ANGIOTENSIN CONVERTING ENZYME AND VASCULAR ENDOTHELIAL GROWTH FACTOR RESPONSES TO EXERCISE TRAINING IN CLAUDICANTS: THE ROLE OF ACE INHIBITION

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April 2009

Presented for the Award of Doctor of Medicine (Research)

ABSTRACT

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Exercise training is well recognised as an effective treatment for intermittent claudication. The mechanism underlying exercise induced improvements is multi-factorial but remains poorly understood. Low angiotensin-converting enzyme (ACE) activity has been associated with enhanced responses to endurance training. Specifically, low ACE activity has been associated with improved muscle metabolism, endothelial function, and suppressed inflammatory responses; processes linked with exercise training benefits in claudicants. Furthermore, pharmacological inhibition of ACE has been associated with enhanced angiogenesis in animal models of ischaemia, secondary to increases in vascular endothelial growth factor (VEGF).

In this study, 11 claudicants were randomised to 8 weeks of supervised exercise training (n=6) or exercise advice (n=5). Walking ability was recorded before and after this period, and blood samples taken. Reverse transcription polymerase chain reaction (RT-PCR) was used to determine the effects of exercise training on ACE, VEGF and VEGF receptor (VEGFR) gene expression, and enzyme-linked immunosorbant assays (ELISA) measured changes in ACE and VEGF protein levels. In another experiment, a cell culture model of hypoxia, utilising ECV 304 cells and diethylenetriamine-nitric oxide (DETA-NO), was used to study the effects of the ACE inhibitor ramiprilat on ACE and VEGF responses to hypoxia, using RT-PCR and ELISA.

Supervised exercise improved claudication distance by 105 metres ($p < 0.05$) and maximum walking distance by 141 metres ($p < 0.05$). ACE mRNA expression increased 30%, $VEGF_{121}$ expression 43% and $VEGF_{165}$ expression 70% (all $p < 0.05$). Soluble VEGFR-1 mRNA expression increased by 63% and VEGFR-2 72% (both $p < 0.05$).

ACE and VEGF protein levels remained comparatively stable. In the cell culture experiments, ramiprilat increased VEGF protein levels in hypoxia. Although a lack of experimental runs prevented statistical analysis, the results also suggest that ramiprilat has a stimulatory effect on ACE mRNA expression in hypoxia.

Improvements in walking ability after exercise training are associated with increases in both VEGF and VEGF receptor expression. ACE inhibitors could play a role in improving claudication by potentiating increases in VEGF in addition to their known action of suppressing ACE activity.

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

I would like to thank the following people who have all helped to a great extent in completing this thesis: Dr Simon Hollingsworth, for giving me the opportunity to conduct the thesis, and for his always astute supervision and guidance. I am also very grateful to Mr Jason Lee, for both his collaboration and camaraderie in the laboratory, and to Dr Neil Jones and Mr Garth Powell, for their invaluable help and technical advice.

STATEMENT OF ORIGINALITY

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The work presented in this thesis is the sole undertaking of Mr Paul Ng. The results have not been presented for the award of any other degree, apart from the data relating to the effects of the ACE polymorphism and ramipril on training responses in claudicants, which was taken from a thesis submitted for an MSc degree previously undertaken by the author.

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ABBREVIATIONS

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ABPI	Ankle brachial pressure index
ACE	Angiotensin-converting enzyme
Ang-II	Angiotensin II
ANOVA	Analysis of Variance
AP-1	Activating protein 1
AT_1	Angiotensin II receptor Type 1
AT_2	Angiotensin II receptor Type 2
ATP	Adenosine triphosphate
BK	Bradykinin
Bp	Base pair
BSA	Bovine serum albumin
CD	Claudication distance
cDNA	Complimentary deoxyribonucleic acid
CK2	Casein kinase 2
COX-2	Cyclo-oxygenase 2
CRP	C-reactive protein
DETA-NO	Diethylenetriamine-nitric oxide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbant assay
FCS	Foetal calf serum
FGF	Fibroblast growth factor
GAPDH-3	Glyceraldehyde-3-phosphate dehydrogenase

HBSS	Hanks' balanced salt solution
HIF	Hypoxia-inducible factor
HUVECs	Human umbilical vein endothelial cells
IL-6	Interleukin-6
IOD	Integrated optical density
M-MLV	Moloney Murine Leukaemia virus
mRNA	Messenger ribonucleic acid
MWD	Maximum walking distance
NO	Nitric oxide
PBMN	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCTA	Percutaneous transluminal angioplasty
PKC	Protein kinase C
PlGF	Placental growth factor
RAS	Renin-angiotensin system
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
SEM	Standard error of the mean
TB	Trypan blue
Tm	Annealing temperature
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

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INTRODUCTION

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1.1 INTERMITTENT CLAUDICATION

Peripheral arterial disease is caused by atherosclerotic lesions in the arteries supplying the lower limbs, which result in a reduction of blood flow to the extremities. It is a significant cause of functional impairment, mortality and morbidity in the western world, and in the UK alone affects up to 20% of people over the age of 75.[49;243] With incidence increasing with age, and the continued growth of the elderly population, its prevalence is likely to rise considerably over the next 20 years.[43]

A wide spectrum of disease severity exists. Gangrene and limb loss occur in the most severe cases, whilst those with mild disease may be completely asymptomatic. Many individuals with mild disease experience pain on walking. This phenomenon has been termed intermittent *claudication*, a word derived from the Latin word 'claudicatio' meaning 'limping.' It was first coined by physiologists who, after ligating the femoral arteries of horses, noted that they limped before stopping *intermittently* as they ran in the field.[71] The term intermittent claudication is now used to describe muscular leg pain brought on by walking which, with continued walking causes the patient to stop, but is relieved within minutes by rest. The pain can be in one or both legs, and is typically felt in the calf muscle. Claudication affects 15 to 40% of patients with peripheral arterial disease. It is associated with decreased mobility, a reduced ability to perform normal daily activities, and can have a considerable negative impact on quality of life.[229;244]

1.1.1 Pathophysiology

Although intermittent claudication may be caused by conditions such as popliteal artery entrapment, fibromuscular dysplasia and external compression syndromes, these causes

are rare with the pathology underlying almost all cases being atherosclerosis.[43] Cigarette smoking is the greatest risk factor for developing atherosclerosis, with other risk factors including old age, diabetes, hypertension and hyperlipidaemia. The formation of atherosclerotic plaques involves a complex interaction between lipids, the endothelium, inflammatory cells, platelets and vascular smooth muscle cells. In brief, injury to the endothelium, for example by cigarette smoking or low density lipoproteins, causes an inflammatory response characterised by the infiltration of inflammatory cells into the arterial wall, smooth muscle cell proliferation and lipid deposition.[211] The consequence of this process are occlusive or stenotic atherosclerotic lesions which may occur anywhere in the arterial tree, but primarily in large and medium sized vessels.

In healthy subjects, the onset of exercise produces a series of physiological responses to enable the metabolic demands of exercise to be met. Almost immediately, cardiac output and respiratory rate increase, and peripherally vasodilatation occurs in active muscles.[125;132] The net result is a large increase in blood flow to the active muscles, resulting in increased oxygen delivery and carbon dioxide removal, thereby allowing exercise to be sustained.[125] However, in claudicants atherosclerotic lesions in the main arteries supplying the legs interfere with blood flow to the leg muscles sufficiently to limit blood flow increases after the onset of exercise. Thus, the failure to increase oxygen delivery in response to exercise leads to an imbalance in the demand for and the supply of oxygen. Subsequently, the supply of adenosine triphosphate (ATP) synthesised by aerobic metabolism becomes insufficient to sustain the level of exertion, causing anaerobic metabolism to predominate. This reliance on anaerobic pathways during walking leads to an accumulation of lactic acid and phosphate ions in the leg muscles, causing muscular pain and fatigue.[255]

Although the pathogenesis of intermittent claudication is initiated by atherosclerosis, there is a poor correlation between the pressure drop across the limb and ischaemic symptoms and function.[113] This is principally due to the complex pathophysiology of intermittent claudication, with several other pathological processes contributing to the condition causing a further loss of walking performance (Figure 1.1).[86] Repeated episodes of ischaemia and reperfusion, with the associated generation of reactive oxygen species, damages skeletal muscle causing neurological, muscular and metabolic disturbances.[117;284] Distal axonal denervation of muscle fibres, muscle fibre atrophy and loss of muscle strength have all been described.[56;199] Oxidative metabolism is also considerably impaired, with studies showing a decrease in the level of aerobic enzymes, a reduction in capillary density with increasing severity of disease, and a reciprocal increase in anaerobic enzyme levels.[56] Furthermore, abnormal muscle metabolism leads to the accumulation of intermediates of oxidative metabolism such as the acylcarnitines in the muscle. These are formed from acetyl-CoA intermediates in the oxidation of fatty acids, proteins, and carbohydrates, and are detrimental to muscle function.[115] In addition, abnormalities in endothelial function may lead to a misdistribution of blood flow in the microcirculation, with blood flow increased through arterio-venous shunts, and decreased through nutritional capillaries.[1] Changes in blood viscosity, microthrombi, white blood cell and platelet activation also contribute to decreased regional blood flow and ischaemia.[83;86] Finally, another major issue is general physical deconditioning caused by the relative inactivity of these patients.[71]

1.1.2 Natural History

In addition to causing severe functional disability and reducing quality of life, intermittent claudication is also a significant marker for systemic atherosclerosis, with a

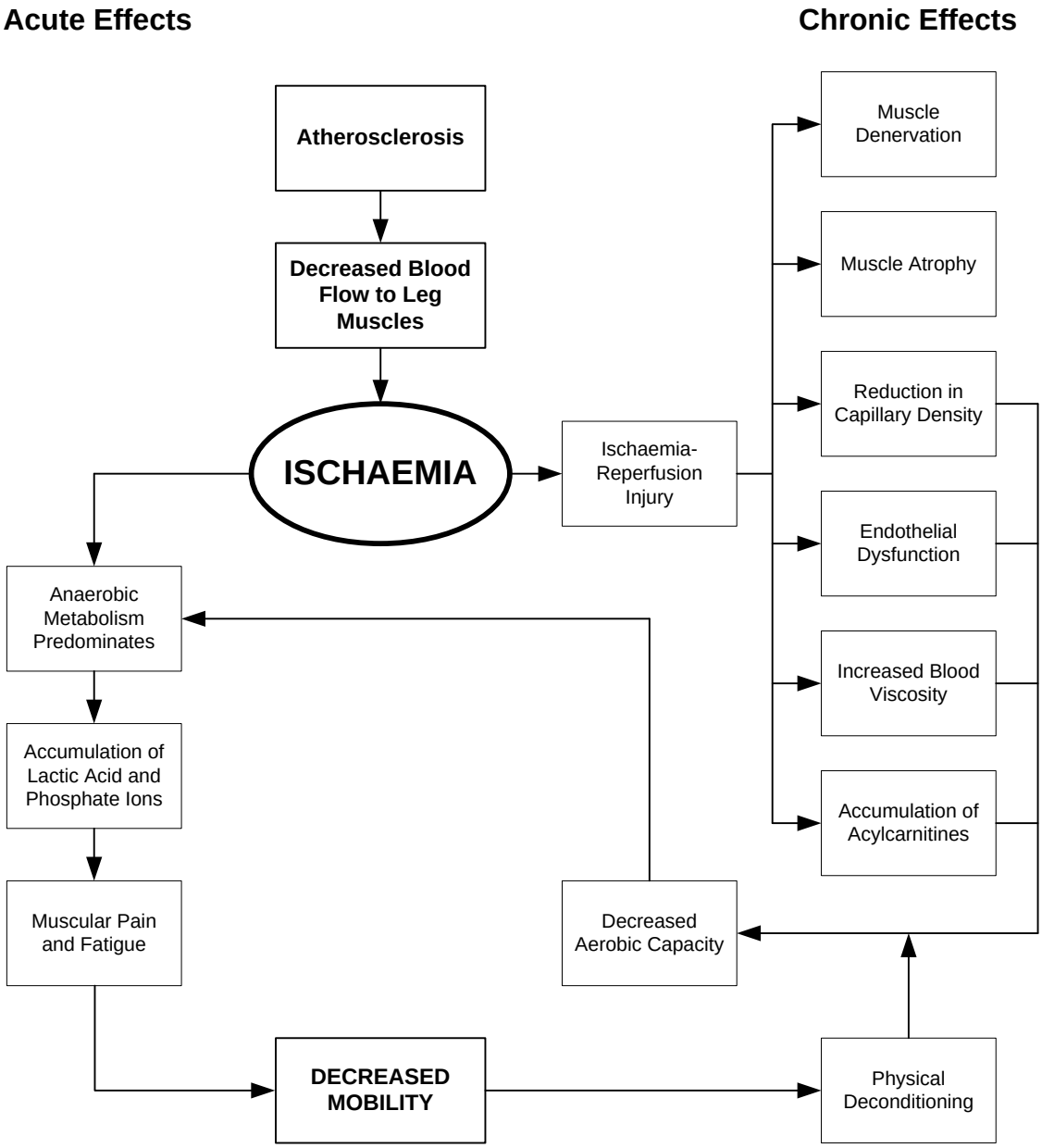

Figure 1.1 Summary of the Pathophysiological Processes Involved in Intermittent Claudication.

large proportion of patients also having coronary artery and cerebrovascular disease. Overall, 50 percent of claudicants will die within 10 years, more than three times that of an age matched normal population. The commonest causes of death are myocardial infarction and stroke. Indeed, a claudicant is more likely to die from myocardial infarction than a comparable patient with angina.[229] In contrast, the natural history of arterial disease of the lower limbs is relatively benign. The majority of patients can be managed conservatively, and do not require interventional procedures. Up to 75 percent of patients with intermittent claudication will have limb symptoms that remain stable or improve. Only 25 percent deteriorate, and only 5 percent will ever need intervention in the form of endovascular procedures or surgery. About 2 percent of patients with intermittent claudication, usually diabetics and heavy smokers, will eventually need a major amputation.[71]

1.1.3 Management

Given the significant vascular mortality and morbidity in patients with peripheral arterial disease, a key part of management involves secondary prevention in optimising cardiovascular risk factors to reduce the number of cardiac and cerebral events. This includes encouraging smoking cessation, and ensuring good control of blood pressure and diabetes. Patients should also be started on anti-platelet therapy, such as aspirin or clopidogrel, and unless any contra-indications are present be started on a statin to reduce cholesterol.[43]

Regarding the specific treatment of peripheral arterial disease, the severity of disease determines both symptoms and management. In the presence of critical ischaemia, in which the viability of a limb is threatened, surgery or endovascular procedures such as

angioplasty are the widely accepted management modalities.[71] The treatment options for patients with less severe symptoms such as intermittent claudication include bypass surgery, endovascular procedures, drug therapy and exercise.[229;255] At present, there is no consensus about management and intervention rates vary greatly between centres. Surgery is generally reserved for those with debilitating symptoms not suitable for angioplasty and unresponsive to conservative measures, but is associated with increased morbidity and mortality.[43] Several drug therapies have been used to try to improve the symptoms of claudication with generally limited success.[111] The main exception is cilostazol, a phosphodiesterase inhibitor, which has been shown to increase both pain-free and maximal treadmill walking distance, and improve quality of life. However, common side-effects include headache, diarrhoea and palpitations, and the drug is contra-indicated in the presence of heart failure.[13] There is also evidence that statins increase pain-free walking distance in addition to lowering cholesterol.[163]

The continual development of percutaneous transluminal angioplasty (PCTA) has led to its widespread use in the treatment of intermittent claudication. However, its role is still controversial.[43] The recent trend in the treatment of intermittent claudication has been towards a more conservative approach, and in those with relatively mild symptoms, exercise still remains the cornerstone of treatment.[49]

1.2 EXERCISE FOR INTERMITTENT CLAUDICATION

The benefits of exercise for claudicants have been known for many years. Improvements in walking after exercise training were first described by Erb in 1898. In 1988, Housley described the treatment of intermittent claudication with the 5 words: ‘stop smoking and keep walking’.[255] More recently, the TransAtlantic Inter-Society

Consensus (TASC) working group publication made a recommendation that 'a programme of exercise therapy (preferably supervised) should always be considered as part of the initial treatment for patients with intermittent claudication'.[71] To date, a large number of clinical trials have demonstrated the efficacy of exercise for improving walking distance, functional capacity and quality of life.

