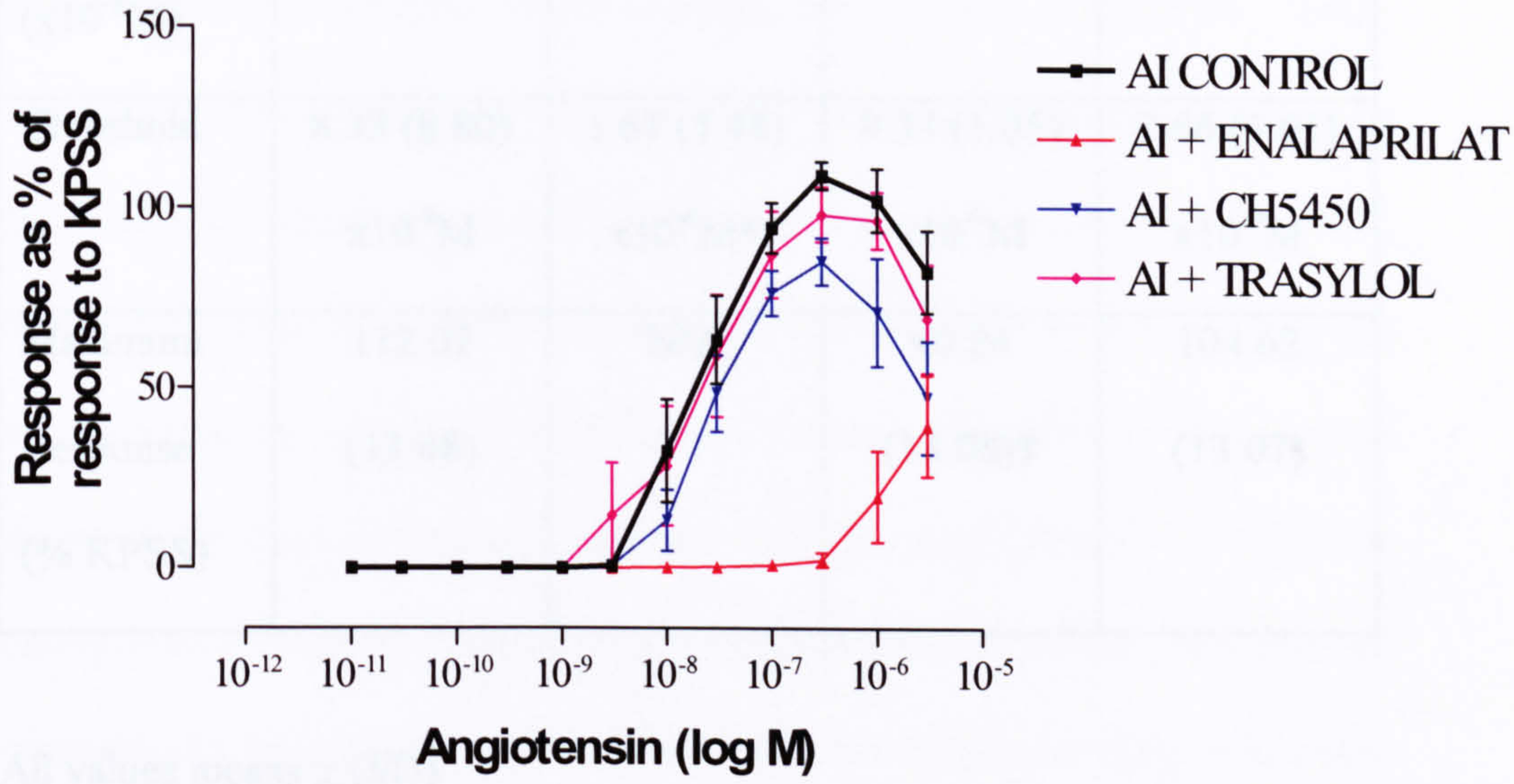



Figure 3.14 shows the cumulative concentration-response curve to AI in the presence of inhibitors compared to control. Only enalaprilat significantly inhibited the response to AI.

Table 3.9: Potency of AI in rabbit cutaneous resistance arteries

| | Control<br>n=9 | Enalaprilat<br>n=8 | CH 5450<br>n=6 | Trasylol<br>n=6 |
|---|---|---|---|---|
| EC50<br>($x10^{-8}M$) | 4.24 (4.84) | N/A | 3.25 (2.42) | 5.00 (6.63) |
| Threshold | 8.33 (8.80)<br>$x10^{-9}M$ | 1.61 (1.48)<br>$x10^{-6}M$* | 9.33 (1.05)<br>$x10^{-9}M$ | 7.66 (3.61)<br>$x10^{-9}M$ |
| Maximum<br>Response<br>(% KPSS) | 112.02<br>(13.48) | N/A | 90.26<br>(13.08)† | 104.62<br>(13.07) |

All values means ± (SD)

*$p<0.001$ compared to control

†$p<0.01$ compared to control

## 3.4 Discussion

In the introductory review I discussed the possibility of the existence of local AII generation in a variety of tissues. In the preliminary experiments described in this chapter I showed that AI stimulates a contractile response in human resistance arteries, which is completely blocked by losartan. Thus, the data I have presented here suggests that, when supplied with the precursor AI, human resistance arteries are also capable of generating AII, which then acts in a paracrine fashion to regulate vessel tone through activation of the AT1R. Furthermore, the data presented in this chapter demonstrate that in-vitro blockade of ACE is insufficient to prevent the contraction of human subcutaneous resistance arteries to AI. In contrast, responses to AI in rabbit resistance arteries were almost completely inhibited by enalaprilat. Lastly I have demonstrated that enalaprilat potentiates the response to exogenous BK in human resistance arteries. I shall begin this discussion by considering the response to BK.

ACE is identical to kininase II, the enzyme responsible for the degradation of kinins, and it has been suggested that potentiation of BK may be partly responsible for the actions of ACEi. This, however, remains controversial. Although plasma kinin concentrations have been shown to increase in humans after administration of quinapril, other studies have not confirmed this.[284, 285] This may be due to technical difficulties in measuring kinin levels accurately. Physiological studies do suggest an

interaction between ACE inhibitors and kinins. BK has been shown to be responsible for coronary artery vasodilation observed in dogs after administration of ramiprilat.[286] Similarly, BK-mediated vasodilation of human coronary arteries is potentiated by enalaprilat.[287] However, whether the cardiac effects of ACE-inhibition are mediated by potentiation of kinins is unclear. A recent study found no increase in tissue kinin levels in the right atrial appendage after administration of an ACEi orally prior to open-heart surgery.[288]

In these experiments I have demonstrated that enalaprilat can potentiate the response to exogenously administered BK in human resistance arteries. This raises the possibility that the hypotensive actions of ACEi and, perhaps, their effect on peripheral resistance may be mediated through the potentiation of endogenous kinins. Gainer et al studied the effect of a specific BK-receptor antagonist, HOE 140, on the blood pressure response to ACE inhibition and AT1R antagonism in sodium deplete normotensive and hypertensive subjects.[289] HOE 140 reduced the hypotensive effect of captopril by 53%, but did not alter the response to losartan. As expected, captopril reduced renal vascular resistance, but this was unaffected by HOE 140. Thus, while potentiation of endogenous kinins appeared to contribute to the hypotensive action of captopril, they did not appear to contribute to the regulation of renal vascular resistance. Further studies are required to investigate the contribution of endogenous kinins to the regulation of peripheral vascular resistance.

The most important observation in this study was the failure of enalaprilat to reduce AI-mediated contractions in human resistance arteries. Several alternative explanations for this need to be considered. One possibility is that enalaprilat did not fully inhibit tissue ACE in human vessels. This seems unlikely for two reasons: firstly there was an obvious effect of enalaprilat in rabbit vessels, which were of very similar size to their human equivalents, and secondly enalaprilat potentiated BK in human vessels. The possibility that the apparent effect of the inhibitors on AI in human and rabbit vessels might have been due to differences in their contractile abilities, or to endothelial denudation, is rendered remote by the very similar responses to KPSS, NE and ACh.

The most likely explanation for the failure of enalaprilat to significantly inhibit AI-mediated responses is the presence of an alternative enzymatic pathway (or pathways) for AII generation. This has been described extensively in the myocardium and in large and medium sized blood vessels in man and in animals. Urata observed that SBTI reduced AII generation in human heart homogenates by 80%, while captopril reduced AII generation by only 11%.[182] This led to the identification of human heart chymase.[183] Similarly Takai showed that the contraction of human gastroepiploic arteries could be reduced by 96% in the presence of chymostatin, but only 30% in the presence of lisinopril.[208] These data suggest that, far from being mediated by ACE, the predominant route for AII generation in the human myocardium and vasculature is through the action of

chymase. The data presented above suggest that, in human resistance arteries, neither inhibition of ACE, nor chymase significantly attenuates the response to AI and thus differ from previous studies. In contrast, the combination of the two inhibitors suggests that there is a dual pathway for AII generation in human resistance arteries, mediated by ACE and a chymostatin-sensitive enzyme, which is probably HHC. These pathways appear to be equally able to generate AII, so that significant inhibition of the response to AI was only achieved by combining inhibitors of both ACE and chymase.

The comparison with rabbit vessels is of interest: here enalaprilat inhibited the response to AI, to the same degree as combination treatment in human vessels. There was a slight reduction in the maximum response in the presence of a chymase inhibitor, with no change in the EC50 or threshold. This result is difficult to interpret and may be artefactual. The broad serine protease inhibitor, trasylol (which inhibits kallikrein) had no effect. These results are consistent with an early study, which investigated the effect of the ACE inhibitor, teprotide, on AI and AII-mediated contraction of isolated rabbit aortic rings.[290] Teprotide completely inhibited contraction to AI but had no effect on AII. Thus a species difference exists in the response to AI and it would appear that the local production of AII in rabbit subcutaneous arteries is ACE-dependent. Moreover these rabbit vessels consistently failed to vasodilate when challenged with BK. Such species differences are clearly of importance in choosing suitable animal models of

CHF, hypertension or chronic renal failure, in which ACE is an important therapeutic target.

It is possible that these in-vitro experiments exaggerate the importance of alternative AII generating pathways. In humans, as in dogs, a dichotomy exists, with chymase appearing to be responsible for the majority of AII generation in homogenised myocardial tissue *in-vitro*, but ACE being the predominant enzyme *in-vivo*. This problem has been noted before and raises the possibility that the importance of non-ACE pathways may exaggerated by in-vitro preparations which expose AI to an enzyme from which it is normally hidden.[204] *In-vivo*, AI will be exposed to both circulating and endothelial ACE and thus may be unavailable for conversion by chymase. Furthermore, in our experiments, reagents were added to the solution in the organ bath and therefore delivered to the vessel abluminally. This may expose adventitial and interstitial chymase to an unphysiologically high concentration of AI.

Although the presence of non-ACE AII generation seems the most plausible explanation for the data described in this chapter, another possibility is that chymase degrades an endogenous vasodilator, just as ACE degrades BK. Inhibition of ACE and chymase may thus appear to result in inhibition of the contractile response to AI due to the potentiation of counter-regulatory vasodilators.

Why then is redundancy observed in the final limb of the AII generating pathway? One possibility is that that AII generated locally by

non-ACE pathways has a different role from AII generated by circulating and endothelial ACE. AII is known to regulate cellular hypertrophy and hyperplasia and contribute to vascular remodelling.[291] Thus, the contraction of resistance arteries to AII, generated by chymase, could be an epiphenomenon. *In-vivo* AII, generated by chymase, may have a trophic role. One model of the possible trophic role of vascular AII is the balloon-injured vessel. It has been shown that ACEi reduce restenosis in rodent models of balloon angioplasty.[292] However this does not appear to be the case in larger animals, such as the dog.[212] A recent study confirmed that cilazapril did not prevent restenosis after coronary angioplasty in humans.[293] In contrast inhibition of the AT1R does reduce intimal hyperplasia after balloon injury in dogs.[215] It therefore seems reasonable to speculate that the failure of ACEi to reduce restensosis after vascular injury in large animals and man is due to the continuing generation of AII through the action of chymase. The potential importance of the trophic actions of AII and chymase has been emphasised by a recent study of chymase expression in the human atherosclerotic aorta. This study compared chymase expression (identified by immunohistochemistry) and AII-forming activity in normal, atherosclerotic and aneurysmal aortae from human subjects obtained either at autopsy, or during vascular surgery.[294] AII formation, largely due to chymase, was greater in homogenates from diseased arteries compared to controls and there was a slight increase in the density of chymase-positive mast cells. A further study investigated chymase

and ACE-dependent AII formation in human internal thoracic arteries and showed a significant correlation between chymase expression and serum total and LDL cholesterol.[295] Thus hypercholesterolaemia and atherosclerosis may be associated with greater non-ACE AII formation.

The relevance of non-ACE AII generation in-vivo is difficult to test since there are no specific chymase inhibitors licensed for this application. However, studies have shown that the exercise-induced rise in AII levels measured in the veins draining the human leg could be inhibited by nafamostat, a serine protease inhibitor, but not captopril.[296, 297] In ischaemic conditions AII generation may be mediated by a serine protease - and nafamostat has also shown to increase lower limb blood flow and exercise capacity in patients with peripheral vascular disease.[298]

A number of approaches could be used to resolve these questions. In the technique of perfusion myography vessels are cannulated at both ends and then perfused at a controlled pressure, allowing a distinction to be made between drugs applied luminally or abluminally.[299] Additionally, in-vivo studies using forearm occlusion plethysmography to study the effects of ACEi on the contraction to AI would give an indication of global vascular bed AI - AII conversion, but would not give information about specific parts of the vascular tree and are limited by the absence of specific chymase inhibitors licensed for use in man. A further possibility is the use of AI-isopeptides, which will liberate AII by the action of chymase, but not ACE (or vice-versa).[206] $Pro^{11}$ $D\text{-}Ala^{12}$ AI is created by adding a terminal

dipeptide, Pro-DAla, to the COOH terminus of native AI. This peptide is resistant to ACE, due to the presence of Proline at the penultimate position, and to carboxy-peptidases, due to the terminal D-Alanine. It is, however, cleaved by chymase to liberate AII and has been shown to induce a pressor response in the conscious baboon. A similar peptide, $Pro^{10}$-AI has been described that is resistant to chymase, but sensitive to ACE.[300] It may thus be possible to use specific isopeptides to dissect further the relative contributions of ACE and chymase to AII generation in the human vasculature.

The techniques of molecular biology could also be used in a complementary approach to investigate the regulation of ACE and chymase expression in vascular and other tissue. A pathophysiological role of chymase would be made more likely if increased expression of this enzyme could be demonstrated in diseased tissue (such as atherosclerotic arteries) and after ACE-inhibition. Finally, the possibility of potentiation of endogenous vasodilators by chymase can be tested in two ways; firstly by investigating the effect of chymase (and ACE) inhibition on vasoconstrictors other than AI, and secondly by studying the response to a panel of known vasodilators in the presence of chymostatin and/or enalaprilat.

In summary, our data indicates that AII generation in human subcutaneous arteries is mediated by a dual enzymatic pathway and provides a strong basis for further investigation of these mechanisms and for novel therapeutic strategies in order to facilitate blockade of the RAS in man.

# Chapter 4

# Responses to angiotensin I in arteries from normal human subjects: the role of the ACE I/D polymorphism

## 4.1 Introduction

In section 1.3 I discussed the associations between polymorphisms in genes encoding the RAS and cardiovascular disease. One recurring theme in that discussion was the relative paucity of studies demonstrating an intermediate phenotype for these polymorphisms. Ueda et al showed that the pressor response to infused AI was greater in subjects who were homozygous for the D allele of the ACE gene, compared to I homozygotes.[144] These data suggested that an intermediate phenotype for the ACE I/D polymorphism is greater AII generation associated with the D allele. However this result has bot been confirmed by other recent studies.[147, 148]

In this chapter I describe the responses to AI in resistance arteries from normal human subjects, analysed according to their ACE genotype. The aim of this analysis was to test the hypothesis that possession of the D allele of the ACE gene would result in a greater contractile response of human resistance arteries to AI due to greater conversion of AI to AII.

## 4.2 Volunteers and genotypes

The data reported in this chapter pertains to the same cohort of normal volunteers described in Chapter 3. Thus, patient selection and clinical characteristics are the same. In total 47 patients were screened for entry into the study. DNA was available for 40 subjects who were therefore genotyped

for the ACE I/D polymorphism, using the techniques described in appendix 1 to methods. The numbers of subjects with each genotype are given in Table 4.1.

Table 4.1: ACE Genotypes (all subjects)

| Genotype | **II** | **ID** | **DD** |
|---|---|---|---|
| Number of Subjects | 12 | 18 | 10 |

As described in methods, the initial aim of this project was to study responses to AI in subjects homozygous for the ACE genotype. Initial experiments therefore excluded heterozygotes. In some subjects who underwent gluteal biopsy no arteries could be identified and thus do not form part of this analysis; a further group of patients were excluded from analysis because of poor artery responses, according to the criteria described in methods (these were vessels that either failed to contract to KPSS and NA, or failed to vasodilate to ACh, during the standard activation). As the project proceeded the number of biopsies that failed to yield usable arteries fell, due to improved operator technique. As the number of homozygotes recruited was fairly small, it was decided, after genotyping the first batch, to include heterozygous subjects in the study. For these reasons the proportion of arteries studied from patients with each genotype was uneven.

The numbers of patients with each genotype, for whom data regarding AI-mediated contraction are available, are shown in table 4.2. Clinical data for these patients is also shown (these data are obviously a subset of the clinical data given in table 3.2). The two groups were well matched for all parameters – though there was a trend to older subjects in the II homozygous group, this was non-significant.

Table 4.2: ACE Genotype and Clinical Characteristics of Patients Studied

| | **ACE Genotype**<br><br>All data mean (±SD) | |
|---|---|---|
| | **ID** | **II** |
| Number | 11 | 4 |
| Age | 27.59 ( 6.36) | 46.25 (13.00) |
| Creatinine | 92.09 (7.67) | 90.00 (9.54) |
| BP | 128.8/74.4<br><br>(12.7/11.3) | 131.0/81.5<br><br>(3.5/5.0) |
| Glucose | 4.91 (0.54) | 4.67 (0.58) |
| Cholesterol | 4.88 (0.83) | 5.00 (1.41) |
| Haemoglobin | 14.86 (1.07) | 15.67 (1.15) |

## 4.3 Responses to Angiotensin I According to Genotype

The lack of DD homozygotes limited the analysis of responses by genotype to a comparison of heterozygotes with subjects homozygous at the I allele. Concentration-response curves to AI were obtained in 11 heterozygotes and 4 II homozygotes. The effect of enalaprilat 1μM was studied in a further 8 arteries from heterozygotes, but only 2 vessels were available from II homozygotes. Figure 4.1 shows the response to AI in subjects with each genotype. In heterozygotes the maximum response to AI (normalised to the response to KPSS) appeared to be greater than the response in homozygotes [106.28 (71.57) vs. 68.81 (36.51) %]. However, this difference did not achieve significance ($p=0.382$ by unpaired t-test). There was no difference in the EC50 for AI in ID compared to II [$1.21 \times 10^{-8}$ vs. $1.10 \times 10^{-8}$M, $p=0.919$). Figure 4.2 shows the response to AI in the presence of enalaprilat in heterozygotes and homozygotes. The maximum response in arteries from ID patients in the presence of enalaprilat was 82.47 (28.97)%, and the EC50 was $2.35 \times 10^{-8}$ M. In heterozygotes enalaprilat did not significantly reduce the maximum response ($p=0.102$) or alter the EC50 ($p=0.664$). There were insufficient arteries from II subjects treated with enalaprilat for these data to be analysed.

Figure 4.1: Response to AI in the absence of inhibitors: effect of genotype

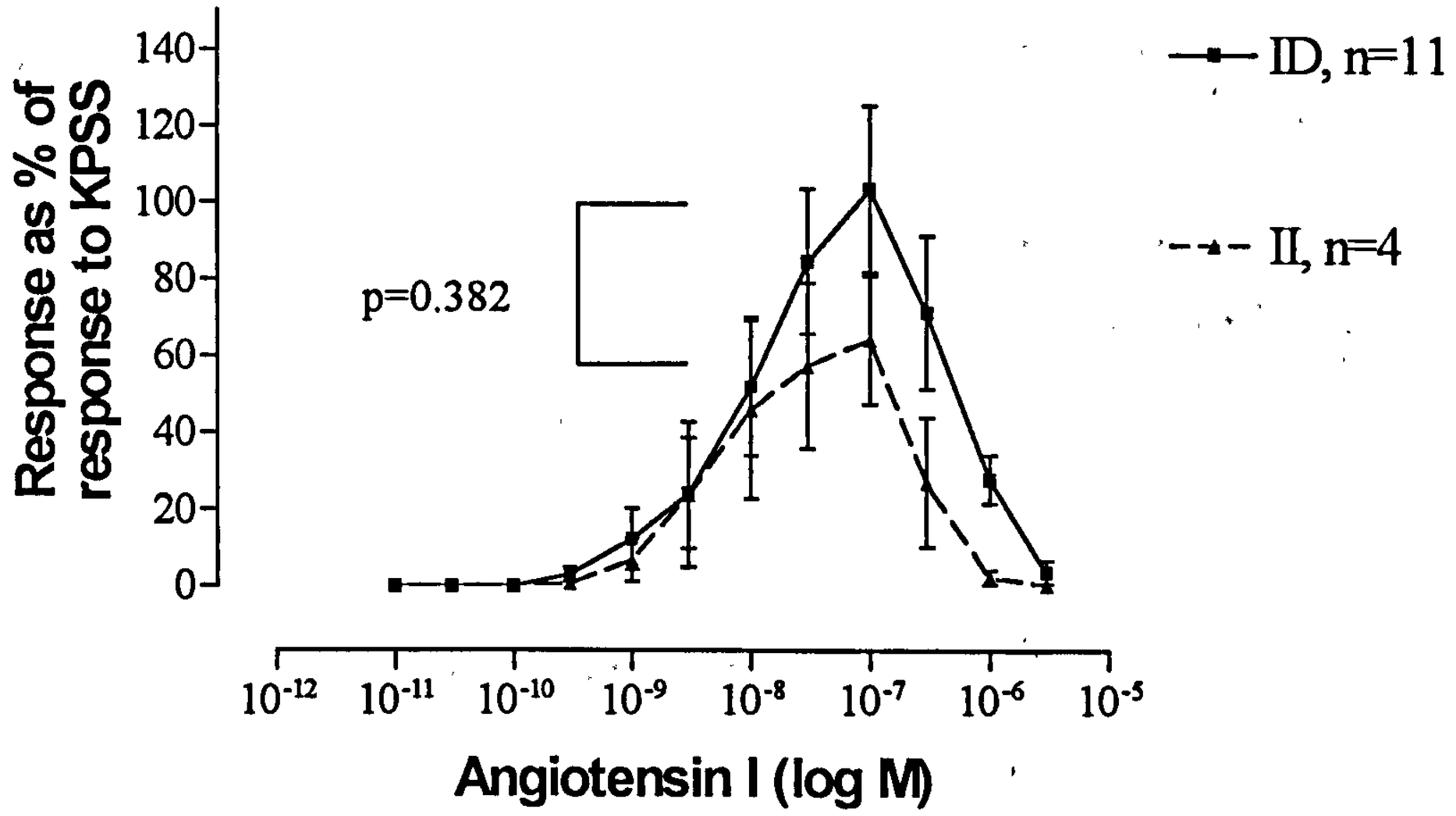



Figure 4.1 shows concentration-response curves to AI in subjects either heterozygous or homozygous at the I/D locus. There was no significant difference in either the maximum response or the EC50.

Figure 4.2: Response to AI: effect of genotype on response to enalaprilat

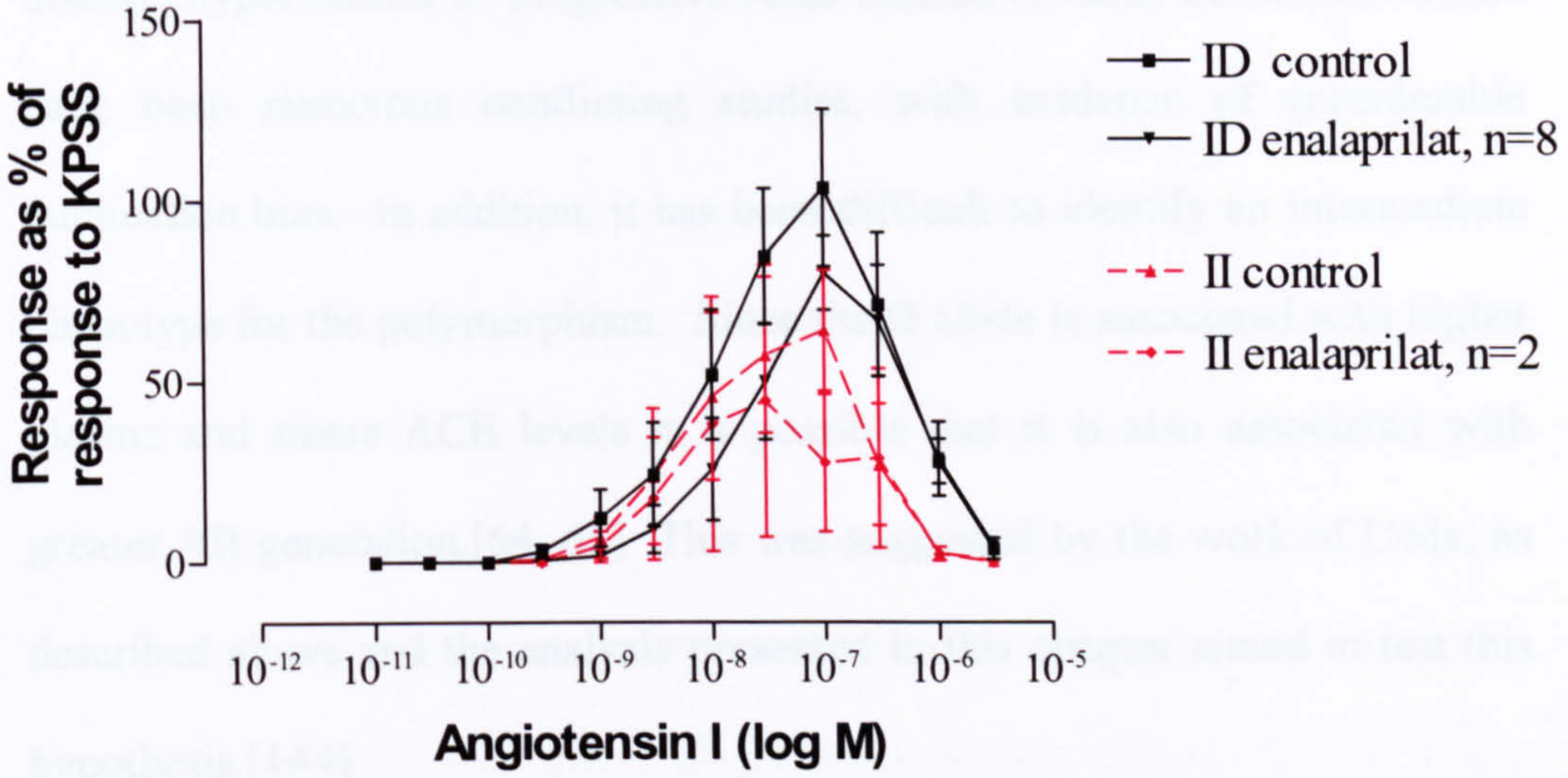



Figure 4.2 shows response to AI in the presence and absence of enalaprilat in subjects with the ID and II genotypes. Enalaprilat did not significantly alter either the maximum response or EC50 to AI in ID heterozygotes.

## 4.4 Discussion

Whether the ACE I/D polymorphism is associated with cardiovascular disease, hypertension or progressive renal disease remains debatable. There have been numerous conflicting studies, with evidence of considerable publication bias. In addition, it has been difficult to identify an intermediate phenotype for the polymorphism. Since the D allele is associated with higher plasma and tissue ACE levels it is possible that it is also associated with greater AII generation.[64, 65] This was suggested by the work of Ueda, as described above and the analysis presented in this chapter aimed to test this hypothesis.[144]

Possession of the D allele was not associated with a greater response to AI. Though there may have been a trend to a higher maximum response to AI in arteries from ID heterozygotes, it did not reach significance. Similarly, there was no difference in the sensitivity to AI according to genotype. Thus, these data do not support the hypothesis that the D allele is associated with greater AII generation at a tissue level.

The data presented above is, however, very limited. There were no data describing responses in DD homozygotes. Thus, it is possible that the trend towards an increased response to AI, associated with possession of the D allele, would have reached significance if DD homozygotes had been compared to II homozygotes. The effect of enalaprilat would also have been interesting in this regard, since it might be expected that the higher ACE

levels, associated with the D allele, would render subjects resistant to ACE-inhibition. It was therefore disappointing that so little data on the effect of enalaprilat was obtained.

In chapter 3 I suggested that a dual pathway exists for AII generation in human resistance arteries, mediated by ACE and chymase. The lack of inhibition seen with enalaprilat suggests that chymase is able to compensate fully when ACE is blocked and the same situation arises when chymase in blocked. Neither ACE nor chymase can therefore be seen as rate-limiting for AII generation. If this is so, it is difficult to understand how variation in the ACE gene could result in detectable differences in AII generation. Thus, the failure to demonstrate an effect of genotype, though difficult to interpret in such a small study, is compatible with other data presented in this thesis. Studies of the intermediate phenotype of the I/D polymorphism may be confounded by the presence of non-ACE AII generation and this possibility needs to be considered when designing such experiments. An elegant solution to this problem was demonstrated by Steeds et al, who used an ACE specific AI-isopeptide, $Pro^{10}$-AI (which is not hydrolysed by chymase) to study the role of the ACE I/D polymorphism on vascular reactivity in resistance arteries from 70 normal human subjects.[300] They could show no difference in the response to the isopeptide according to ACE genotype.

In summary, the data described in this chapter are not consistent with any discernible effect of ACE genotype on AII generation in human resistance arteries. While it is possible that this polymorphism is associated

with phenotypic variation, a much larger study would be required to demonstrate it. Studies using AII levels, or generation, should take into account the possibility of non-ACE pathways.