1.2.1 Supervised and Unsupervised Exercise Programmes

In clinical practice, exercise training in claudicants ranges from simple advice, in which patients are encouraged to walk regularly and to persist through the claudication pain as much as possible, to more formal supervised exercise programmes. In the UK, the majority of patients are only given exercise advice and few supervised programmes are available, due to the fact that they are very labour intensive and expensive.[243]

The majority of trials examining the effectiveness of exercise training have studied supervised training programmes. A meta-analysis of 21 exercise rehabilitation programmes demonstrated that the distance walked to the onset of claudication pain on a treadmill increased by 179 percent and the maximal walking distance by 122 percent following a period of exercise training. Furthermore, the components of an exercise programme that led to the greatest improvements in walking distances were: training session greater than 30 minutes duration, frequency of training sessions at least 3 times per week, walking used as the mode of training, use of near maximal pain during training as the end point, and programme length of greater than 6 months.[99] This meta-analysis included non-controlled observational studies. A Cochrane review analysed 10 randomised controlled trials involving nearly 250 patients enrolled mainly in supervised walking programmes. This analysis demonstrated a significantly increased maximal

walking time of 6.5 minutes after exercise therapy, with an improvement in walking distance of about 150 percent.[148]

Some studies have made comparisons between the benefits of supervised exercise and unsupervised exercise. In one trial, patients were randomised to either a supervised weekly exercise class or exercise advice alone. After 6 months the supervised exercise group had improved their walking distance by 129 percent compared to only 69 percent for the exercise advice only group.[49] Several other studies also show limited benefits for unsupervised exercise, with the bulk of evidence showing that supervised exercise programmes provide the most symptomatic benefits.[197;243] A recent Cochrane review of 8 randomised controlled trials showed a significant difference in improvement with supervised exercise training, with an approximately 150 metre greater increase in walking distance compared to unsupervised training. This difference was maintained at 6 months.[18] However, despite this evidence unsupervised training is likely to be more effective than no training at all.[71] There are several reasons why supervised training may be more beneficial than unsupervised training. Patients enrolled on a structured supervised exercise programme are much more likely to comply with treatment compared to those only given advice. In addition, supervised training regimes consist mainly of treadmill walking, which is able to produce higher workloads compared to walking on level ground at normal speed. These higher workloads potentially stimulate greater adaptations to training, including cardiovascular adaptations.[18]

Other trials have shown treadmill training to be more effective than strength training in improving walking performance in claudicants.[116] In addition to walking ability, exercise programmes have been shown to produce improvements in functional status,

physical activity levels, and quality of life.[97;198] Moreover, the benefits and effectiveness of exercise are similar in both smokers and non-smokers.[98] Exercise rehabilitation programmes have been shown to be safe, with no associated morbidity or mortality. Initial studies have also shown improvements in glucose metabolism, reduction in cholesterol levels, and enhanced smoking cessation. These benefits are not seen with other interventions and could potentially decrease cardiovascular risk and mortality.[71;148]

1.2.2 Exercise Compared to Angioplasty for Intermittent Claudication

While the mainstay of treatment for intermittent claudication remains conservative therapy, improvements in the safety and technique of PCTA have resulted in an increase in the number of these procedures to treat patients with relatively mild peripheral arterial disease. PCTA involves dilating a stenosed or occluded artery, most commonly using a balloon with or without leaving a stent in-situ. However, the role of PCTA in treating patients without critical ischaemia is still controversial. Guidelines published by the American Heart Association and American College of Cardiology in 2005 stated that patients with claudication should only be considered for revascularisation if conservative measures had failed or were predicted to fail, and only then in the presence of severe disability.[43]

Although PCTA has been shown to improve walking distance and quality of life compared to conservative treatment, more recent trials have compared the long-term efficacy of PCTA with exercise training.[43;44] A Cochrane review demonstrated PCTA to have short term benefits that were not sustained in the long-term.[96] Another randomised-controlled trial showed patients in an exercise group to have greater

improvements at 15 months compared to a PCTA group. In addition, claudicants with superficial femoral artery disease seem to benefit more from exercise, compared to those with aortoiliac disease.[188] A further Cochrane review showed no significant benefits from the placing of a stent compared to angioplasty alone in claudicants.[12]

In summary, a large number of trials have shown exercise training to be a safe, non-invasive and effective method to increase the walking ability, quality of life and general health of claudicants. It is beneficial to all patients who are able to exercise, is more effective than PCTA in the long-term and should remain as the principal treatment for intermittent claudication.

1.2.3 Mechanisms for Improvement with Exercise

Although the benefits of exercise are well recognised, the precise mechanisms that lead to an improvement in walking ability are not fully understood, and have recently been identified as an area requiring further research.[86] However, it appears that a combination of adaptive mechanisms, some not observed in healthy individuals, account for the benefits seen after training (Figure 1.2) These act by reversing some of the pathophysiological processes underlying the condition, and are described below:

Increased Blood Flow

Traditionally, improvements in walking ability following exercise have been attributed to an increase in blood flow secondary to the development of collateral vessels that 'bypass' the arterial stenoses or occlusions. The development of these collateral vessels is a universal response to muscle ischaemia, but is inconsistent and the vessels produce variable amounts of flow.[209] However, in some cases these collaterals may compensate

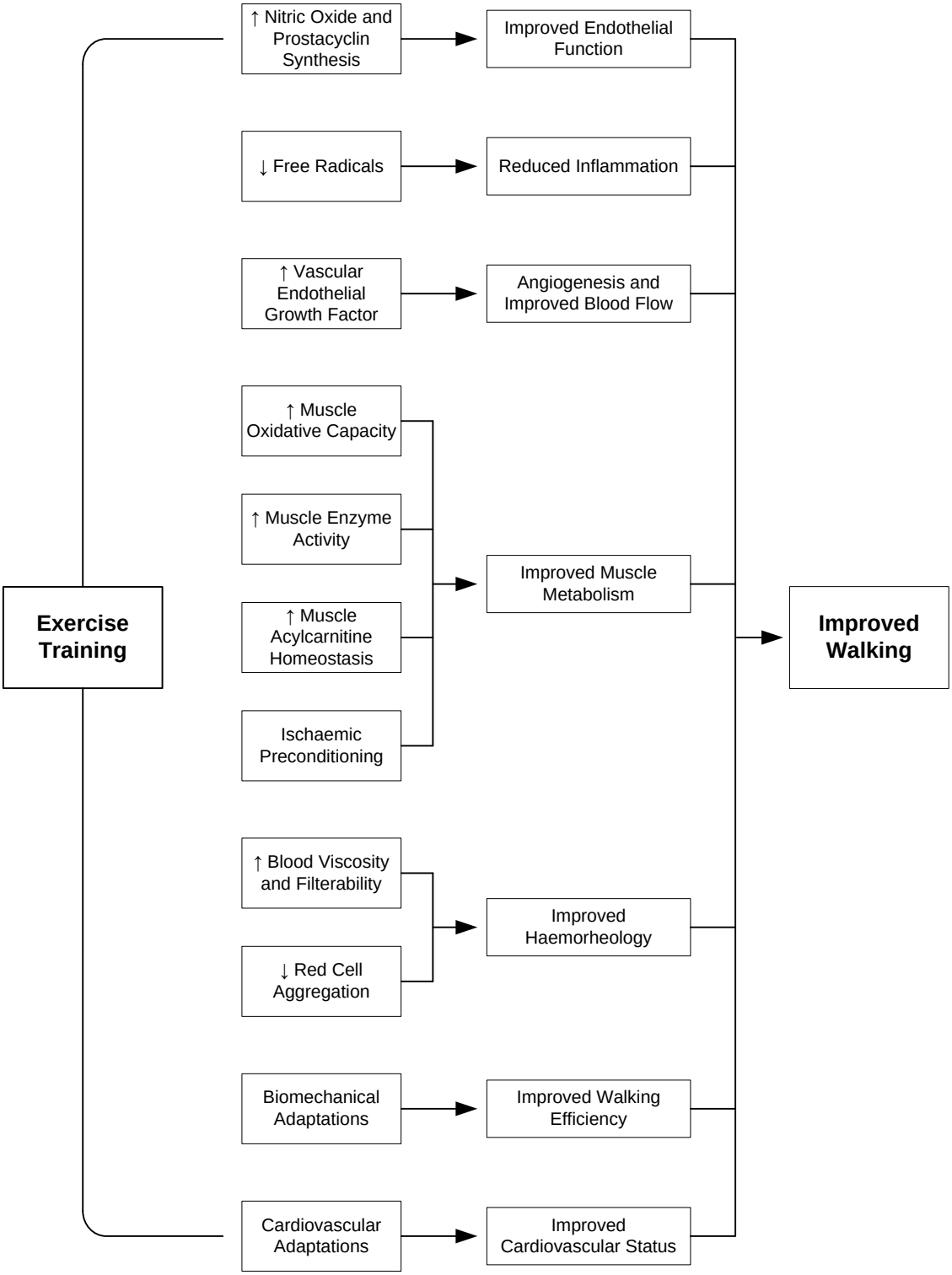

Figure 1.2 Summary of Potential Mechanisms Leading to Improved Walking Ability with Exercise Training.

At present, data are insufficient to accurately quantify the relative importance of each mechanism. Adapted from Stewart *et al.*[244]

for the arterial stenoses enough to minimise or even eliminate ischaemic symptoms, with proximal collateral vessels appearing to be more effective than distal collaterals in reducing symptoms.[86] The development of collateral vessels involves the enlargement and remodeling of pre-existing arterioles into mature vessels, a process called arteriogenesis.[222] The mechanism underlying arteriogenesis is poorly understood, but may involve increased sheer stress in the arterioles, caused by the redistributed blood flow, activating the endothelium and stimulating the release of factors such as placental growth factor (PlGF).[157;222] Vascular endothelial growth factor (VEGF) is not thought to play a major role.[110] Other studies have shown that muscle capillaries may also become arteriolised- a process associated with increased fibroblast growth factor-2 expression.[15]

Despite the significant evidence for collateral vessel formation, the development of collateral vessels and an increase in gross blood flow appears to play a limited role in improving walking ability with exercise training in claudicants. The majority of studies in claudicants have shown little or no increase in blood flow following an exercise programme, even when significant improvements in walking ability have been recorded.[244;255] When increases in blood flow have been detected, they are poorly correlated to improvements in walking distance.[71;114] Similarly, studies have consistently shown a poor correlation between leg blood flow and walking ability.[255] PCTA, which results in a patent artery and increased blood flow, is associated with less marked increases in walking ability than exercise.[188] These findings might be explained by animal models of ischaemia, which show that arteriogenesis occurs 10 days after femoral artery ligation, peaks at 20-40 days, and is not temporally associated with ischaemia.[110] One might speculate that in stable claudicants (the subjects of most exercise studies), collateral development has already occurred and thus would be

unlikely to contribute to improvements in walking ability. Furthermore, since ischaemia *per se* does not appear to be the stimulus for arteriogenesis, exercise programmes that involve repeated episodes of ischaemia would be unlikely to activate the pathways linked to arteriogenesis.

As there is currently little evidence to support an increase in blood flow as a major factor in the increase in walking ability after training, other mechanisms must account for the improvements seen. Some have proposed that while overall blood flow does not increase, training may result in a redistribution of blood from inactive to exercising muscles. Another finding is increased capillary density in the muscles.[261] The sprouting of new capillaries in the muscles is a separate phenomenon to arteriogenesis, and is termed angiogenesis.[253] Unlike arteriogenesis, angiogenesis is mediated by ischaemia and it has been suggested that repeated training, causing transient hypoxia, may increase the expression of VEGF resulting in endogenous angiogenesis.[244] Several animal models have shown upregulation of VEGF with ischaemia.[151] The effects of increased capillary density in active muscles may include a redistribution of blood flow, improved substrate delivery and the enhanced removal of metabolites. This is supported by findings showing a reduction in femoral venous oxygen saturation in claudicants after training, indicating increased oxygen uptake in the muscles.[239] Therefore, while gross increases in blood flow do not appear to be important in the improvement in walking ability with exercise training, the improved local delivery and distribution of the limited blood supply due to angiogenesis could be a much more important mechanism underlying improvements. Moreover, the mechanism underlying angiogenesis is promoted by ischaemia, and will thus be stimulated by an exercise programme. VEGF and angiogenesis will be discussed in more detail in Section 1.4.

Changes in Muscle Metabolism

Several adaptations occur in skeletal muscle following exercise training that lead to an improvement in the aerobic capacity of the muscles and hence a reversal of the metabolic dysfunction seen in claudicants prior to training. Animal models have shown that these adaptations only occur with a combination of training and hypoxia, and that hypoxia alone does not induce any change.[255] Studies of muscle biopsies before and after an exercise training programme have revealed significant increases in oxidative enzymes such as cytochrome oxidase and succinic oxidase, with smaller increases in citrate synthase after training.[121] Fatty acid oxidative metabolism is also enhanced, as shown by the decrease in respiratory exchange ratio for a given workload after training.[114] This increase in fatty acid metabolism reflects an increase in mitochondrial function and/or better oxygen delivery, but is generally indicative of increased oxidative capacity. In contrast, no changes are seen in anaerobic capacity.[255] The decreased reliance on anaerobic pathways has been observed in studies examining venous blood samples in claudicants, with decreased lactic acid levels at similar workloads noted after a period of training.[239;254] An increase in arteriovenous oxygen gradient is also noted, indicating increased muscle oxygen extraction, possibly related to increased muscle myoglobin concentration.[122;144] Furthermore, exercise training has also been shown to reduce the level of acylcarnitines that accumulate in the leg muscles of claudicants secondary to defective oxidative metabolism, with the extent of removal correlated to improvements in walking ability.[114;115]

Another metabolic effect potentially underlying exercise related improvements in claudicants is ischaemic preconditioning, a phenomenon whereby short periods of ischaemia offer protection from a later episode of prolonged ischaemia. This was first

described in the heart, but has now been demonstrated in many other tissues including skeletal muscle.[301] Several short periods of ischaemia have been shown to improve the subsequent ischaemic tolerance of skeletal muscle, with preservation of force production, decrease in lactate accumulation, decreased ATP depletion and reduction of necrosis all observed.[2;32] The mechanism for this protection is thought to relate to the activation of Protein kinase C by triggers such as adenosine and bradykinin. This leads to altered nuclear transcription and the opening of mitochondrial K_{ATP} channels, resulting in preserved ATP generation in low oxygen conditions.[300] Whether these effects are reflected in less severe models of ischemia such as intermittent claudication or produce long-term benefits remain to be seen. However, in one study claudicants 'preconditioned' by performing several submaximal walking exercises performed better in a subsequent maximal walking test.[37]

Changes in Endothelial Function and the Microcirculation

Endothelial dysfunction has been demonstrated in patients with peripheral arterial disease, secondary to a decrease in nitric oxide (NO) synthesis.[299] This causes a decrease in exercise induced hyperaemia, and hence impaired substrate delivery to the muscles. Short-term exercise has been shown to stimulate endothelium-dependent vasodilatation. In the long-term, the sheer stress caused by increased blood flow from repeated episodes of exercise increases the endothelial expression of NO synthase and prostacyclin, improving endothelial function and vasodilatation in response to exercise.[179] Although few studies of endothelial function in claudicants have been performed, one study has shown an increase in endothelial dependent vasodilatation of 61% and an increase in post-occlusive reactive hyperaemic blood flow of 15%, following a 6 month period of exercise rehabilitation.[27]

Reduction of Exercise-Induced Inflammation

It has been suggested that exercise training could actually be harmful to patients, by promoting a systemic inflammatory response that decreases muscle function and promotes atherosclerosis.[268] Indeed, in claudicants baseline levels of inflammatory markers are raised, and exercise to the point of muscle pain generates free radicals, activates neutrophils and produces an inflammatory reaction.[267] However, in the long-term exercise training causes an attenuation of these inflammatory markers, with training resulting in a decrease in C-reactive protein and serum amyloid levels.[266] This decrease in the inflammatory response may lead to improvements in muscle function and walking ability, and concerns that exercise training might potentiate vascular inflammation and atherosclerosis appear to be unjustified.[267]

Changes in Haemorheology

Haemorheology describes the behaviour and fluidity of blood, which is abnormal in claudicants with many having elevated blood viscosity.[254] Exercise training has been shown to reverse these abnormalities, with a reduction of blood and plasma viscosity, an increase in red cell filterability, and a decrease in red cell aggregation noted.[83] These changes increase the fluidity of blood and are thought to allow increased oxygen delivery to exercising muscles, thus enhancing walking performance. However, other studies have revealed no benefits after an exercise programme.[52;254]

Changes in the Cardiorespiratory System and Biomechanics of Walking

Some of the benefits of exercise training may be due to an improvement in systemic cardiovascular status. Exercise trained patients have lower resting and exercise heart rates compared to untrained patients. Peak oxygen consumption also increases.[114] One

group has demonstrated that upper limb exercise training in claudicants, resulting in an increase in cardiovascular fitness, also produces significant improvements in walking distance similar to those observed after lower limb training.[282;312] Another observation noted after walking programmes is a decrease in steady-state oxygen consumption for equivalent workloads during treadmill testing.[115;254] This implies that the oxygen cost for the same workload has decreased.[255] However, although central cardiovascular adaptations may improve walking ability, significant improvements in walking distance may occur in the absence of any increase in cardiovascular fitness.[178]

Another potential factor leading to improvements after an exercise programme is an improvement in the biomechanics and mechanical efficiency of walking after training.[244;255] Patients with claudication pain often adapt their gait to favour stability at the expense of velocity. This maladaptation increases the oxygen cost of walking, but may be reversed by exercise training.[244] This theory is supported by evidence which shows that after 4 months training, less oxygen is used at a given workload on a treadmill.[290] Other studies have also reported that oxygen uptake, respiratory exchange ratio, lactate levels, and heart rate were all lower in exercise trained claudicants.[114;116] Whilst this may reflect improved biomechanics and mechanical efficiency, an increase in muscular metabolic efficiency may also contribute to this effect, and may also help to explain increases in walking ability noted after upper limb training.[282] One factor that has been shown to enhance muscular efficiency in response to training is a polymorphism of the angiotensin converting enzyme (ACE) gene, which forms part of the renin-angiotensin system.[288] In addition, this polymorphism is allied to improved endurance, reduced inflammation and enhanced endothelial function, all factors known to be important for increasing walking ability in claudicants after training.

1.3 ANGIOTENSIN-CONVERTING ENZYME

ACE forms an integral part of the renin-angiotensin system (RAS). Its main role is in the production of angiotensin II (Ang-II) from angiotensin I, and the degradation of bradykinin (BK). Ang-II and BK are the key physiologically active components of the RAS, and thus their rate of production or degradation is one of the major determinants of the activity of the RAS. A functional polymorphism of the ACE gene has been described that is linked to variation in ACE activity, and has been shown to have widespread physiological effects. The effects of ACE and the ACE I/D polymorphism will be discussed in this section.

1.3.1 Renin-Angiotensin System: Overview

The Circulating Renin-Angiotensin System

The discovery of a metabolic pathway that contributed a major role to circulatory homeostasis was made in the 1950's.[54] This pathway, termed the circulating RAS, is important in regulating blood pressure, sodium homeostasis and fluid balance.[107] In the circulating RAS, a decrease in renal perfusion pressure stimulates the production and release of renin, a proteolytic enzyme, from the juxta-glomerular cells of the kidney into the circulation. Renin cleaves angiotensinogen, a circulating α_2-globulin synthesised by the liver, to form the decapeptide angiotensin-I. ACE, which is found both as a membrane bound protein at the cell surface and in circulating plasma (soluble ACE), subsequently further cleaves angiotensin-I to form the octapeptide Ang-II.[145] Ang-II exerts several effects including salt and water retention via the release of aldosterone from the adrenal cortex, and vasoconstriction.[54] Moreover, another important role of ACE is in the degradation and inactivation of BK, which otherwise has potent vasodilatory effects and influences muscle metabolism. Finally, Ang-II can be further

degraded to form angiotensin III, angiotensin IV and angiotensin-(1-7). These biologically active peptides may contribute some of the effects of RAS, although their relative contribution is unclear.[36] The key components of the circulating RAS are summarised in Figure 1.3.