# Chapter 5

# Studies of resistance arteries in patients with Chronic Heart Failure and Coronary Heart Disease

## 5.1 Introduction

CHF is associated with neurohormonal dysfunction and activation of the RAS.[301] It is now felt that the activation of the RAS (and other neurohormonal systems) is a maladaptive response that contributes to the progression of CHF through a variety of mechanisms, not restricted to the heart itself.[165] In addition to contributing to abnormal central and peripheral haemodynamics, neurohormonal activation contributes to cardiac remodelling, with fibrosis and myocyte apoptosis.[302] Furthermore, endothelial dysfunction and peripheral muscle abnormalities appear and both renal and pulmonary homeostatic mechanisms are affected. Thus, while neurohormonal activation may be beneficial initially, it then contributes to the progression of the syndrome. The logical conclusion from this hypothesis is that treatments that reduce neurohormonal activation may not only improve symptoms, but also slow the progression of the syndrome. This hypothesis has been tested in a number of large clinical trials which have uniformly shown that ACEi reduce mortality and improve symptoms in CHF.[163]

While ACEi are undoubtedly beneficial in CHF, the mortality from this syndrome remains depressingly high. Thus, in CONSENSUS I mortality at one year was 36% and in Ve HeFT II the 4 year cumulative mortality was 41%, despite enalapril therapy.[169, 303] One explanation for the poor prognosis in CHF, despite treatment with ACEi, is that the suppression of the RAS due to these drugs is incomplete. Thus, progressive left ventricular

dysfunction has been associated with the failure to suppress plasma AII levels with ACEi.[170] In section 1.4.1 I discussed the evidence that ACEi do not suppress plasma AII fully and, in section 1.4.2, I suggested that this phenomenon may be due to both the presence of non-ACE AII generating pathways and the pharmacokinetic properties of ACEi.

In previous chapters I have described responses to AI and BK in normal human subjects and suggested that there may be a dual pathway for AII generation. This pathway appears to consist of ACE and chymase, as evinced by the inhibition of the contractile response to AI in the presence of both enalaprilat and chymostatin. However, while inhibition of AII generation seems the most plausible explanation of the results described in previous chapters, another possibility is that chymase may degrade endogenous vasodilators, just as ACE degrades BK. The potential significance of non-ACE AII generation in human resistance arteries is greatest in patients treated with ACEi. In such people continuing AII generation may contribute to disease progression and it is possible that non-ACE pathways are actually upregulated as a result of treatment.

The studies described in this chapter were therefore designed to investigate the possibility of non-ACE AII generation in patients treated with ACEi. The subjects chosen were patients with CHF and they were compared with a group of ACEi-naïve patients who had documented CHD. Thus the experimental group differed from control in only 2 respects: treatment with ACEi and the presence of left ventricular systolic dysfunction. While the

basic design of the experiments was similar to those previously described, they were more comprehensive. Thus, the effects of enalaprilat and chymostatin on the responses to the vasoconstrictors, AII and NE, and the vasodilators, ACh and BK were also studied, for the reasons given above.

## 5.2 Patient selection

For all clinical studies patients with diabetes mellitus or renal failure (creatinine > 200 μmol/l) were excluded. Written informed consent was obtained from each patient and all protocols were approved by the Hospital Ethics Committee. Two groups of patients were studied; patients with chronic heart failure (CHF), established on ACE inhibitor therapy, and patients with coronary heart disease (CHD) who were not taking such agents. Male and female patients were invited to take part in this study.

On the morning of study, after 15 minutes supine rest, blood pressure was recorded in the right arm, with a standard mercury sphygmomanometer (Accoson UK). Blood was then drawn from a cannula in an ante-cubital vein for estimation of renal function, serum cholesterol, serum ACE and plasma neurohormones. Finally patients underwent a gluteal biopsy, as described in chapter 2.

Patients with CHF

Ambulatory patients with New York Heart association II/III CHF, who were attending the outpatient clinic, were studied. All were on long term (>3 month) ACE inhibitor and diuretic treatment. The aetiology of CHF was coronary heart disease in all cases and each patient had a left ventricular ejection fraction of less than 40%, as assessed by transthoracic echocardiography (Accuson 128XT/10C, Simpson's biplane method). All patients had suffered a previous myocardial infarction. The patient's usual medication (including ACE inhibitor therapy) was taken on the study morning.

Patients with CHD

These were patients with chronic stable angina attending outpatient clinics. All patients had preserved left ventricular function, determined as an echocardiographic left ventricular ejection fraction of 40 per cent or more (Simpson's biplane), and none were treated with an ACE inhibitor.

## 5.3 Experimental protocols

Immediately after the gluteal biospy had been taken, resistance arteries were dissected free of subcutaneous fat. Any blood remaining in the lumen was extruded by gentle pressure with watch-makers forceps and the vessels were cleaned of adherent connective tissue. They were then stored in

Kreb's solution at 4°C overnight. Experiments were performed the next day and arteries were mounted in a four-channel myograph, normalised and subjected to the same standard activation procedure as described in chapter 2. Experiments were therefore performed 24 hrs after biopsy and, in the case of patients with CHF, 24hrs after their last dose of ACEi.

In one set of experiments the responses of the arteries to AI was then investigated following the same protocol as described in chapter 3. Thus, the arteries were incubated for 30 minutes with either no inhibitor (vessel 1, control), enalaprilat 1μM (vessel 2), chymostatin 10μM (vessel 3) or both enalaprilat 1μM and chymostatin 10μM (vessel 4). A cumulative concentration response curve for AI was then performed.

In a second set of experiments the response of resistance arteries to AII, from 0.01 nM to 0.3 μM, was investigated in the presence of the same inhibitors. Unfortunately tachyphylaxis to AI and AII precluded performing curves to each agonist in the same vessel.

After exposure to AI or AII vessels were washed with Kreb's solution to re-establish baseline and inhibitors were again added, maintaining the same relationship between vessel and inhibitor. The responses of the arteries to norepinephrine (NE), bradykinin (BK) and acetylcholine (ACh) were then investigated. Thus, after 30 minutes a CRC was constructed to NE from 1 nM to 30 μM in log molar increments. Thereafter the vessels were again washed with Kreb's solution to re-establish baseline, inhibitors were reapplied and, after a further 30 minutes a CRC to BK from 0.1 nM to 3 μM

was performed. Finally a CRC to ACh was performed from 1 nM to 30 μM in the same manner. Pre-contraction for the CRCs to ACh and BK was achieved with NE 10μM and vasodilators were added once plateau was reached (defined, arbitrarily as a maintained contraction, stable at the same level, for 2 minutes). Experimental protocols are summarised in table 5.1. The order of experimental protocols was held constant throughout this study.

Table 5.1 Experimental protocol for resistance arteries from patients with CHF and CHD

| | Vessel | | | |
|---|---|---|---|---|
| | **1** | **2** | **3** | **4** |
| **"Standard Activation"** | KPSS<br>KPSS<br>NE 10μM<br>ACh $3\times10^{-6}$μM | | | |
| **Incubation 30 mins** | Control Vehicle | Enalaprilat 1μM | Chymostatin 10μM | Enalaprilat +Chymostatin (same concentrations ) |
| **CRC to AI** | AI concentrations0.01nM, 0.03nM, 0.1nM, 0.3nM etc … to 3μM | | | |
| **Wash to baseline** | | | | |
| **Incubate 30 mins** | Control Vehicle | Enalaprilat 1μM | Chymostatin 10μM | Enalaprilat 1μM + Chymostatin 10μM |
| **CRC to NE** | NE 1nM, 3nM, 10nM etc … to 30μM | | | |
| **Wash to baseline, reapply inhibitors maintaining same relationship to vessel Incubate 30 mins, Pre-constrict with NE 10 μM** | | | | |
| **CRC to BK** | BK 0.1nM, 1nM, 10nM etc… to 3μM | | | |
| **Wash to baseline, reapply inhibitors maintaining same relationship to vessel Incubate 30 mins, Pre-constrict with NE 10 μM** | | | | |
| **CRC to ACh** | ACh 0.1nM, 1nM, 10nM etc… to 3μM | | | |

A similar set of experiments was performed using AII instead of AI. The concentration range for AII was 0.01 nM to 0.3 μM.

## 5.4 Patient Characteristics

Gluteal biopsies were performed in 47 patients, 21 of whom had CHF, the rest CHD. I was unable to obtain arteries, or was forced to discard arteries according to my pre-defined criteria, from 11 patients. Resistance arteries were therefore studied from 18 patients with CHF and 18 patients with CHD. Clinical characteristics of these patients are shown in table 5.2. In this table patients are subdivided according to the primary experiment performed – either a CRC to AI or to AII. As discussed in methods and further in Chapter 3, tachyphylaxis to AI precluded repeated CRCs to this peptide, or to AII, in the same artery. Responses to AI and AII were therefore studied in separate patients.

The patients studied had mild-moderate CHF according to NYHA functional class, but all had reduced EF by transthoracic echocardiography. The aetiology of CHF was ischaemic in almost all cases - indeed 17/18 patients with CHF had suffered a myocardial infarction in the past and 8/18 had undergone previous coronary bypass surgery. All patients with CHF, but none with CHD, were receiving treatment with an ACE-inhibitor. Baseline characteristics of patients with CHD and CHF differed in only two respects (except their previously defined differences in left ventricular function and ACE inhibitor and diuretic prescriptions). Firstly, CHF patients were on treatment with digoxin more often than CHD patients and more CHD patients than CHF patients were on treatment with beta-blockers. Secondly, AI

concentration curves were constructed in CHF patients who were older than their CHD counterparts (p=0.004).

All patients who attended for the study had blood samples drawn for estimation of plasma renin, AII and aldosterone and serum ACE. These values are shown in table 5.3 (a, b). Values for individual patients are shown to highlight the wide variation in results. The mean plasma AII value (mean ± SD) in patients with CHF, who were receiving therapy with ACEi, was 16.41 (14.60) pg/ml, which is greater than the upper limit of the standard reference range. However some patients suppressed their AII to undetectable levels. In patients with CHF the mean plasma aldosterone was 14.33 (8.54) ng/100ml and the mean plasma renin was 70.85 (56.12) μU/ml, but again there was a wide variation in levels. The mean plasma AII in patients with CHD was 6.56 (5.34) pg/ml, which is within the reference range, though again there was wide variation in levels. Plasma AII was significantly higher in patients with CHF despite ACE-inhibition (p=0.045, by 2-tailed t-test). Similarly plasma renin, at 11.0 (13.32) μU/ml, was significantly lower in patients with CHD (p=0.003, by 2-tailed t-test). There was no difference in plasma aldosterone (p=0.93).

Plasma ACE levels are also shown in tables 5.3 (a) and (b). Of the patients with CHF 9/13, for whom data is available, had suppressed their plasma ACE to undetectable levels. However, suppression of serum ACE did not appear to associate with suppression of plasma AII. Figure 5.1 shows plasma AII plotted against serum ACE in those patients with CHF and CHD

for whom matched values were available. There was no correlation between serum ACE and AII in either patient group. In patients with CHD plasma ACE was not suppressed, and was significantly higher than in patients with CHF (p=0.026).

Table 5.2: Clinical characteristics of patients with CHF and CHD

| **Concentration response curves** | angiotensin I | | angiotensin II | |
|---|---|---|---|---|
| | CHF | CHD | CHF | CHD |
| **Number of patients** | 10 | 10 | 8 | 8 |
| **Sex (M/F)** | 8/2 | 9/1 | 6/2 | 7/1 |
| **Age** | 70.1 (+/-6.1)) | 60.3 (+/-7.4) | 70.5 (+/-11.1) | 66.2 (+/-6.3) |
| **Previous MI** | 9 | 2 | 8 | 2 |
| **Previous CABG** | 6 | 1 | 2 | 1 |
| **NYHA functional class** | 5 III; 5 II | NA | 4 II; 4III | NA |
| **Hypertension** | 1 | 1 | 2 | 3 |
| **NIDDM** | 0 | 0 | 0 | 0 |
| **Ejection fraction** | 24.5 (+/-6.1) | 59.3 (+/-8.4) | 21.4 (+/-8.6) | 57.9 (+/-9.3) |
| **Drug therapy** | | | | |
| ACE inhibitor | 10 | 0 | 8 | 0 |
| diuretic | 10 | 0 | 8 | 0 |
| digoxin | 3 | 0 | 2 | 0 |
| calcium channel blocker | 0 | 2 | 1 | 3 |
| nitrate | 4 | 4 | 3 | 4 |
| beta blocker | 1 | 9 | 2 | 7 |
| HMG Co A reductase inhibitor | 5 | 8 | 4 | 5 |
| aspirin | 8 | 10 | 4 | 8 |
| **Systolic BP** | 125 (+/-18) | 149.2 (+/-9.6) | 131 (+/-19) | 151 (+/-17) |
| **Diastolic BP** | 70 (+/-9) | 82.6 (+/-0.9) | 80 (+/-8) | 79.5 (+/-2.1) |
| **Glucose** | 5.5 (+/-1.8) | 5.9 (+/-1.7) | 5.5 (+/-1.0) | 5.3 (+/-0.9) |
| **Cholesterol** | 5.0 (+/-0.9) | 4.7 (+/-0.8) | 4.7 (+/-1.1) | 5.0 (+/-1.1) |
| **Creatinine** | 116 (+/-17) | 89 (+/-13) | 100 (+/-28) | 94 (+/-12) |

CABG – coronary artery bypass grafting; NYHA – New York Heart Association; MI – myocardial infarction; CHD – coronary heart disease; CHF – chronic heart failure; ACE – angiotensin converting enzyme; HMG CoA – 3-hydroxy – 3 – methylglutaryl coenzyme A; NIDDM – non insulin dependent diabetes mellitus (all values mean ± SD).

Table 5.3: Plasma renin, AII, aldosterone and ACE from patients with CHF and CHD

a) Patients with CHF

| Patients with CHF | | | | |
|---|---|---|---|---|
| Patient ID | Renin (μU/ml) | AII (pg/ml) | Aldo (ng/100ml) | ACE U/l |
| 3 | 21 | 2.8 | 7 | <10 |
| 4 | 59 | 11.3 | 11 | 31 |
| 7 | 129 | 30.3 | 32 | 30 |
| 8 | | | 6 | <10 |
| 9 | 36 | 4.3 | 11 | 58 |
| 10 | 212 | <1 | 14 | <10 |
| 14 | 49 | <1 | 10 | <10 |
| 18 | 143 | 22 | 36 | 51 |
| 33 | 54 | 22.4 | 12 | <10 |
| 34 | 74 | 21.4 | 19 | <10 |
| 35 | | | 13 | <10 |
| 36 | 5 | 52.4 | 7 | |
| 37 | 76 | 16.2 | 11 | <10 |
| 40 | 18 | 3 | 18 | |
| 41 | 45 | 27.2 | 8 | <10 |
| Total | Mean (SD) | Mean (SD) | (Mean (SD) | Mean (SD) |
| 15 | 70.85 (56.12) | 16.41 (14.60) | 14.33 (8.34) | 13.07 (20.76) |

Neurohormone levels for patients with CHF, whose vessels were studied. All were thought to be taking an ACE inhibitor. Means were calculated taking values below detection threshold (i.e. '<1', '<10') to equal zero. Standard reference ranges are: renin (5-50 μU/ml), AII (2-12 pg/ml), aldosterone (<25ng/100ml) and ACE (<88U/l).

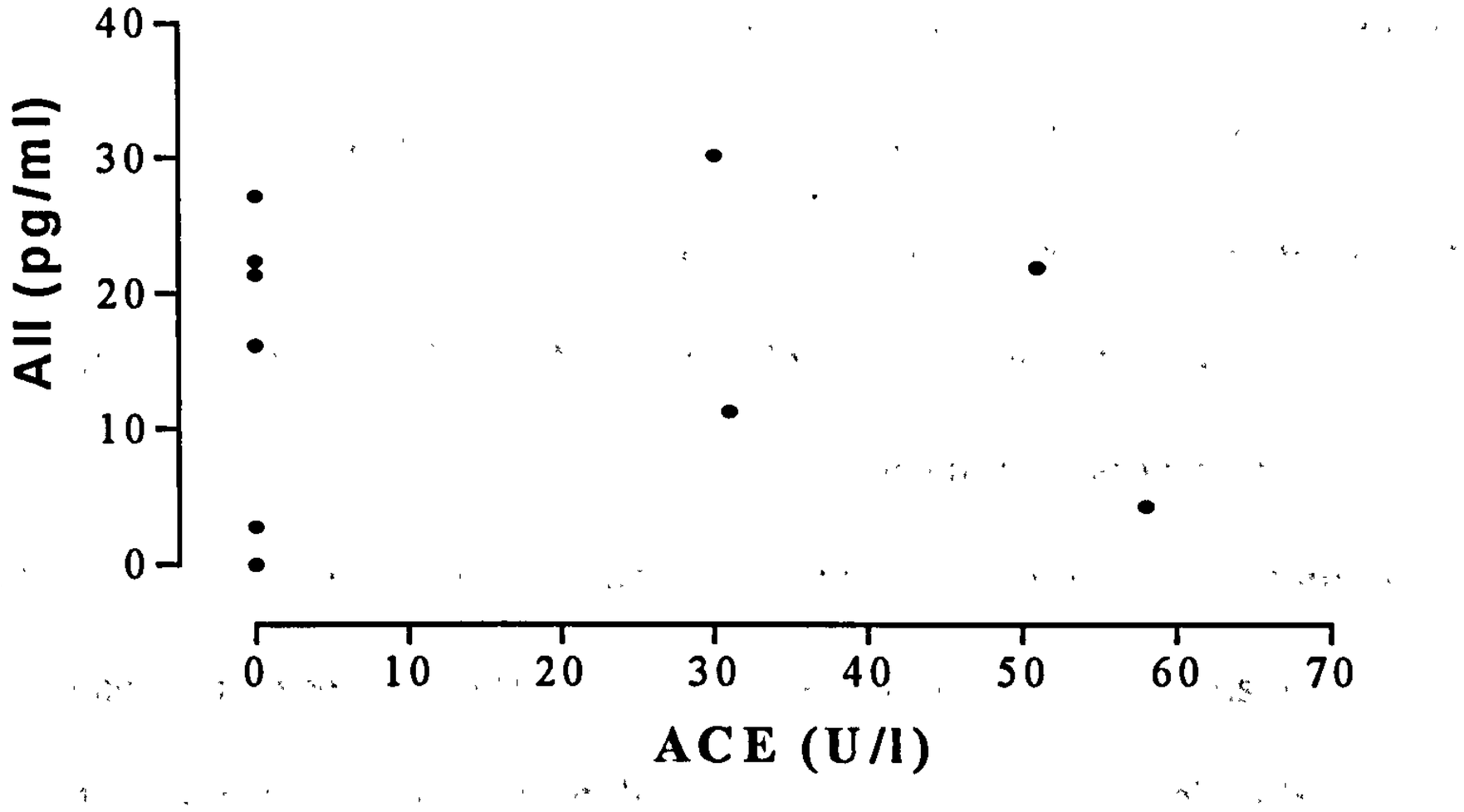
b) Patients with CHD

Patients with CHD

| Patient ID | Renin (μU/ml) | AII (pg/ml) | Aldo (ng/100ml) | ACE U/l |
|---|---|---|---|---|
| 2 | | | | 19 |
| 6 | 52 | <1 | 38 | 13 |
| 13 | 21 | 11.5 | 12 | 34 |
| 17 | 4 | 1.2 | 3 | 39 |
| 21 | 4 | 6 | 8 | 10 |
| 22 | 5 | <1 | 7 | 27 |
| 23 | 3 | 4 | 77 | 32 |
| 24 | 11 | 16.3 | 11 | 51 |
| 25 | 5 | 5 | 9 | 51 |
| 26 | 5 | 9.5 | 0 | 33 |
| 29 | 18 | | 7 | 38 |
| 30 | 16 | 15.8 | 6 | 10 |
| 31 | | | 10 | 32 |
| 45 | 3 | 5 | 4 | |
| 46 | 5 | 5 | 5 | 28 |
| 47 | 2 | 6 | 11 | 28 |
| Total | Mean (SD) | Mean (SD) | Mean (SD) | Mean (SD) |
| 15 | 11.0 (13.32) | 6.56 (5.34) | 13.87 (19.46) | 29.67 (12.78) |

Figure 5.1: Plasma ACE and AII levels in patients with CHF and CHD

a) Patients with CHF




b) Patients with CHD

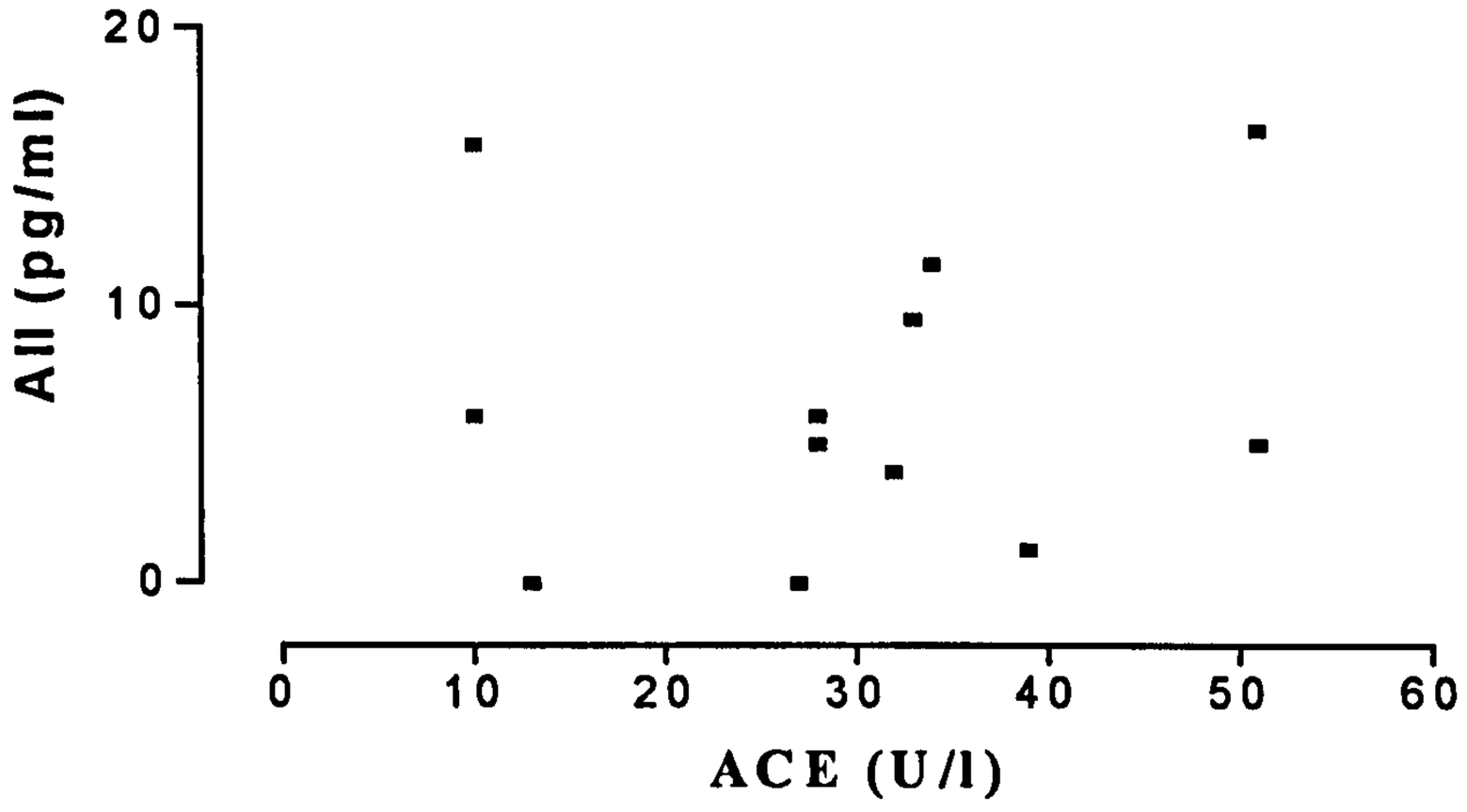



Figure 5.1 shows plasma AII levels plotted against serum ACE. Values below the detection thresholds were taken to be zero.

## 5.5 Characteristics of Arteries Studied

As in the previous study in normal volunteers it was frequently not possible to obtain 4 arteries from biopsies and a considerable number were discarded because of lack of response to KPSS or ACh. Resistance arteries were studied in 4 groups – arteries from patients with CHF or CHD, exposed to AI or AII respectively. The number of vessels studied, together with their sizes and responses to KPSS, NE and ACh, are shown in tables 5.4, 5.5, 5.6 and 5.7. A minimum of 6 arteries in each subgroup was arbitrarily regarded as the minimum necessary to complete a data-set. There were no significant differences between control and experimental groups in terms of vessel diameter, contractile ability to either NE or KPSS, or endothelial-dependent vasodilatation to ACh.

Table 5.4: Vessel diameters and responses to KPSS, NE and ACh in resistance arteries from patients with CHF, exposed to AI

| | Control n=9 | Enalaprilat n=6 | Chymostatin n=6 | Combinatio n n=8 |
|---|---|---|---|---|
| $L_1$ (μm) | 335.96 (68.79) | 330.95 (93.41) | 332.19 (56.23) | 309.98 (65.04) |
| Response to KPSS (kPa) | 18.53 (4.77) | 20.52 (3.75) | 16.08 (7.49) | 16.42 (3.72) |
| Response to NE (kPa) | 19.03 (3.77) | 20.48 (2.28) | 15.25 (6.64) | 17.32 (3.43) |
| Response to ACh (% of pre-contraction to NE) | 86.01 (11.04) | 73.51 (29.82) | 93.03 (6.16) | 84.02 (15.64) |

Table 5.4 shows group responses to KPSS, NE and ACh (means ± SD) in vessels from patients with CHF exposed to AI. There were no significant differences between these parameters for control and each experimental group by unpaired t-test.

Table 5.5: Vessel diameters and responses to KPSS, NE and ACh in resistance arteries from patients with CHD, exposed to AI

| | Control n=7 | Enalaprilat n=6 | Chymostatin n=6 | Combination n=6 |
|---|---|---|---|---|
| $L_1$ (μm) | 281.75 (13.77) | 324.24 (41.70) | 327.00 (47.25) | 294.47 (71.35) |
| Response to KPSS (kPa) | 18.79 (3.97) | 20.03 (5.10) | 16.31 (8.50) | 17.78 (4.74) |
| Response to NE (kPa) | 19.84 (3.93) | 21.31 (4.50) | 16.10 (8.14) | 19.58 (4.62) |
| Response to ACh (% of pre-contraction to NE) | 63.77 (37.83) | 75.28 (21.59) | 62.26 (34.977) | 66.17 (29.17) |

Table 5.5 shows group responses to KPSS, NE and ACh (means ± SD) in vessels from patients with CHD exposed to AI. There were no significant differences between these parameters for control and each experimental group by unpaired t-test.

Table 5.6: Vessel diameters and responses to KPSS, NE and ACh in human resistance arteries from patients with CHF, exposed to AII

| | Control<br>n=7 | Enalaprilat<br>n=8 | Chymostatin<br>n=7 | Combination<br>n=7 |
|---|---|---|---|---|
| $L_1$ (μm) | 278.86<br>(31.88) | 259.60<br>(56.68) | 296.36<br>(42.97) | 283.17<br>(41.91) |
| Response to KPSS (kPa) | 18.63<br>(5.89) | 15.11 (6.23) | 15.16 (4.69) | 17.82 (5.86) |
| Response to NE (kPa) | 20.78<br>(5.73) | 18.84 (7.43) | 18.78 (6.21) | 23.11 (7.48) |
| Response to ACh (% of pre-contraction to NE) | 61.75<br>(34.26) | 54.30<br>(35.10) | 66.59 (31.31) | 70.63 (29.27) |

Table 5.6 shows group responses to KPSS, NE and ACh (means ± SD) in vessels from patients with CHF exposed to AII. There were no significant differences between these parameters for control and each experimental group by unpaired t-test.

Table 5.7: Vessel diameters and responses to KPSS, NE and ACh in resistance arteries from patients with CHD, exposed to AII

| | Control n=6 | Enalaprilat n=6 | Chymostatin n=8 | Combination n=8 |
|---|---|---|---|---|
| $L_1$ (μm) | 371.98 (83.20) | 327.37 (131.05) | 323.32 (86.25) | 326.94 (107.13) |
| Response to KPSS (kPa) | 20.22 (8.11) | 17.92 (10.01) | 18.46 (9.51) | 21.04 (7.96) |
| Response to NE (kPa) | 14.67 (8.41) | 16.16 (9.51) | 17.03 (8.47) | 22.67 (9.28) |
| Response to ACh (% of pre-contraction to NE) | 64.21 (34.14) | 82.70 (21.28) | 69.64 (31.47) | 78.19 (29.09) |

Table 5.7 shows group responses to KPSS, NE and ACh (means ± SD) in vessels from patients with CHF exposed to AII. There were no significant differences between these parameters for control and each experimental group by unpaired t-test.

## 5.6 Response to AI in patients with CHF and CHD

Resistance arteries from patients with CHF and CHD were subjected to the same "warm-up" as described in Chapter 3 for normal volunteers and were discarded according to the same criteria. CRCs to AI were performed according to the same protocol i.e. after co-incubation with either vehicle (vessel 1: control), enalaprilat 1μM (vessel 2), chymostatin 10μM (vessel 3) or both inhibitors together (vessel 4). Dose response curves to AI are shown in Figures 5.2 to 5.6. AI induced a dose-dependent contraction in arteries from patients with CHF and CHD. In the absence of any inhibitor, the threshold (defined as the concentration at which a contraction was first observed) was [mean ±(SD)] 5.03 (3.82) $x10^{-10}$M and 2.76 (3.36) $x10^{-9}$M in arteries from patients with CHF and CHD, respectively (Figure 5.2). Thus arteries from patients with CHF appeared more sensitive to AI than those from patients with CHD, or normal volunteers. The maximum responses (expressed as the % contraction to KPSS) were 69.01 (16.83) and 65.81 (22.41)% in vessels from patients with CHF and CHD, respectively, and occurred at 0.1μM in both. Maximum responses, EC50 and AUC values for all vessels exposed to AI are given in table 5.7.