Local Renin-Angiotensin Systems

In addition to the circulating RAS, local RASs have also been described in various tissues including the heart, brain, lung, pancreas and adipose tissue.[131] A local RAS has now been discovered in skeletal muscle.[72;221] This local skeletal muscle RAS depends on a combination of in-situ synthesis of all the RAS components and the uptake of renin or prorenin from the circulation.[63] ACE has been identified on both skeletal muscle membranes and on endothelial cells of the capillary bed.[72;221] In animal models, local skeletal muscle ACE accounts for up to 50% of BK hydrolysis, and angiotensin II generated locally has been shown to be of functional importance.[72;131] The role of this local RAS is still debated but may include growth stimulation, neovascularisation, and the regulation of inflammation.[126] The importance of tissue RAS as a potential therapeutic target comes from evidence that the anti-hypertensive properties of ACE inhibitors correlate better with the inhibition of tissue ACE, rather than plasma ACE. Furthermore, the additional benefits of ACE inhibitors such as improved endothelial function and reduction in inflammation, appear to be independent of changes in blood pressure, and are thought to relate to effects on local RAS systems.[75]

Angiotensin-II Receptors

Ang-II produces its effects via two main receptors, type I (AT_1) and type II (AT_2), which were originally classified according to their ability to bind with various

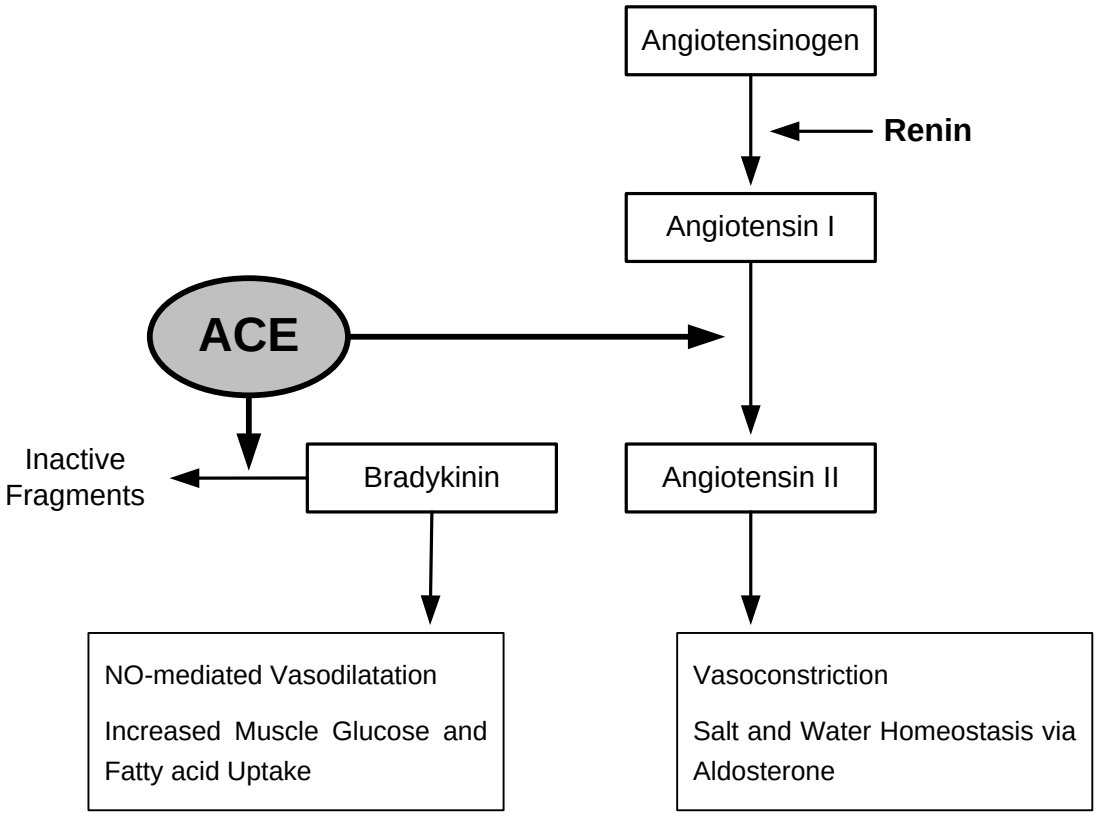

Figure 1.3 Summary of the Renin-Angiotensin System

ACE catalyses the production of Ang-II and the degradation of BK. Thus, a decrease in ACE activity will lead to lower levels of Ang-II and increased levels of BK.

antagonists.[264] More recently, two other angiotensin II receptors (AT_3 and AT_4) have been described, but their role has yet to be clearly defined.[150] AT_1 and AT_2 receptors are both seven transmembrane domain G-protein coupled receptors, although they appear to have quite different pharmacology. The AT_1 receptor is expressed ubiquitously and appears to mediate most of the well known biological effects of Ang-II.[150] In contrast, the role of the AT_2 receptor is less well understood. Although it is highly expressed in foetal tissues during development, its expression decreases markedly after birth. However, the AT_2 receptor can be upregulated in pathological conditions, for example after injury, during wound healing and by ischaemia.[150;236] It appears to have an opposing role to the AT_1 receptor, antagonising its actions.[127]

1.3.2 The ACE I/D Polymorphism

Since ACE acts as the primary catalyst for Ang-II production, any factor affecting ACE activity will have substantial effects on the RAS. The ACE gene is located on chromosome 17q23, spanning 21 kilobases and containing 26 exons. Two isoforms of ACE are produced by this gene: gonadal ACE is expressed only in the testes and will not be considered further, while somatic ACE is expressed throughout the body.[124] ACE levels have been shown to vary considerably between individuals, but less so within families, indicating a genetic influence on ACE levels.[34]

Rigat *et al.* discovered a functional polymorphism of the ACE gene consisting of the presence (insertion, I-allele) or absence (deletion, d-allele) of a 287 base pair fragment at intron 16 on the ACE gene (Figure 1.4).[203] Presence of the I-allele is known to affect both serum and tissue ACE, and is associated with reduced ACE activity.[64;203] Thus, three genotypes are possible: II (with low ACE activity), ID (with intermediate ACE

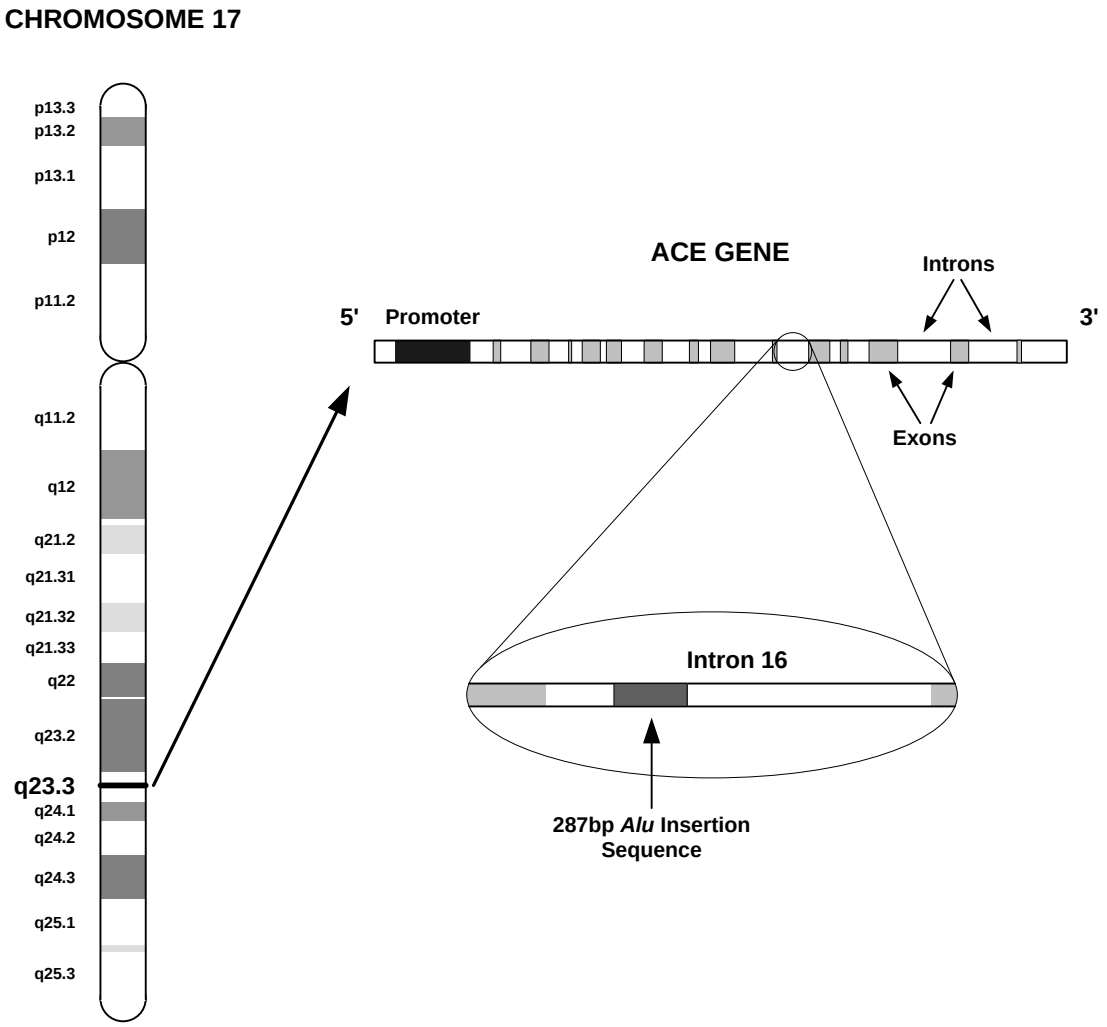

Figure 1.4 Schematic Diagram of the ACE Gene Illustrating the Insertion/Deletion Polymorphism

The ACE gene is found on the long arm of chromosome 17. The I/D polymorphism consists of the presence (I-allele) or absence (D-allele) of a 287bp *Alu* repeat sequence at intron 16 of the ACE gene.

activity) and DD (with high ACE activity). In Caucasians, the I- and D-alleles are equally common. Thus, 25% of the population will have the II genotype, 50% ID, and 25% the DD genotype.[131;167]

Although the polymorphism occurs within an intron, it is a very strong marker for ACE activity and accounts for up to 47% of the variance in serum ACE activity.[203] The insertion consists of a repeat *alu* sequence.[204] These *alu* insertional elements are abundant and occur throughout the human genome, and can reduce the transcription of adjacent regions in the genome by a variety of mechanisms, for example by inducing methylation or by promoting local nucleosome assembly.[213] Indeed, in heterozygote ID individuals, more ACE mRNA has been shown to originate from the D-allele than the I-allele.[246] However, it also remains possible that the I/D polymorphism is only a marker for ACE activity, and that it is in strong linkage disequilibrium with another polymorphism which may be directly responsible for the variations in ACE.[186]

The ACE I/D polymorphism has been associated with many pathological conditions, including diabetic renal disease, atherosclerosis and cardiac growth.[25;35;169] Low ACE activity has also been linked to many functional and physiological changes, which could be of significance in considering adaptations seen in claudicants in response to training.

1.3.3 ACE Polymorphism: Effects on Endurance Training

A number of recent studies have suggested that presence of the I-allele is associated with increased endurance performance, with an excess of I-alleles occurring in elite endurance athletes. These studies are summarised in Table 1.1. In addition, the benefits of the I-allele relate to an improved training response rather than endowing an

Table 1.1 Studies Showing an Association Between ACE I/D Genotype and Physical Performance

Reference	Subjects	End-point	Findings
Montgomery *et al.*[170] (1998)	British male Caucasian army recruits (n=123)	Repetitive elbow flexion with a 15kg barbell after a 10 week general physical training program	I-allele associated with improved response to endurance training
Montgomery *et al.*[170] (1998)	Elite Mountain Climbers (n=25)	Genotype distribution among elite climbers compared to controls	An excess of II- and deficiency of DD-genotypes in the elite climbers
Gayagay *et al.*[101] (1998)	Australian Caucasian Olympic trial male and female rowers (n=64)	Allele and genotype distribution in elite rowers compared to matched controls	I-allele and II genotype frequency significantly increased in elite rowers
Myerson *et al.*[173] (1999)	British male and female Olympic standard runners of mixed distances (n=79)	Allele frequency and genotype distribution compared to distance run and matched controls	I-allele associated with endurance events and D-allele associated with power events
Woods *et al.*[293] (2001)	Caucasian elite male and female swimmers (n=56)	Allele and genotype distribution in elite swimmers compared to non-elite swimmers	DD genotype and D-allele associated with elite power-orientated short distance swimmers
Nazarov *et al.*[175] (2001)	Russian athletes of both sexes from various sports (n=217)	Allele and genotype distribution of 'outstanding' athletes stratified by event duration compared to matched controls	An excess of the D-allele associated with outstanding short distance athletes, and an excess of the I-allele associated with outstanding middle distance athletes
Tsianos *et al.*[272] (2004)	Elite male and female long distance swimmers (n=35)	Comparison of ACE genotype frequency with swimming event longevity	I allele associated with longer distance and D allele with shorter distance swimmers
Hruskovicova *et al.*[123] (2006)	Successful long distance runners and skaters (n=455)	Genotype and allele frequency compared to sedentary controls	An excess of the I-allele and II genotype in successful marathon runners and skaters

individual with innate endurance capacity, as prior to training performance is independent of genotype.[170] In contrast to the I-allele, the D-allele appears to be associated with increased power-orientated performance (Table 1.1). In addition, several studies, summarised in Table 1.2, have found no association between ACE genotype and athletic performance. However, a common attribute of these studies of elite athletes was that they were selected from a wide range of disciplines, and often from events in which endurance performance alone was not a pre-requisite for success. Such heterogeneous groups of athletes were unlikely to produce reliable information. The studies of response to training in army recruits also used subjects from broad backgrounds, and exposed them to diverse training regimes and were thus unlikely to find a significant correlation.[238]

ACE Polymorphism and Skeletal Muscle Efficiency

In searching for a potential physiological interaction between the I-allele and enhanced endurance trainability, some investigators have measured maximal oxygen uptake (VO_2 max), a standard measure of cardiorespiratory fitness.[130] Several studies have shown no relationship between ACE genotype or improved endurance with VO_2 max.[130;131;295] Indeed, individuals with similar VO_2 max can differ widely in endurance performance.[60] It is thus unlikely that the enhanced endurance performance associated with the I-allele is related to changes in cardiovascular capacity. The lack of association between VO_2 max and endurance may also explain some of the negative studies reported in Table 1.2.

Another theory proposes a local muscular effect as the underlying physiological link between the I-allele and endurance performance.[288] One study revealed that oxygen consumption at a fixed workload in army recruits decreased significantly after general

Table 1.2 Studies Showing No Association Between ACE I/D Genotype and Physical Performance

Reference	Subjects	End-point	Findings
Taylor *et al.*[260] (1999)	Australian Caucasian national standard aerobic athletes of both sexes and various sports (n=120)	ACE I/D polymorphism genotype distribution among athletes compared to matched controls	No difference in ACE genotype frequencies between athletes and controls
Rankinen *et al.*[196] (2000)	Male endurance athletes from various sports (n=192)	I/D allele and genotype distribution, and maximal oxygen uptake in athletes compared to sedentary controls	I/D allele and genotype frequencies similar between athletes and controls. No association between I-allele and maximal oxygen uptake
Rankinen *et al.*[195] (2000)	Healthy Caucasian and black sedentary subjects of both sexes (n=476)	Changes in cardiorespiratory endurance parameters following a personalised 20 week training program	No association between ACE genotype and responses to endurance training, except in Caucasians where DD homozygotes had greatest improvements
Sonna *et al.*[238] (2001)	Male and female ethnically diverse US Army recruits (n=147)	Changes in peak oxygen uptake and performance in Army Physical Fitness Test (APFT) following an 8 week physical training programme	No significant association between ACE genotype and peak oxygen uptake or APFT score before and after training
Scott *et al.*[224] (2005)	International Kenyan distance runners (n=70)	Comparison of I/D allele frequency with national athletes and matched controls	ACE I/D polymorphism not associated with elite endurance status in Kenyans

training. This reduction was more marked in subjects with II genotype compared with DD subjects, suggesting an ability after training to perform 'more work for less oxygen'.[295] Delta efficiency, which represents the efficiency of muscular contraction, was also examined in these recruits. Prior to training, delta efficiency was independent of genotype, but after training delta efficiency increased in II subjects, whereas no changes were seen in DD recruits.[288] At least some of these effects on muscular efficiency are thought to be mediated by elevation of BK activity.[287] Another study has shown that the II genotype leads to a relative sparing of energy reserves during a period of intense training, suggesting enhanced metabolic efficiency.[168] Thus, in summary the low ACE activity related I-allele is associated with improved responses to endurance training, a benefit which seems to be allied to an improvement in muscular efficiency.

1.3.4 ACE Polymorphism: Effects on Inflammation

Reduction of inflammation has previously been postulated as a cause for improvements in claudicants with exercise (Section 1.2.3). Moreover, increased inflammatory markers are associated with worse physical performance in elderly.[47] The RAS has an important function in regulating inflammatory responses and ACE has been shown to have a pro-inflammatory role in almost every stage of acute inflammation.[166] An increase in local ACE expression is associated with macrophage activation, and may play a crucial role in conducting local tissue inflammatory responses.[146] ACE may also be important in regulating systemic inflammation via the Ang-II stimulation of interleukin-6.[31]

Reduction in ACE activity has been shown to attenuate systemic inflammatory responses.[166] Likewise, AT_1 receptor antagonists have been shown to reduce levels of C-reactive protein and pro-inflammatory factors in normal individuals.[62] Conversely, in

inflammatory diseases such as acute respiratory distress syndrome (ARDS), patients have both elevated ACE activity and Ang-II levels, and the D-allele is associated with increased incidence and poorer outcome.[160;285] Similarly, in meningococcal septicaemia the ACE DD genotype is associated with increased severity of sepsis and higher mortality.[108]

1.3.5 ACE Polymorphism: Other Associations

In addition to muscular efficiency and inflammation, the ACE genotype is allied to other associations that may augment the response to exercise training in claudicants. Low ACE activity associated with the I-allele has been suggested to mediate some of its effects by improving endothelial function through the increase in half-life of BK.[131] Supporting this theory is evidence which shows that endothelial dependent vasodilatation is significantly higher in endurance athletes with the II genotype, compared to those of DD genotype.[256] The DD genotype has also been associated with endothelial dysfunction and reduced vasodilatory responses in the coronary and peripheral arteries of normal subjects.[33;172] However, one study has shown no effect of the I/D polymorphism on endothelial function.[212] An increase in vasodilatation has already been proposed as a mechanism for improvements in walking ability with training, and thus changes in ACE activity may modulate this effect.