<u>Effect of ACE and chymase inhibition on responses to AI</u>

As described in chapter 2 (section 2.1.9) the absence of a clear maximum precluded the comparison of curves in terms of either EC50 or

maximum response. Thus, the threshold concentration was used to compare the effect of ACE and chymase inhibition on the responses to AI in arteries from patients with CHD and CHF. Figure 5.3 illustrates the effect of enalaprilat on AI responses. In arteries from patients with CHD the threshold concentration in the presence of enalaprilat was little different to control, at 2.20 (3.85) $x10^{-8}$M ($p>0.05$, control vs. enalaprilat), and there was no significant shift in the dose response curve, or reduction in the maximum response. However, in arteries from patients with CHF incubation with enalaprilat induced a reduction in the maximum response to 38.15 (19.17)% ($p>0.05$, compared to control) and a shift in the dose-response curve to the right. The threshold increased to 2.44 (3.86) $x10^{-8}$M ($p<0.05$ compared to control), and the EC50 increased from $1.38(0.12)\times10^{-8}$M to $5.75\ (0.47)\times10^{-8}$M when arteries were co-incubated with enalaprilat.

Figure 5.4 illustrates the effect of chymostatin on AI-mediated responses. In the presence of chymostatin the threshold concentrations were 0.56 (1.20) $x10^{-8}$M and 1.32 (1.33) $x10^{-9}$M in arteries from patients with CHF and CHD, respectively. Chymostatin did not significantly inhibit the response to AI in arteries from either patient group ($p>0.05$).

Figure 5.5 shows the effect of combining enalaprilat and chymostatin on AI-mediated responses. The threshold concentrations in the presence of both inhibitors were 0.77 (1.07) $x10^{-6}$M and 2.90 (2.70) $x10^{-7}$M in arteries from patients with CHF and CHD, respectively. The combination was therefore associated with a marked inhibition of the response in arteries from

patients with both CHF and CHD ($p<0.001$ in CHF and $p<0.01$ in CHD, respectively). The degree of inhibition was similar to that which was found in normal vessels (see Chapter 3), with a large shift to the right and no clear maximum response. In arteries from patients with CHF the addition of chymostatin to enalaprilat significantly enhanced the degree of inhibition ($p<0.01$, enalaprilat vs. combination). These results are summarised in table 5.8.

In order to be consistent with previous analyses AUC values were also calculated for control and each experimental curve in arteries from patients with CHD and CHF. An Anderson-Darling test suggested that these data were not normally distributed. Hence a Mann Whitney U test was used to compare groups. This showed that in arteries from patients with CHF, enalaprilat and the combination of enalaprilat and chymostatin significantly inhibited the response to AI ($p=0.0039$, $p=0.001$, respectively). The addition of chymostatin to enalaprilat enhanced the degree of inhibition ($p=0.038$). In contrast, in CHD, enalaprilat did not inhibit the response to AI and the combination just failed to reach significance ($p=0.054$).

Figure 5.2: Response to AI in arteries from patients with CHF and CHD

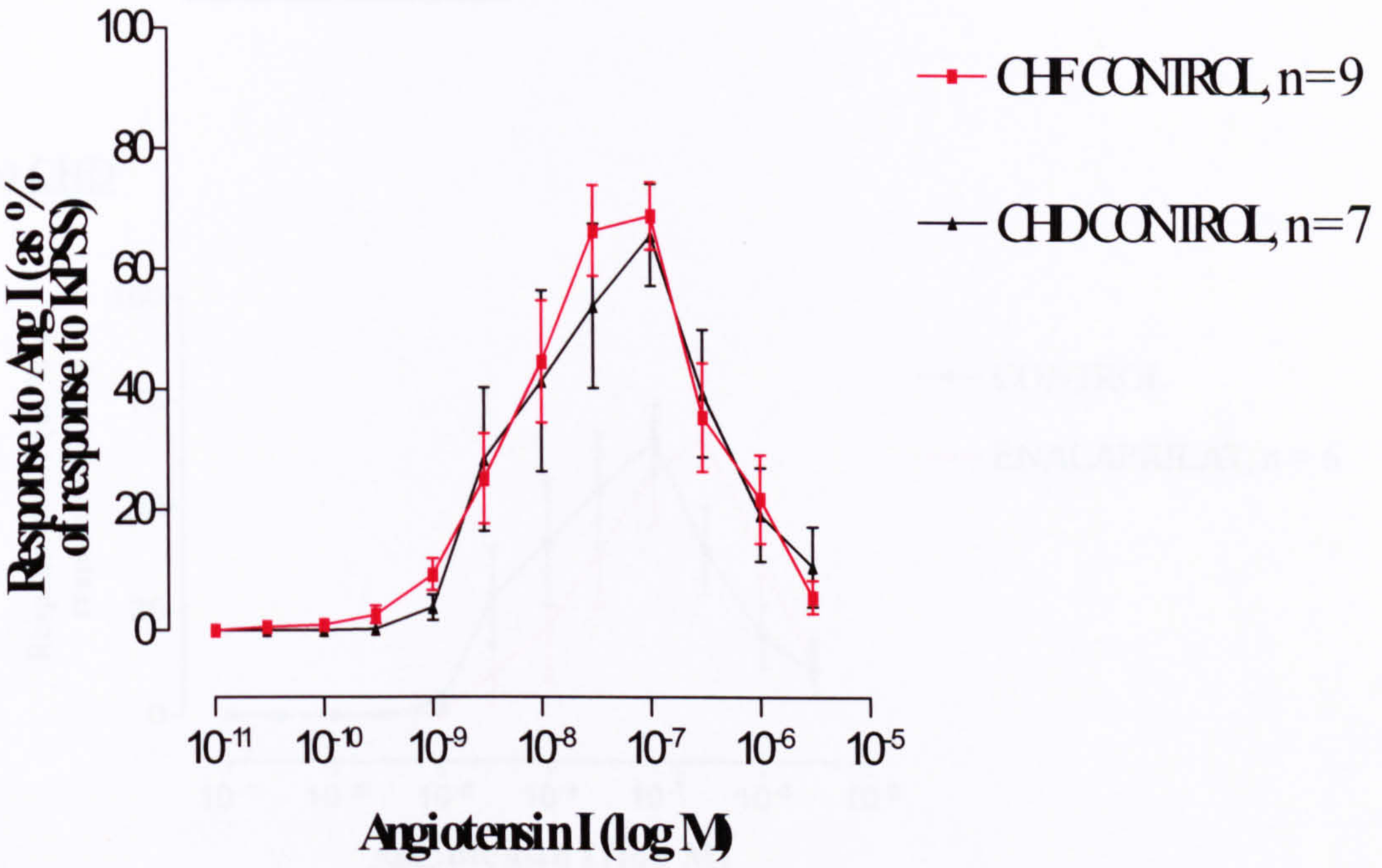
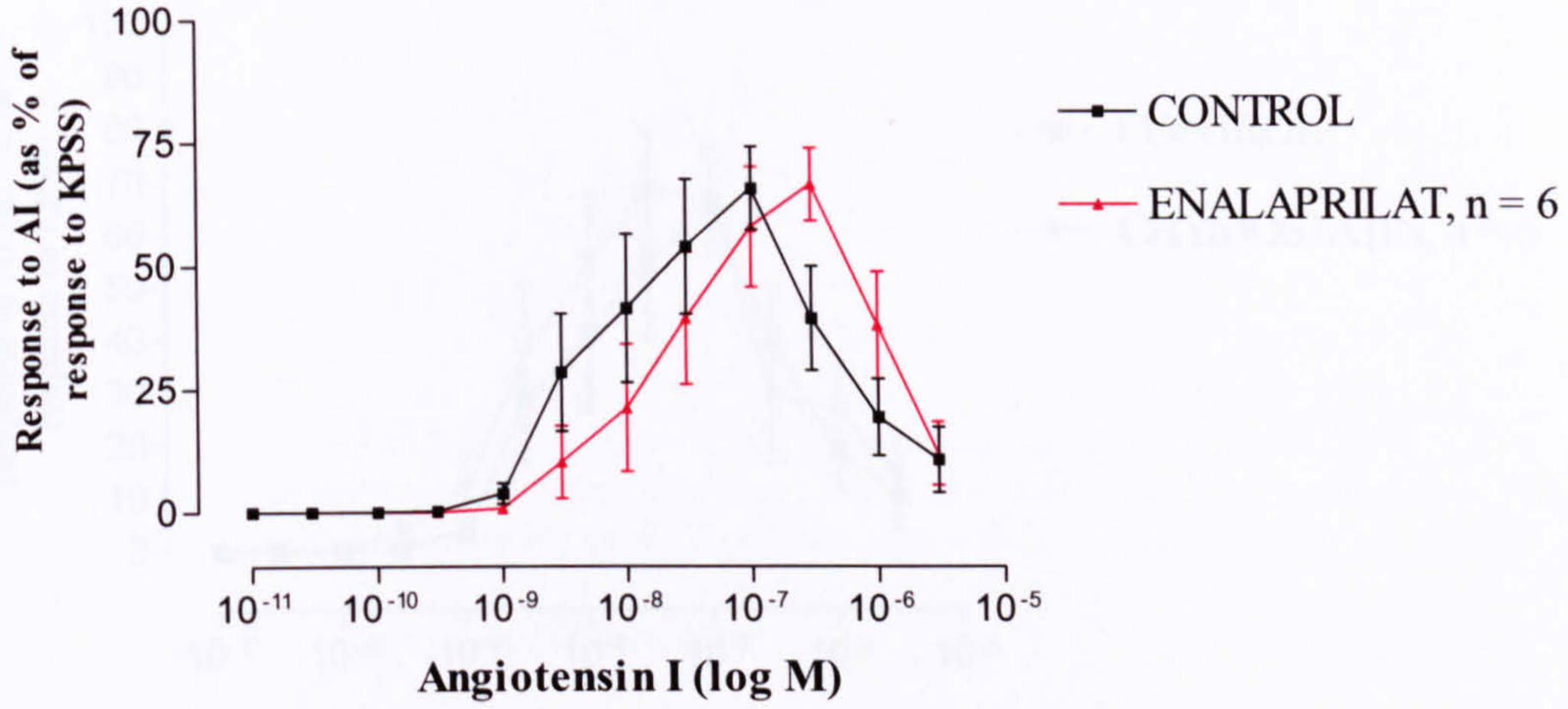



Figure 5.2 shows cumulative concentration-response curves to AI in arteries from patients with CHF and CHD. There was no difference between the curves in terms of maximum response, EC50 or AUC.

Figure 5.3: Response to AI in arteries from patients with CHF and CHD: effect of enalaprilat

a) CHD




b) CHF

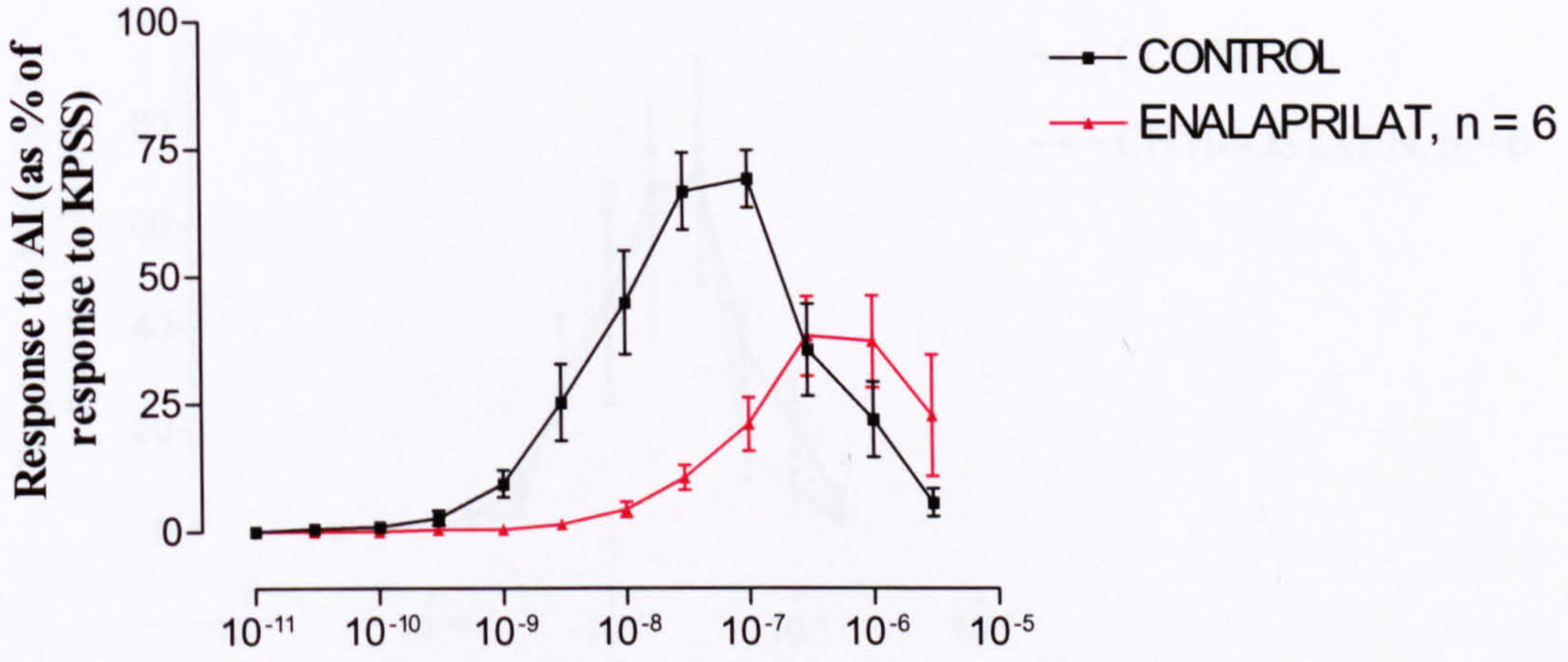
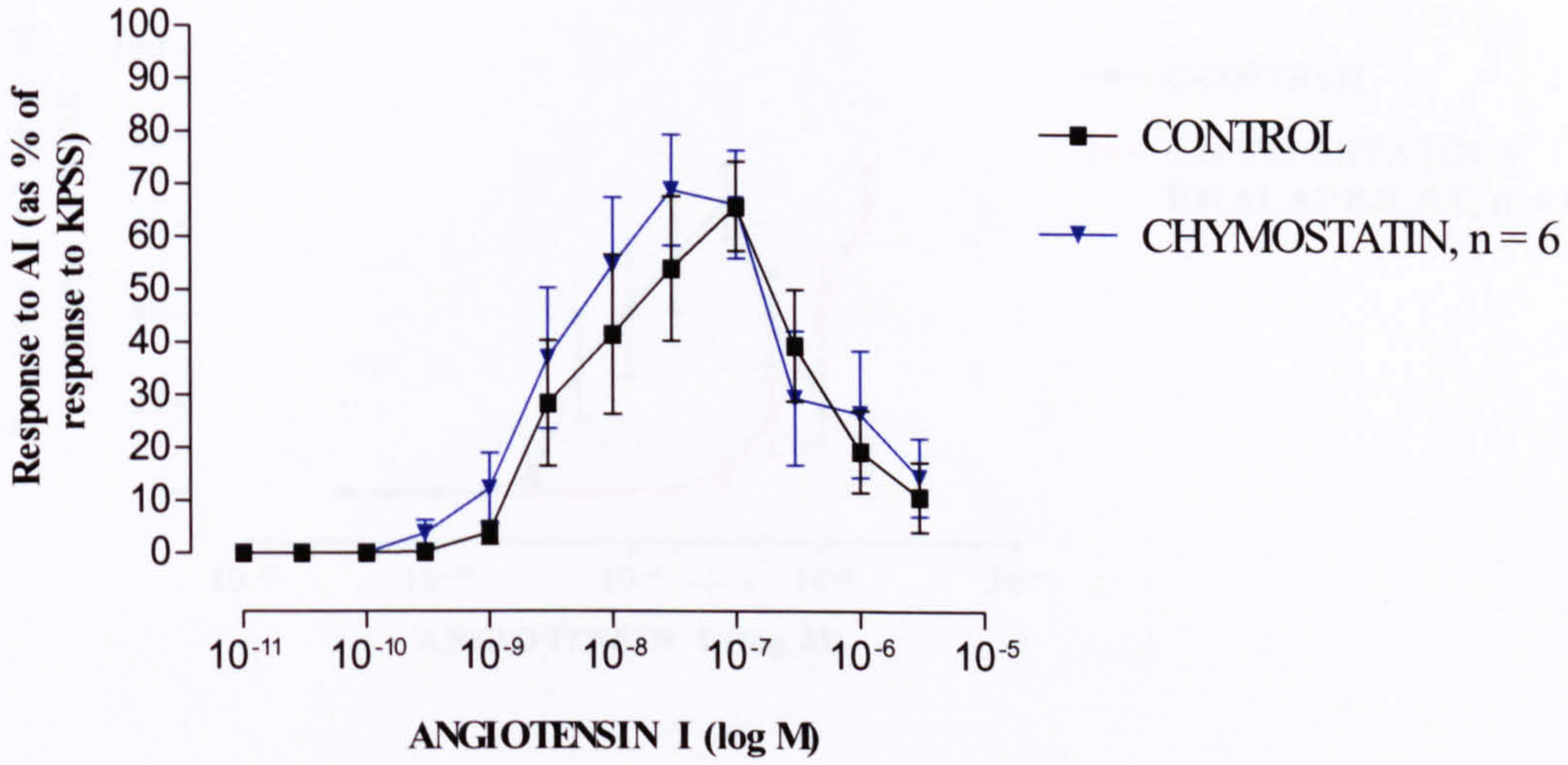



Figure 5.3 shows cumulative concentration response curves to AI in arteries from patients with CHF and CHD in the presence of enalaprilat. Enalaprilat significantly increased the threshold to AI in CHF ($p<0.05$), but not CHD ($p>0.05$).

Figure 5.4: Response to AI in arteries from patients with CHF and CHD: effect of chymostatin

a) CHD




b) CHF

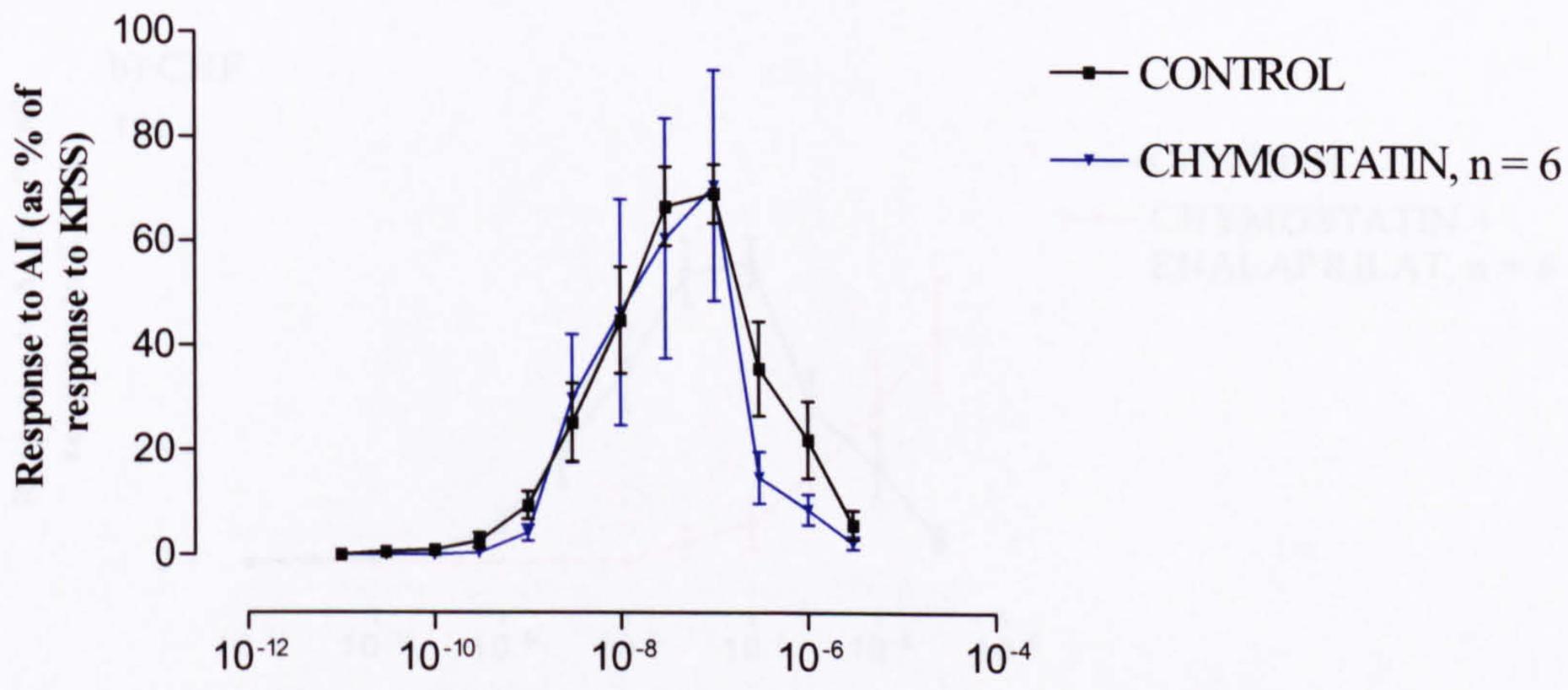



Figure 5.4 shows cumulative concentration response curves to AI in arteries from patients with CHF and CHD in the presence of chymostatin. In neither was there any apparent inhibition.

Figure 5.5: Response to AI in arteries from patients with CHF and CHD: effect of chymostatin and enalaprilat

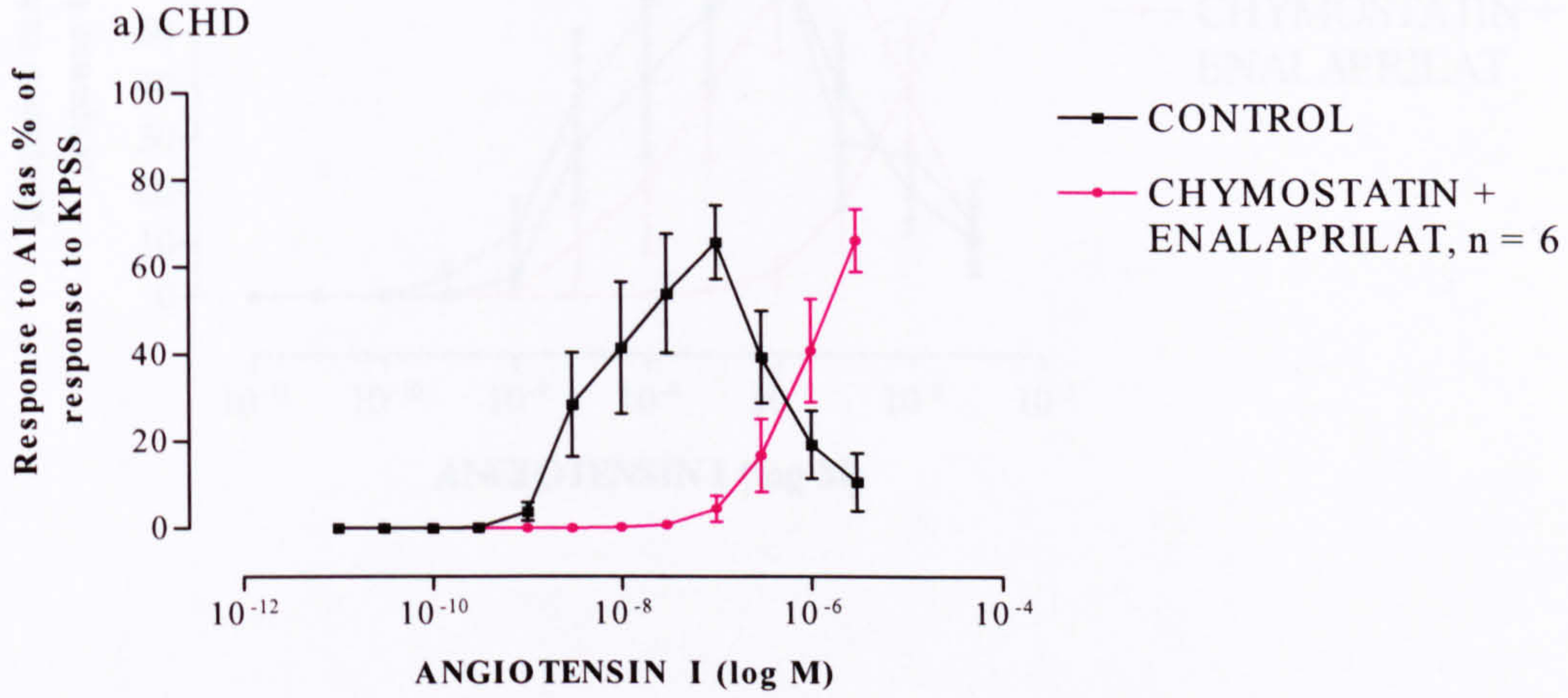
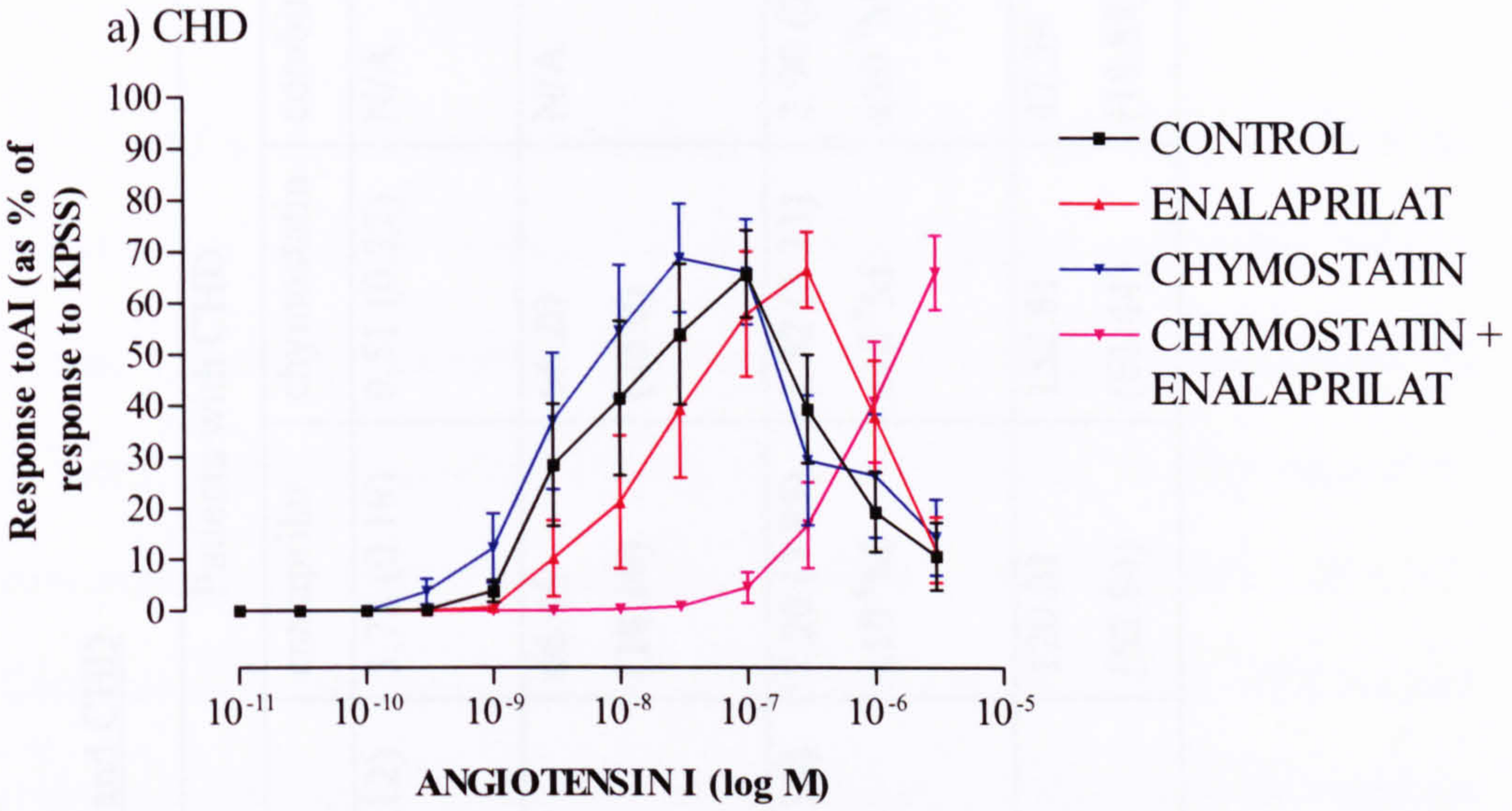



Figure 5.5 shows the effect of the combination of enalaprilat and chymostatin on the responses to AI in arteries from patients with CHF and CHD. The threshold to AI was increased significantly in both CHD and CHF ($p<0.001$, $p<0.01$, respectively).

Figure 5.6: Response to AI in arteries from patients with CHF and CHD: summary



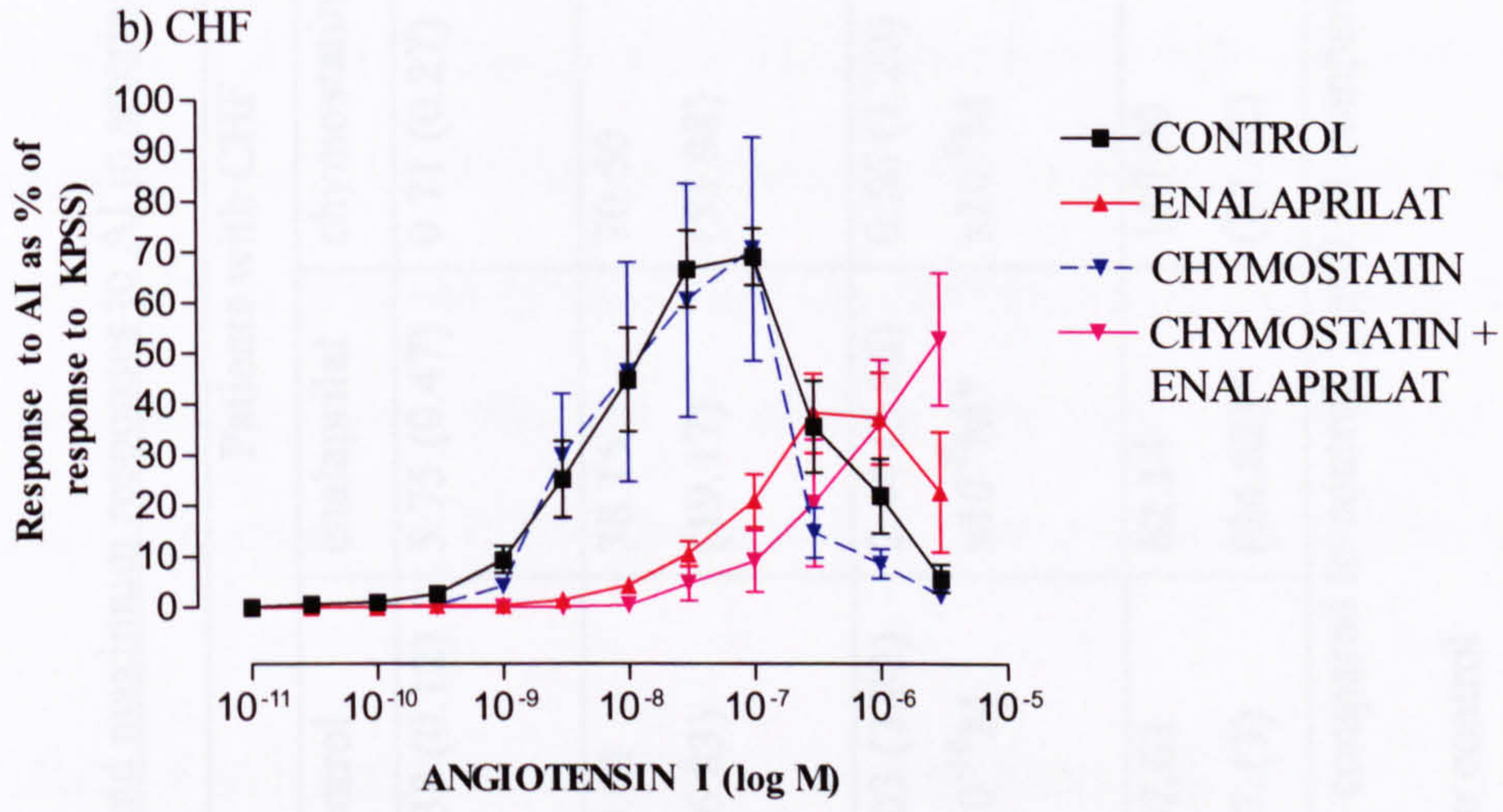




Figure 5.6 summarises figures 5.2-5.5. In arteries from patients with CHF, the combination of enalaprilat and chymostatin increased the threshold for AI compared to enalaprilat alone ($p<0.05$).

Table 5.8: Potency and maximum responses to AI in arteries from patients with CHF and CHD

| | Patients with CHF | | | | Patients with CHD | | | |
|---|---|---|---|---|---|---|---|---|
| Means (± SD) | control | enalaprilat | chymostatin | combination | control | enalaprilat | chymostatin | combination |
| EC50 ($\times 10^{-8}$M) | 1.38 (0.12) | 5.75 (0.47) | 0.71 (0.27) | N/A | 1.70 (0.12) | 3.72 (0.19) | 0.51 (0.22) | N/A |
| Max Response (% response to KPSS) | 69.01 (16.83) | 38.15 (19.17) | 70.50 (53.98) | N/A | 65.81 (22.41) | 66.45 (18.10) | 66.20 (25.0) | N/A |
| Threshold concentration | 5.03 (3.82) $\times 10^{-10}$M | 2.44 (3.86) $\times 10^{-8}$M* | 0.56 (1.20) $\times 10^{-8}$M | 0.77 (1.07) $\times 10^{-6}$M†‡ | 2.76 (3.36) $\times 10^{-9}$M | 2.20 (3.85) $\times 10^{-8}$M | 1.32 (1.33) $\times 10^{-9}$M | 2.90 (2.70) $\times 10^{-7}$M† |
| AUC | 132.63 (37.13) | 62.14 (24.62)* | 117.90 (87.42) | 30.08 (17.76) | 128.87 (80.50) | 120.01 (62.84) | 152.81 (61.44) | 47.59 (18.88) |

*p<0.05, † p<0..001 compared to control; ‡ p<0.01 compared to enalaprilat

†p<0.01 compared to control

## 5.7 Response to AII in patients with CHF and CHD

CRCs to AII were performed according to the same protocol as described in previous sections i.e. after co-incubation with either vehicle (vessel 1: control), enalaprilat 1μM (vessel 2), chymostatin 10μM (vessel 3) or both inhibitors together (vessel 4). AII induced a dose-dependent contraction in resistance arteries from patients with both CHD and CHF. Concentration response curves were similar to those seen for AI, with marked tachyphylaxis and an "inverted U" shape. Figure 5.7 illustrates the response to AII in the absence of any inhibitors. The threshold contraction [mean ± (SD)] was at 2.03 (3.91) $x10^{-10}$M and 2.27 (3.54) $x10^{-10}$M AII for CHD and CHF, respectively. The maximum response in CHD (mean ± SD) was 49.99 (27.79)% at 10nM. In contrast the maximum response in CHF was 77.48 (9.68)% at 30nM. Theses values were significantly different (p=0.028, by unpaired t-test). The EC50s for CHD and CHF, respectively, were 1.55 (0.1) nM and 3.80 (0.2) nM (p=ns). Thus the maximum response to AII, but not the sensitivity, was enhanced in patients with CHF compared to CHD. Maximum responses, EC50 values and AUCs are summarised in table 5.8.

<u>Effect of ACE and chymase inhibition on responses to AII</u>

Figure 5.8 illustrates the effect of enalaprilat, chymostatin and the combination of both inhibitors on responses to AII in arteries from patients with CHD. Although a slight shift in the dose-response curve to the right is

evident, there were no significant differences between control and experimental curves in terms of maximum response or EC50 ($p>0.05$ by ANOVA). For the sake of consistency with previous analyses of AI curves, AUC was also calculated and used as a comparator, with similarly negative results. Thus there was no apparent attenuation of the response to AII when either ACE or chymase were inhibited singly or in combination.

Figure 5.9 shows analogous data for arteries from patients with CHF. As previously noted for the control curve, the maximum responses to AII were consistently greater than observed in arteries from patients with CHD. However neither enalaprilat, chymostatin, or their combination significantly attenuated AII responses. Again these curves were compared in terms maximum response, EC50 and AUC. These data are summarised in table 5.9.

Figure 5.7: Response to AII in arteries from patients with CHD and CHF in absence of inhibitors

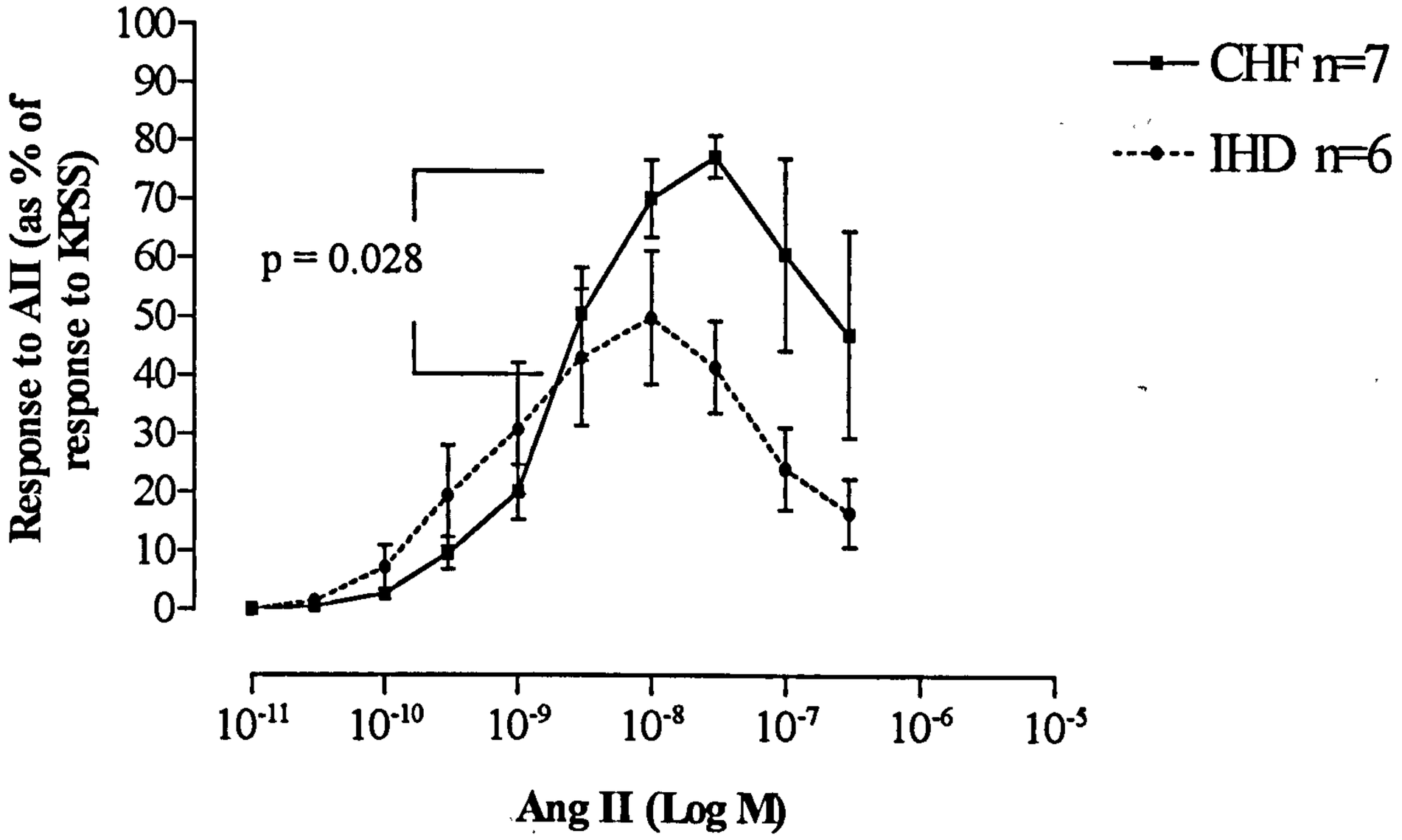



Figure 5.7 shows the response to AII in control vessels. The maximum response was greater in arteries from patients with CHF ($p = 0.028$), but there was no difference in EC50.

Figure 5.8: Response to AII in resistance arteries from patients with CHD: effect of ACE and Chymase inhibition

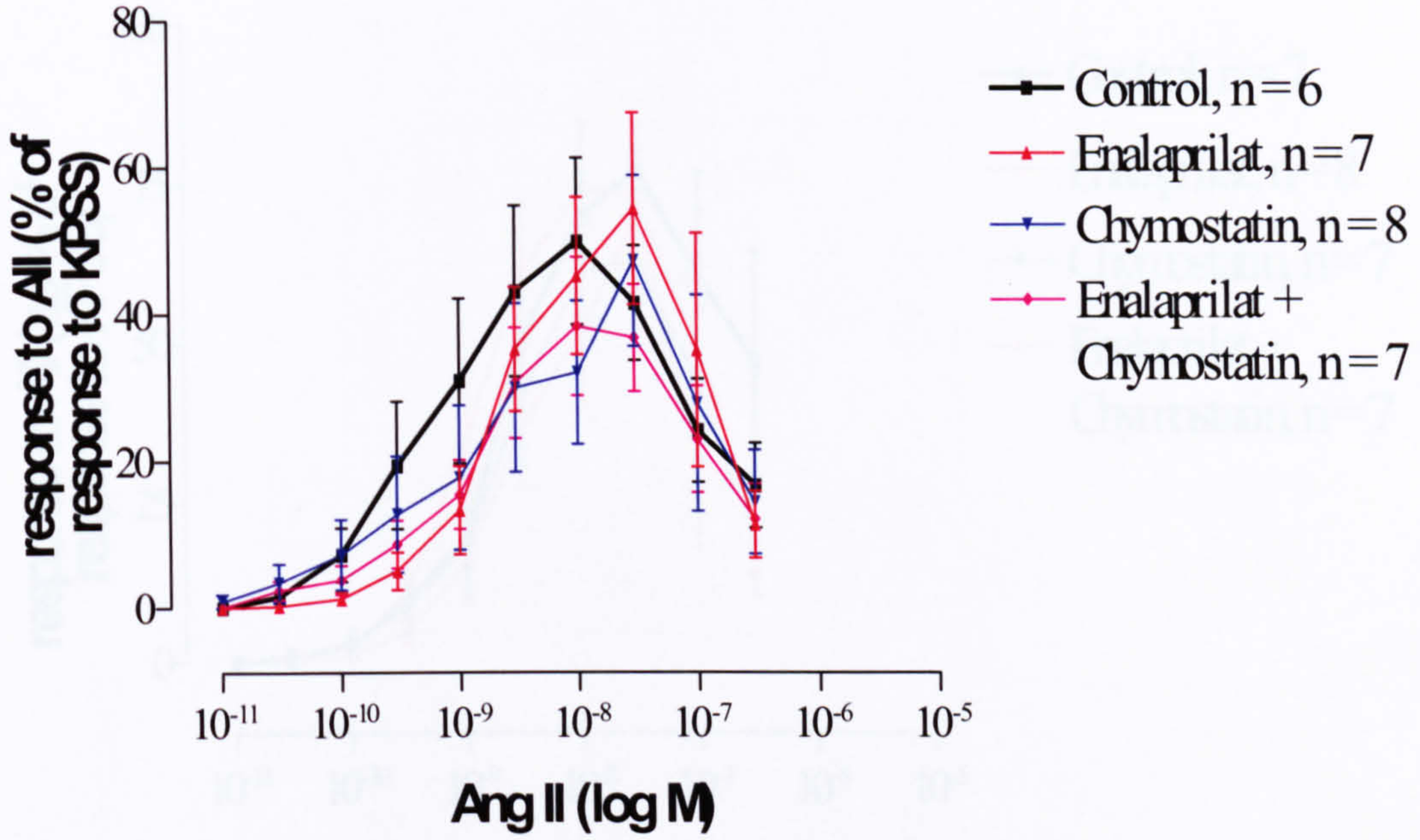



Figure 5.8 shows the responses to AII in arteries from patients with CHD. Neither enalaprilat nor chymostatin, alone or in combination, significantly inhibited the response.

Figure 5.9: Response to AII in resistance arteries from patients with CHF: effect of ACE and Chymase inhibition

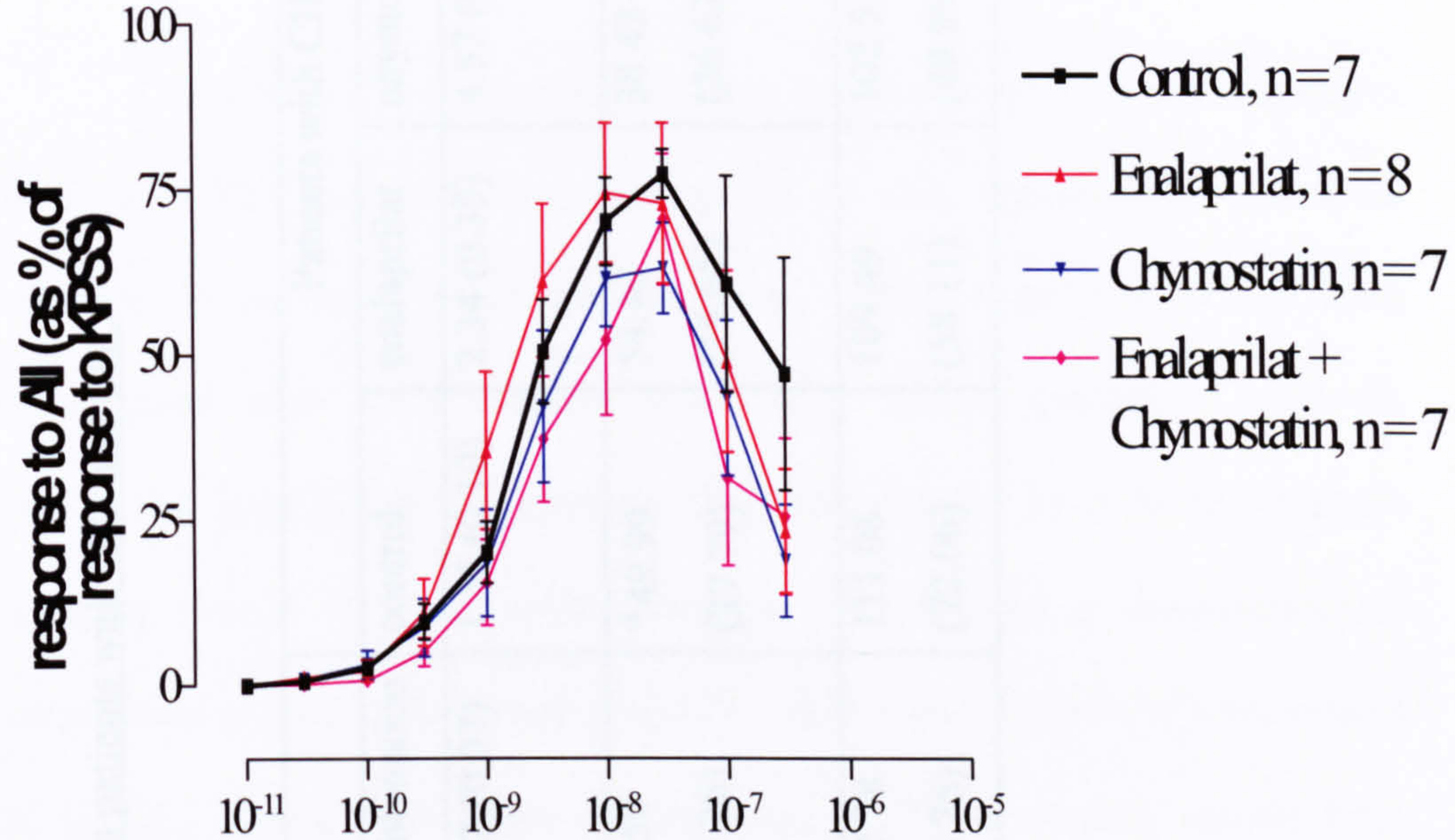



Figure 5.9 shows the responses to AII in arteries from patients with CHF. Neither enalaprilat nor chymostatin, alone or in combination, significantly inhibited the response. The maximum response to AII was significantly greater than in CHD, regardless of the presence of ACEi or chymostatin.

Table 5.9: Potency and maximum responses to AII in arteries from patients with CHF and CHD

| | Patients with CHF | | | | Patients with CHD | | | |
|---|---|---|---|---|---|---|---|---|
| Means (± SD) | control | enalaprilat | chymostatin | combination | control | enalaprilat | chymostatin | combination |
| EC50 ($\times 10^{-9}$M) | 3.80 (0.20) | 2.75 (0.14) | 7.08 (0.08) | 28.2 (0.07) | 1.55 (0.10) | 2.34 (0.35) | 4.57 (0.07) | 2.34 (0.29) |
| Max Response (% response to KPSS) | 77.48 (9.68) | 74.60 (29.97) | 63.20 (18.06) | 70.58 (26.26) | *49.99 (27.79) | 54.46 (34.65) | 38.42 (26.63) | N/A |
| AUC | 157.56 (27.91) | 161.00 (85.21) | 125.67 (49.99) | 112.78 (68.76) | 111.08 (72.06) | 109.49 (58.11) | 102.51 (49.99) | 81.17 (50.47) |

*p = 0.028, max response CHD vs. max response CHF

## 5.8 Responses to norepinephrine, acetylcholine and bradykinin in arteries from patients with CHF and CHD

### 5.8.1 Responses to norepinephrine

CRCs to NE were performed after arteries from patients with either CHF or CHD had been exposed to AI or AII, maintaining the same relationship between vessel and inhibitor. Thus responses to NE were investigated after co-incubation with either vehicle (vessel 1: control), enalaprilat 1μM (vessel 2), chymostatin 10μM (vessel 3) or both inhibitors together (vessel 4). In control vessels NE induced a dose-dependent contraction in arteries from patients with a threshold of 1nM and a maximum at 10μM. Figure 5.10 shows the response to NE in control vessels from patients with CHF and CHD, expressed as the absolute increase in internal pressure above baseline and not normalised to a standard vasoconstrictor. Thus, the maximum response to NE (mean ± SD) in arteries from patients with CHF was 21.03 (5.77)kPa compared to 21.45 (6.04) kPa in those from patients with CHD ($p = 0.786$). There was no difference in the EC50 for NE in the control vessels, as can be seen in the figure.

<u>Effect of ACE and chymase inhibition on responses to NE</u>

Figure 5.11 illustrates the response to NE in arteries from patients with CHD in the presence of enalaprilat, chymostatin and their combination. The maximum response seemed to be slightly attenuated, with a rightward

shift in the curve, in those vessels exposed to enalaprilat or chymostatin, but not the combination. However these differences were not statistically significant ($p>0.05$ by ANOVA). Maximum responses and EC50 values are shown in table 5.10. Figure 5.12 illustrates the response to NE in patients with CHF. Again there seemed to be a slight reduction in the maximum response in the presence of either enalaprilat or chymostatin alone, but not with the combination. However these differences were not significant. Similarly, there were no significant differences in the EC50 values.

Table 5.10: Maximum response and EC50 to NE

| **CHD** | | | | |
|---|---|---|---|---|
| | Control | Enalaprilat | Chymostatin | Combination |
| Max Response (kPa) | 21.45 (6.04) | 17.49 (7.58) | 15.42 (7.59) | 21.03 (7.68) |
| EC50 (M, $\times 10^{-7}$) | 1.12 (0.27) | 2.19 (0.32) | 1.48 (0.35) | 1.48 (0.43) |

| **CHF** | | | | |
|---|---|---|---|---|
| | Control | Enalaprilat | Chymostatin | Combination |
| Max Response (kPa) | 21.03 (5.77) | 17.88 (3.88) | 16.23 (3.27) | 18.33 (5.86) |
| EC50 (M, $\times 10^{-7}$) | 1.86 (0.44) | 3.16 (0.29) | 1.70 (0.47) | 2.04 (0.35) |

All values means (± SD)

There were no significant differences between control and experimental groups in either CHD or CHF by ANOVA.

Figure 5.10: Responses to NE in arteries from patients with CHF and CHD

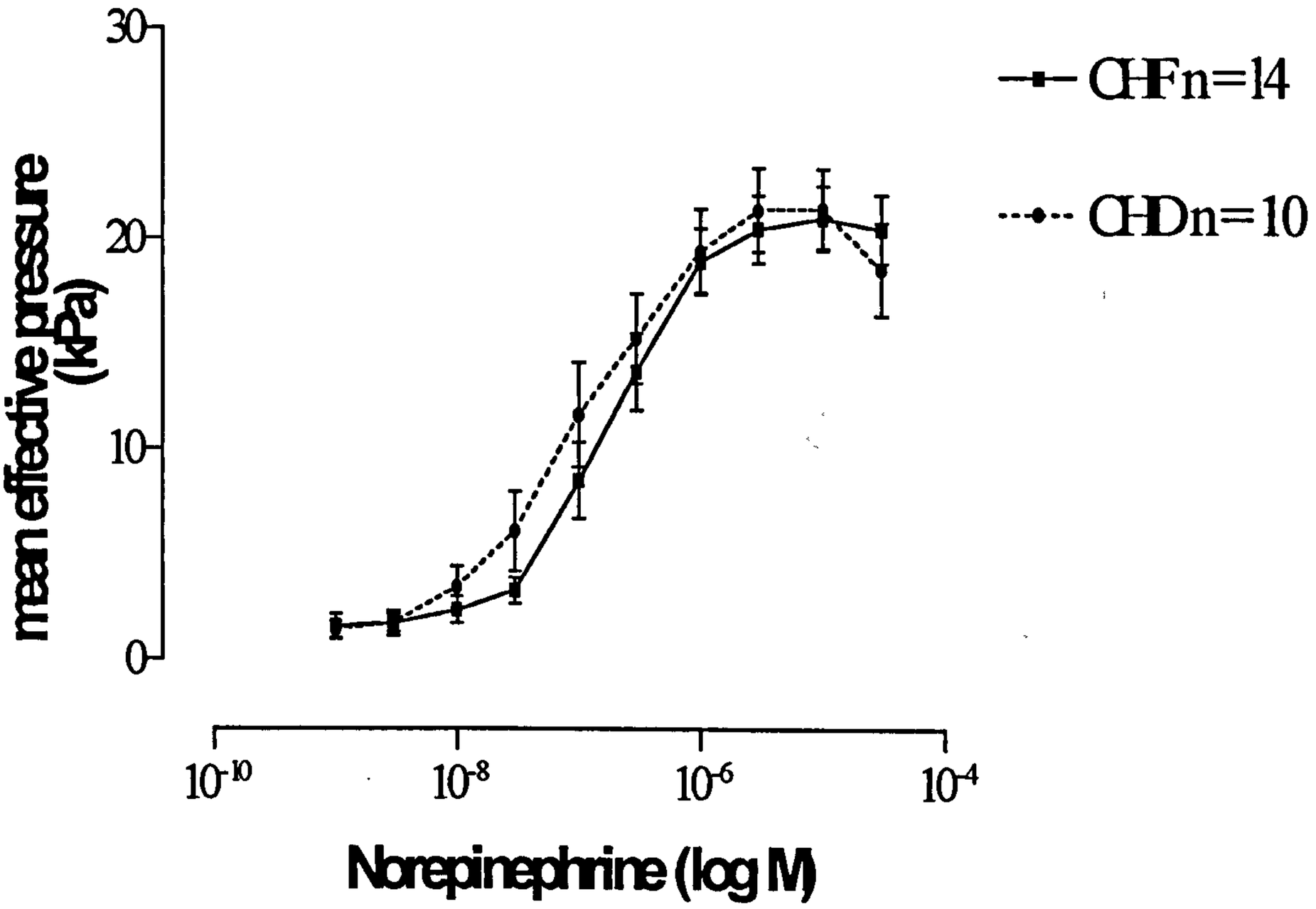



Figure 5.10 demonstrates the response to NE in arteries from patients with CHF and CHD in the absence of any inhibitors. These responses were not different by EC50 or maximum.

Figure 5.11: Responses to NE in arteries from patients with CHD: effect of ACE and chymase inhibition

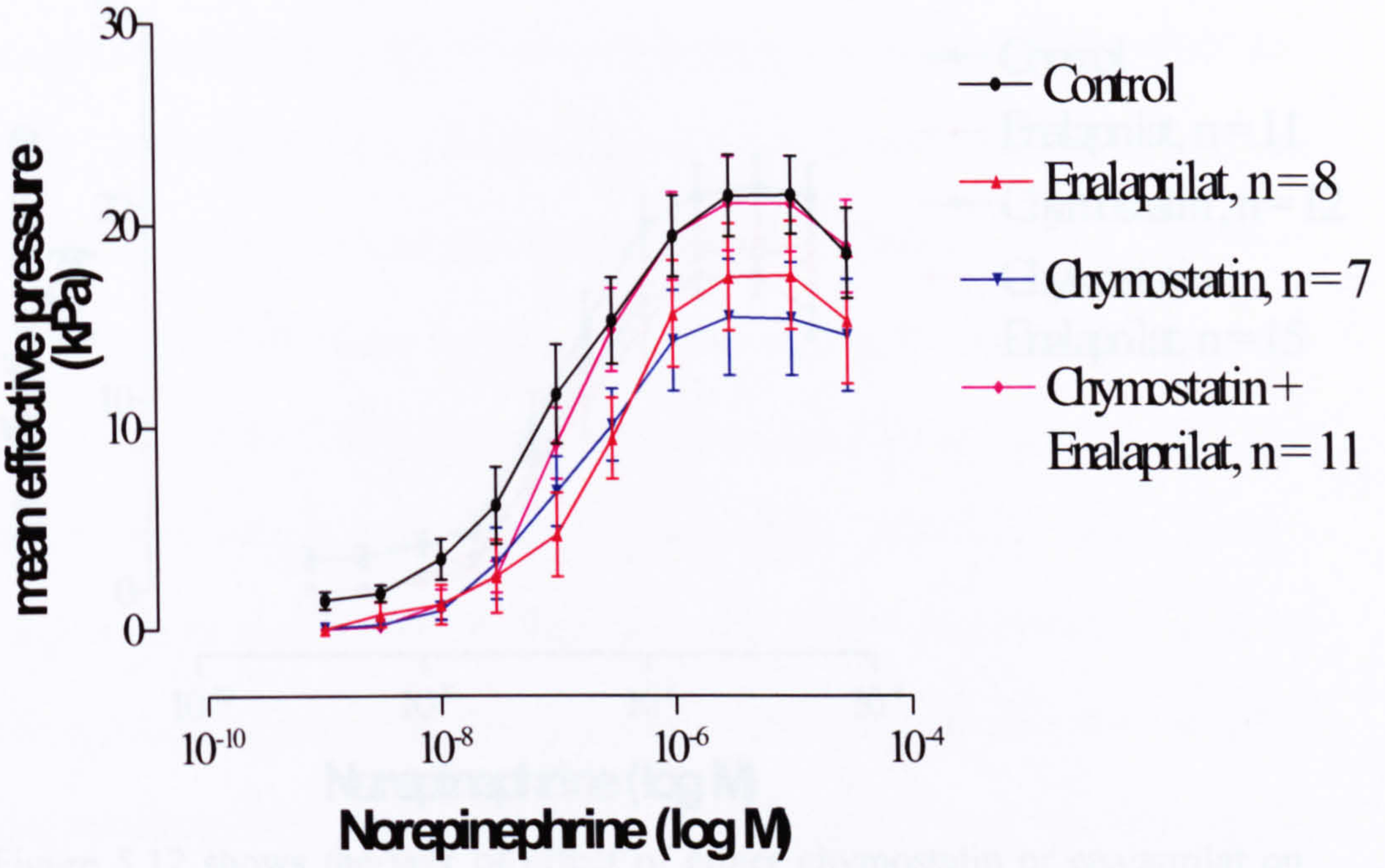



Figure 5.11 shows the effect of chymostatin and enalaprilat on responses to NE in arteries from patients with CHD. There were no significant differences between control and experimental groups ($p>0.05$ by ANOVA).