The I-allele is also associated with an increase in slow twitch, fatigue resistant type I muscle fibres, and a reciprocal decrease in fast twitch, fatigable type IIb muscle fibres.[310] Likewise, elite rowers shown to have an excess of the I-allele also have a tendency to possess a high proportion of type I muscle fibres.[131] Although low ACE activity related to the I-allele has been related to many benefits that could improve

claudicants' responses to exercise training, as yet no evidence is available that associates the I/D polymorphism with angiogenesis. However, the RAS has been shown to be involved in the angiogenic process, with ACE inhibitors demonstrated to modulate the process in contrasting ways within different tissues and disease states.

1.4 ACE AND ANGIOGENESIS

The sprouting of new capillaries in muscle, termed angiogenesis, has been suggested as a mechanism for improvement in claudicants after exercise training.[253;261;292] The beneficial effects of increased capillary density in active muscles could include a redistribution of blood flow with less shunting, improved substrate delivery and the enhanced removal of metabolites, enabling best use of the limited blood supply.

1.4.1 Angiogenesis: Cellular Events

Angiogenesis is characterised by the sprouting of new capillaries from the ends or sides of pre-existing vessels (Figure 1.5). Several distinct events have been established and are described below:

Increased Vascular Permeability and Breakdown of Cell Contacts

Angiogenesis is initiated by vasodilatation and an increase in vascular permeability. This allows the extravasation of plasma proteins, which provide a provisional scaffold for migrating endothelial cells.[39;78] Inter-endothelial cell contacts are relaxed, the underlying matrix loosens and smooth muscle cells detach, allowing the endothelial cells to migrate.[158] Proteinases such as the matrix metalloproteinases and plasminogen activators are also released, which degrade the extracellular matrix, causing the release of more growth factors and creating room for endothelial cells to migrate into.[176]

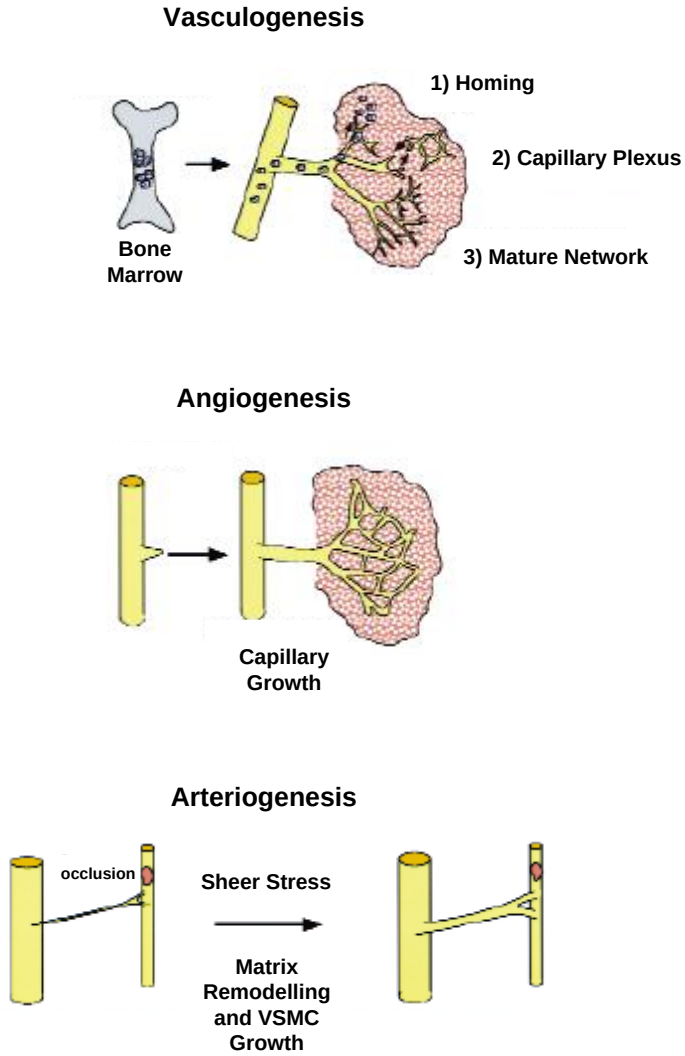

Figure 1.5 Vascular Growth in the Adult

Vascular growth may occur via *Vasculogenesis* (the mobilisation of angioblasts from the bone marrow), *Angiogenesis* (capillary sprouting) or by *Arteriogenesis* (collateral growth from existing vessels). Taken from *Carmeliet*.[39]

Migration and Lumen Development

With the physical barriers to angiogenesis degraded, endothelial cells proliferate and migrate into the cleared path. As they migrate into the extracellular matrix, the endothelial cells initially assemble into solid cords, which subsequently develop a lumen. This is achieved by intercalation and thinning of the cells, and is accompanied by fusion with pre-existing vessels to increase their diameter and length.[39;57]

Survival and Remodelling

Once the new vessels are assembled, the endothelial cells become quiescent and can survive for years. With time remodelling occurs and the vessels mature into a structured network of branching vessels.[38]

The angiogenic process is mediated by many different angiogenic factors including placental growth factor (PlGF), platelet derived growth factor (PDGF), fibroblast growth factors (FGF) and Angiopoietin-2.[38;153;158;189] Another crucial factor regulating angiogenesis, which is involved in many stages of the process, is VEGF.

1.4.2 Vascular Endothelial Growth Factor

Vascular endothelial growth factor-A (VEGF) is the most important mediator of neovascularisation associated with human diseases.[89] It is a multi-tasking cytokine, affecting diverse tissues and stimulating a variety of events.[308] VEGF belongs to a family of related growth factors, which also includes VEGF-B, VEGF-C, VEGF-D and PlGF. VEGF-C and VEGF-D are primarily lymphangiogenic factors, while PlGF has a primary role in arteriogenesis.[253] The role of VEGF-B *in vivo* is not known, but it may act a weak angiogenic factor.[235]

VEGF Isoforms

The VEGF gene is located on chromosome 6p23.1 and spans approximately 14 kilobases, containing 8 exons and 7 introns.[208;265] As a result of differential splicing and proteolytic processing, at least six isoforms are produced from the gene, which are named according to the number of amino acid residues: 121, 145, 165, 183, 189 and 206. Of these $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ are the major forms secreted by most cells types.[208] $VEGF_{121}$ lacks the amino acid residues encoded by exons 6 and 7, and exists as a freely diffusible protein as it does not bind to cell surface heparins. Although $VEGF_{121}$ mRNA transcripts are the most abundant, $VEGF_{165}$ is the most common and biologically active protein.[201;309] $VEGF_{165}$ lacks the residues encoded by exon 6, and has intermediate properties with approximately half binding to the extracellular matrix and half freely diffusible. The importance of the $VEGF_{165}$ isoform as the principle effector of VEGF is underlined by studies showing that mice lacking the equivalent of $VEGF_{165}$ tend to die soon after birth, whilst those only expressing $VEGF_{165}$ and no other isoforms are healthy.[42;240] Finally, $VEGF_{189}$ is almost completely bound to the extracellular matrix, making it a reservoir for VEGF that can be mobilised by proteolysis.[90]

VEGF Receptors

The biological effects of VEGF are mediated via two related receptor tyrosine kinases, VEGFR-1 (fms-like tyrosine kinase, Flt-1) and VEGFR-2 (kinase-insert domain receptor, KDR) found on the vascular endothelium.[231;262] The precise function of VEGFR-1 is still a matter for debate. However, the evidence suggests that the properties of VEGFR-1 vary depending on the developmental stage, pathology and tissue type.[90] In some circumstances, VEGFR-1 is thought to act as a 'decoy' receptor, negatively regulating the actions of VEGF by binding it but not transmitting an intracellular signal.

In other situations, VEGFR-1 is capable of VEGF signalling, but only weakly.[308] In addition, a soluble form of VEGFR-1 exists (sVEGFR-1), created by alternative splicing of VEGFR-1 mRNA and consisting of the extracellular domain of the VEGFR-1.[135] sVEGFR-1 acts purely as a decoy, binding free VEGF to form an inactive complex and thereby inhibiting its actions.[89] Conversely, VEGFR-2 is the most biologically important of the VEGF receptors, and is the primary receptor mediating the mitogenic, angiogenic and permeability enhancing effects of VEGF in the endothelium.[90;253] Indeed, mice lacking the equivalent of VEGFR-2 completely fail to develop a vasculature and die *in utero*.[228] Lastly, VEGF may also bind to neuropilin receptors.[137] These are non-tyrosine kinase receptors, and due to the lack of any associated signalling function are not thought to be functional *per se*.[308] The relationship between the major VEGF isoforms and receptors are summarised in Figure 1.6.

1.4.3 VEGF and Angiogenesis

The link between VEGF and angiogenesis has been demonstrated in many studies, with VEGF playing a major role in several stages of the process. As well as its importance as a factor in angiogenesis in the developing embryo, VEGF also mediates pathological angiogenesis in the adult. The effects of VEGF in angiogenesis are regulated almost entirely by VEGFR-2.[46] However, more recent data have also indicated an essential role for VEGFR-1 in pathological angiogenesis, which acts by potentiating VEGFR-2 signalling.[46;185]

In the initial stages of angiogenesis, VEGF mediates an increase in vascular permeability by redistributing intercellular adhesion molecules and altering cell membrane structure.[78] In fact, VEGF was originally termed vascular permeability

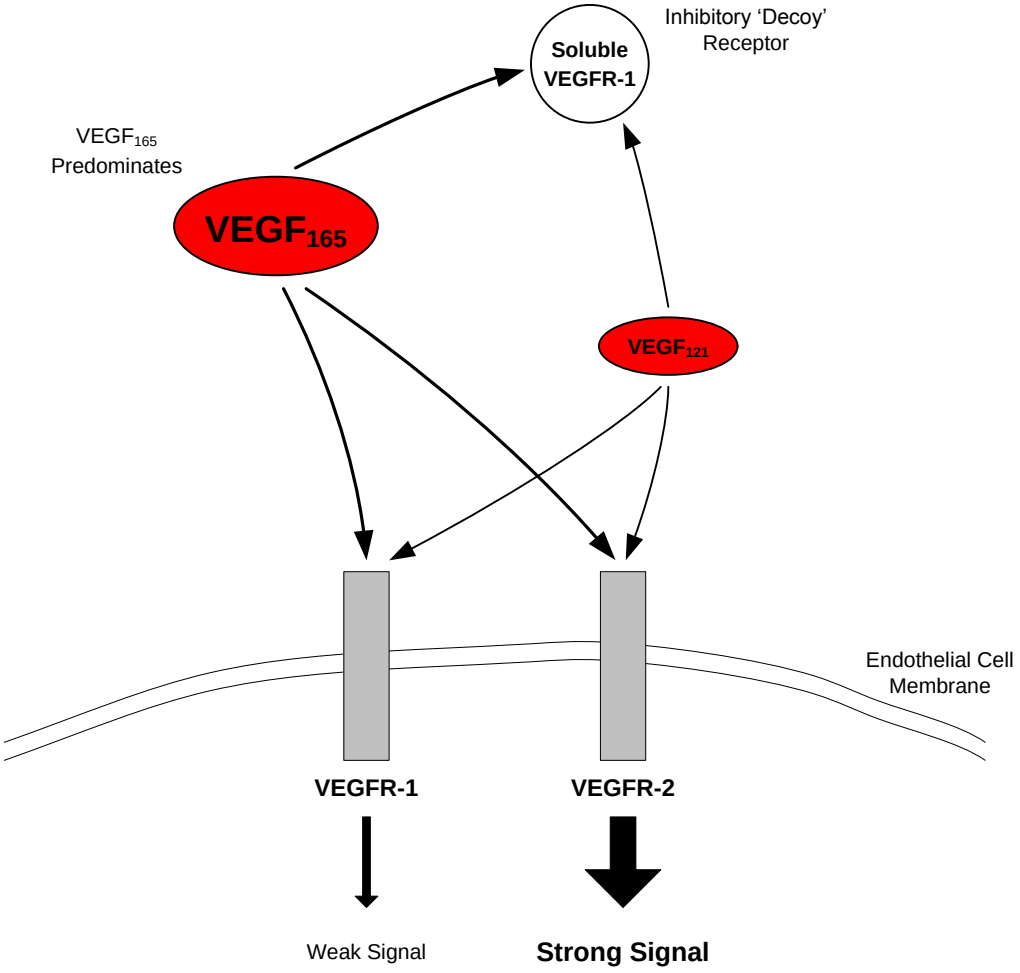

Figure 1.6 Overview of the Interactions Between the Major VEGF Isoforms and Receptors Involved in Angiogenesis

The majority of the biological effects of VEGF are mediated by the binding of $VEGF_{165}$ to VEGFR-2 receptors. VEGFR-1 receptors can sometimes produce a weak intracellular signal, whilst sVEGFR-1 receptors are inhibitory and act by binding free VEGF.

factor.[227] VEGF also subsequently stimulates the proliferation and migration of endothelial cells from existing vessels to new sites.[88] Moreover, $VEGF_{121}$ and $VEGF_{165}$ in combination with Angiopoietin-1 are critical in lumen formation and increasing vessel diameter.[247] In the longer term, VEGF acts as a survival factor for endothelial cells, with a reduction in VEGF level associated with vessel regression.[8;41] In concert with the local environment, VEGF is also important in the differentiation of the endothelium according to local needs, and the remodelling of new vessels into functional networks.[40;205]

The strong link between VEGF and angiogenesis has led to attempts to manipulate VEGF in an attempt to enhance angiogenesis and produce clinical improvements in ischaemia. Activation of VEGF in rabbits with ischaemic legs has been shown to increase capillary density and improve blood flow.[61] Recent trials using adenoviral VEGF gene transfer techniques to upregulate VEGF and stimulate angiogenesis have also been successful in animal models. However, human trials including the recent Regional Angiogenesis with Vascular Endothelial growth factor (RAVE) trial, using similar adenoviral gene transfer techniques to upregulate VEGF, have not been related to any clinical improvements in humans.[194] The results of this trial illustrate the complex processes underlying VEGF regulation.

1.4.4 VEGF Regulation

Hypoxia-Inducible Factor (HIF)-1

The major stimulus underlying VEGF transcription is hypoxia. VEGF and VEGFR regulation in hypoxia is summarised in Figure 1.7. Hypoxia triggers numerous responses in a cell including an increase in VEGF mRNA, and it has now been

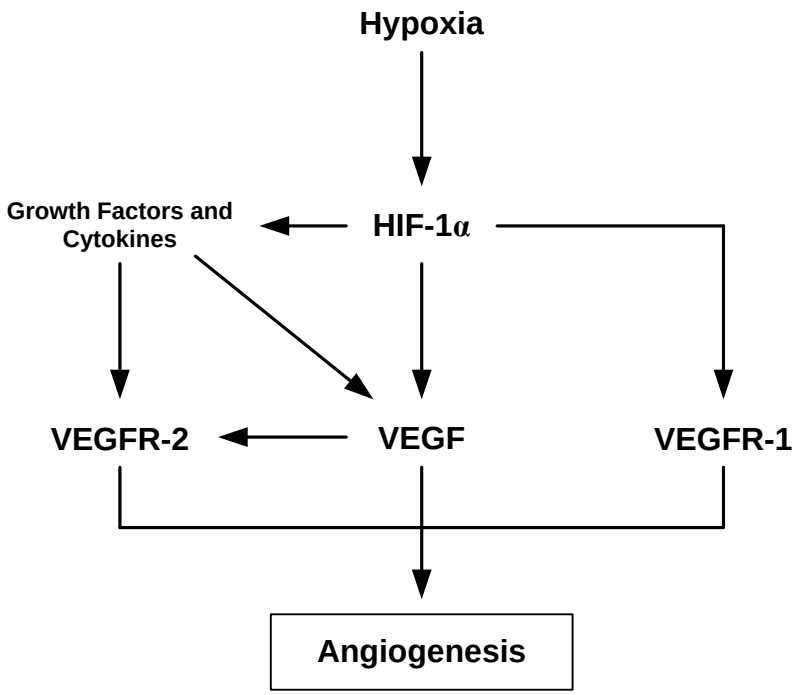

Figure 1.7 VEGF and VEGF Receptor Regulation in Hypoxia

Hypoxia leads to stabilisation of HIF-1α and increased HIF-1 levels. HIF-1 directly upregulates VEGF and VEGFR-1 expression. Growth factors and cytokines induced by HIF-1 may in turn upregulate VEGF and VEGFR-2. VEGFR-2 is also upregulated by VEGF itself.

established that hypoxia-inducible factor (HIF)-1 is the key mediator of these hypoxic responses.[226] HIF-1 is a transcriptional factor which acts as the master regulator of oxygen homeostasis and has been linked to over a hundred target genes.[192;225] It consists of a heterodimer composed of a constitutively expressed HIF-1β subunit and an inducibly expressed HIF-1α subunit, and is regulated by cellular oxygen levels and growth factors.[283] The HIF-1α subunit is usually subject to rapid degradation in non-hypoxic conditions. However, this process is inhibited by hypoxia resulting in an exponential increase in HIF-1α levels as oxygen concentrations decrease.[305] This stabilization of HIF-1α increases the formation of HIF-1 heterodimers, which in turn bind to hypoxia-responsive elements (HRE) in the promoters of hypoxia inducible genes, including VEGF and VEGFR-1, initiating transcription.[95;102] However, the VEGFR-2 promoter lacks this site, although its expression is also increased in hypoxia. VEGFR-2 expression is autoregulated, with levels increased by VEGF and bFGF.[187;230]

VEGF Expression in Ischaemic Skeletal Muscle

Although ischaemia has been demonstrated to increase VEGF expression via HIF-1, studies of human skeletal muscle from patients with leg ischaemia have revealed a more complex picture. VEGF and VEGFR-2 have both been shown to be downregulated in subjects with chronic ischaemia.[274;278] However, in cases of acute-on-chronic ischaemia HIF1-α, VEGF and VEGFR-2 gene expression are all significantly upregulated by 3-, 7- and 2-fold respectively.[274;278] In other words, different gene expression patterns occur in different states, with increases in HIF-1α, VEGF and VEGFR-2 associated only with acute ischaemia. Indeed, one might speculate that the repeated episodes of acute ischaemia produced by an exercise training programme could over time stimulate increases in the VEGF/VEGFR-2 axis and produce angiogenesis. However, one study

of claudicants showed no acute rise in VEGF protein levels in response to acute exercise, or any increase in VEGF levels after 6 weeks training.[292] As yet, no study in humans has examined the effects of exercise training in claudicants on VEGF/VEGFR gene expression.