Figure 5.12: Responses to NE in arteries from patients with CHF: effect of ACE and chymase inhibition

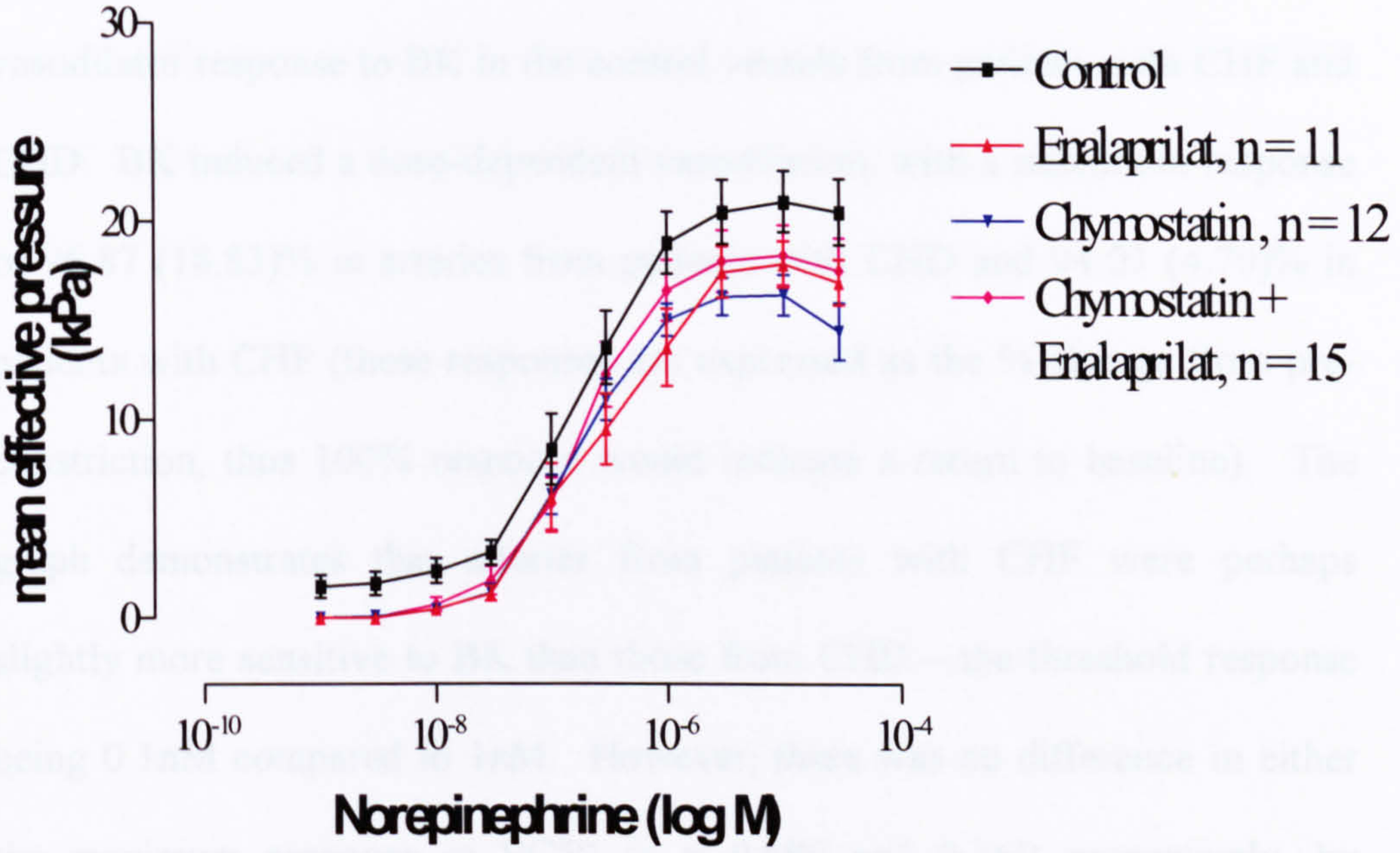



Figure 5.12 shows the lack of effect of either chymostatin or enalaprilat on responses to NE in arteries from patients with CHF. There were no significant differences between control and experimental groups ($p>0.05$ by ANOVA).

### 5.8.2 Responses to bradykinin

After CRC s had been performed to NE arteries were washed with Kreb's to re-establish baseline and then incubated with enalaprilat etc as described above. After 30 minutes vessels were pre-constricted with NE 10μM and CRCs to BK were performed. Figure 5.13 illustrates the vasodilator response to BK in the control vessels from patients with CHF and CHD. BK induced a dose-dependent vasodilation, with a maximum response of 96.87 (18.83)% in arteries from patients with CHD and 94.03 (4.70)% in patients with CHF (these responses are expressed as the % change from pre-constriction, thus 100% response would indicate a return to baseline). The graph demonstrates that arteries from patients with CHF were perhaps slightly more sensitive to BK than those from CHD – the threshold response being 0.1nM compared to 1nM. However, there was no difference in either the maximum response or EC50 ($p = 0.689$ and 0.169, respectively, by unpaired t-test).

<u>Effect of ACE and chymase inhibition on responses to BK</u>

Figure 5.14 illustrates the responses to BK in the presence of ACE and chymase inhibitors in arteries from patients with CHD. As expected there was a shift to the left in the dose-response curve in the presence of enalaprilat, but not with chymostatin. However when expressed as EC50 this did not reach significance ($p>0.05$ by ANOVA), though the trend is obvious.

There was no significant difference in the maximum responses in any of the groups. These results are summarised in table 5.11.

Figure 5.15 illustrates responses to BK in arteries from patients with CHF. Again there was a trend towards potentiation in the presence of enalaprilat, but this failed to reach significance ($p>0.05$ by ANOVA). There was no difference in the maximum response between groups. Actual values are given in table 5.11. Figure 5.16 summarises these data.

Table 5.11: Responses to BK

| CHD<br>All values mean ± (SD) | | | | |
|---|---|---|---|---|
| | Control | Enalaprilat | Chymostatin | Combination |
| Max Response (% change) | 96.87 (18.83) | 84.84 (13.63) | 89.20 (5.15) | 88.63 (15.43) |
| EC50 (M, $\times 10^{-8}$) | 19.90 (3.30) | *3.09 (0.14) | 30.90 (1.30) | 5.25 (0.24) |

| CHF<br>All values mean ± (SD) | | | | |
|---|---|---|---|---|
| | Control | Enalaprilat | Chymostatin | Combination |
| Max Response (% change) | 94.03 (4.70) | 95.27 (5.21) | 94.41 (6.53) | 94.38 (6.12) |
| EC50 (M, $\times 10^{-8}$) | 17.8 (2.3) | 4.07 (0.12) | 12.70 (3.20) | ⇧5.57 (0.13) |

ANOVA did not reveal significant differences between control and experimental groups for either EC50 or maximum response

*, ⇧ $p>0.05$

Figure 5.13: Response to BK in arteries from patients with CHF and CHD

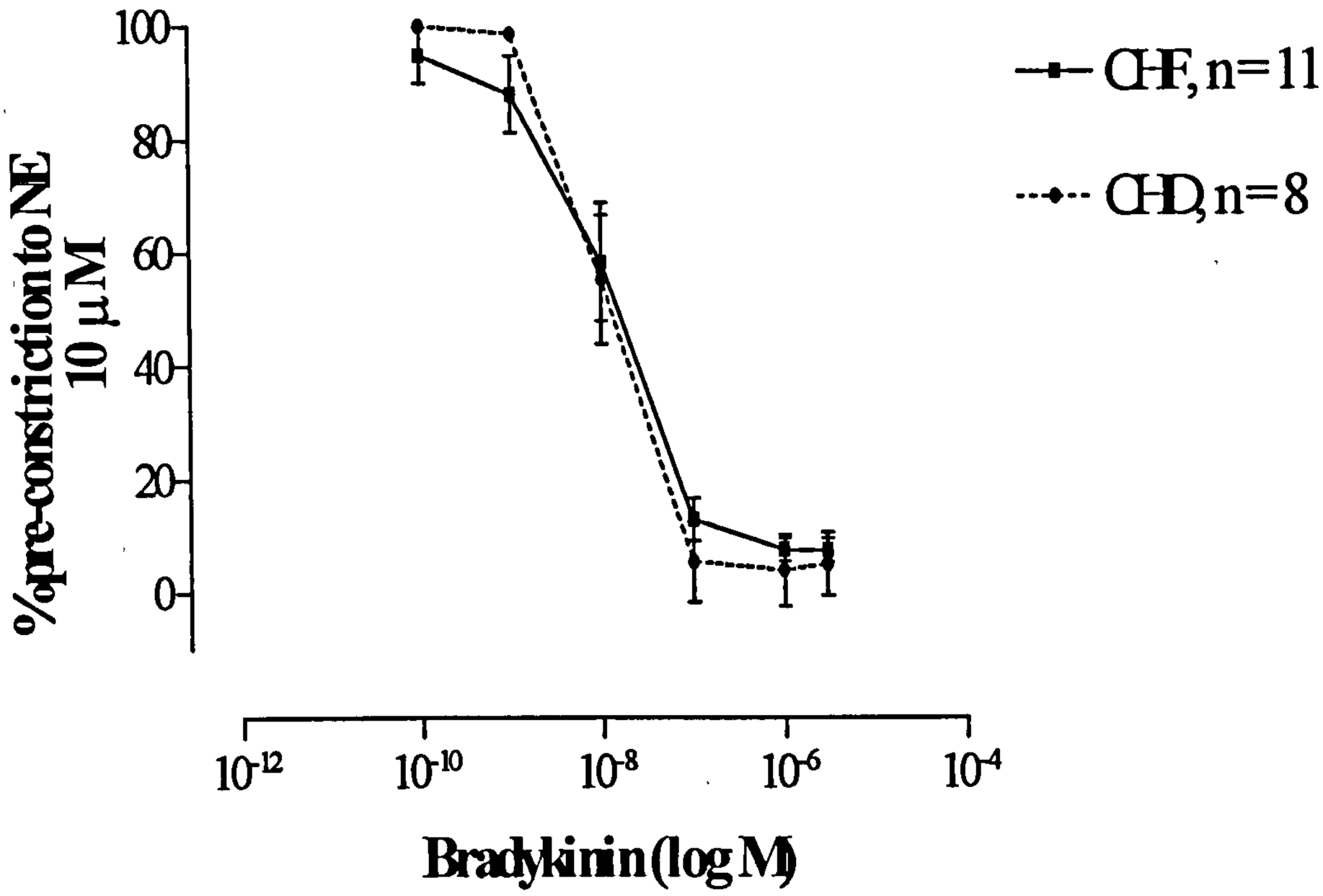



Figure 5.13 shows the response to BK in arteries from patients with CHD and CHF in the absence of any inhibitors. There was no significant difference between the 2 groups.

Figure 5.14: Response to BK in arteries from patients with CHD: effect of ACE inhibition

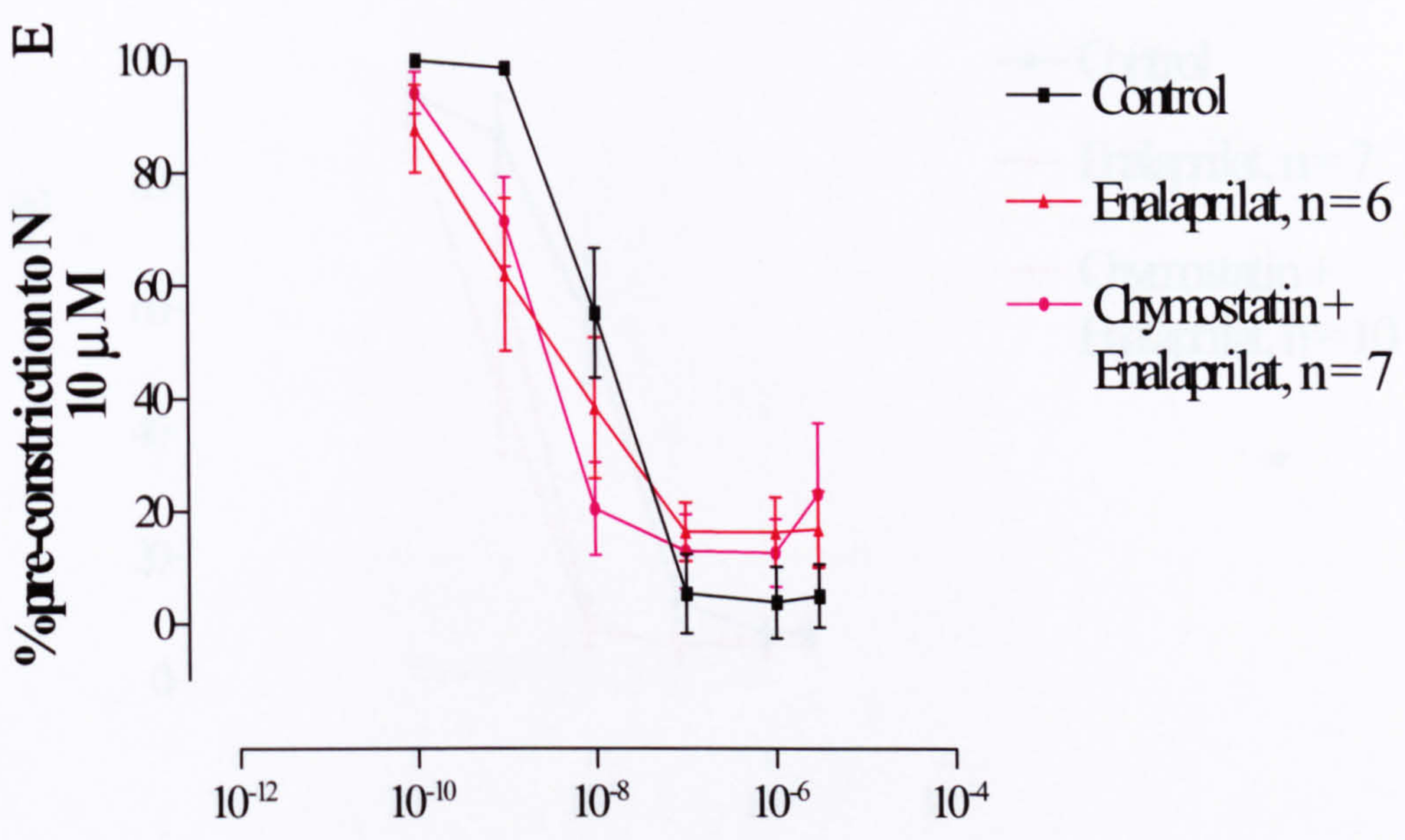



Figure 5.14 illustrates the effect of ACE and combined ACE and chymase inhibition on the responses to BK in arteries from patients with CHD. There was a trend towards potentiation in the presence of enalaprilat, but this did not reach significance ($p>0.05$, EC50, control vs. enalaprilat).

Figure 5.15: Response to BK in arteries from patients with CHF: effect of ACE inhibition

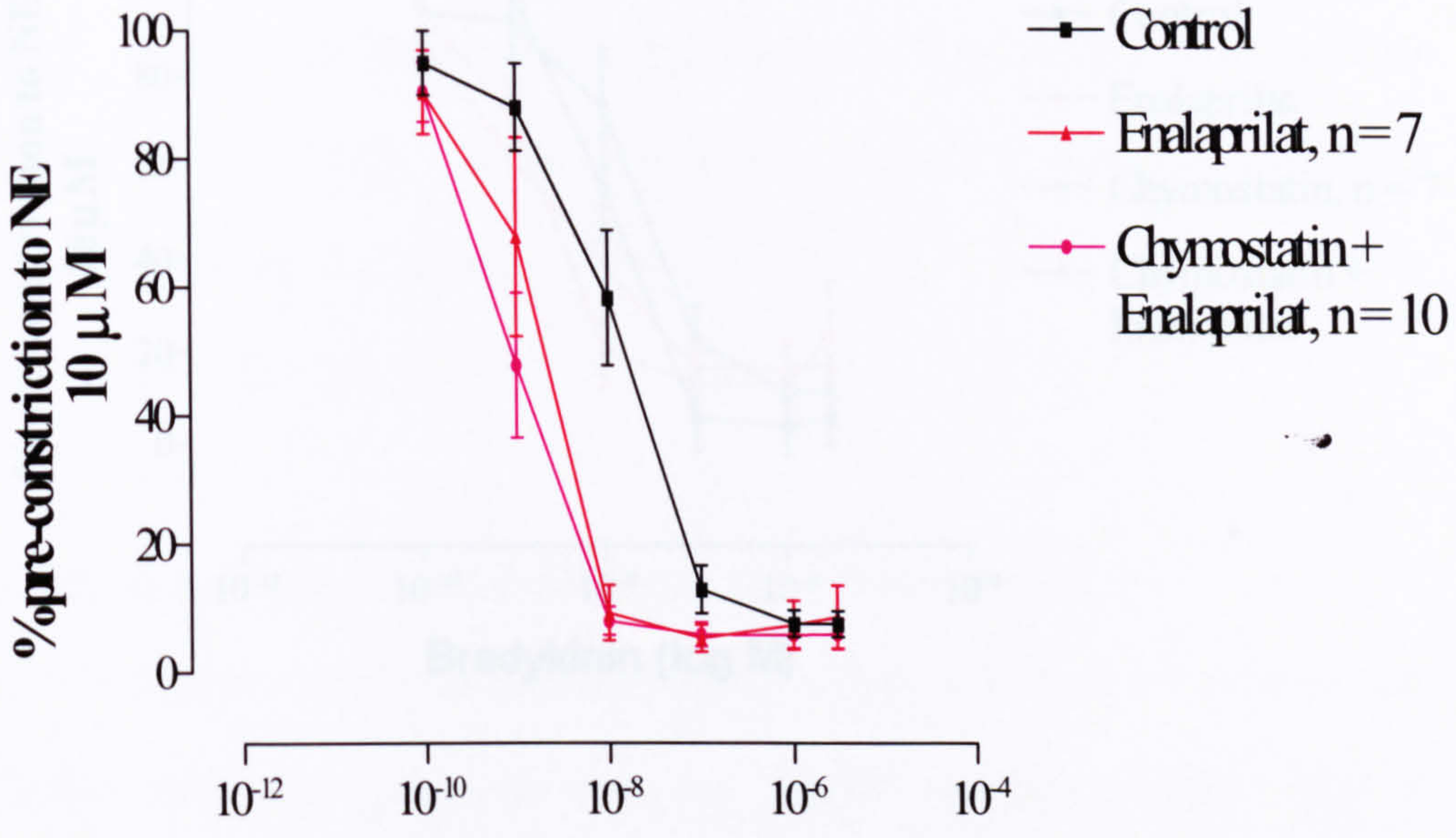
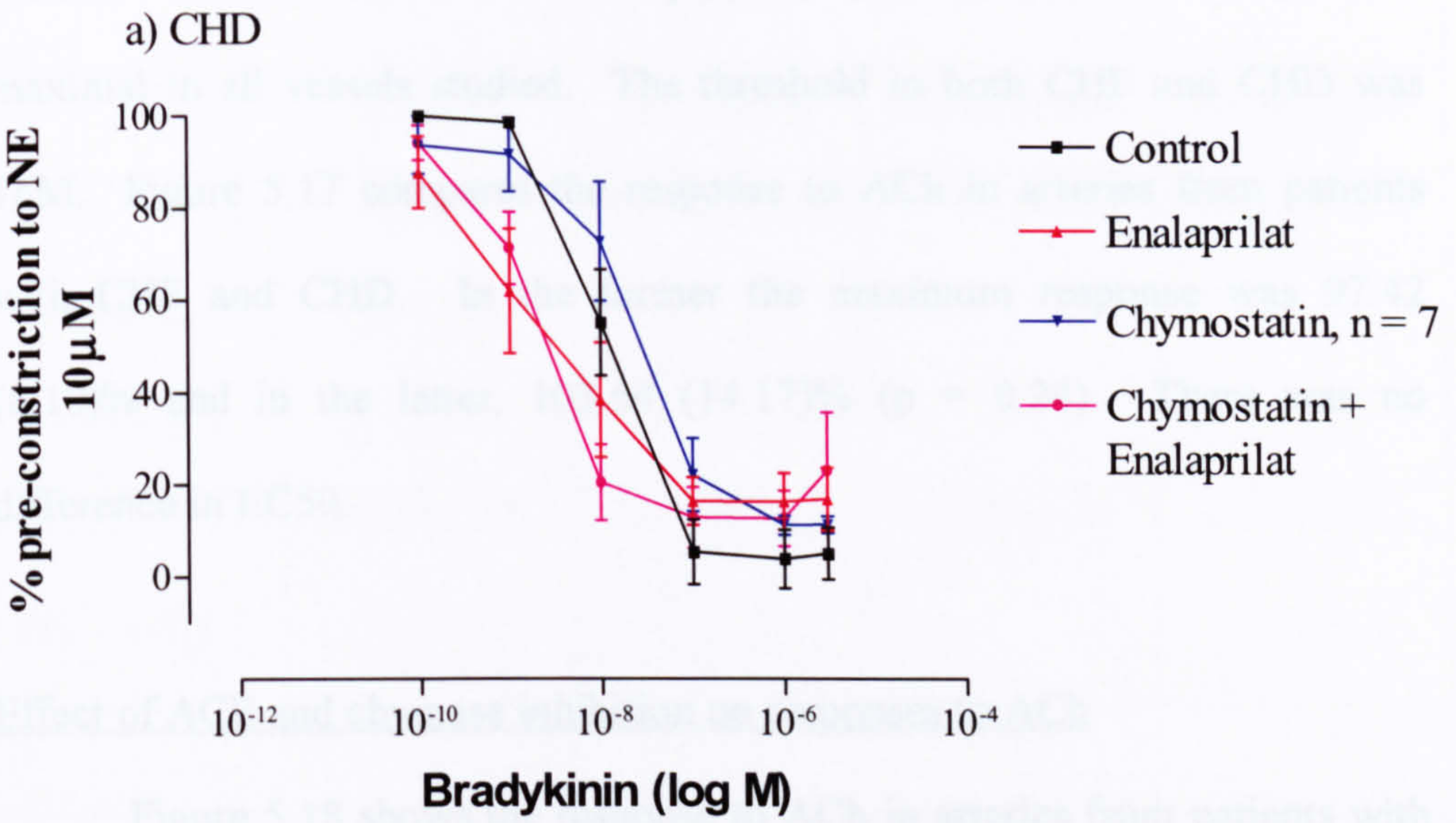



Figure 5.15 illustrates the effect of ACE and combined ACE and chymase inhibition on the responses to BK in arteries from patients with CHF. There was a trend towards potentiation in the presence of enalaprilat, but again this did not reach significance.

Figure 5.16: Response to BK in arteries from patients with CHF and CHD:

effect of ACE and chymase inhibition



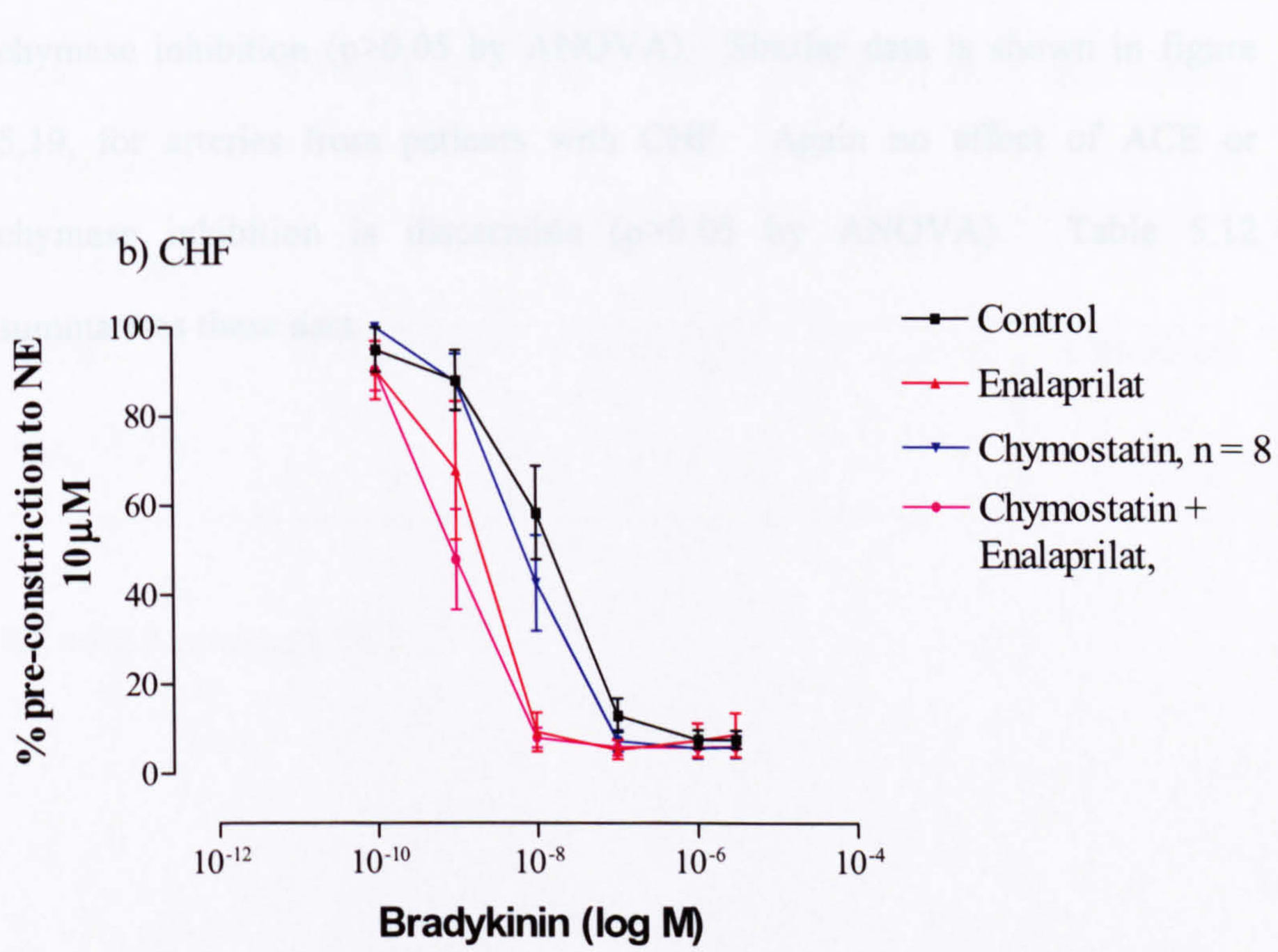




Figure 5.16 summarises figures 5.14 and 5.15

### 5.8.3 Responses to acetylcholine

CRCs to ACh were constructed after those to BK, and in the same fashion. ACh induced a dose-dependent vasodilation, which was near maximal in all vessels studied. The threshold in both CHF and CHD was 1nM. Figure 5.17 compares the response to ACh in arteries from patients with CHF and CHD. In the former the maximum response was 97.42 (8.10)% and in the latter, 103.64 (14.17)% ($p = 0.24$). There was no difference in EC50.

<u>Effect of ACE and chymase inhibition on responses to ACh</u>

Figure 5.18 shows the response to ACh in arteries from patients with CHD. There did not appear to be any significant effect of either ACE or chymase inhibition ($p>0.05$ by ANOVA). Similar data is shown in figure 5.19, for arteries from patients with CHF. Again no effect of ACE or chymase inhibition is discernible ($p>0.05$ by ANOVA). Table 5.12 summarises these data.

Table 5.12: Responses to ACh

| CHD | | | | |
|---|---|---|---|---|
| | Control | Enalaprilat | Chymostatin | Combination |
| Max Response (% change) | 103.64 (14.17) | 98.08 (5.54) | 97.83 (3.81) | 96.67 (7.65) |
| EC50 (M, $\times 10^{-8}$) | 10.0 (1.90) | 7.24 (0.42) | 40.70 (1.1) | 8.91 (0.23) |

| CHF | | | | |
|---|---|---|---|---|
| | Control | Enalaprilat | Chymostatin | Combination |
| Max Response (% change) | 97.42 (8.10) | 95.07 (8.81) | 94.08 (11.63) | 98.22 (6.01) |
| EC50 (M, $\times 10^{-8}$) | 11.50 (1.90) | 7.94 (0.27) | 10.0 (1.50) | 7.24 (0.36) |

All values means (± SD)

Figure 5.17: Response to ACh in arteries from patients with CHF and CHD

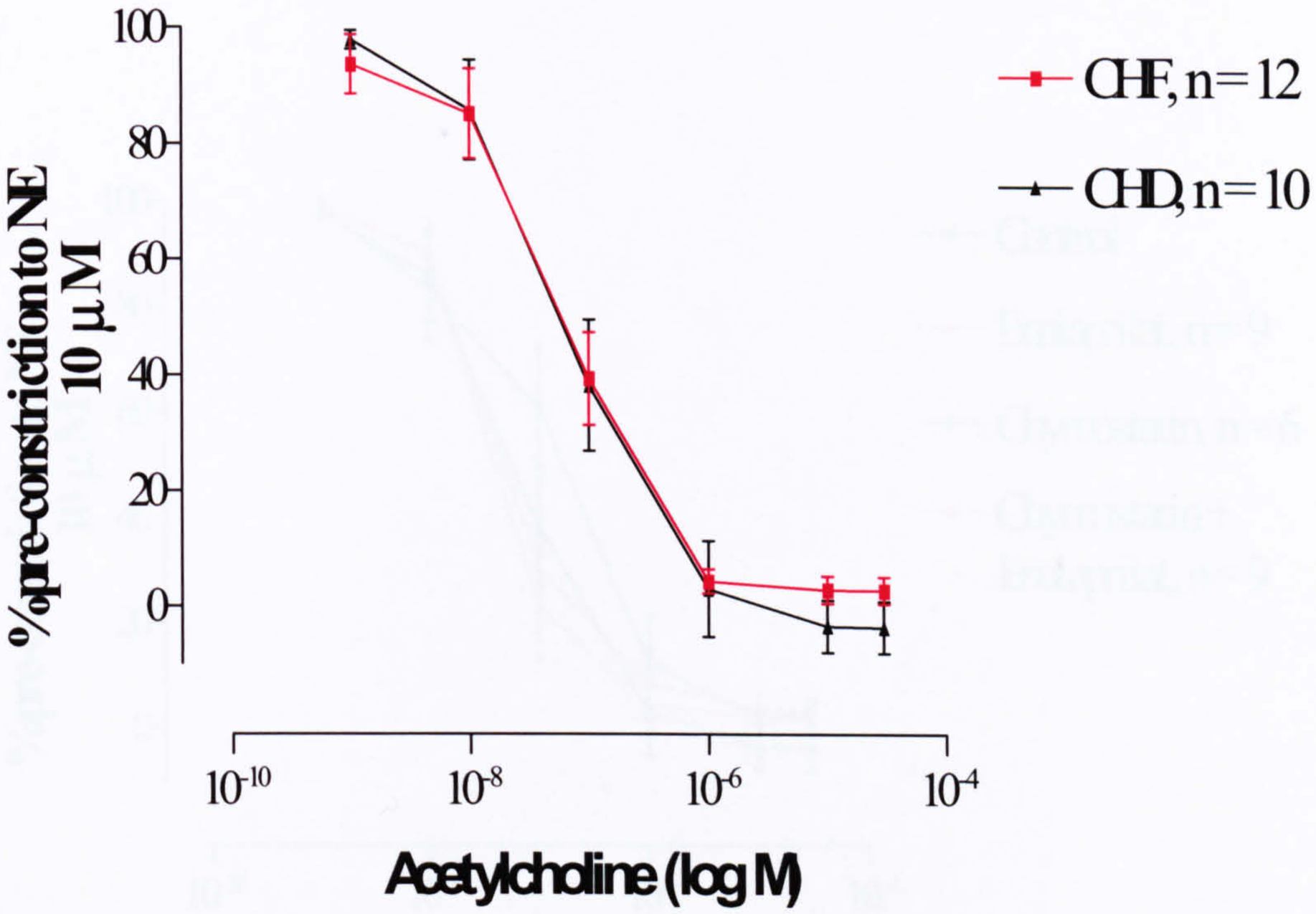



Figure 5.17 illustrates the effect of ACh in arteries from patients with CHF and CHD, in the absence of either ACE or chymase inhibition.