Growth Factor and Cytokine Regulation

In addition to hypoxia and HIF-1, VEGF production is also regulated by the paracrine or autocrine release of growth factors including epidermal growth factor, transforming growth factor (TGF)-α, TGF-β, keratinocyte growth factor, insulin-like growth factor-1, FGF and PDGF.[91;177] Inflammatory cytokines such as Il-1α and Il-6 also induce VEGF expression in some cell types.[177] Finally, Ang-II and BK, which are both closely modulated by ACE and whose activity is altered by ACE inhibition, have also been demonstrated to regulate VEGF release.[233;289]

1.4.5 Effects of ACE Inhibition on VEGF and Angiogenesis

The role of the RAS and of ACE in angiogenesis has recently been the subject of many studies, and is not yet fully understood. The evidence accumulated to date has often been conflicting, frequently with opposite effects observed depending on the tissues examined and experimental protocols used. Many of these studies have examined the effects of ACE inhibitors on angiogenesis. These are outlined below:

Effects of ACE Inhibitors in Cancer

The suggestion that ACE inhibitors could decrease the incidence of cancer led to the instigation of many studies examining their effects on tumour growth and angiogenesis.[149] One of the initial studies revealed that captopril inhibited angiogenesis

and tumour growth in rats.[281] Further studies have shown that perindopril significantly inhibits angiogenesis *in vivo* in mouse models of hepatocellular carcinoma and in head and neck squamous cell carcinoma.[180;298] Perindopril also inhibits angiogenesis in hepatocellular carcinoma in association with a concomitant suppression of VEGF expression.[302] Decreased angiogenesis mediated by perindopril has been associated with a reduction in Ang-II production, reduced VEGF promoter activity and decreased VEGF transcription.[298] Moreover, decreases in both Ang-II and VEGF have been suggested as key mechanisms underlying reduced angiogenesis following ACE inhibition.[303]

The relationship between Ang-II and VEGF has been investigated extensively. Ang-II has been found to stimulate angiogenesis in animal models.[87;193] Ang-II can also induce the secretion of VEGF in several human cell types including tumour cells by upregulating HIF-1α expression.[184;193;289] VEGF is similarly able to induce ACE transcription and protein levels, indicating a synergistic relationship between the RAS and VEGF.[219] The induction of VEGF by Ang-II is mediated primarily via the AT_1 receptor, although blockade of the AT_1 receptor does not inhibit angiogenesis, illustrating the complexity of the process.[51;302]

Thus, the effects of ACE inhibitors in tumour angiogenesis are likely to be result of multiple pathways, and not just VEGF transcription.[298] It is also important to consider that tumour cells are quite different to normal cells, representing a unique environment with an often chaotic and unbalanced expression of angiogenic factors and molecular signals.[57] Tumour vessels are also structurally and functionally disorganised compared to vessels in normal tissues.[57]

Effects of ACE Inhibitors in Proliferative Diabetic Retinopathy

The discovery that lisinopril could decrease the progression of diabetic proliferative retinopathy led to many studies examining the role of the RAS in retinal angiogenesis.[48] In diabetes the retinal RAS is over-activated, with the severity of retinopathy correlating with the activity of the RAS.[65] VEGF is also significantly overexpressed.[4] Moreover, Ang-II is a stimulus for VEGF responses in the retina, as Ang-II increases VEGFR-2 expression.[182] *In vivo* studies have also shown that ACE inhibition reduces VEGF expression in the retina, which is otherwise upregulated up to four fold.[103] Thus, it has been suggested that the overactive RAS in diabetic retinas upregulates VEGF most likely via Ang-II acting on AT_1 receptors, and that ACE inhibition reduces angiogenesis by decreasing Ang-II signalling.[77;103] However, retinal VEGF expression is also closely linked to blood pressure, and it may be a reduction in blood pressure that underlies these effects rather than a direct effect of the RAS.[103;286] Evidence showing that β-blockers are equally efficacious in reducing diabetic retinopathy support this theory.[275]

Effects of ACE Inhibitors in Ischaemic tissues

In contrast to the studies of tumour and retinal angiogenesis, studies of ischaemic tissues have demonstrated a completely opposite picture, with ACE inhibition related to increased angiogenesis in both ischaemic limbs and cardiac tissue. This differential effect was clearly illustrated in a study of diabetic mice, where perindopril was observed to increase angiogenesis in ischaemic hindlimbs but reduce vessel growth in the diabetic retina.[77] In separate studies, quinaprilat has been shown to potentiate angiogenesis in a rabbit model of hindlimb ischaemia, perindopril to improve angiogenesis and blood flow hypertensive rats (independent of changes in blood pressure), and perindopril to have a pro-angiogenic effect in ischaemic mice hindlimbs.[85;233;250] Furthermore,

lisinopril has also been demonstrated to increase capillarisation in the hypertensive rat myocardium, an increase potentiated further in response to exercise training.[311] The timing of ACE inhibition is also crucial, with ACE inhibition prior to an acute ischaemic insult impairing reparative angiogenesis compared to the positive effects of inhibition in established chronic ischaemia.[81]

The mechanism by which ACE inhibition increases angiogenesis in ischaemia is complex, and has not been fully elucidated. However, the evidence indicates that VEGF up-regulation plays an important role. In a rat ischaemic model, low dose perindopril was shown to induce angiogenesis in association with a rise in VEGF, but addition of a VEGF-neutralising antibody abolished this effect.[234] Other studies have also shown a rise in VEGF associated with ACE inhibitor induced angiogenesis.[77;233]

1.4.6 Role of Ang-II and BK in Ischaemia Induced Angiogenesis

ACE activity plays an important role in regulating skeletal muscle angiogenesis. In chronic heart failure, a condition characterised by poor muscle perfusion, ACE expression is inversely related to skeletal muscle capillary density.[221] The actions of ACE are to produce Ang-II and degrade BK. The actions of these molecules in angiogenesis are described below:

Angiotensin II

Ang-II has a predominantly pro-angiogenic role in ischaemia, similar to that described previously in tumours. Rats with ischaemic limbs treated with Ang-II have increased angiogenesis allied to increases in VEGF. Furthermore, Ang-II mediated angiogenesis is prevented by VEGF neutralising antibodies.[252] Ang-II mediates angiogenesis via its

AT_1 receptor, as blockade of AT_1 inhibits Ang-II related angiogenesis.[220;251;252] AT_1 receptor knockout mice also have impaired angiogenic responses to ischaemia, an effect independent of reduced blood pressure, but related to a decrease in VEGF expression.[220] In contrast, the AT_2 receptor has anti-angiogenic effects in ischaemia. AT_2 receptor knockout mice have greater angiogenesis in response to ischaemia than wild-type mice.[236] These anti-angiogenic properties of the AT_2 receptor are partly mediated by its pro-apoptotic effects.[236] In addition, although the AT_2 receptor does not alter VEGF levels, it has been shown to inhibit VEGF-mediated endothelial cell migration and tube formation by stopping VEGF signalling at the level of akt phosphorylation.[19;236]

Ischaemia also modulates Ang-II receptor expression, causing marked rises in AT_2 levels but no change in AT_1 expression. This change in relative receptor level creates a potential increase in anti-angiogenic signalling by Ang-II, and could partly explain the pro-angiogenic role of ACE inhibition in ischaemia compared to tumour and diabetic retinal angiogenesis.[19;236] However, the role of Ang-II in angiogenesis is further complicated by reports which illustrate that Ang-II levels during chronic ACE inhibition can be normal or even raised, due to increased feedback to raise renin levels and to Ang-II generation by alternative pathways.[277] Overall, this evidence suggests that although Ang-II can modulate angiogenesis in opposing ways via its 2 main receptors, it is unlikely to be a key factor in ACE inhibitor induced angiogenesis in ischaemia.

Bradykinin

A secondary effect of ACE inhibition is decreased BK degradation, leading to raised BK levels. Interestingly, BK has been shown to be a potent activator of ischaemia induced angiogenesis. Upregulation of BK by kallikrein gene transfer enhances

angiogenesis in ischaemic limbs.[79;80] Furthermore, ischaemia induces the expression of BK_1 receptors and blockade of BK_1 or BK_2 receptors blunts angiogenesis in ischaemia.[79] As a result, it has been suggested that ACE inhibitors may stimulate angiogenesis via a BK-mediated pathway.[85;250] In support of this, it has been demonstrated that ACE inhibitor induced angiogenesis is completely blunted in BK_2 receptor deficient mice.[77;233] In addition, BK_2 receptor deficient mice also lack the rises in VEGF observed with ACE inhibition.[77] Stimulation of the BK pathway is thought to mediate angiogenesis by inducing endothelial NO synthase expression and stimulating NO signalling pathways. BK is also a pro-inflammatory cytokine, and can upregulate inflammatory factors such as cyclo-oxygenase 2 (COX-2). This induction of inflammation by BK could be another pathway to increase angiogenesis.[77;233]

In summary, the role of the RAS in angiogenesis is unclear with ACE inhibitors having opposite effects on angiogenesis depending on the tissues involved. These differences may be a product of the various pathways that ACE effects, and the relative importance and activation of each pathway in each tissue and disease state. For example, in the same animal model ACE inhibition has been shown to decrease the otherwise over-expressed VEGF in the retina whilst increasing the relatively weakly expressed VEGF in ischaemic muscle.[77] In ischaemic muscle, the BK pathway appears to be the key factor in stimulating angiogenesis in response to ACE inhibition, whereas in cancer and diabetes, inhibition of the Ang-II pathway appears to be more important. The effects of ACE inhibition on VEGF and angiogenesis in ischaemic muscle are summarised in Figure 1.8. Although the crucial role of VEGF in angiogenesis has now been established, to date there have been no reports on how VEGF or its receptors change in response to an exercise program in claudicants. In addition, although ACE inhibitors

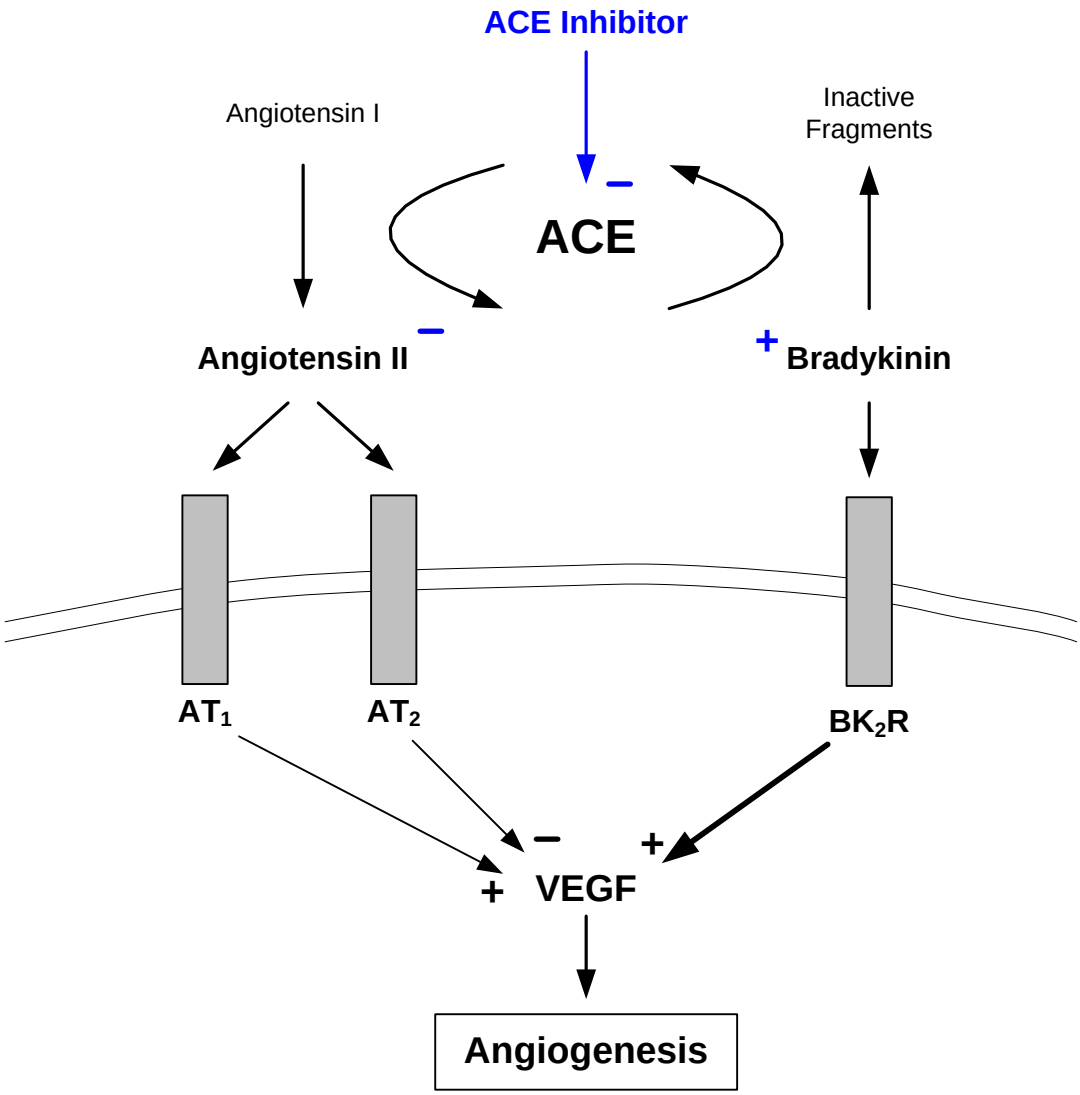

Figure 1.8 Effects of ACE Inhibition on Angiotensin II and Bradykinin in VEGF-mediated Angiogenesis in Ischaemia

In ischaemia, Ang-II produces pro-angiogenic effects via AT_1 and anti-angiogenic effects via AT_2, whilst BK is pro-angiogenic. ACE inhibition decreases the conversion of Ang-I to Ang-II and increases BK levels. In ischaemia, potentiation of the BK signalling pathway is thought to be the most important factor in ACE inhibitor induced angiogenesis.

have been shown in animal models of ischaemia to increase angiogenesis via VEGF, there are no studies illustrating the effects of ACE inhibition on VEGF in a human model of ischaemia. Finally, although the benefits of ACE inhibitors have traditionally been attributed to a decrease in Ang-II and increase in BK, recent evidence suggests that a novel ACE signalling pathway that could also explain some of the events.

1.5 ACE REGULATION AND SIGNALLING

1.5.1 ACE Regulation

Although ACE is a critical component of the RAS, there are limited data available on its regulation at a cellular or tissue level. Drugs such as dexamethasone, bleomycin and aldosterone have been reported to induce ACE expression.[66;68] In addition, several growth factors and cytokines, including endothelin-1, FGF, hepatocyte growth factor (HGF), oncostatin M and atrial natriuretic peptide (ANP) have all been demonstrated to induce ACE expression.[14;67;92;216;218] Conversely, tumour necrosis factor-α, interleukin-1β and Ang-II are reported to downregulate ACE expression.[217;223]

The intra-cellular signalling pathways regulating ACE are poorly understood. However, the evidence suggests that increases in ACE mRNA are mediated by mitogen-activated protein kinase (MAPK) and protein kinase C (PKC), which activate various transcription factors.[68;279] Both bleomycin and HGF have been shown to activate ACE gene transcription via activation of the transcription factor early growth response-1 (Egr-1), for which a binding site exists in the ACE gene promoter.[67;68] In addition, the transcription factor activating protein 1 (AP-1) is also activated by PKC and binds in the ACE promoter upregulating ACE expression.[84] Both Egr-1 and AP-1 are activated by PKC acting on the extracellular signal-regulated kinase 1/2 signalling pathway.[84]

ACE and Hypoxia

ACE is known to be up-regulated by hypoxia, with studies showing an increase in ACE synthesis in endothelial cells, and increased activity and expression in pulmonary artery smooth muscle cells.[136;171;306] Hypoxia has also been shown to increase ACE, Ang-II and AT_1 in pulmonary artery fibroblasts via HIF-1, with Ang-II subsequently potentiating HIF-1 and cellular hypoxic responses in a positive feedback loop.[141] The effects of repeated episodes of acute-on-chronic hypoxia, as observed in exercising claudicants on ACE expression has not yet been studied.

1.5.2 ACE Inhibitors and Cell Signalling

Another well described finding associated with chronic ACE inhibition is increased ACE gene expression and protein secretion, an effect which is independent of changes in Ang-II or BK levels.[58] In addition, some of the beneficial effects of ACE inhibition cannot be attributed to a decrease in Ang-II generation or an accumulation of BK.[94] Subsequent investigation has led to the elucidation of a novel signalling pathway with membrane bound ACE acting as a key signalling molecule.