Figure 5.18: Response to ACh in arteries from patients with CHD: effect of ACE and chymase inhibition

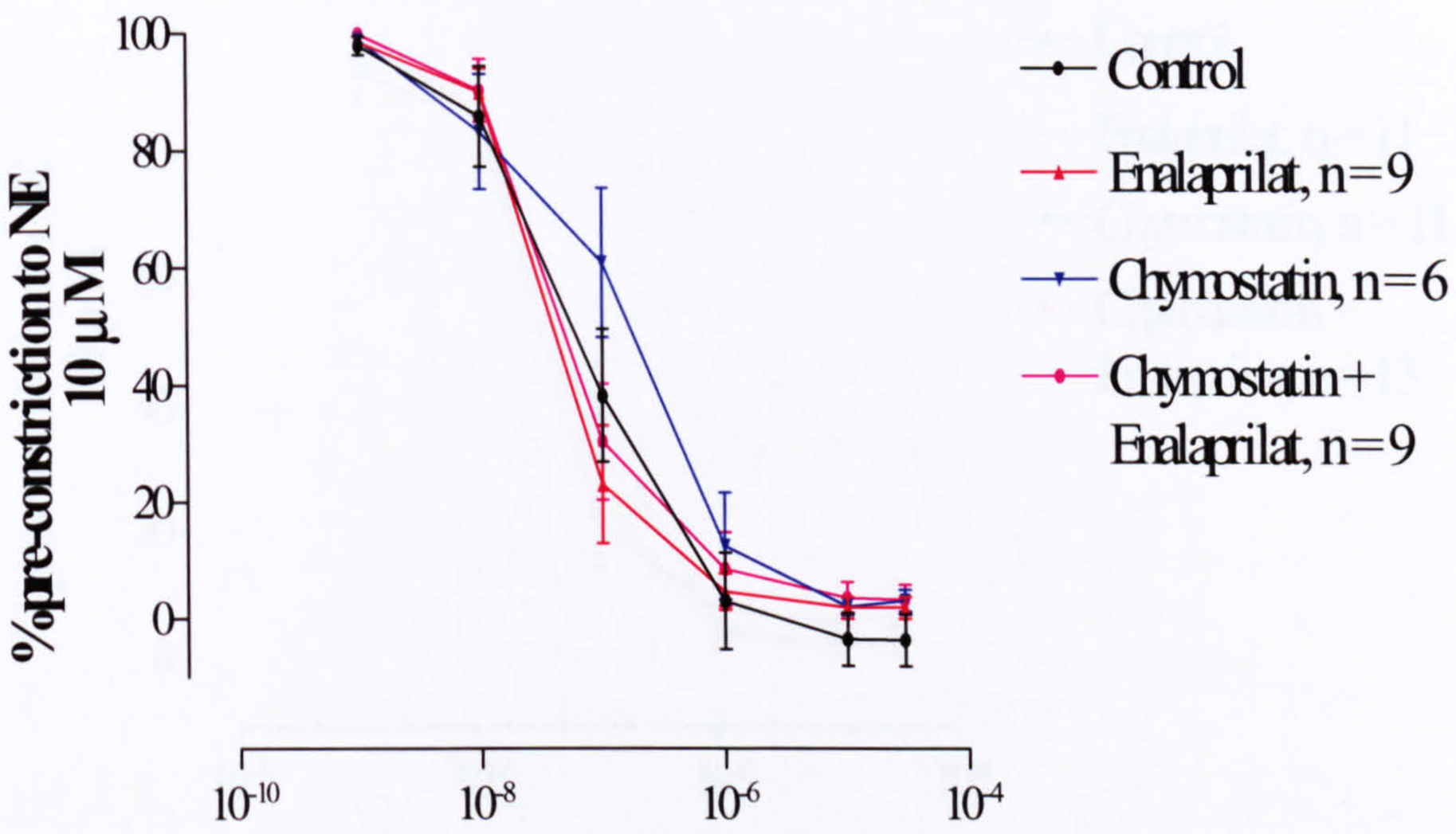



Figure 5.18 illustrates the response to ACh in arteries from patients with CHD in the presence of ACE and chymase inhibitors.

Figure 5.19: Response to ACh in arteries from patients with CHF: effect of ACE and chymase inhibition

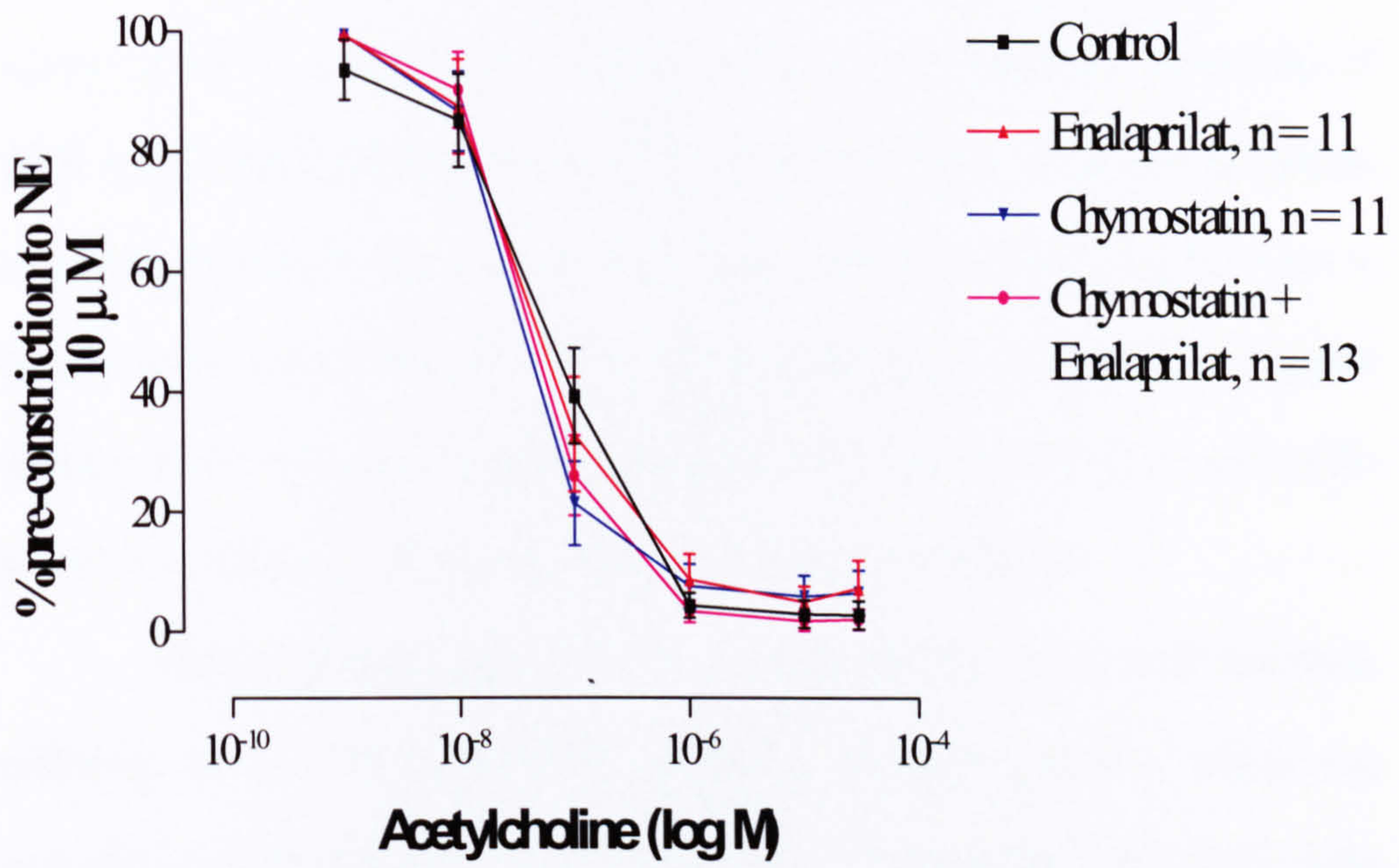



Figure 5.19 illustrates the response to ACh in arteries from patients with CHF in the presence of ACE and chymase inhibitors.

## 5.9 Discussion

In chapter 3 I demonstrated that AI induces a dose-dependent contraction in human resistance arteries. A surprising finding was that the response to AI, though dependent on AII formation, was not dependent on the action of ACE. Instead there appears to be a dual pathway, consisting of ACE and a chymostatin-sensitive enzyme (most likely chymase) in human resistance arteries. The aim of the studies described in this chapter was to extend these experiments to patients receiving ACEi (compared to similar patients who were not) and to investigate the effect of chymase and ACE-inhibition on the action of other vasoconstrictors and dilators.

Patient characteristics: The patients studied were well matched, differing only in the pre-defined criteria of ejection fraction, which was reduced in those with CHF, and medication. All patients with CHF were receiving ACEi. Patients with CHF were also older than patients with CHD, as might be expected. Since all the patients were instructed to take their medication (including ACEi in those patients with CHF) on the morning of study, plasma AII and serum ACE levels are illuminating. Most of the patients with CHF had suppressed serum ACE, suggesting that they had taken their ACEi on the morning of study. Consistent with that suggestion, plasma renin levels were elevated in patients with CHF, though with a wide spread of values. However, despite compliance with ACEi therapy, plasma AII levels were elevated in patients with CHF. In contrast, patients with CHD were not

hyper-reninaemic and had lower AII levels than patients with CHF. These data are consistent with other studies in CHF in demonstrating failure of suppression of AII despite ACEi therapy and might be due to the presence of alternative enzyme pathways. However, they should be interpreted with caution, since plasma AI was not measured.[282] In the assays used in this study there is an approximately 1% cross-reactivity between AI and AII. Thus apparent failure to suppress AII could be due to very high AI levels, detected in the AII assay.

Myography Data: In arteries from CHD the effects of enalaprilat and chymostatin on the dose-response curve to AI were qualitatively very similar to those described in Chapter 3 for normal volunteers. Neither enalaprilat nor chymostatin significantly inhibited the response to AI, but there was a pronounced trend towards inhibition in the combination group. These results are consistent with those of Voors et al, who also described a dual pathway for AII generation in internal mammary arteries taken from patients with CHD, and they also extend my previous findings in small resistance arteries from healthy volunteers to patients with CHD.[210]

Collectively, the available data in healthy volunteers and patients with CHD, show that neither an ACE inhibitor alone, nor a chymase inhibitor alone, substantially inhibit the conversion of AI to AII in small arteries, medium sized arteries or veins. Only the combination of an ACE inhibitor and a chymase inhibitor effectively blocks AI to AII conversion. These findings imply that both the limbs of the dual pathway for conversion of AI to

AII have a high capacity and that substrate can normally be rapidly and completely shunted through one limb, should the other be blocked.

It is important to note that the response of small resistance vessels to AII was not affected by enalaprilat, chymostatin or the combination of the two in arteries from patients with either CHF or CHD. Similarly the responses to NE in the presence or absence of the inhibitors were very similar. Thus there is no evidence that potentiation of the action of vasodilator substances by any of these inhibitors indirectly influences the response to AI. The responses to AII do, however, merit further consideration. Figure 5.7 shows the response to AII in the absence of inhibitors in arteries from each patient group. The response to AII was enhanced in arteries from patients with CHF. Stephens et al studied the response to AII and NE in a subset of patients with impaired left ventricular function after MI.[274] These patients were randomised to receive ramipril or placebo as part of the AIRE trial.[275] They showed that the maximum response to AII was enhanced in patients who received the ACE-inhibitor, compared to patients who received placebo, or normal controls. In contrast, while there was no difference in the maximum response, arteries from patients who received ramipril exhibited greater sensitivity to NE. Therefore, they hypothesised that the increased response to AII was due to an interaction between NE and AII. My findings confirm the exaggerated response to AII, but I did not show an altered sensitivity to NE.

In both CHF and CHD subjects, there was a pronounced trend towards potentiation of dilator responses to bradykinin in the presence of enalaprilat. This was consistent with the effect of enalaprilat on bradykinin responses described in chapter 3. Chymostatin did not appear to enhance the effect of enalaprilat, suggesting that chymase plays little, or no, role in the degradation of kinins in human resistance arteries.

I also showed that vessels from patients with CHF and CHD exhibited a vasodilator response to ACh. There was no apparent difference between the patient groups, suggesting that endothelial function in CHF does not differ from CHD. However, in the absence of a normal control group, it is not possible to make any inference about vascular endothelial function in these patients with cardiovascular disease compared to healthy subjects. Our results do raise the possibility that previous reports of endothelial dysfunction in CHF may in fact reflect endothelial dysfunction in coronary heart disease rather than in CHF per se. Neither enalaprilat nor chymostatin had any apparent effect on responses to ACh.

In contrast to the responses seen in arteries from CHD patients, the response to AI in arteries from patients with CHF proved to be quite different. Here enalaprilat significantly inhibited the response to AI, an effect that was possibly enhanced in the presence of chymostatin. As in previous experiments, chymostatin alone had no effect on AI-mediated responses. Thus, contrary to my original expectation, I did not find "up-regulation" of the chymostatin sensitive pathway in these subjects and instead, found that

the small resistance arteries from these patients were more sensitive to ACE inhibition than those from healthy volunteers or patients with CHD. It is unlikely that this finding is due to the minor age and therapy differences between CHF and CHD patients. There are, however, at least three potential explanations.

The first possibility is that the chymostatin sensitive non-ACE pathway is actually "down-regulated" in CHF patients treated with an ACE inhibitor. Why this should occur is not clear, though it could occur if the ACE pathway is "up-regulated". The second possibility, that ACE activity is induced, could also explain our findings in CHF and it is known that both CHF and ACE inhibitor treatment, experimentally, can induce ACE. A third possibility is that tissue ACE is not fully inhibited when arteries are incubated with enalaprilat for 30 minutes. The inhibition of AI responses in the presence of enalaprilat, observed in patients with CHF, might then reflect the effect of residual ACE-inhibition due to their medication, enhanced by adding the inhibitor in the bath. This explanation seems unlikely because the experiments with BK clearly demonstrated that the addition of enalaprilat to the bath exerted a physiological effect. In addition, if the enalaprilat in the bath failed to inhibit vascular ACE, then the response to AI in the presence of the combination of chymostatin and enalaprilat should not have been different from chymostatin alone.

The results of the experiments described here cannot be due to altered ACE activity alone. If there is an alternative pathway for AII generation, AII

should continue to be generated by this enzyme (or enzymes) in the presence of an ACE-inhibitor. My findings, therefore, imply that the chymase pathway is "down-regulated" alone, or in conjunction with induction of ACE, in CHF.

These findings differ from those obtained by Wolny et al, who studied the responses to AI in coronary arteries from patients with CHF treated with ACE inhibitors.[204] In these vessels cilazaprilat did not inhibit the response to AI, but chymostatin did inhibit it by 78%, and the combination of chymostatin and cilazaprilat inhibited the response by 97%. Thus, it would appear that in coronary arteries the chymase pathway is predominant, though the incremental inhibition obtained by combining chymostatin and cilazaprilat suggests that some of the conversion of AI to AII is mediated by ACE. It is possible that the results obtained by Wolny are due to a difference in the physiology of coronary arteries compared to resistance arteries - and reflect the relative contribution of ACE and chymase to AII generation in these tissues. However, in Wolny's experiments the effect of the inhibitors was only studied at 1μM AI, which is supra-physiological, and not over a whole concentration range. It is therefore difficult to make a direct comparison between their results and mine.

One limitation of the study presented here is that I was not able to distinguish between the effect of ACE inhibition and the presence of CHF *per se* i.e. all the CHF patients were also on an ACE inhibitor. This question could be resolved in two ways: one approach might be to study arteries obtained from ACE inhibitor-naïve patients with CHF and a second would be

to study arteries from CHD patients pre and post ACE-inhibition. Despite this limitation, however, these findings are important, since they suggest that physiological escape from ACE inhibition can occur in a relevant clinical situation.

Even though the chymase pathway may be less active in CHF, I did find an incremental and statistically significant inhibition of AI to AII conversion with the combination of chymostatin and enalaprilat compared to enalaprilat alone in these patients. This finding provides a rationale for using drugs which inhibit the action of AII directly in the treatment of patients with CHD and CHF; an alternative, and potentially worthwhile approach, would be the development of agents that inhibit non-ACE AII generating pathways.

# Chapter 6

# Organ bath preparations of internal mammary arteries from patients with coronary heart disease

## 6.1 Introduction

In previous chapters I have examined the responses to AI in human resistance arteries from normal subjects, patients with CHF and patients with CHD. As discussed in section 1.4, ACE-independent AII generation has been shown in a number of animals and in a variety of tissues. In man non-ACE AII generation has been demonstrated in the heart and in the vasculature. The aim of the study presented in this chapter was to investigate whether non-ACE AII generation occurs in human medium-sized arteries. Accordingly I studied the responses to AI in human internal mammary arteries which were readily available from cardiac theatre.

## 6.2 Patient selection

Human internal mammary arteries (IMAs) were obtained from male and female patients undergoing coronary artery bypass grafting (CABG). Vessels were obtained from cardiac theatre and were segments discarded after the procedure had finished. Since the use of the vessels was at the discretion of the surgeon, it was not possible to select patients in advance. There were, therefore, no specific exclusion criteria and patients with both hypertension and diabetes mellitus were included. Similarly it was not possible to screen the patients for left ventricular dysfunction in advance and this (and other) clinical information was derived from the case notes (comments about LV

function were recorded at the time of cardiac catheterization). Approval to use the discarded IMAs was granted by the Hospital Ethics Committee. These experiments were performed by Dr Mark Petrie, as part of a collaborative study with myself. Protocols were based on my previous work, as described above, and it was agreed in advance that the study would be included in this thesis.

## 6.3 Human internal mammary artery preparation

IMAs were dissected from the thoracic wall by the surgeon, using a no touch technique, leaving the vessels surrounded by internal thoracic fascia. The discarded distal end of the IMA (1-2cm) was carefully removed and placed in ice-cold physiological salt solution (PSS), and the vessels were immediately transferred to the laboratory.

The vessels were cleaned of connective tissue and cut into four 2-3mm long segments. Rings were suspended on wires in 10ml organ chambers filled with physiological salt solution (PSS), maintained at $37^0$C, and aerated with a 95% $O_2$-5% $CO_2$ mixture. The rings were connected to force transducers, and changes in isometric tension were recorded. The PSS (pH 7.4+/-0.1) had the following composition (in mM): NaCl 130, KCl 4.9, $NaHCO_3$ 14.9, $KH_2PO_4$ 1.18, glucose 5.5, $MgSO_4.7H_2O$ 1.17, Ca $Cl_2..H_2O$ 1.6, EDTA 0.03, indomethacin 0.02, dissolved in DMSO.

## 6.4 Experimental protocol

After an incubation period of 45 minutes vessels were initially activated with phenylephrine (PE) $3\times10^{-6}$M and subsequently with KCl 80mM. Vessels were then incubated for a further 30 minutes in either PSS (control, vessel 1) or with enalaprilat $10^{-6}$M (vessel 2), chymostatin $10^{-5}$M (vessel 3) or both enalaprilat $10^{-6}$ and chymostatin $10^{-5}$M (vessel 4). Cumulative concentration response curves were then performed to AI from 0.01 nM to 3 μM.

## 6.5 Patient Characteristics

Internal mammary arteries were obtained from patients undergoing coronary artery bypass surgery at the Western Infirmary. Unused internal mammary arteries were obtained from cardiac theatre at the discretion of the surgeon. No prior selection of patients studied was therefore made. Clinical data concerning the patients was taken from the case-notes retrospectively. Patient characteristics are shown in table 6.1. As can be seen from the table, patients formed a heterogeneous group. Left ventricular function was preserved in most patients and only one was receiving an ACE-inhibitor.

Table 6.1: Patient from whom internal mammary arteries were harvested for organ bath experiments

| **Number** | 11 |
|---|---|
| **Sex** | 7 males;4females |
| **Age(mean+/-SD)** | 58+/-11 |
| **Left ventricular function at cardiac catheterisation** | 9 "good"<br>1 "fair"<br>1 moderate LVD |
| **Previous MI** | 6 |
| **Hypertension** | 4 |
| **NIDDM** | 2 |
| **Drug therapy** | |
| Ace inhibitor | 1 |
| diuretic | 1 |
| digoxin | 0 |
| calcium channel blocker | 5 |
| nitrate | 8 |
| beta blocker | 8 |
| HMG CoA reductase inhibitor | 7 |
| aspirin | 10 |

SD – standard deviation; NIDDM – non insulin dependent diabetes mellitus; ACE – angiotensin converting enzyme; HMG CoA – 3-hydroxy – 3 – methylglutaryl coenzyme A; LVD – left ventricular dysfunction.

## 6.6 Responses to AI in human internal mammary arteries

AI induced a dose-dependent contraction in human internal mammary arteries, which was qualitatively similar to that observed in human resistance arteries. In the absence of inhibitors, the threshold was 1.61 (1.25) x$10^{-8}$M, the maximum response occurred at 0.1μM and the EC50 was 0.63 (0.51) x$10^{-7}$M. Marked tachyphylaxis was again observed. Compared to those in resistance vessels, the responses observed in these experiments were extremely variable. Thus, responses were again expressed as the % response to KPSS as an internal control.

Responses to AI in the presence of enalaprilat, chymostatin and the combination are shown in figure 6.1. EC50s, threshold concentrations, maximum responses and AUC values are shown in table 6.2. Neither enalaprilat nor chymostatin alone inhibited the response to AI. In fact, there appeared to be a greater maximum response in the presence of both of these inhibitors. In the case of enalaprilat there appeared to be a larger maximum response, with little difference in the EC50. However, neither the maximum response nor the EC50 were significantly different to control ($p>0.05$ by ANOVA). Similarly, although the response in the presence of chymostatin was greater than in control vessels, this was not significant. The combination of chymostatin and enalaprilat had very little effect on the maximum response compared to control but did induce a shift of the dose-response curve to the right, with an EC50 of 8.55 (6.37) x$10^{-7}$M and a threshold of 8.04 (10.5) x$10^{-}$

$^{7}$M. Although there was no significant difference in the EC50 ($p>0.05$ by ANOVA), the threshold concentration was significantly higher ($p<0.01$ by ANOVA). When AUC was used to compare the responses, only the combination appeared to induce significant inhibition ($p = 0.0029$).

Figure 6.1: Response to AI in human internal mammary arteries: effect of ACE and chymase inhibition

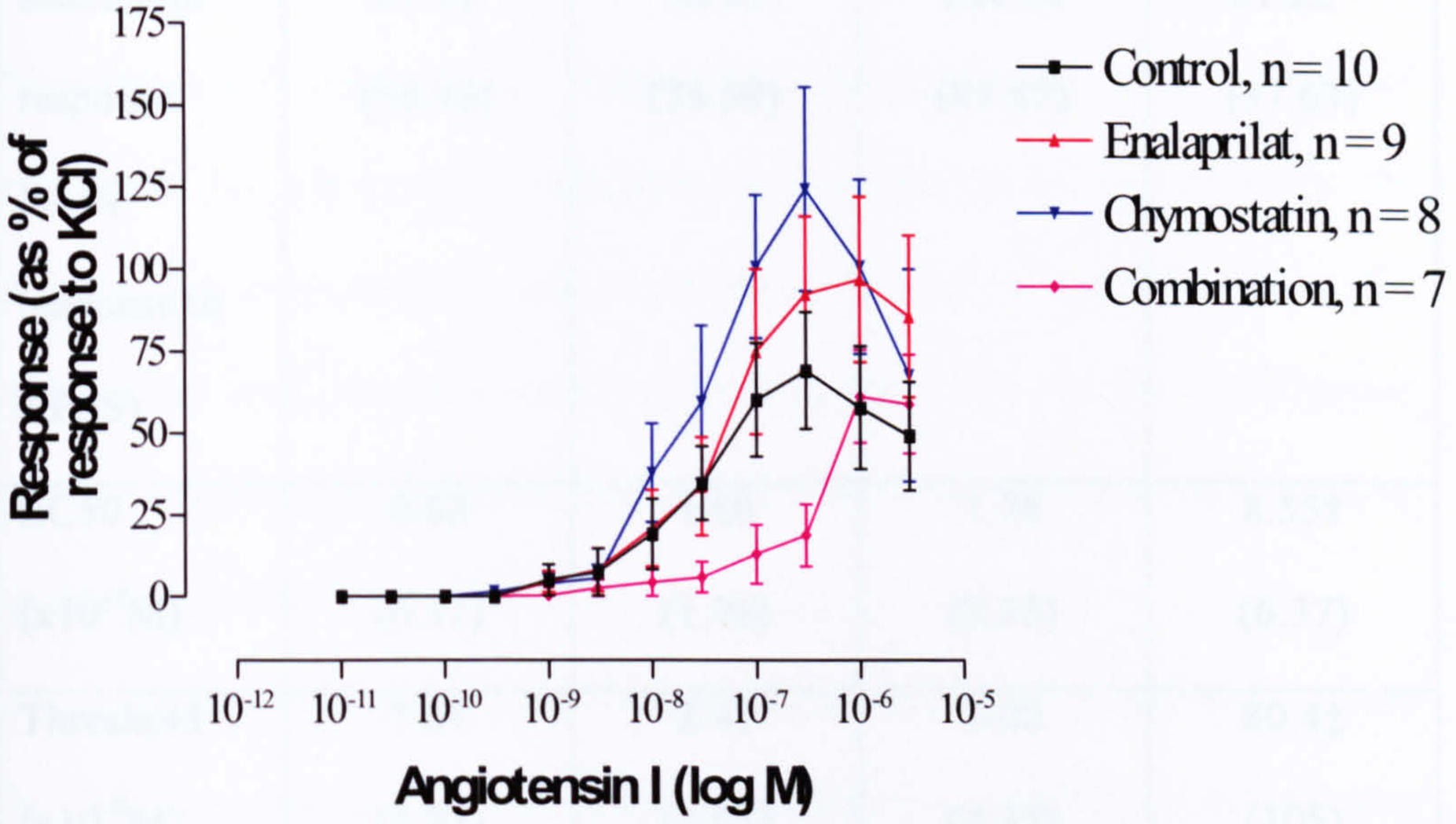



Figure 6.1 shows the response to AI in human internal mammary arteries expressed as the % of response to activation with KCl, 80mmol/l. Neither enalaprilat nor chymostatin alone inhibited the response to AI, however the combination significantly increased the threshold concentration and reduced the AUC ($p<0.01$ by ANOVA, $p=0.0029$).

Table 6.2: Potency, maximum response and AUC to AI in internal mammary arteries from human subjects with CHD

| Means ±SD | Control | Enalaprilat | Chymostatin | Combination |
|---|---|---|---|---|
| Maximum response (as % response to KPSS) | 69.11 (56.48) | 96.95 (75.59) | 124.44 (87.87) | 61.22* (37.63) |
| EC50 ($x10^{-7}M$) | 0.63 (0.51) | 1.68 (1.76) | 1.78 (3.35) | 8.55† (6.37) |
| Threshold ($x10^{-8}M$) | 1.61 (1.25) | 2.47 (3.07) | 3.02 (4.33) | 80.4‡ (105) |
| AUC | 138.61 (135.35) | 184.03 (135.35) | 233.54 (165.74) | 67.24⇧ (61.44) |

Table 6.2: Potency and maximum response to AI in human IMAs. All data is mean ± (SD).

*p = ns compared to control

† p>0.05 compared to control

‡p<0.01 compared to control

⇧ p = 0.0029 compared to control

## 6.7 Discussion

The aim of the experiments presented in this chapter was to investigate whether non-ACE AII generation occurs in medium-sized arteries from human subjects. In these vessels, as in resistance arteries, AI induced a dose-dependent contraction, suggesting conversion to AII. Neither enalaprilat nor chymostatin inhibited the response to AI. In contrast, the combination of enalaprilat and chymostatin induced a marked shift to the right of the dose-response curve. Although the EC50 for the combination was not significantly different from control, the threshold concentration was higher and the AUC was lower.

These experiments were limited by the variability of the responses to AI. This may account for the apparent increase in the maximum response to AI in the presence of enalaprilat and chymostatin (which did not reach significance).

Thus, the effect of ACE and chymase inhibition on the response to AI was qualitatively similar in internal mammary arteries to that observed in resistance arteries. These results are similar to those of Voors et al, who studied the effect of captopril and chymostatin on AI-mediated responses in human internal mammary arteries.[210] In their experiments both AI and the ACE-dependent AI analogue, [$Pro^{10}$]-AI, elicited a contraction that was abolished by irbesartan, conforming dependence on activation of the AT1R. Captopril completely inhibited [$Pro^{10}$]-AI responses and increased the

threshold concentration to AI three-fold. The maximum response to AI in the presence of captopril was slightly higher than control – similar to the data presented in this chapter. Chymostatin had a slight inhibitory effect on AI responses (reducing the maximum response and increasing the threshold). The combination of captopril and chymostatin induced a much greater inhibition of AI responses than either agent alone.

The results of the experiments presented in this chapter, therefore, confirm that a dual pathway for AII generation exists in human internal mammary arteries *in-vitro*. Internal mammary arteries are an order of magnitude larger than resistance arteries and are more proximally placed in the arterial tree. These results may therefore suggest that the phenomenon of non-ACE AII generation may be generalised throughout the vasculature. However, as discussed in chapter 3, these data should be interpreted with caution, since there is a discrepancy between the results of *in-vitro* and *in-vivo* investigations. Further studies are required to determine the significance of non-ACE AII generation *in-vivo*.

# Chapter 7

# Identification of ACE and Chymase in human internal mammary arteries using immunohistochemistry

## 7.1 Introduction

In chapter 3 and chapter 5, I showed that the contractile response to AI in human resistance arteries and internal mammary arteries appeared to be due to generation of AII, mediated by a dual enzymatic pathway. In addition to ACE, an alternative enzyme appeared to be able to generate AII. This enzyme was susceptible to inhibition by chymostatin and, from the literature, is most likely to be chymase. However, the presence of chymase as an AII-generating enzyme in these experiments can only be inferred from the effect of pharmacological inhibition. These experiments therefore, do not unequivocally prove that chymase is the enzyme responsible for ACE-independent AII generation. In particular it is known that cathepsin G is able to generate AII from angiotensinogen directly and that this reaction is inhibited by chymostatin, aprotinin and α-antitrypsin.[208, 209, 304]

The demonstration of the presence of chymase in human blood vessels would provide additional evidence that this enzyme is responsible for vascular non-ACE AII generation. In addition, as discussed in chapter 3, the spatial localisation of chymase and ACE expression may be related to their physiological roles *in-vivo*. Urata has shown that in the human myocardium chymase is synthesised in mast cells, endothelial cells and mesenchymal cells and, on release, localises to the interstitium.[198, 199] In human saphenous veins, immunohistochemistry localises chymase to the adventitia, in association with mast cells, while ACE is found in endothelial cells and diffusely in the smooth muscle cell layer.[211] Chymase has not however,

## 7.1 Introduction

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been identified in human resistance arteries or internal mammary arteries. *In-vitro* experiments on non-ACE AII generation may over-estimate the significance of chymase because they take no account of its localisation. Thus AI introduced to the luminal surface of resistance arteries may be entirely converted to AII by ACE, with little or no contribution from chymase.

The aim of the study presented in this chapter was, therefore, to identify and localise chymase and ACE in human vascular tissue using immunohistochemistry (IHC). Dissected resistance arteries suitable for IHC were not available. Therefore the vessels studied were human internal mammary arteries. In addition, IHC was performed on skin samples taken with gluteal fat biopsies, hoping to identify ACE and chymase expression in arterioles or capillaries therein. Skin mast cells are known to express chymase at relatively high levels and so this tissue provided a positive control for this enzyme.[305] IHC was also performed in sections of normal kidney, kept as part of the collection in the pathology department. ACE is known to be heavily expressed in the kidney and mast cells have also been identified in the renal interstitium.[306]

## 7.2 Patient selection

Human internal mammary arteries (IMAs) and saphenous veins (SV) were obtained from male and female patients undergoing coronary artery bypass grafting (CABG) as described in chapter 6. As described above, the

vessels obtained were those left unused at the end of the procedure and again it was not possible to select the patients in advance. Patient details were thus obtained retrospectively from the case-notes and there were no specific inclusion or exclusion criteria.

Samples of skin used in this study were those taken at the time of gluteal biopsy from normal human volunteers. Details of patient selection are described in chapter 3. After dissection of resistance arteries, the remaining skin was cleaned of fat and divided into two; one sample was placed into liquid nitrogen to allow preparation of frozen sections and the other was placed into formaldehyde.

Sections of normal kidney were available from the collection in the pathology department. Samples used in these experiments were taken from a normal kidney intended for use in renal transplantation but not used for technical reasons.

## 7.3 Experimental protocol

Details of immunohistochemical methods are given in chapter 2 (appendix to methods). Briefly, two-step indirect immunohistochemistry was employed. Chymase and ACE were detected using mouse monoclonal antibodies visualised with standard immunoperoxidase or alkaline phosphatase techniques. Preliminary experiments were performed to identify the optimal dilutions of each antibody. These were performed in both frozen

sections and formaldehyde-fixed specimens. Immunohistochemical specimens were reviewed in association with Dr George Lindop.

## 7.4 Identification of chymase and ACE by IHC

Internal mammary arteries were taken from five patients who had undergone coronary artery bypass grafting. Patient characteristics are shown in table 7.1. Skin biopsies were taken from patients who were normotensive, non-diabetic men, as described in chapter 3.

Representative immunohistochemical preparations are shown in Figures 7.1-7.5. Neither ACE nor chymase were identified in formaldehyde-fixed preparations. However both were detected in frozen sections. These are illustrated in figures 7.1-7.5. Figure 7.1 (A) illustrates immunohistochemical staining for chymase in the kidney. Mast cells staining strongly for chymase were identified in small numbers, scattered throughout the interstitium. Figure 7.1 (B) illustrates staining for chymase in sections of human skin. Chymase was identified scattered throughout the dermis, within mast cells. However, chymase was also identified within mast cells, in close proximity to skin arterioles in the adventitia, as shown in figure 7.2 (A and B). Figure 7.3 illustrates the location of chymase and ACE in internal mammary arteries. Chymase was identified in the adventitia, in association with mast cells, with no staining within the medial layer (figure 7.3 [A]). Little endothelium was present in these sections and no staining for chymase was identified therein. Figure 7.3 (B) shows an internal mammary artery

stained for ACE. While staining for ACE was at a low level, there appeared to be a "blush" of staining throughout the medial layer, at levels too low to localise at a cellular level (figure 7.4 [A]). On a high-power view, the nuclei of endothelial cells were visible, with some staining for ACE possibly seen also. In the skin, ACE was identified within the stratified squamous epithelium and in association with apocrine glands and capillaries. ACE could also be seen on the luminal side of capillaries (figure 7.5 [A]). No vessels resembling resistance arteries were seen in these sections. A representative negative control is shown in figure 7.5 (B).

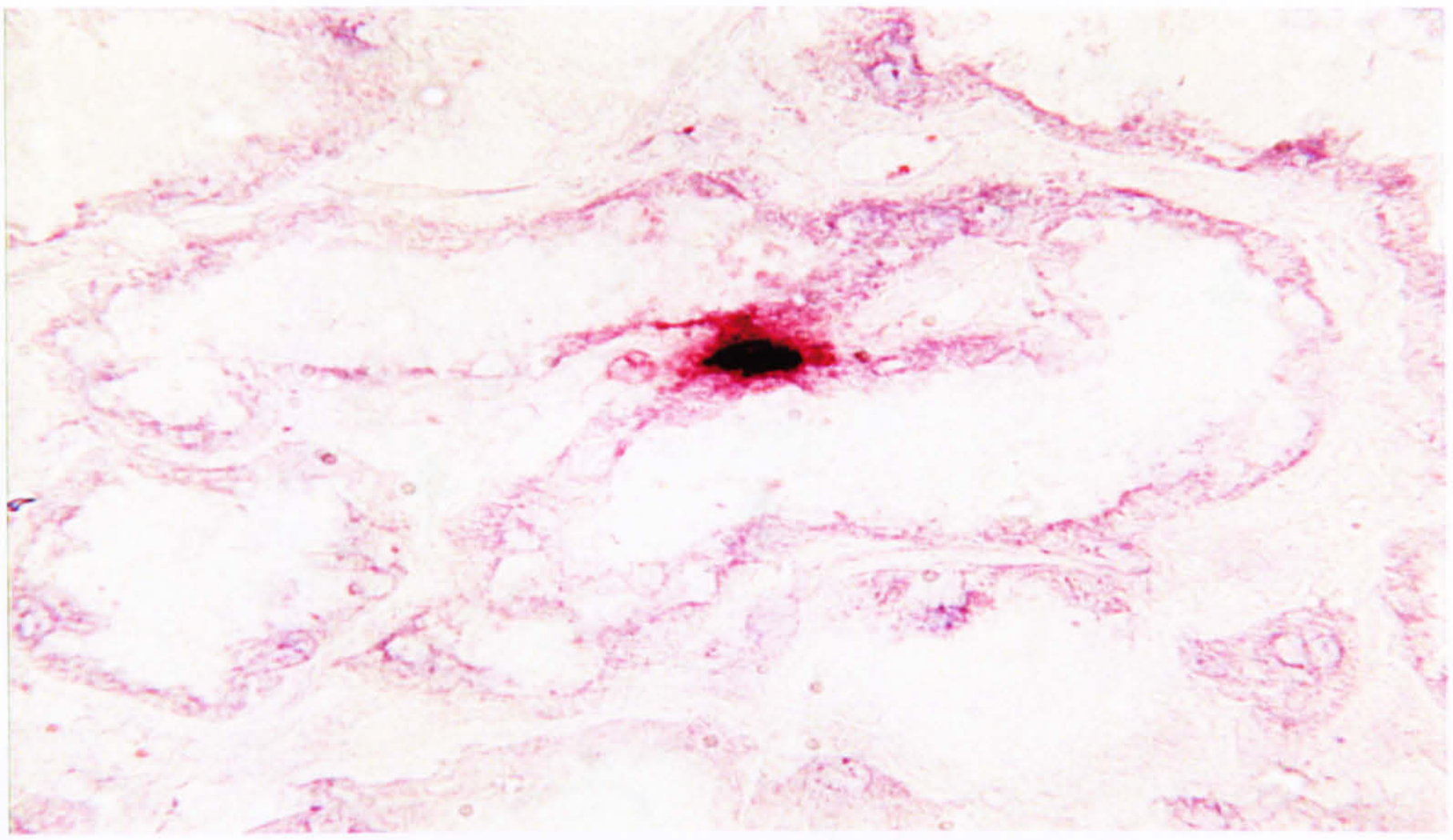
Table 7.1: Patients from whom internal mammary arteries were harvested for IHC

| Patient number | 5 |
|---|---|
| Sex (male:female) | 2:3 |
| Age at time of bypass | 67.6 (8.8) |
| Blood Pressure | 151.4/75.0 (16.1/12.2) |
| Creatinine | 83.2 (6.0) |
| Glucose | 5.1 (0.6) |
| Cholesterol | 6.4 (1.7) |
| HMG Co A reductase inhibitor | 3/5 |

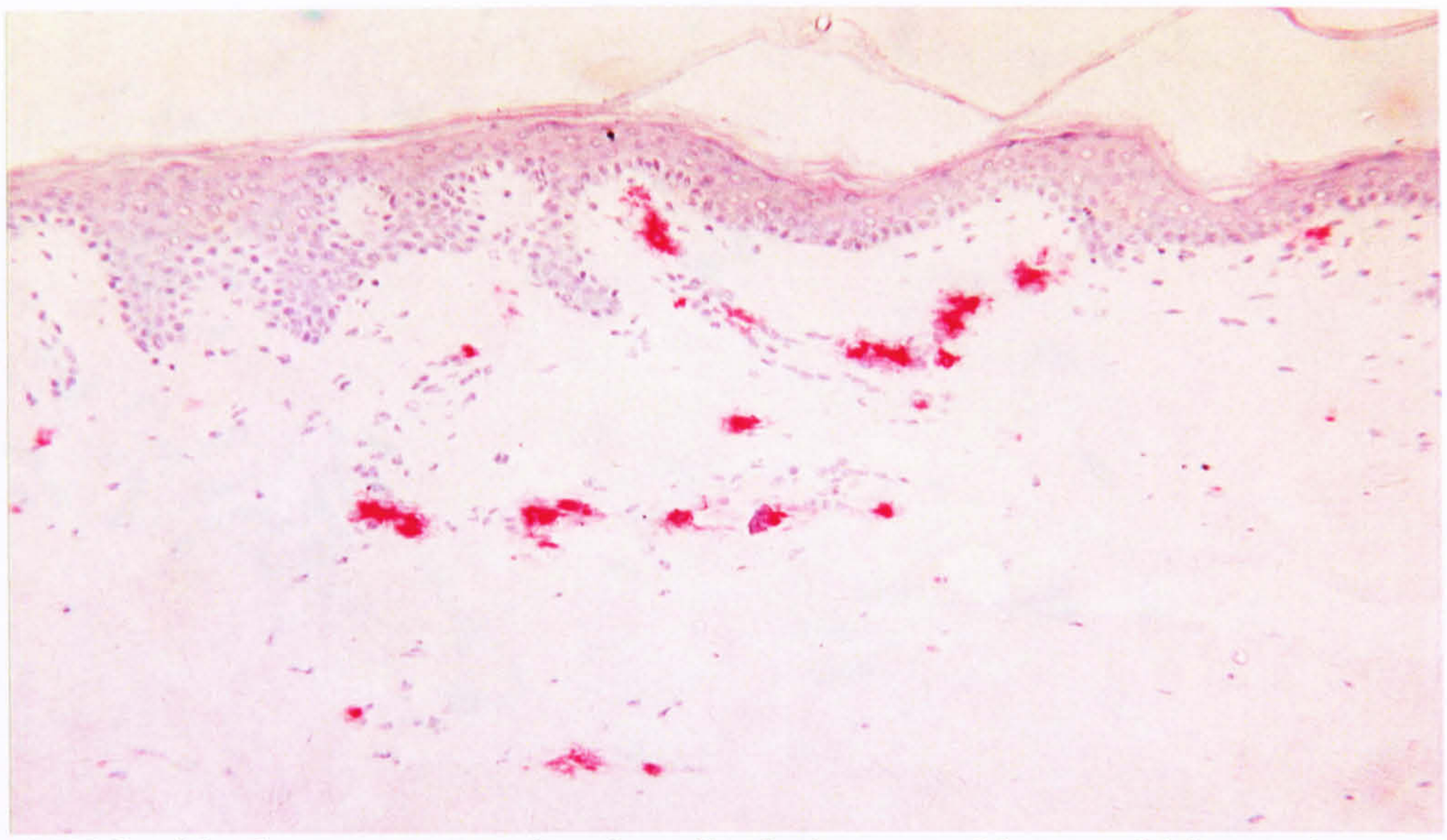
Table 7.1 describes patients from whom internal mammary arteries were harvested. All values are given as mean (± SD). All patients were receiving a β-blocker and aspirin. None were diabetic. Serum biochemistry and blood pressure values were those taken pre-operatively, as recorded in the case-sheet.

Figure 7.1: Immunohistochemistry for chymase in the kidney and skin

A



B

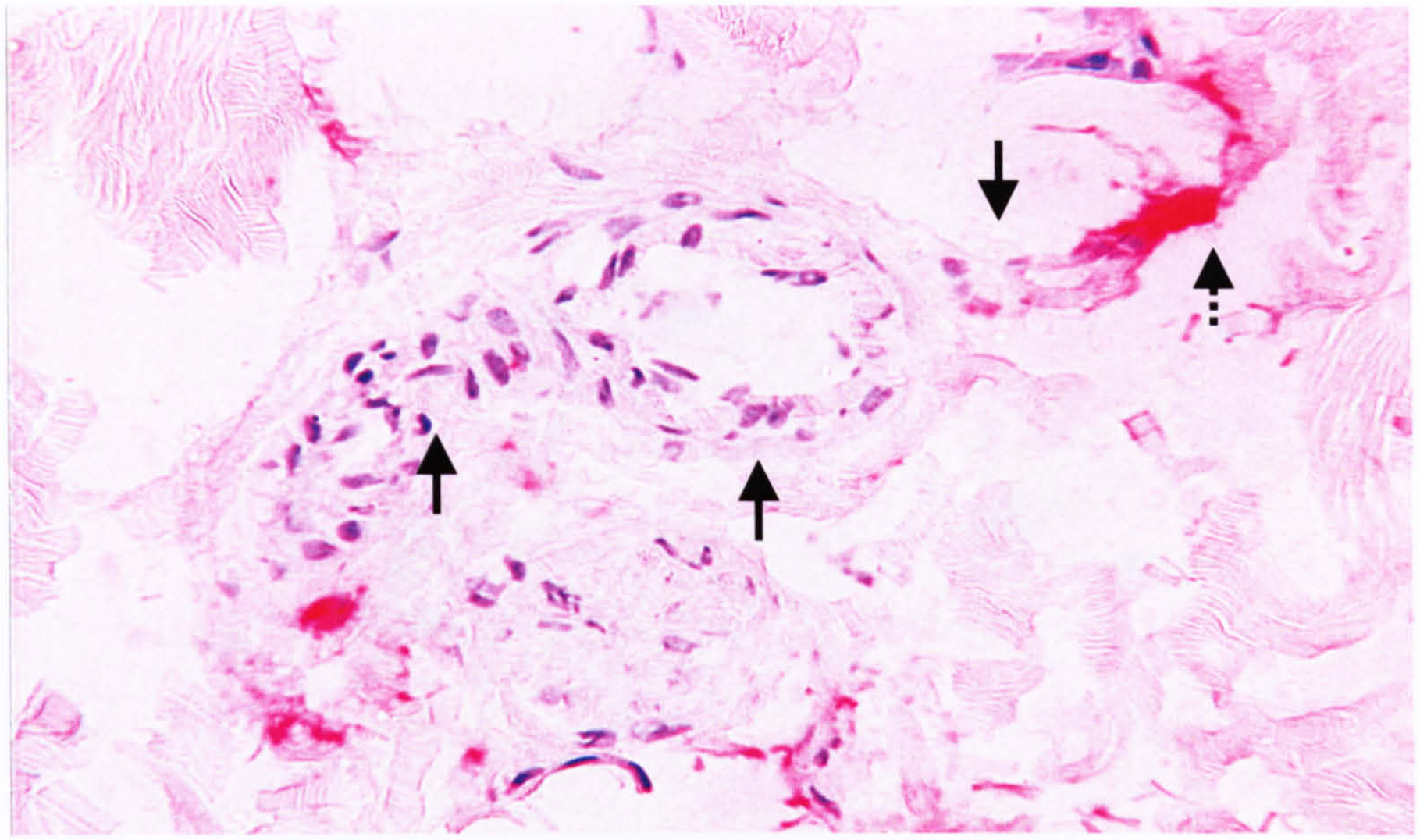


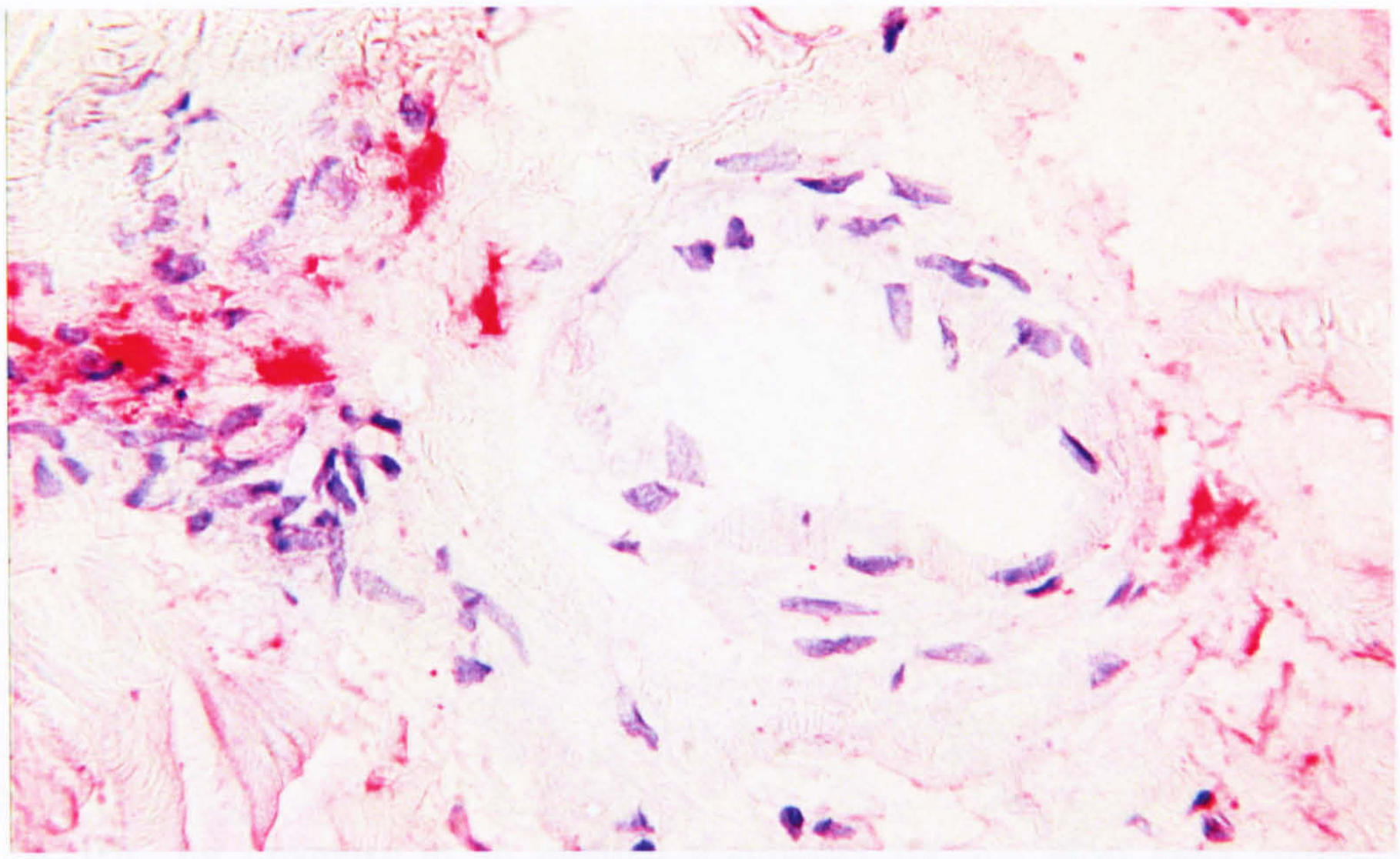
A) IHC with chymase monoclonal antibody in normal human kidney. A mast cell is seen in the interstitium (immunoperoxidase method; 1:400 dilution of primary antibody)

B) IHC with chymase monoclonal antibody in gluteal skin from a normal volunteer. Mast cells are seen in the dermis (alkaline phosphatase method; 1:1000 dilution).

Figure 7.2: Immunohistochemistry for chymase in human skin

A





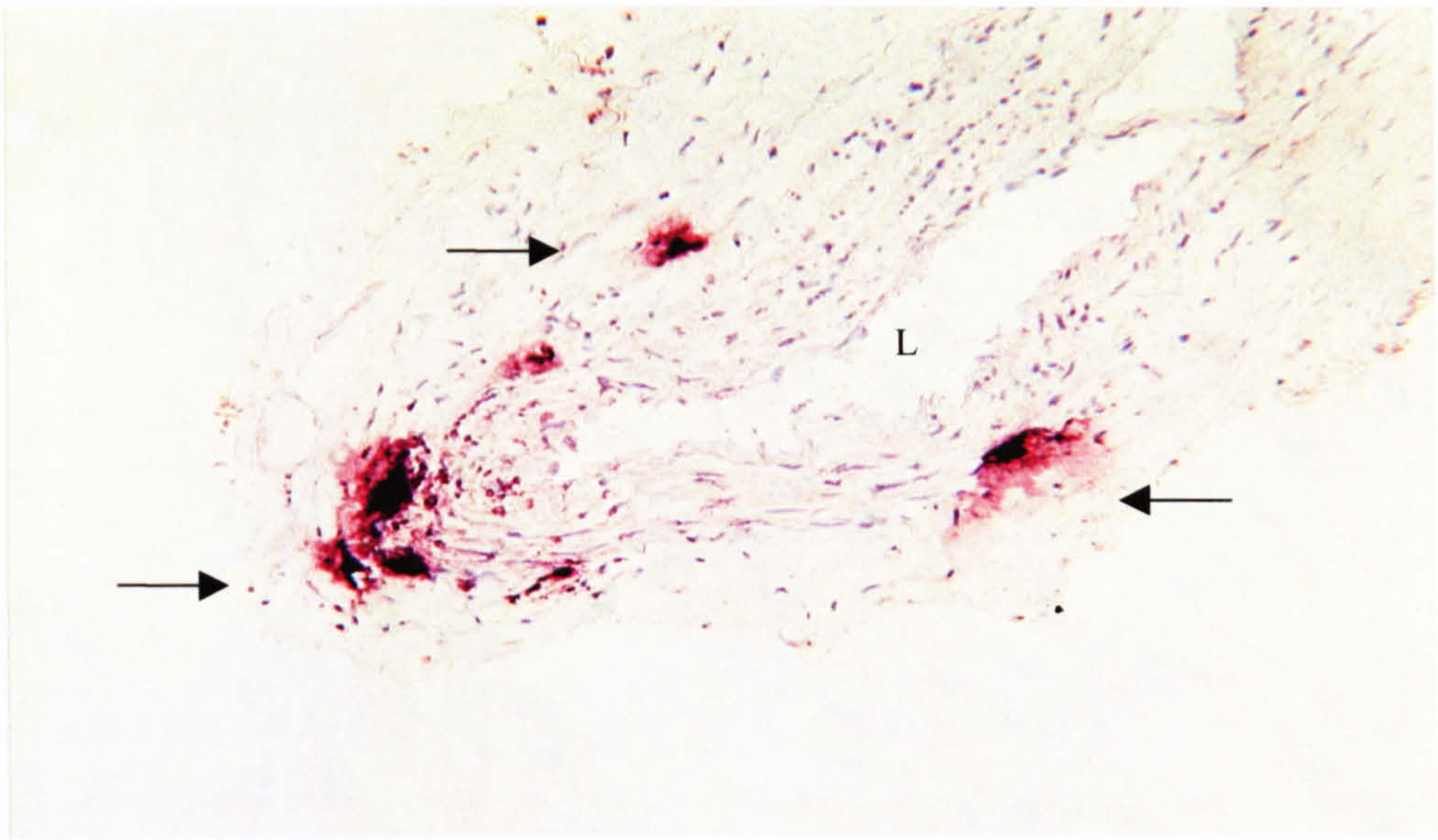
B

A) IHC for chymase in normal gluteal skin. An arteriole is seen with branches (arrowed). Chymase is seen within adventitial mast cells (broken arrow).

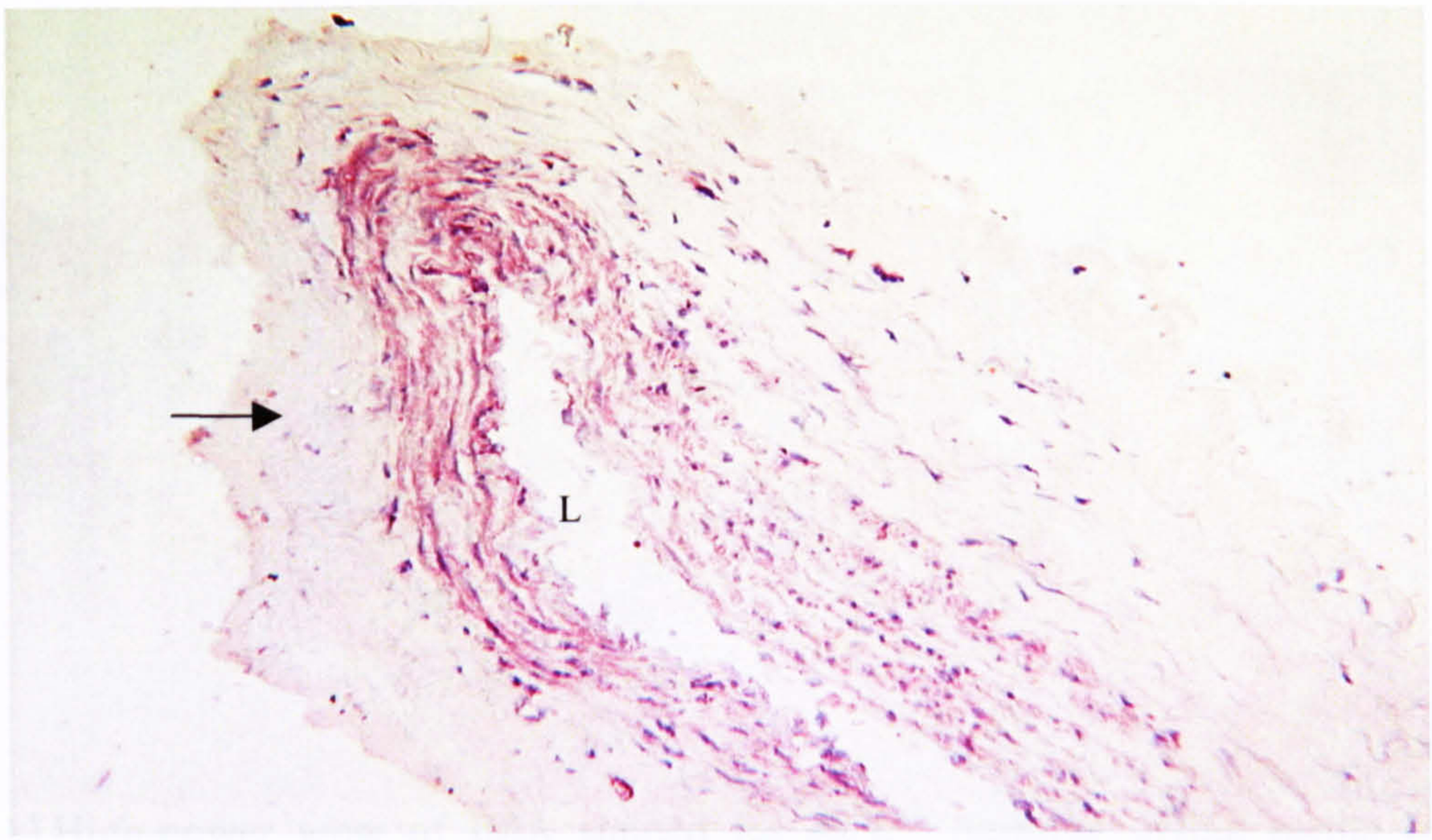
B) IHC for chymase in normal gluteal skin. An arteriole is seen with adventitial mast cells stained for chymase (both alkaline phosphatase method with 1:1000 dilution of primary antibody)

Figure 7.3: Immunohistochemistry for chymase and ACE in internal mammary arteries