The short cytoplasmic tail of membrane bound ACE contains several amino acid sequences that can interact with cellular proteins. For example, casein kinase 2 (CK2) physically interacts with the cytoplasmic tail and phosphorylates a serine residue (Ser^{1270}) on the amino acid chain, stabilising ACE within the cell membrane.[140] Mitogen-activated protein kinase kinase 7 (MKK-7) and c-Jun N-terminal kinase (JNK) also associate with the ACE cytoplasmic tail. Interestingly, binding of an ACE inhibitor (ramiprilat or perindoprilat), or to a lesser degree BK to the extracellular domain of ACE increases CK-2 phosphorylation of Ser^{1270}, activating ACE-associated JNK. This

subsequently leads to the translocation and accumulation of phosphorylated c-Jun in the nucleus, which alters gene expression.[138] This 'outside-in' ACE signalling pathway has been demonstrated to be responsible for increasing ACE expression and COX-2 expression via AP-1, and is summarised in Figure 1.9.[138;139]

Thus, in addition to its traditional roles, ACE acts as a signal transduction molecule activated by binding of an ACE inhibitor. The importance of this pathway has not yet been elucidated, but one could speculate that it could account for some of the benefits of ACE inhibition. COX-2, which is upregulated by this pathway, has been demonstrated to be an important mediator of angiogenesis. COX-2 has been demonstrated to increase VEGF expression, and COX-2 null cells do not produce VEGF.[10;214] Inhibition of COX-2 has also been shown to decrease VEGF expression.[162;304] Reciprocally, VEGF upregulates COX-2 expression.[296] Thus, upregulation of COX-2 expression by the ACE 'outside-in' signalling pathway could potentially enhance VEGF-mediated angiogenesis. Furthermore, upregulation of ACE expression by this pathway, in the continued presence of an ACE inhibitor, could lead to further increases in ACE 'outside-in' signalling.[94]

The discovery of an ACE signalling pathway also leads to the notion that genetically predisposed low ACE activity, linked to the I/D polymorphism as described by Rigat *et al.*, could be a different entity to pharmacologically lowered ACE activity, which in addition to decreasing ACE activity generates other cell signalling properties. ACE inhibitors may thus have dual benefits in ischaemia, by increasing COX-2 (and potentially VEGF) gene expression via 'outside-in' signalling, while maintaining ACE activity (but not ACE level) at a low level.

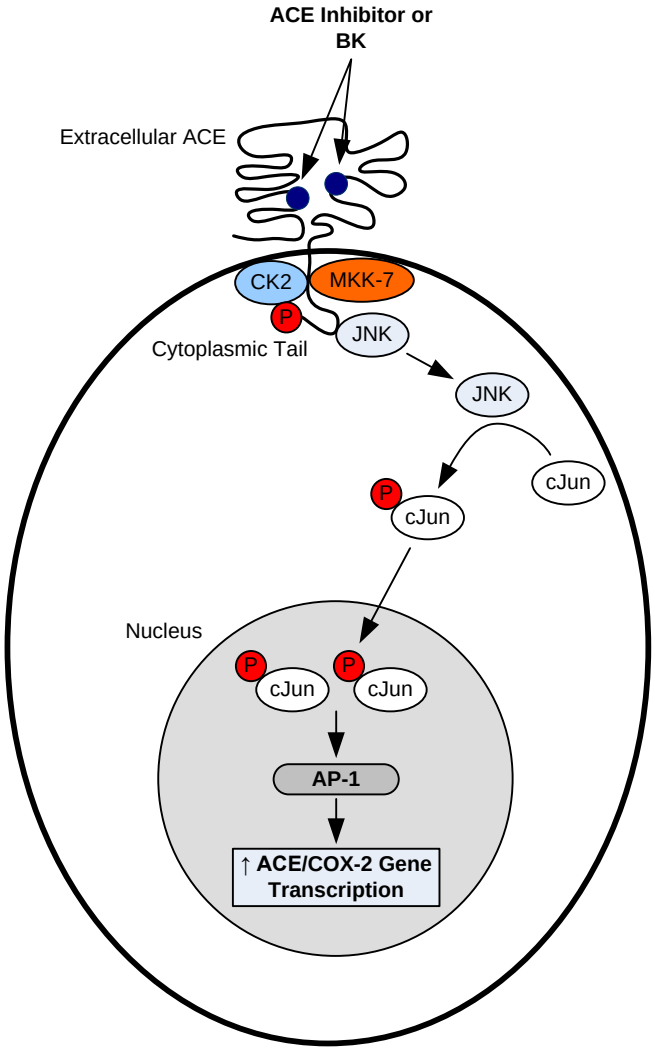

Figure 1.9 The ACE Inhibitor and BK-induced 'Outside-in' ACE Signalling Pathway

ACE inhibitor or BK binding to ACE leads to phosphorylation of ACE Ser^{1270}. Depending on initial phosphorylation, ACE-associated JNK becomes activated, most likely via ACE-associated MKK-7, leading to an accumulation of phosphorylated c-Jun in the nucleus and enhancement of the DNA-binding activity of AP-1. AP-1 activation increases ACE and COX-2 gene expression. Adapted from Ryan *et al.*[215]

1.6 ACE AND INTERMITTENT CLAUDICATION

In summary, besides its traditional role as a mediator of circulatory blood pressure and salt and water homeostasis, the RAS is found locally in many tissues where it regulates many processes including cell metabolism, inflammation and endothelial function. A functional polymorphism of the ACE gene exists, whereby the I-allele is associated with decreased ACE transcription and levels, and hence reduced ACE activity. Physiological links exist whereby variation in ACE activity could modulate responses to exercise training in claudicants: the I-allele and associated low ACE activity is related to improved metabolic efficiency, enhanced response to endurance training, reduced inflammation and improved vasodilatation, all areas that may positively affect the response to training in claudicants. The RAS and ACE have also been implicated in ischaemic angiogenesis, where the RAS contributes a major role in regulating VEGF production, and ACE inhibitors have been revealed to potentiate angiogenesis in ischaemia.

Despite this evidence, the effects of the ACE polymorphism and of ACE inhibition on walking ability in claudicants have not been extensively studied. The studies that have investigated the effects of the ACE polymorphism and ACE inhibitors in intermittent claudication will be discussed below:

1.6.1 ACE I/D Polymorphism and Intermittent Claudication

Very few studies have scrutinized possible links between the ACE I/D polymorphism and peripheral arterial disease. Conflicting reports have been presented regarding a potential association between the ACE polymorphism and the prevalence of peripheral arterial disease, with the majority of studies finding no significant

association.[133;202;258;271] However, given the heterogeneous populations analysed, these studies were unlikely to find a significant link. Only one study of Chinese diabetics associated the D-allele with a significantly increased risk of peripheral arterial disease.[263] Nevertheless, it remains doubtful that the ACE polymorphism is a major risk factor for the development of peripheral arterial disease. In another study, the II genotype was shown to be associated with a significantly reduced risk of atherosclerotic disease progression compared to subjects with the DD genotype.[259] However, a subsequent investigation by the same group showed no relationship between the D-allele and progression of atherosclerosis in patients with peripheral arterial disease.[257] Finally, a study of 65 subjects with hypertension secondary to renal artery stenosis reported an association between the DD genotype and increased severity of peripheral arterial disease. This association was not seen in subjects with essential hypertension and may be related to enhanced activation of the RAS in renovascular hypertension.[156]

As yet no published study has examined the effects of the ACE genotype on responses to exercise training in peripheral arterial disease. However, previous work in our laboratory studied the association of the ACE polymorphism with responses to supervised exercise training in a group of stable claudicants. Pre-training assessments were independent of genotype. However, after training, subjects possessing an I-allele (n=12) improved claudication distance (CD) by 151% and maximum walking distance (MWD) by 251%, compared to only 46% and 29% respectively for subjects without an I-allele (n=3). Thus, the I-allele appeared to be related to improved responses to exercise training. No change in cardiovascular fitness or ankle brachial pressure index (ABPI) was found in the subjects, suggesting a local muscular effect as the mechanism for the improvement (see List of Presentations).

1.6.2 ACE Inhibitors and Intermittent Claudication

The benefits attributed to the I-allele of the ACE polymorphism are thought to be mediated by its associated low ACE activity. In contrast, ACE is known to be up-regulated by hypoxia (see Section 1.5). This activation of the RAS could be detrimental to training responses in claudicants, but a potential means of correcting this would be to suppress ACE activity pharmacologically using ACE inhibitors. These drugs are widely prescribed, and have been used for some time in the treatment of hypertension. Several different ACE inhibitors exist, which have slightly different effects according to their structure, lipid solubility and pharmacokinetics. The majority in use today are pro-drugs which are metabolised by the liver into an active form.[237] They are also effective in decreasing both serum and tissue ACE activity.[82] However, the potential for tissue inhibition for each drug is related to each drug's lipid solubility. For example ramiprilat, the active form of ramipril, is 23 times more lipophilic than enalaprilat and is associated with much more pronounced tissue ACE inhibition.[17]

Interestingly, ACE inhibitors have been discovered to have a variety of clinical benefits unrelated to changes in blood pressure, which may all be related to the effects of tissue inhibition and could have positive implications for claudicants. In the management of heart failure, the benefits gained from ACE inhibitors exceed what can be explained by improvements in cardiovascular status, and it has been suggested that some of the benefits in these patients may be the result of increased muscular efficiency.[167] ACE inhibitors could also potentiate ischaemic pre-conditioning, by increasing the availability of BK, a known key trigger leading to preserved ATP generation in ischaemic conditions.[76;300] In addition, ACE inhibition helps peripherally in chronic heart failure, improving local vasodilatation, oxygen extraction and exercise

performance more than improvements in cardiac output.[74] ACE inhibitors have also been shown to decrease inflammation by preventing Ang-II mediated Il-6 production, an effect that has been linked to improved atherosclerotic plaque stability and a decrease in acute coronary syndromes.[20;105;166] Indeed, the HOPE study has shown that ramipril reduces deaths and cardiac events in a broad range of high risk patients, leading to the widespread prescribing of ACE inhibitors in vascular patients.[307]

Studies of ACE inhibitors in Intermittent Claudication

The majority of reports on the effects of ACE inhibitors in ischaemic muscle have come from animal studies. One study reported that ACE inhibition enhanced blood flow to ischaemic muscles and increased exercise tolerance after training, a benefit potentially relevant to claudicants.[297] Moreover, the effects of ACE inhibitors on angiogenesis has already been discussed in detail in Section 1.4.5.

Only a limited number of studies have examined the effects of ACE inhibitors in claudicants. Several reports demonstrate that captopril preserves or even increases blood flow, and increases walking ability in hypertensive claudicants.[45;152;181;207] These benefits seem to be independent of its blood pressure lowering effects.[118;250] However, other trials have demonstrated captopril to have no benefits in claudicants.[21;206] Furthermore, captopril is one of the first generation of ACE inhibitors, possessing a sulphydryl group which has its own pharmacological properties completely distinct from ACE inhibition.[11;191] Thus, any effect of captopril on claudicants in these studies cannot definitely be attributed purely to ACE inhibition. The sulphydryl group is also associated with several side-effects, and captopril has markedly less tissue activity compared to newer ACE inhibitors, and is thus now rarely prescribed.[17;128] Amongst

other ACE inhibitors, one trial demonstrated an association between lisinopril and improved walking ability, although this was a similar effect to that of bisoprolol, and no placebo or control group was used.[276] Another study of perindopril showed no change in ABPI, a marginal increase in claudication distance and a slight reduction in walking distance compared to placebo.[183]

Many of the trials described above were poor quality studies, often with no placebo control and with low subject numbers. Indeed, a recent Cochrane review on the impact of drug treatment for hypertension on peripheral arterial disease included only 2 out of a possible 46 studies in the final review, with the others excluded due to poor quality.[155] However, a more recent randomised double-blind controlled trial comparing ramipril to placebo in non-diabetic, normotensive claudicants provides more compelling evidence for the benefits of ACE inhibitors in claudication, with a significant increase in both pain free and maximum walking time recorded with ramipril compared to placebo.[3] As has been previously stated, ramipril is noted for its lipid solubility and hence its enhanced tissue activity.[17] One could speculate that it is this tissue ACE inhibition in ischaemic limbs that would provide maximal benefit to claudicants. A summary of the studies performed to date on ACE inhibition and claudication is shown in Table 1.3.

Thus far, no randomised control trial of supervised exercise training and ACE inhibitors in claudicants has been published. However, previous work in this laboratory examined the effects of the low dose ramipril on responses to a supervised exercise training program using a randomised double-blind placebo controlled crossover study (See List of Presentations). In contrast to the study by Ahimastos *et al.*, this trial showed ramipril to be significantly detrimental to training responses. Training on placebo increased CD

Table 1.3 Summary of Studies Performed to Date of ACE Inhibitors in Intermittent Claudication

Reference	Subjects/Drugs	End-point	Findings
Catalano *et al.*[45] (1985)	Captopril and hypertensive claudicants	Pain free walking interval and ABPI	Captopril improved both pain free walking interval and ABPI
Libretti *et al.*[152] (1986)	8 weeks of captopril or chlorthalidone in hypertensive claudicants (n=40)	ABPI, pain free and absolute walking intervals	Captopril improves ABPI, pain free and absolute walking intervals compared to chlorthalidone
Roberts *et al.*[207] (1987)	1 month each of captopril, atenolol, labetolol and pindolol in hypertensive claudicants (n=20)	CD, MWD and blood flow	Captopril preserved (but did not improve) blood flow, CD and MWD compared to placebo. Beta- blockers reduced BF, CD and MWD
Bernardi *et al.*[21] (1988)	2 weeks each of captopril, nicardipine and placebo in normotensive claudicants (n=10)	Pain free walking interval, duration of exercise and ABPI	Captopril improved ABPI but had no effect on walking ability
Roberts *et al.*[206] (1992)	3 months each of captopril and nifedipine in hypertensive claudicants (n=12)	Calf blood flow and treadmill walking ability	No difference in walking ability. Improved blood flow compared to nifedipine
Van der Ven *et al.*[276] (1994)	Effects of lisinopril and bisoprolol on exercise trained hypertensive claudicants (n=11)	Walking ability, blood flow and vascular resistance	Walking ability improved on both lisinopril and bisoprolol. No change in vascular resistance or blood flow
Overlack *et al.*[183] (1994)	6 weeks of perindopril or placebo in hypertensive claudicants (n=54)	ABPI, CD and MWD	No change in ABPI. Marginal increase in CD and reduction in MWD with perindopril
Novo *et al.*[181] (1996)	12 weeks each of captopril, ticlopidine and placebo in hypertensive male claudicants (n=24)	CD and MWD	Captopril increased CD and MWD compared to placebo and ticlopidine
Ahimastos *et al.*[3] (2006)	24 weeks of ramipril or placebo in normotensive claudicants (n=40)	Pain free and maximum walking time	Ramipril improved pain free and maximal walking time compared to placebo

by 90 metres and MWD by 754 metres, whilst after ramipril the improvement in CD was only 35 metres and MWD 225 metres. ABPI was unaffected by either drug treatment. However, this was a pilot study involving only 8 subjects, and the data are not conclusive.

Thus, the question still remains as to whether ACE and VEGF have a significant role in improving walking ability in claudicants after exercise training, and whether reduction of ACE activity with ACE inhibitors could play a role in improving exercise training responses and increasing walking ability.

CHAPTER 2

HYPOTHESIS AND AIMS

CHAPTER 2

2.1 BACKGROUND

Intermittent claudication remains a major and widespread health problem in the western world, and can be associated with a significant impairment of walking ability and quality of life.[243] The role of surgery and angioplasty for intermittent claudication is limited, and until now pharmacological treatments of the condition have provided little or no reduction in symptoms.[229] However, exercise training and supervised programmes in particular, have been associated with significant improvements in symptoms, and exercise therapy continues to be the mainstay for treatment.[244] The mechanism by which exercise improves intermittent claudication is not fully understood and has recently been identified as an area for potential research.[86] It is thought that the mechanism is multi-factorial, and includes angiogenesis, changes in muscle metabolism, improved endothelial function, and a reduction in inflammation.[244;255]

One metabolic pathway that could influence responses to exercise training is the RAS. More specifically low ACE activity, associated either with the I-allele of the ACE I/D polymorphism or pharmacological reduction by ACE inhibitors, has been associated with increased muscular efficiency and endurance, reduced inflammation and enhanced endothelial function.[74;166;256;288] Thus, potential physiological associations exist whereby variation in ACE activity may modulate responses to exercise training in claudicants. Furthermore, previous unpublished work in this laboratory has associated the I-allele with enhanced response to training in claudicants. However, while many studies have examined the link between the ACE polymorphism and exercise training responses or endurance performance, as yet no studies have examined the effects of an exercise training programme on ACE expression and protein levels. These phenotypic responses

(gene expression and protein levels) potentially provide greater information than genotype studies, as ACE activity *per se* can be linked directly to exercise training responses. It is possible that the ACE genotype is in linkage disequilibrium with another as yet undiscovered factor, rather than being the actual factor underlying enhanced training responses. By quantifying the responses of the ACE gene to this environmental stimulus, a clearer picture on whether the gene is of influence can be established. Furthermore, ACE activity can easily be modified pharmacologically using existing widely used drugs, making it an amenable target for potential treatment of claudicants.

The RAS is also involved in regulating angiogenesis via its interaction with VEGF. Angiogenesis provides many potential advantages in claudicants, including a redistribution of blood flow, reduced shunting, improved substrate delivery and the enhanced removal of metabolites. VEGF and its receptors, in particular the $VEGF_{165}$ isoform and VEGFR-2, play a vital role in almost all the stages of angiogenesis, and in ischaemia low ACE activity is related to increased VEGF and angiogenesis.[90] However, attempts to manipulate VEGF so far in humans have produced limited clinical benefits.[194] Nevertheless, the link between the RAS and VEGF in ischaemia provides a possible basis for augmenting angiogenesis in claudicants. In animal models of ischaemia, VEGF is upregulated by ACE inhibitors, most likely via a mechanism primarily involving BK rather than Ang-II, leading to improved blood flow and endurance performance.[233;297] Furthermore, other studies have shown that ACE inhibitors and BK (itself raised in association with low ACE activity) possess signalling properties via membrane bound ACE, and can cause increases in ACE and COX-2 (and potentially VEGF) expression.[93] Thus, ACE inhibitors may have dual benefits in ischaemia, by increasing gene expression via 'outside-in' signalling, while also

maintaining low ACE activity. It is possible that some of the beneficial effects of ACE inhibition could be accounted for by this mechanism. The effects of exercise training in claudicants, or of ACE inhibition in a human model of ischaemia, on differential VEGF isoform and receptor gene expression and protein levels has not yet been studied.

Thus, the evidence points towards low ACE activity, whether genetically inherited or pharmacologically manipulated, to be potentially of benefit to claudicants and walking ability, with an increase in VEGF-mediated angiogenesis one of the major possible improvements. However, clinical trials of ACE inhibitors in claudicants have shown mixed results, and the possible benefit from using ACE inhibitors to treat intermittent claudication remains unclear. Previous work in this laboratory indicated that ACE inhibitors were detrimental to training responses in claudicants, whilst another recently published trial showed ramipril to be related to a significant improvement in walking ability.[3] One means to better understand the potential role of ACE, VEGF and ACE inhibition in claudicants would be to assess how exercise training in claudicants affects ACE and VEGF biology, as determined by mRNA expression and protein levels, and relate these molecular changes to improvements in walking ability. In addition, the effects of ACE inhibition in hypoxia on ACE and VEGF could also be studied. Thus, the following questions will be addressed by this thesis:

1. How does repetitive exercise training in claudicants affect ACE and VEGF gene expression and protein levels? Are these changes related to improvements in walking ability?
2. Is VEGF receptor expression affected in the same way as VEGF?
3. How do ACE inhibitors affect ACE and VEGF in a human model of ischaemia?

In considering the first question, whilst it is known that acute exercise or hypoxia leads to an increase in ACE activity and Ang-II, the effects of repetitive exercise training and hypoxia on ACE expression are unknown.[171;294] Interestingly, when examining inflammatory responses in claudicants, acute training is known to promote inflammation, but after a prolonged period of repetitive training inflammation becomes attenuated.[267] As ACE acts as a pro-inflammatory cytokine, it is possible that this reduction in inflammation could be related to a concurrent decrease in ACE expression.

Secondly, VEGF is known to be increased in acute hypoxia. However, when examining the effects of exercise training on VEGF, it is important to study all the various VEGF isoforms and receptor types, as these all have varying and often opposing roles in VEGF biology, and could respond differently to training. Furthermore, the reported association between ACE and VEGF suggests a potential synergistic effect, with a decrease in ACE activity likely to amplify any increase in VEGF.

Finally, in considering the effects of ACE inhibition on ACE and VEGF in a human model of ischaemia, results of studies to date in animal models suggest that ACE inhibitors will increase both ACE protein expression and VEGF activity (while maintaining low ACE activity by its effects on binding to ACE).

2.2 HYPOTHESIS AND AIMS

The central hypothesis of this thesis is that low ACE activity is related to improved responses to exercise training in claudicants, by a mechanism involving its interaction with VEGF (in addition to its general effects on endurance performance). This thesis will concentrate specifically on the effects of exercise training in claudicants on ACE

and VEGF, the interaction between ACE and VEGF, and on the effects of ACE inhibition on ACE and VEGF responses to hypoxia. A summary of the experimental hypothesis is illustrated in Figure 2.1. In more specific detail, the aims of this thesis are to:

1. Examine the effects of a supervised exercise training programme on walking ability in a group of claudicants.
2. Examine the effects of the exercise training programme on ACE gene and protein expression, together with VEGF isoform/VEGF-R gene expression and VEGF protein levels. In addition, examine the influence of these molecular changes on the physiological responses to the training programme, and investigate the link between changes in ACE and VEGF expression.
3. Examine in ACE I/D heterozygotes, the differential baseline allele expression and the individual responses of the I- and D-alleles to training.
4. Develop a stable human endothelial cell culture model of hypoxia in which ACE and VEGF are reliably expressed.
5. Assess how ACE inhibitors modulate ACE/VEGF expression and protein levels in a model of human tissue hypoxia.

To address these aims, the following experiments were performed.

2.3 EXPERIMENTAL DESIGN

To address the first 3 aims, a randomised controlled clinical trial was developed to examine the effects of an exercise training programme on walking ability and ACE, VEGF and VEGF-R gene activity in patients with intermittent claudication. Patients were recruited to the trial and randomised to a treatment group (8 weeks supervised

training) or a control group (exercise advice only). Prior to training, a set of baseline measurements was recorded. Walking ability was assessed by an incremental treadmill walking test and by a repetitive heel raise test. ACE, $VEGF_{121}$, $VEGF_{165}$, VEGF-R1, sVEGFR-1 and VEGFR-2 mRNA expression were all determined by reverse transcriptase polymerase chain reaction (RT-PCR) of mRNA obtained from peripheral blood mononuclear cells (PBMNs) isolated from whole blood. ACE genotype was confirmed by PCR of genomic DNA isolated from PBMNs. Plasma levels of ACE and VEGF were measured using a sandwich enzyme-linked immunosorbant assay (ELISA). Differential expression of the D- and I- alleles in ACE I/D heterozygote subjects was assessed by RT-PCR as described above, using a specific primer set and restriction enzyme protocol. This protocol exploited a known linkage between the ACE I/D polymorphism and a single nucleotide polymorphism involving a G/A substitution that creates a restriction enzyme site. In the treatment group, the 8 week training period consisted of three 30 minute sessions of supervised treadmill walking exercise per week. The control group was given standard verbal advice to do as much walking as possible, and to try to walk through the claudication pain. Following the 8 week treatment period, all the initial assessments were repeated.

To address the final 2 aims, a laboratory cell culture model of hypoxia was developed using a human umbilical vein endothelial cell line (ECV 304) treated with Diethylenetriamine-nitric oxide (DETA-NO). DETA-NO is a nitric oxide donor which has been shown previously to create a ‘metabolic hypoxia’ by inhibiting the electron transport chain and cellular respiration, thus driving cells into anaerobic metabolism.[6] Once a stable and viable model had been established the effects of hypoxia alone, ACE inhibition alone, and of ACE inhibition combined with hypoxia on ACE and VEGF

gene activity was measured. ACE, $VEGF_{121}$ and $VEGF_{165}$ mRNA expression was determined by RT-PCR of mRNA extracted from cell lysates. ACE and VEGF protein levels were measured by ELISA of cellular protein extracted from the same cell lysates.

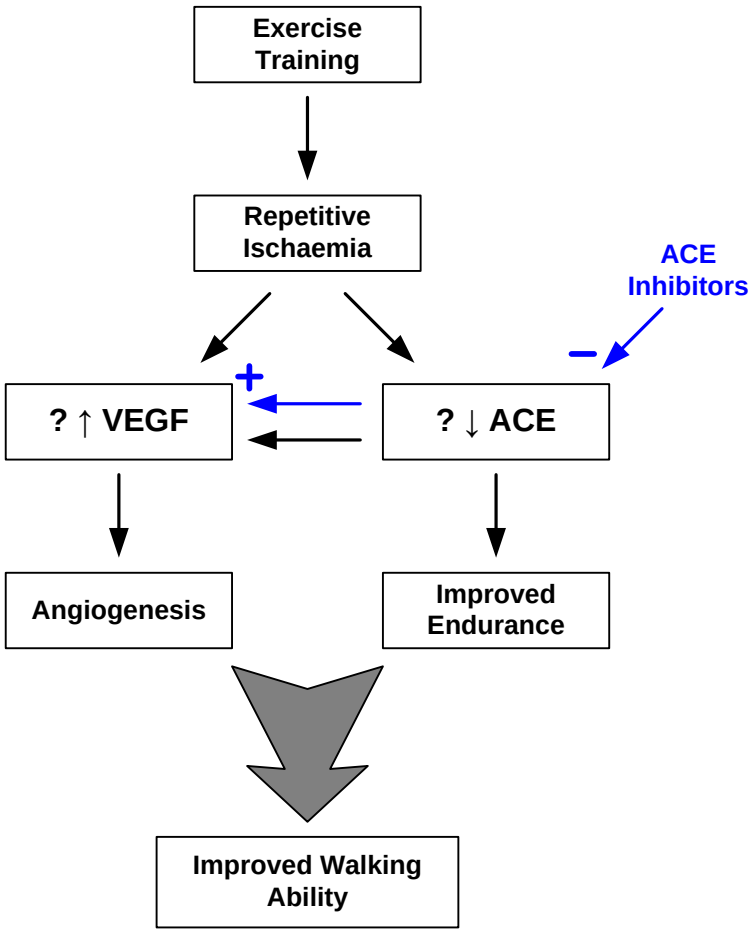

Figure 2.1 Summary of Hypothesis

Both ACE and VEGF potentially play a role in the improvements in walking ability noted after exercise training. However, the effects of exercise training in claudicants on ACE and VEGF are unknown. Furthermore, evidence suggests that inhibition of ACE could potentiate VEGF responses in addition to decreasing ACE activity, both effects that could increase the benefits of training in claudicants. Thus, the central aim of this thesis was to examine the effects of exercise training on ACE and VEGF, and to assess the effects of ACE inhibition on ACE and VEGF responses in ischaemia.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

3.1 CLINICAL TRIAL: TRAINING RESPONSES IN CLAUDICANTS

The aim of this trial was to examine the responses of a cohort of claudicants to a supervised exercise training programme, specifically looking at measurements of walking ability in relation to changes in ACE, VEGF and VEGF receptor expression and protein levels. A separate group of claudicants who were given exercise advice alone were used as a control.

3.1.1 Ethical Approval and Study Outline

Ethical approval for the study was granted by the Joint UCL/UCLH Ethics Committee in Ethics of Human Research: Committee A. An overall outline of the study is illustrated in Figure 3.1. In summary, patients fulfilling the study inclusion criteria were recruited. A series of baseline measurements were initially made. Demographic data and general measurements including height, weight, blood pressure and ABPI were recorded. Walking ability was assessed on a treadmill using an incremental treadmill test and calf endurance by repetitive calf raises. A blood sample was taken for DNA, RNA and protein analysis. ACE, $VEGF_{121}$, $VEGF_{165}$, VEGF-R1, sVEGFR-1 and VEGFR-2 mRNA expression were all determined by RT-PCR of mRNA obtained from PBMNs isolated from blood. ACE genotype was confirmed by PCR of genomic DNA also isolated from PBMNs. Plasma levels of ACE and VEGF protein were measured using an ELISA. Differential expression of the D- and I- alleles in ACE I/D heterozygote subjects was assessed by RT-PCR and a restriction enzyme protocol. Subjects were then randomised to receive either supervised treadmill exercise training three times a week or exercise advice alone for 8 weeks, following which all the baseline assessments were repeated.

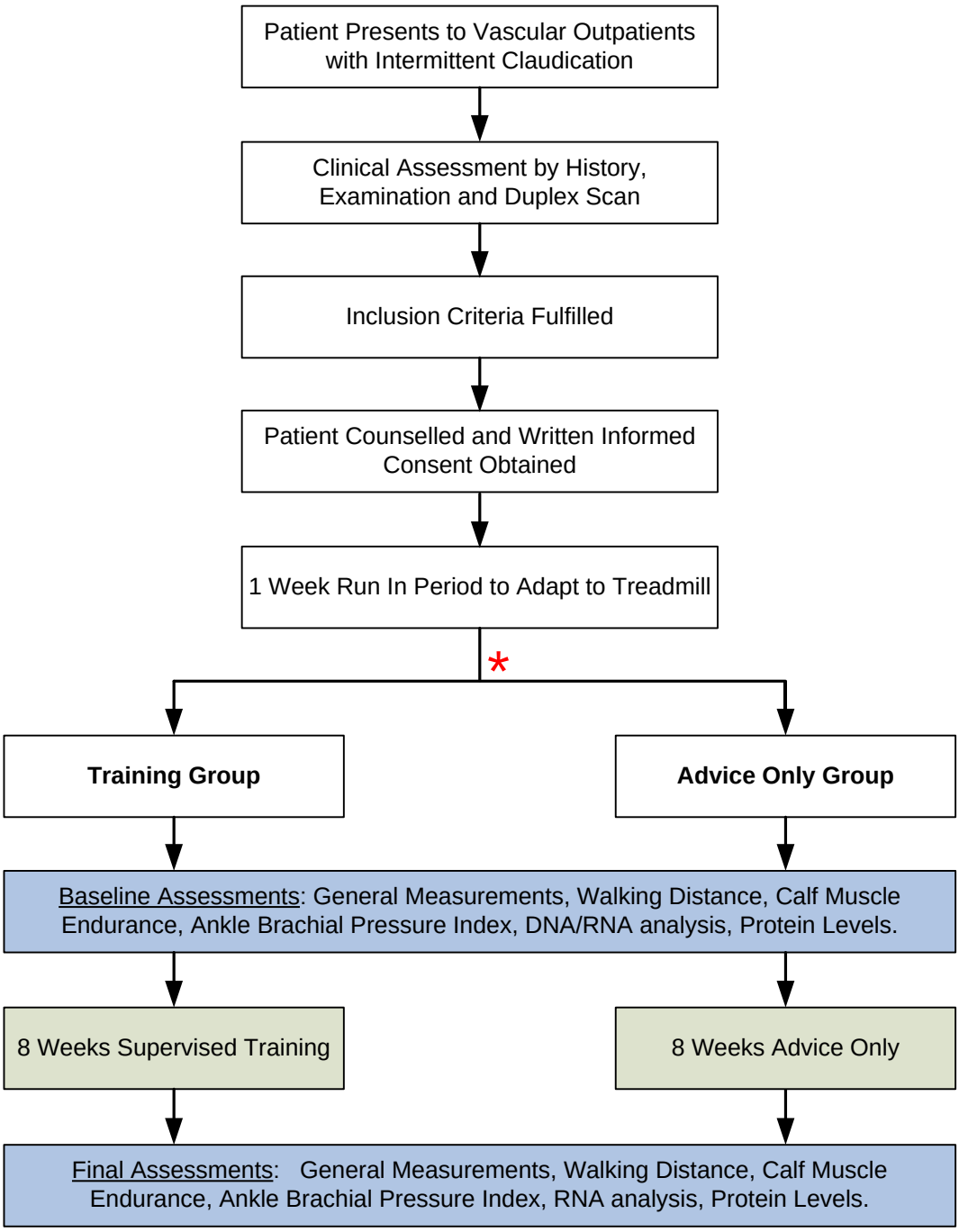

Figure 3.1 Study Outline

* Patients Randomized to Treadmill Training or Exercise Advice Only For 8 Weeks.

3.1.2 Patient Recruitment and Randomisation

Patients were identified and recruited from vascular surgery clinics at the Middlesex and University College Hospitals, London. Some subjects were also referred directly by their GPs for assessment. Subjects with stable but limiting intermittent claudication were evaluated to take part in the trial. Eligibility was assessed by taking a full medical history, clinical examination and arterial duplex scanning. Only patients with a good history for intermittent claudication, signs of arterial disease on examination, an ankle-brachial pressure index (ABPI) of ≤ 0.9, and an arterial duplex scan confirming arterial stenoses were included. Intermittent claudication was defined by a history of muscular leg pain associated with walking, which increased on further walking and subsided within a few minutes on resting. Subjects with evidence of other significant disease processes that could limit walking distance or the subsequent response to an exercise programme were excluded. Patients prescribed ACE inhibitors or angiotensin II receptor antagonists, which potentially alter ACE and VEGF expression, were also excluded. Full inclusion and exclusion criteria are shown in Table 3.1. Once the patient had been identified as suitable for the trial, they were informed and given an information leaflet about the trial, and offered an appointment to attend the exercise laboratory at the Middlesex Hospital. At this appointment, they were briefed in detail about the trial, assessed for their ability to walk on a treadmill and if in agreement full written informed consent was obtained.

On entry, demographic data such as age, sex, and cardiovascular risk factors were recorded. A detailed vascular history including estimated walking distance, affected leg(s) and previous vascular interventions was taken. The disease distribution on arterial duplex scan was noted. Each subject then completed a 1 week run-in period, consisting

Table 3.1 Study Inclusion and Exclusion Criteria

INCLUSION CRITERIA

History and examination findings consistent with intermittent claudication

Arterial duplex scan confirming arterial stenoses and resting ABPI ≤0.9

Claudication symptoms stable for > 3 months

EXCLUSION CRITERIA

Unlimited walking distance

Unable to walk on a treadmill

Evidence of critical ischaemia (rest pain or ulceration)

Severe cardiac or respiratory disease

Walking distance limited by other factors such as osteoarthritis or back pain

Patient prescribed ACE inhibitors or angiotensin II receptor antagonists

Angioplasty or surgical intervention within the last 6 months

Diabetic neuropathy

Claudication symptoms not stable

of 3 half hour sessions walking on a treadmill (Cardiokinetics Meditrack), allowing them to familiarise themselves with the treadmill prior to taking the initial walking test. This important adaptation period reduced the potential error in measuring a subject's initial walking distance, which could have been underestimated due to the subject's unfamiliarity with walking on a treadmill. These sessions took the format of a training session, as described in Section 3.1.5. Subjects were subsequently randomly assigned to either the treatment group (supervised training) or control group (exercise advice). Randomisation was achieved using identical sealed envelopes containing a card marked either 'Training Group' or 'Control Group'. Twenty five of each card was initially produced and placed in an envelope. Once sealed, the envelopes were randomly marked from 1 to 50, and the envelopes allocated in numerical order to each recruited patient. Although a large number of patients were screened for inclusion in the study, only 11 subjects were recruited, 6 of whom were randomised to the training group and 5 to the control group. Although chronic obstructive airways disease (and hypoxia) is known to have adverse effects on skeletal muscle performance and metabolism, it was not excluded as this would have severely limited available recruits.[159] Nevertheless, none of the recruited subjects had significant symptomatic pulmonary disease.

3.1.3 Patient Assessments

At initial and final assessments, a blood sample was taken and general measurements recorded first. This was followed by measurement of walking ability on a treadmill and after a 5-10 minute break, calf muscle endurance. The protocols are described below:

General Measurements

Height, weight, resting heart rate and blood pressure were recorded in each subject.