**A**




**B**

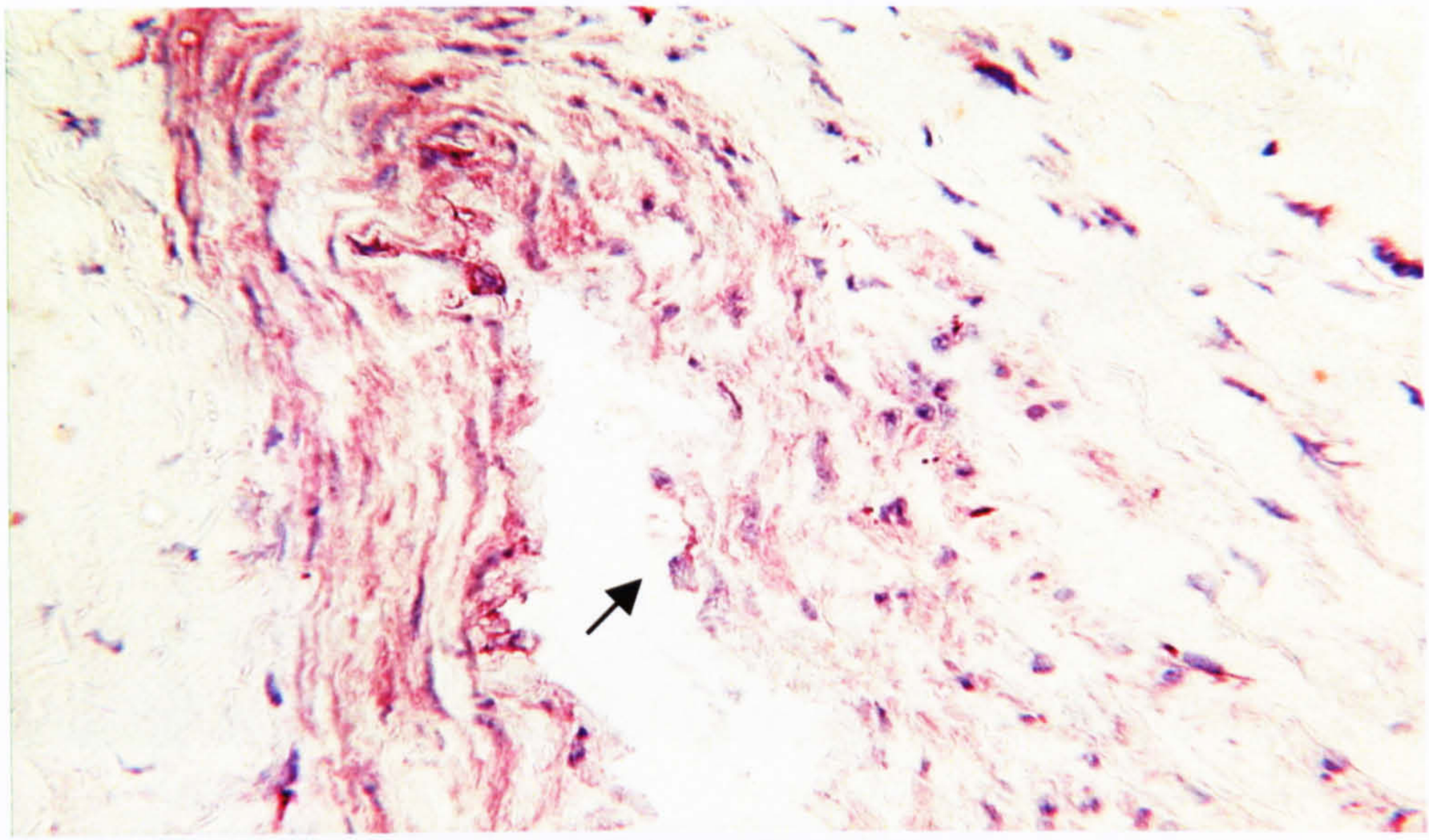



A) IHC for chymase in an internal mammary artery. Mast cells (arrows) stained for chymase are visible in the adventitia, but not the media (1:400 dilution)

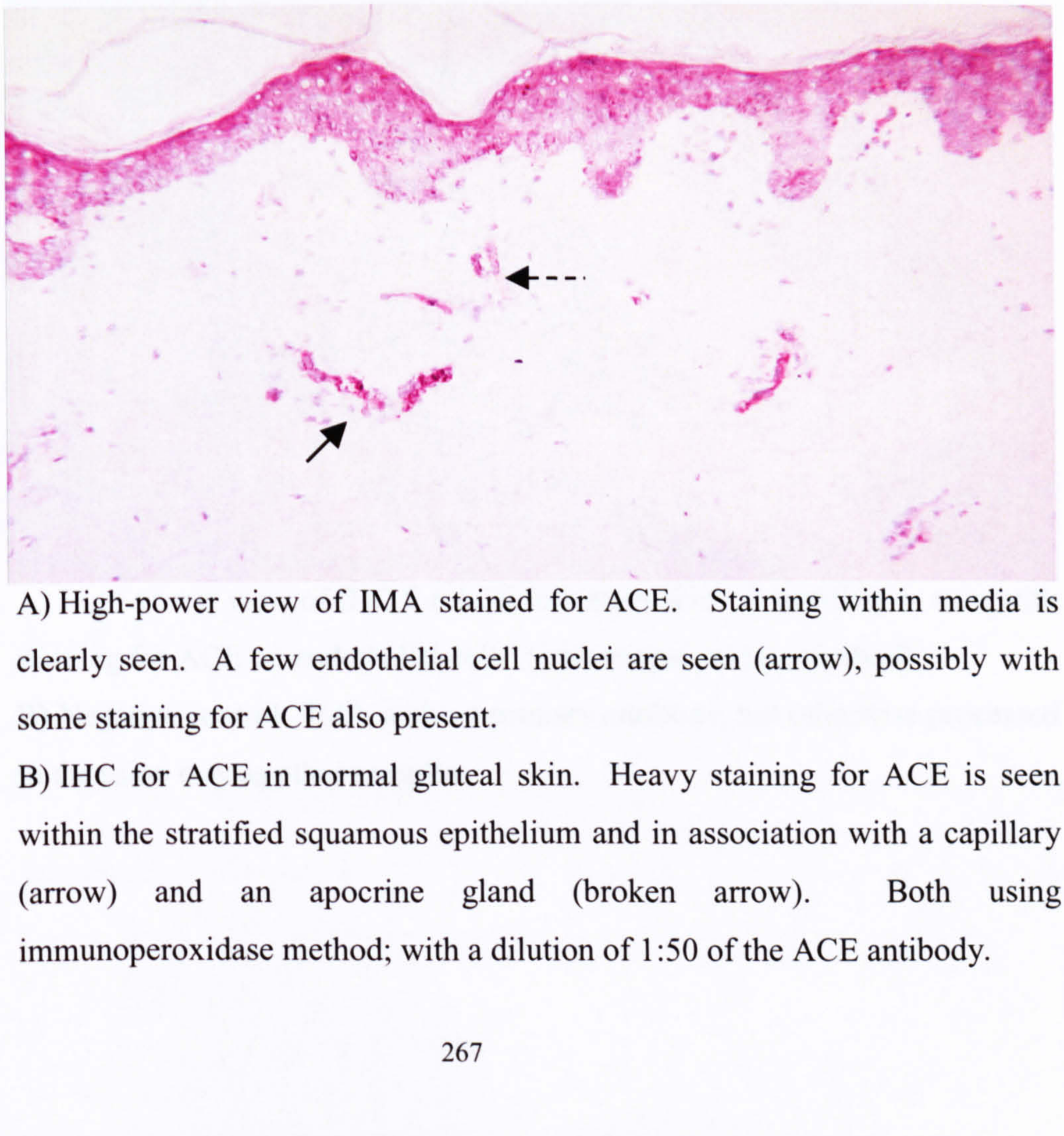
B) IHC for ACE in an internal mammary artery. A "blush" of staining for ACE is visible in the media (arrow), but not the adventitia. The endothelium is not intact (both samples by immunoperoxidase method; ACE at dilution of 1:100). L = lumen.

Figure 7.4: Immunohistochemistry for ACE in the internal mammary artery and skin

**A**



**B**

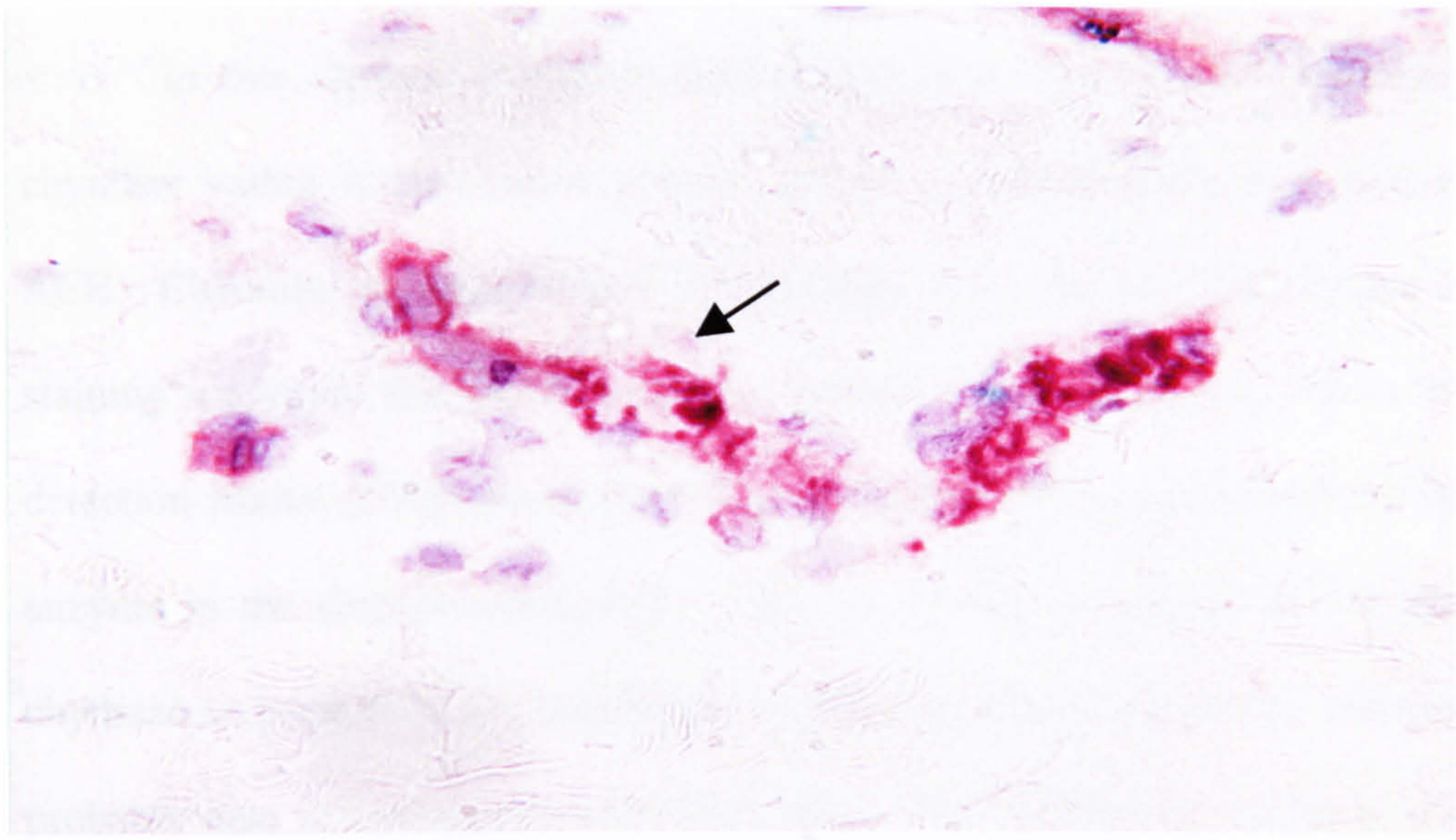


A) High-power view of IMA stained for ACE. Staining within media is clearly seen. A few endothelial cell nuclei are seen (arrow), possibly with some staining for ACE also present.

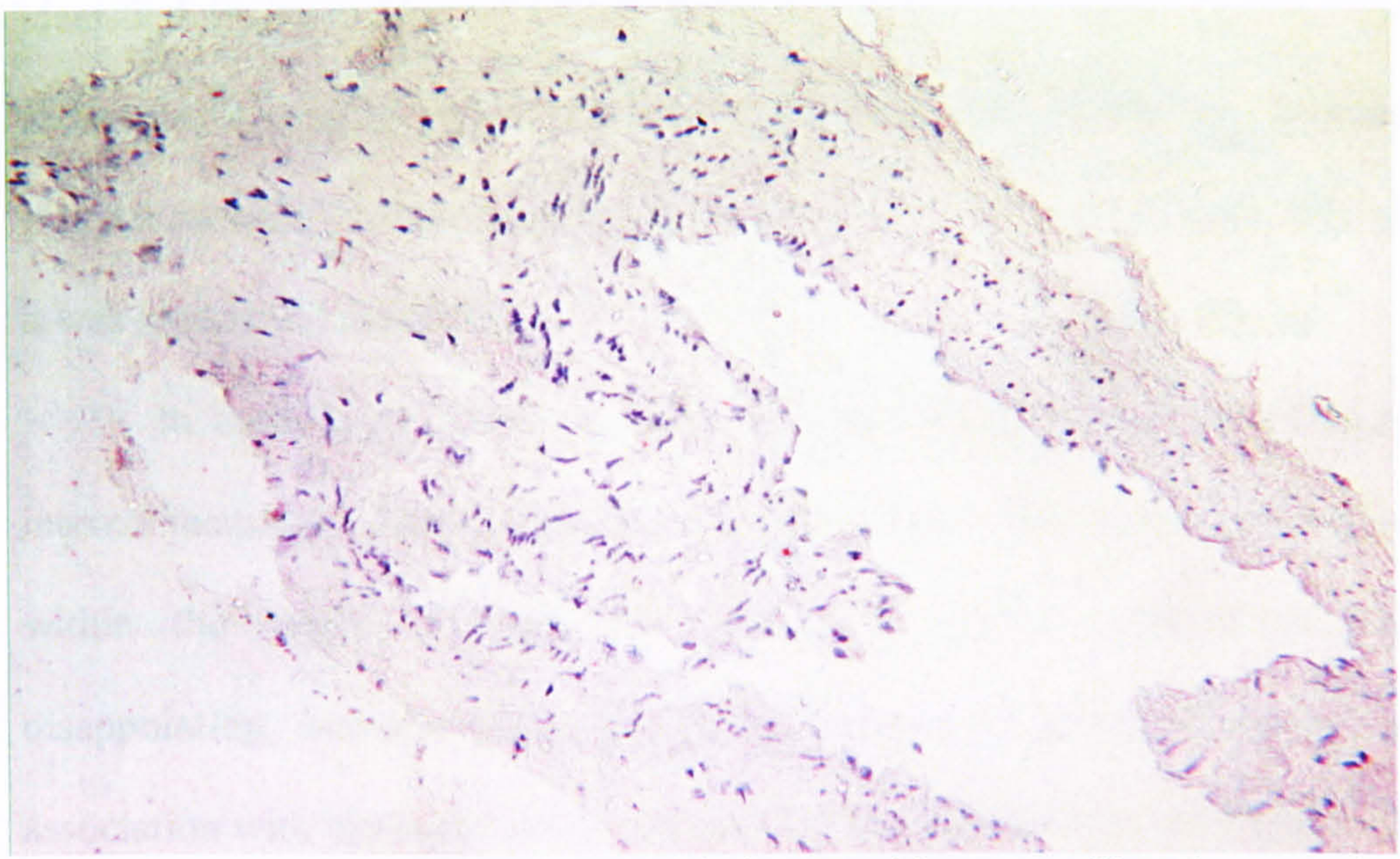
B) IHC for ACE in normal gluteal skin. Heavy staining for ACE is seen within the stratified squamous epithelium and in association with a capillary (arrow) and an apocrine gland (broken arrow). Both using immunoperoxidase method; with a dilution of 1:50 of the ACE antibody.

Figure 7.5: Immunohistochemistry for ACE in the skin and negative control

**A**



**B**



A) High-power view of IHC for ACE in human skin. A capillary is seen with staining for ACE on endothelial cells. (immunoperoxidase method)

B) Negative control. IMA without primary antibody, but otherwise processed in the same way as other samples.

## 7.5 Discussion

In this chapter immunohistochemistry was used to try to identify chymase within human tissue and to compare its distribution with that of ACE. Chymase was identified in human skin, as expected. The pattern of staining suggested that the enzyme was located in mast cells and, within the detection limits of this study, there did not appear to be expression of the enzyme in the dermal interstitium. The key finding presented here is that chymase is present in the adventitia of human internal mammary arteries, probably also in association with mast cells. No staining for chymase was identified in the media. Unfortunately, the absence of intact endothelium means that it is not possible to exclude expression there. However, chymase was not identified on the luminal surface of small skin blood vessels, though it was seen in the adventitia.

In contrast to chymase, ACE was not found in the adventitia of internal mammary arteries, but there appeared to be a low level of expression within the media. Again the absence of intact endothelium was disappointing, however there may have been a low level of staining in association with the remnants of endothelial cells. In the skin, ACE was seen on the luminal side of capillaries, in apocrine glands and in the squamous epithelium.

The results of this study are descriptive and are limited by poor tissue preparation. The absence of endothelium probably reflected tissue handling and the long delay between removal of the vessel and placement to liquid

nitrogen. This severely limits the conclusions that can be drawn. In particular it is not possible to comment definitively on the presence or absence of chymase in the endothelium, though it appears unlikely. The identification of chymase within the adventitia is consistent with previous studies.[198, 211] The failure to identify chymase, except in association with mast cells may be due to the sensitivity of the technique. It is noteworthy that Urata identified interstitial chymase using immunogold precipitation and electron microscopy.[198] Thus, I cannot exclude the presence of chymase, below the detection threshold for this technique, within the vascular interstitium.

It was also disappointing that identification of ACE and chymase was not possible in formaldehyde-fixed sections. Had this been possible these experiments could have been repeated on a wider range of specimens within the departmental collection. This problem was probably due to antigen unavailability after the fixation procedure and may be solved in future by antigen-retrieval techniques, such as heating the tissue or partial trypsin digestion. Furthermore, no attempt was made to quantitate ACE or chymase expression and so no conclusions can be drawn as to their regulation in these tissues. For this molecular techniques would be more suitable, though semi-quantitative immunohistochemistry is possible. Of particular interest is the question of whether ACE-inhibition leads to up-regulation of ACE and/or down-regulation of chymase, as suggested in chapter 5. The regulation of ACE and chymase in disease states would also be of interest, since there is

some evidence that chymase expression is increased in hypercholesterolaemia.[295]

Despite these reservations, both ACE and chymase were identified in human vascular tissue. The localisation of the enzymes was consistent with previous studies and suggests that the two enzymes may have different roles. Chymase is a strong candidate as the enzyme responsible for non-ACE AII generation in the human vasculature identified in previous chapters. The functional significance of this requires further investigation using a combination of physiological and molecular techniques.

# Chapter 8

# Final Discussion

## 8.0 Final discussion

Although it is now over one hundred years since the first demonstration of the pressor action of AII, the RAS remains pivotal to our understanding of the regulation of the cardiovascular system. During this time the biology of the RAS has been studied in great detail. These investigations have culminated in the development of drugs that interrupt the system at key points. Following a number of large clinical trials, the use of ACE inhibitors is now virtually mandatory in the treatment of CHF and these drugs are widely used in the treatment of hypertension and renal disease.[163, 164] To these have been added angiotensin receptor antagonists, which are now being investigated in a number of disease states.[167] Use of the aldosterone antagonist, spironolactone, is also increasing, following the demonstration of its efficacy in severe CHF.[307] Since AII stimulates aldosterone secretion, this constitutes a form of functional inhibition of the RAS.

Despite these facts there are still many areas of controversy concerning the RAS. In particular, there is uncertainty about whether there is a local RAS regulated independently of the systemic RAS. Related to this issue is the question of whether there is significant AII generation by tissue-based pathways other than ACE and, if so, whether they are induced when the RAS is subject to pharmacological interruption. It is also unclear to what extent genetic variation alters the physiology of the RAS and whether this has

pathophysiological significance – for example in determining cardiovascular risk or contributing to the development of essential hypertension.

The studies presented in this thesis have concentrated on the actions of AI and AII in the human vasculature and particularly in human small arteries. There were a number of reasons for choosing this tissue. Regulation of the peripheral resistance contributes to the control of blood pressure and abnormalities of these blood vessels have been identified in hypertension, CHF and uraemia. In terming the vessels that I studied "resistance arteries," I have followed the definition suggested by Mulvany and Aalkjaer i.e. arteries with internal diameters of less than 500μm.[236] However, studies of the pressure drop across the human vascular bed have not been performed and, while it is assumed that these vessels contribute significantly to the regulation of peripheral resistance, this is not known for sure. Moreover, the majority of vessels studied in these experiments were obtained from subcutaneous gluteal fat biopsies. Though this is a well-established technique, a systematic study of the properties of arteries from different vascular beds has not been performed in man. It is not known whether the properties of gluteal subcutaneous resistance arteries, which must play an important role in thermoregulation, are representative of vessels elsewhere. A similar situation pertains to arteries from the abdominal wall. Caution is therefore necessary when interpreting the results of these experiments and extrapolating their significance to the vasculature as a whole. It is assumed throughout this

thesis that the properties of gluteal subcutaneous resistance arteries are similar to those of vessels found in other organs.

The major conclusion from these studies is that, in *in-vitro* preparations of human tissue, the generation of AII is mediated through a dual pathway. In contrast, AII generation in resistance arteries from the rabbit was ACE-dependent. Thus the mechanism of AII generation in resistance arteries appears to be species-specific. This dual pathway appears to be present in both resistance arteries and internal mammary arteries and may be ubiquitous in the human vasculature. One limb of this pathway is ACE. The other limb is an enzyme (or enzymes) susceptible to inhibition with chymostatin. This chymostatin-sensitive component of AII generation ("CAGE") is thought to represent the action of the enzyme chymase. In resistance arteries from normal volunteers and patients with CHD there appeared to be sufficient capacity in each arm of the pathway to generate a normal response to AI, when either ACE or chymase was blocked. The same situation appeared to exist in internal mammary arteries from patients with CHD. Thus it appears that AI can be "shunted" between the enzymes responsible for AII generation. In contrast, in arteries from patients with CHF, enalaprilat exerted a significant inhibitory effect on AI responses. Though this was enhanced by the addition of chymostatin, it would appear that the non-ACE pathway was less important (and had, perhaps, been down-regulated) in these arteries. Whether this was due to treatment of the patients with ACEi, or to their underlying heart failure, is not clear.

There are a number of ways of resolving this issue. One would be to study resistance artery responses to AI in ACE inhibitor-naïve patients and then to repeat the experiments in the same patients after administration of this medication. A complementary approach would be to study patients with CHD and normal left ventricular function before and after administration of an ACE inhibitor. A third possible approach would be to use AI-isopeptides to dissect the relative contributions of ACE and chymase to AII generation. [Pro$^{11}$ D-Ala$^{12}$] AI is a synthetic peptide that is resistant to cleavage by ACE but is hydrolysed to form AII by the action of chymase.[206] Similarly [Pro$^{10}$] AI is susceptible to cleavage by ACE but not chymase.[300] Each of these strategies would represent a functional approach to identify whether the increased sensitivity to enalaprilat seen in CHF patients was due to the syndrome or to the fact that they were receiving ACEi.

Other experiments in resistance arteries were designed to study the mechanism of inhibition of AI responses exerted by enalaprilat and chymostatin. The failure to significantly inhibit responses to AII and NE suggested that there was no non-specific inhibition of contractile responses to standard vasoconstrictors due to stimulation of counter-regulatory vasodilators. Thus, while enalaprilat did potentiate BK, chymostatin did not, and neither influenced the response to ACh. It was an interesting observation that there was no apparent difference in the response to ACh in arteries from CHF compared to CHD patients – suggesting that endothelial dysfunction in the former was not specific to the syndrome itself. However, the absence of

an age and sex-matched control group without vascular disease makes this difficult to interpret. Similarly the enhanced response to AII in arteries from CHF patients was an interesting observation. As discussed in chapter 5, this may be due to an interaction between the sympathetic nervous system and AII. Whatever the mechanism, this observation may have implications for patients with CHF, especially if AII plasma levels are not fully suppressed despite ACE-inhibition.

In chapter 4 I reported an analysis of the role of the ACE I/D polymorphism in determining the responses to AI. This analysis was limited by lack of DD homozygotes, and it did not support the suggestion that variation at the ACE locus may influence vascular AII generation. Indeed, if there is a functional bypass pathway and the conversion of AI to AII is not rate-limiting, then variation in the expression of ACE could be expected to play little part in determining AII generation. In contrast, ACE seemed to be more important in arteries from patients with CHF, since the alternative pathway was unable to compensate fully in the presence of enalaprilat - so variation at the ACE gene locus may be an important determinant of local AII generation in specific situations when the RAS is activated. This question could be addressed by using molecular techniques to quantitate ACE and chymase expression in resistance arteries. Chymase has recently been detected by PCR in human adipose tissue and it might be possible to extend this technique to human blood vessels.[308] Immunohistochemistry has also been employed to identify chymase and ACE expression in human

atherosclerotic aorta in a semi-quantitative manner.[294] In this study AII formation (largely chymase-mediated) was increased in diseased arteries and the number of chymase-positive cells observed in atherosclerotic and aneurysmal lesions was higher than in normal arteries. Similarly ACE expression has been localised within atherosclerotic plaques from human carotid arteries by a combination of immunohistochemistry and in-situ hybridisation.[309] This study showed that ACE appeared to be up-regulated in diseased vessels. Thus there is evidence that expression of both ACE and chymase are increased in diseased large and medium sized arteries. There is, however, little information concerning the regulation of chymase and ACE in small arteries. While it is theoretically possible to identify ACE and chymase gene expression in resistance arteries using the same techniques, the very small quantity of tissue available and the time taken to dissect vessels free renders mRNA difficult to detect. Nonetheless, a study of ACE and chymase gene expression in resistance arteries from patients with CHF, or taking ACEi would be of great interest.

The conclusion that there is a dual pathway for AII generation in human blood vessels is largely dependent on pharmacological experiments. Thus the action of ACE and chymase is inferred from observations of the effect of blocking these enzymes. However, information about the spatial organisation of the enzymes within the vessel wall, together with an understanding of their regulation at a molecular level, is also necessary to understand their physiological and potential pathophysiological role. In

chapter 7 I demonstrated that ACE is found in the medial layer of internal mammary arteries (and is known to be expressed on the surface of endothelial cells), while chymase is located within mast cells in the adventitia. This separation may be functionally significant. Thus, *in-vitro* studies of non-ACE AII generation in excised vessels may have over-estimated its significance by introducing AI abluminally, so exposing adventitial chymase to a substrate from which it is normally hidden. Similarly, the release or activation of previously inactive or intracellular chymase may confound biochemical studies of AII generation in homogenised tissue. There have been no *in-vivo* studies quantifying the contributions of ACE and chymase to AII generation directly, mainly because there is no specific chymase inhibitor available for use in man. However, there have been studies that have assessed AII generation across defined vascular beds. Zisman et al infused $^{125}$I-AI into the coronary circulation and showed that enalaprilat almost completely abolished AII generation. This suggested that chymase contributed little to AII generation within the blood compartment, though it did not exclude non-ACE AII generation within cardiac tissue.[201]. A recent study used venous occlusion plethysmography to study the relative importance of local AII generation and blood-delivered AII for vasoconstriction in the forearm.[310] Enalaprilat inhibited both vasoconstriction and the conversion of AI into AII – the latter by 99%. AI and AII induced vasoconstriction with similar potencies despite a fractional conversion of only 36%. Based on these observations it was suggested that local AII generation was very significant

but was ACE-dependent. A similar conclusion was arrived at by Webb, who also studied AII generation in the forearm using plethysmography.[51] Thus, the mode of delivery of AI to an artery may determine which enzyme is largely responsible for its conversion.

There may be two reasons for the discrepancies between *in-vitro* and *in-vivo* studies of AII generation. The first is that chymase-mediated AII generation may be an artefact that occurs *in-vitro* due to experimental conditions and that this phenomenon is not clinically significant. The importance of experimental conditions was recently emphasised in a study of AI-responses in rat arteries.[311] Chymase dependent conversion of AI was identified, but only at very high substrate concentrations and the authors therefore questioned whether this phenomenon had any *in-vivo* significance. Another explanation is however, that vasoconstriction to AII generated by chymase may be an epiphenomenon of the enzyme. Thus, chymase may indeed mediate local AII generation, but this does not primarily influence vascular tone. It is possible that the spatial dissociation of ACE and chymase within the vessel is reflected in a functional dissociation. In a recent editorial Miyazaki and Takai proposed that chymase may play an important role in vascular proliferation and remodelling.[312] Chymase-mediated AII generation may not be important in normal healthy tissue, but it may be significant when there is inflammation or damage to blood vessels. Evidence to support this notion comes from studies on balloon injured arteries in dogs, where chymase has been shown to be induced.[212] In this system

neointimal hyperplasia can be inhibited by an angiotensin-receptor antagonist, but not an ACE-inhibitor.[214, 215] The significance of the potential trophic role of chymase is emphasised by a recent study that showed an increase in expression associated with hypercholesterolaemia.[295] One could therefore speculate that when blood vessels are damaged, mast cells are recruited, resulting in increased local AII generation, mediated by chymase.

There are a number of ways in which the role of chymase within the vasculature could be clarified. In perfusion myography, resistance vessels are cannulated at each end and isobaric responses (rather than the isometric responses of wire myography) are studied.[299] There are a number of potential advantages associated with this technique. Perfusion mimics *in-vivo* conditions more precisely and the vessels are allowed to maintain a normal shape. The more physiological nature of the preparation may be reflected in observations that pressurised vessels are more sensitive to agonists such as NE.[313] Thus, a comparison of responses to AII in wire and pressure mounted vessels demonstrated that low concentrations of AII induced a sustained contraction in the latter but not the former.[314] The perfusion system may be more appropriate for studies of AI and AII responses in resistance arteries. A further potential advantage of the perfusion system is that this may allow agonists to be introduced to the lumen of the vessel directly. It may then be possible to investigate the relative contributions of ACE and chymase to AII generation when AI is introduced luminally or abluminally.

In summary, in this thesis I have demonstrated that AI acts locally in human resistance arteries to generate AII. This conversion is apparently mediated through the action of ACE and chymase, which together form a dual pathway for AII generation. Studies in arteries from patients with CHD and CHF suggest that the relative contributions of ACE and chymase to AII generation may be modified by either the disease or the treatment. Studies in internal mammary arteries confirm the existence of this dual pathway in a different vascular bed and additionally suggest that the enzymes are spatially distinct within the vessel wall. Whether this spatial organisation is reflected in the functions of the two enzymes is not fully understood and further studies are required to clarify the role of chymase in man *in-vivo*. Of particular importance is the issue of whether chymase expression is increased in disease states such as CHF and atherosclerosis and whether this contributes to the pathophysiology of these syndromes. Chymase itself may be an important therapeutic target and strategies to maximise inhibition of the renin-angiotensin system should take into account the possibility of non-ACE angiotensin II generation.

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