Walking Distance

A graded treadmill protocol was chosen to assess patient walking ability, using testing protocols based on those recommended by the TASC working group.[142] Graded treadmill protocols have advantages compared to constant protocols. The results of a graded test are more reproducible and the same test can also be applied to claudicants with a broad range of walking ability.[112;143] Both the distance to the onset of claudication pain (claudication distance, CD) and the distance at which the pain caused the subject to stop (maximum walking distance, MWD) were recorded. Prior to assessment, subjects were given advice to avoid vigorous activity, caffeine and excessive alcohol intake for 24 hours. All tests were performed in the morning, and one person conducted all the tests in this study. The treadmill speed was set at 3.2 km/hr, and the gradient initially set at 0% and increased by 2% every 2 minutes. The subject was asked to report the onset of claudication symptoms, and then encouraged to walk as far as possible through the pain. Use of the handrails was avoided, as this has been shown to potentially reduce the reliability of measurements.[100] Maximum heart rate was recorded at the end of the test. Perceived leg pain and perceived exertion scores were also recorded using Borg scales (RPE for exertion and CR10 for pain; Appendix 1). These are reliable and reproducible measures of perceived exertion and pain, and enabled the perceived exertion and pain to be assessed between different tests.[24]

Calf Muscle Endurance

Another endpoint measured was calf muscle endurance. Subjects were asked to perform repetitive heel raises until forced to stop by calf pain. This technique has been suggested as a screening test for peripheral arterial disease, and has been shown to have a similar effect on ankle pressures as treadmill walking.[9] Changes in the number of heel raises

also correlate to changes in walking distance, but could have the added benefit over treadmill testing of not having a 'learning curve' or be affected by changes in biomechanics that occur after a period of treadmill training.[112;178] Subjects were required to perform heel raises at a standardised rate of 20 per minute. At the end of each test, perceived pain and exertion were recorded using Borg scales. This test was performed about 5-10 minutes after the treadmill test. This non-standardised time delay could have affected the results of this test. In addition, the previous test of maximal walking distance could have subsequently affected this test, due to ischaemic preconditioning.

Ankle-Brachial Pressure Index

The ankle-brachial pressure index (ABPI) was used as an outcome measure as a simple estimator of leg perfusion. A sphygmomanometer blood pressure cuff and doppler probe (Huntleigh Diagnostics Dopplex II) were used to measure systolic blood pressures in both arms and ankles, from which ABPIs were calculated.

3.1.4 Blood Sample Collection and Processing

In order to determine genotype and measure changes in gene expression and protein levels, blood was collected from each patient at the beginning of each assessment session. Peripheral blood mononuclear cells (PBMNs) were isolated from the blood for DNA and RNA extraction, and plasma taken for measurement of protein levels. Ideally, it would have been preferable to have taken muscle biopsy specimens from each subject to examine for local tissue changes in gene expression and protein levels. However, this would not have been practical or acceptable for the patients. Thus, PBMNs and plasma were used, and should give an indication of changes occurring locally in the tissues.

Approximately 8 ml of blood was taken into Vacutainer® EDTA Blood tubes (Becton Dickinson). These samples were all processed within 20 minutes of collection, using a sterile polysaccharide (5.7% w/v) and sodium diatrizoate (9.1% w/v) solution (Lymphoprep™; Axis Shield) to separate the blood into its constituent components. The following protocol was used:

Centrifugation

Three ml of Lymphoprep™ was added to two sterile 15 ml centrifuge tubes (Corning). Four ml of blood was then carefully layered on top of the Lymphoprep™ layer (Figure 3.2), and centrifuged (ALC PK120 centrifuge) at 400 g for 30 minutes at room temperature. Lymphoprep™ caused the erythrocytes and granulocytes to aggregate, increasing their sedimentation rate significantly, while having little effect on PBMN sedimentation.[109] Thus, after centrifugation the mixture separated into distinct bands (Figure 3.2), with the plasma uppermost, the PBMNs in a thin band or 'buffy coat' at the plasma-medium interface, and the red blood cells and granulocytes in the bottom layers.

Plasma Extraction

The upper plasma layer was aspirated into a 15 ml centrifuge tube, spun at 800 g for 5 minutes to remove cellular debris, transferred to and frozen in a 1.5 ml Eppendorf tube (Starsted) at -20^{o}C.

Extraction and Lysis of PBMNs

The 'buffy coat' layer containing PBMNs was carefully aspirated using a Pasteur pipette, added to a 15 ml centrifuge tube containing 3 ml of Phosphate buffered saline

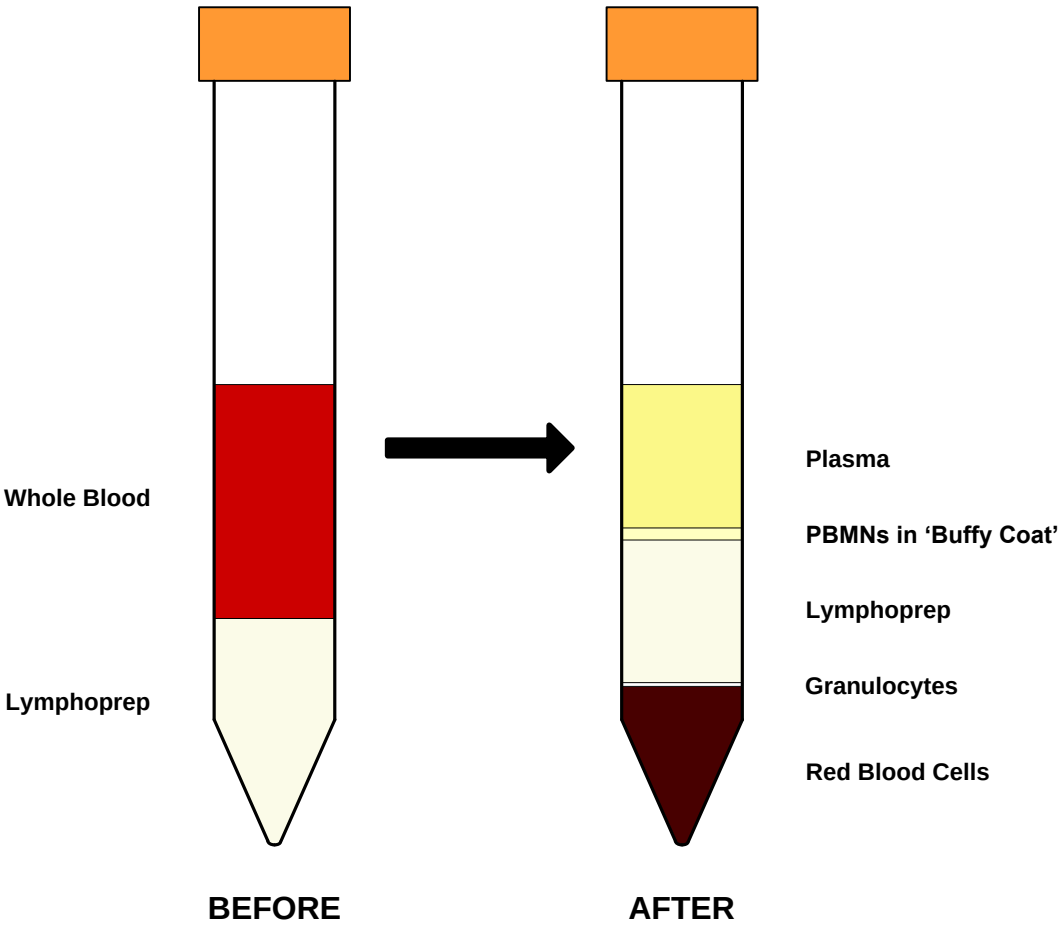

Figure 3.2 Schematic Diagram of the Blood/Lymphoprep™ Layers Before and After Centrifugation

Whole blood was layered onto Lymphoprep™ in a centrifuge tube. Following centrifugation, the mixture separated out into distinct layers containing different cell types.

(PBS; Sigma®), and made up to 10 ml with more PBS. Following centrifugation at 250 g for 10 minutes the supernatant was discarded, the PBMN pellet re-suspended in another 10 ml of PBS and spun again at 250 g for 10 minutes. These washes served to remove extraneous platelets. After discarding the supernatant, the pellet was re-suspended in 200 µl PBS by gentle agitation and 3 ml of TRI reagent™ (Sigma®) was added. This mixture was vortexed for 10 seconds (Vortex-Genie™; Scientific Industries) to lyse the cells and dissolve the RNA and DNA for future isolation. The solution was transferred to sterile 1.5 ml Eppendorf tubes and frozen at -20 ^{o}C. Protocols for the extraction of DNA and RNA from TRI reagent™, and the subsequent measurement of genotype, gene expression and protein levels are described in Sections 3.3 and 3.4.

3.1.5 Supervised Training Programme

Following the baseline assessments, subjects randomised to the treatment group entered an 8 week supervised training programme. A supervised programme was chosen as they are known to be more effective than unsupervised training, and would therefore produce maximal increases in walking distance.[18;49;243] Patient compliance was also ensured. Each subject was required to train 3 times a week, with each training session lasting approximately 30 minutes. The sessions consisted of 10 cycles of 90 seconds walking on a treadmill followed by 90 seconds rest. The aim of this training protocol was to stimulate a moderate to high level of claudication pain during each walking phase, and to allow enough time for the pain to subside between each walk. Training intensity was tailored to each patient according to their original walking ability and the following equation used as a guide to determine initial training speed:

Speed (km/hr) = (Claudication Distance (m)/90 s) x 3.6

This equation simply used the subject's measured CD (taken at initial assessment) to calculate the necessary speed to reach CD in 90 seconds. Treadmill gradient was initially set to 1%. For some individuals with mild symptoms, this equation required a speed that was unacceptably high. For these subjects, treadmill speed was set as high as the individual could tolerate (typically 5km/hour) and the gradient increased accordingly to generate claudication within the 90 seconds.

As a subject's symptoms improved over the training period, treadmill speed was increased to continue to stimulate claudication pain. If the subject improved to a degree such that claudication pain was not stimulated despite using high treadmill speeds and that treadmill speed could not be increased further (to prevent running- typically at 5km/hour), treadmill gradient was increased in 0.5% increments. No other advice was given to the subjects. This training protocol is similar to those that have been recommended to provide greatest improvements in walking.[71;99] Although some groups have recommended 6 month training programmes, shorter programs (6 weeks) have also been shown to work.[99;282] This exact programme has also been shown to be effective in previous studies in this laboratory.[178] During each session, heart rate was continuously monitored (Polar S-series precision toolkit), and the session halted if the heart rate exceeded 80% of the maximal predicted heart rate.

3.1.6 Exercise Advice

Subjects randomised to the control group were given verbal advice on taking exercise similar to what they would have been advised in a vascular outpatients clinic. They were encouraged to take walks 3 times a week, if possible for 20 minutes or more, and to try to walk through the claudication pain as much as possible.

3.2 CELL CULTURE EXPERIMENTS

In the second part of this thesis, a cell culture model of hypoxia was developed using the nitric oxide (NO) donor Diethylenetriamine-NO (DETA-NO) to produce a 'metabolic hypoxia' in an endothelial cell line. The effects of hypoxia on ACE and VEGF in these cells, and on cell viability were examined. Subsequently, the manner by which presence of the ACE inhibitor ramiprilat modulated these responses was then examined. ACE, $VEGF_{121}$ and $VEGF_{165}$ mRNA expression was determined by RT-PCR of mRNA extracted from cell lysates. ACE and VEGF protein levels were measured by ELISA of cell protein obtained from cell lysates. The use of a cell culture model has several advantages over a clinical trial using claudicants. One of the major limitations affecting most clinical trials in claudicants is the heterogeneity of the subjects used. To state a few factors, subjects vary immensely in age, co-morbidities, severity of disease, baseline activity levels and lifestyle. To overcome these limitations large sample sizes are preferable, but claudicants are difficult to recruit to trials.[69] Cell culture experiments have the advantage of using a single population of cells treated identically apart from the specific treatments, allowing a much 'cleaner' experiment to be performed. However, a limiting factor is the loss of interaction between different cell types that would occur in vivo.

3.2.1 Culture Conditions

Materials

A cell line, ECV 304 (Rayne Institute, UCL) was used for these experiments. The ECV 304 cell line were originally reported to have derived from HUVECs by spontaneous transformation, and have been used for over a decade as an endothelial cell model.[249] However, recent studies have shown that they were in fact derived from the bladder

cancer epithelial cell line T24.[30] Despite these findings, ECV 304 cells still show many characteristics of endothelial cells, have been shown to express ACE activity, and are still considered an attractive in vitro model for endothelia, given the scarcity of other suitable endothelial cell lines.[245] Thus they were chosen to be used in these experiments. However, as yet no evidence is available to confirm that ECV 304 cells express bradykinin, a vital link in the RAS. In the following experiments, unless stated otherwise, ECV 304 cells were cultured in medium consisting of Medium 199 with Earle's Salts and Glutamax™ (Gibco/Invitrogen) supplemented with 10% sterile foetal calf serum (FCS; First link (UK) Ltd), 100 u/ml penicillin and 100 μg/ml of streptomycin (Gibco BRL®/Life Technologies). Culture medium and all reagents were warmed to 37°C prior to use.

Resurrecting Cells from Storage

A vial of ECV 304 cells frozen at passage 174 was removed from storage and thawed in a water bath at 37°C. Using a sterile pipette, these cells were transferred to a 50 ml centrifuge tube (Techno Plastic Products AG; TPP®) containing 19 ml of warm culture medium to wash the cells, and centrifuged at 200 g for 5 minutes to form a pellet. The medium was discarded, the cells re-suspended in 5 ml of fresh medium and added to a 75 cm^2 culture flask (BD Falcon™) containing 25 ml of culture medium. The cells were incubated in a humidified chamber at 37°C and 5% CO_2.

Passaging Cells

These cells were sub-cultured at 70 hours at about 80% confluency. The medium was carefully aspirated from the flask and the cells rinsed with 15 ml sterile Hanks' balanced salt solution (HBSS; Sigma) without magnesium or calcium. This rinse removed

residual FCS which could have inhibited the subsequent trypsin reaction. Magnesium and calcium free HBSS was used to facilitate the dissociation process, as these ions allow cells to stick together. A cell dissociation solution consisting of 0.25x Trypsin with EDTA (Gibco BRL®/Life Technologies) in Sigma cell dissociation solution with PBS was used. After removing the HBSS, 13 ml of the dissociation solution was added and the flask incubated for up to 15 minutes at 37°C. During this time, the cells were intermittently monitored for rounding and detachment under a microscope. Once this was evident, the cells were released by a sharp tap on the flask, and 35 ml medium added to the flask to neutralise the trypsin and prevent further activity that may have damaged the cells. This solution was aspirated, added to a 50 ml centrifuge tube and centrifuged at 200 g for 5 minutes to pellet the cells. After discarding the medium, the pellet was re-suspended in a further 10 ml medium, and 5 ml added to each of two 175 cm^2 flasks (Nunc filter cap). These flasks were made up to 40 ml total volume with medium and incubated humidified at 37°C and 5% CO_2.

Flasks were monitored routinely under the microscope to check for contamination and confluency. Subsequently passages were performed every 48-72 hours, when the cells reached about 80% confluency. Typically, each 175 cm^2 flask was split into 3 flasks, using the same protocol as above. At intervals, some cells were frozen in liquid nitrogen for storage.

Freezing Down Cells for Storage

When passaging the cells, some cells were removed and suspended in medium at a density of $2x10^6$ cells/ml (see Section 3.2.2 for counting cells). Glycerol (Sigma), a cryoprotectant that reduces cell rupture due to ice crystal formation, was added to make

up 10% by volume and mixed well. One ml aliquots were then added to freezer tubes, which were placed in a bath of isopropanol overnight in a -80^{o}C freezer. This bath allowed a controlled decrease in temperature of 1^{o}C/minute, and prevented cell disruption by ice crystals. The following day the tubes were transferred into liquid nitrogen for storage.

3.2.2 Preparation of Cells for Experiments

Only clean confluent cultures from 175 cm^2 flasks were used. All experiments in this thesis were performed on cells between passages 178 and 190. Microscopic examination revealed the ECV cells to grow in monolayers with the typical 'cobblestone appearance' of endothelial cells.

Harvesting Cells

Cells were harvested using the same dissociation protocol as for passaging the cells. However, after the initial removal of the cells and centrifugation to form a pellet, the cells were resuspended in 5 ml medium containing either 10% FCS or 1% FCS. Cell viability and number was then measured.

Counting the Total Number and the Percentage of Viable Cells

Cells were counted using a haemocytometer combined with a Trypan blue (TB) exclusion assay to allow cell density and the percentage of viable cells to be determined. A haemocytometer consists of a slide with a grid marked on of known area. There are 9 large squares on the grid, each with a volume of 0.1 mm^3 once a cover slip is placed on top. Thus if the mean number of cells in a square is counted, it is possible to determine cell density and hence the total number of cells. TB is taken up by dead cells, turning

them blue and thus allowing the proportion of dead to viable cells to be calculated. Half a ml of 0.4% TB (Sigma) was combined with 0.4 ml of PBS, to which was added 0.1 ml of the cell suspension to be counted. This solution was mixed well and incubated for 5 minutes at room temperature, allowing the dead cells to absorb the dye. After placing the cover slip onto the haemocytometer, 10 µl of the cell/TB mixture was placed onto the area between the cover slip and slide and allowed to be drawn under the cover slip by capillary action. The number of unstained and blue stained cells on 5 quadrants (the centre and 4 corner squares) of the haemocytometer grid was counted and a mean figure recorded. A specific counting pattern was used to avoid bias. For cells overlapping a boundary, a cell was counted if it overlapped the top or left grid border, but not if it overlapped the bottom or right grid border (Figure 3.3).

The following equation was used to calculate the density of the original cell suspension:

Cells/ml= Mean number of cells (in 0.1 mm^3) x 10000 x Dilution Factor in TB/PBS

To calculate the total number of cells present, the number of cells/ml was multiplied by the total volume of the original cell suspension. To determine the percentage of viable cells, the following equation was used:

% Viable Cells= (Number of Unstained Cells/Total Number of Cells) x 100

Only cultures with greater than 90% viability were used for experiments, and from each 175 cm^2 flask the yield was typically between 8-10 million cells.

Plating Out Cells

After counting the cells, working solutions of $1x10^6$ cells/ml were prepared by adding an appropriate volume of culture medium. For cell viability assays, cells were plated out onto 96 well plates (Techno Plastic Products AG) with the appropriate drug(s) and the

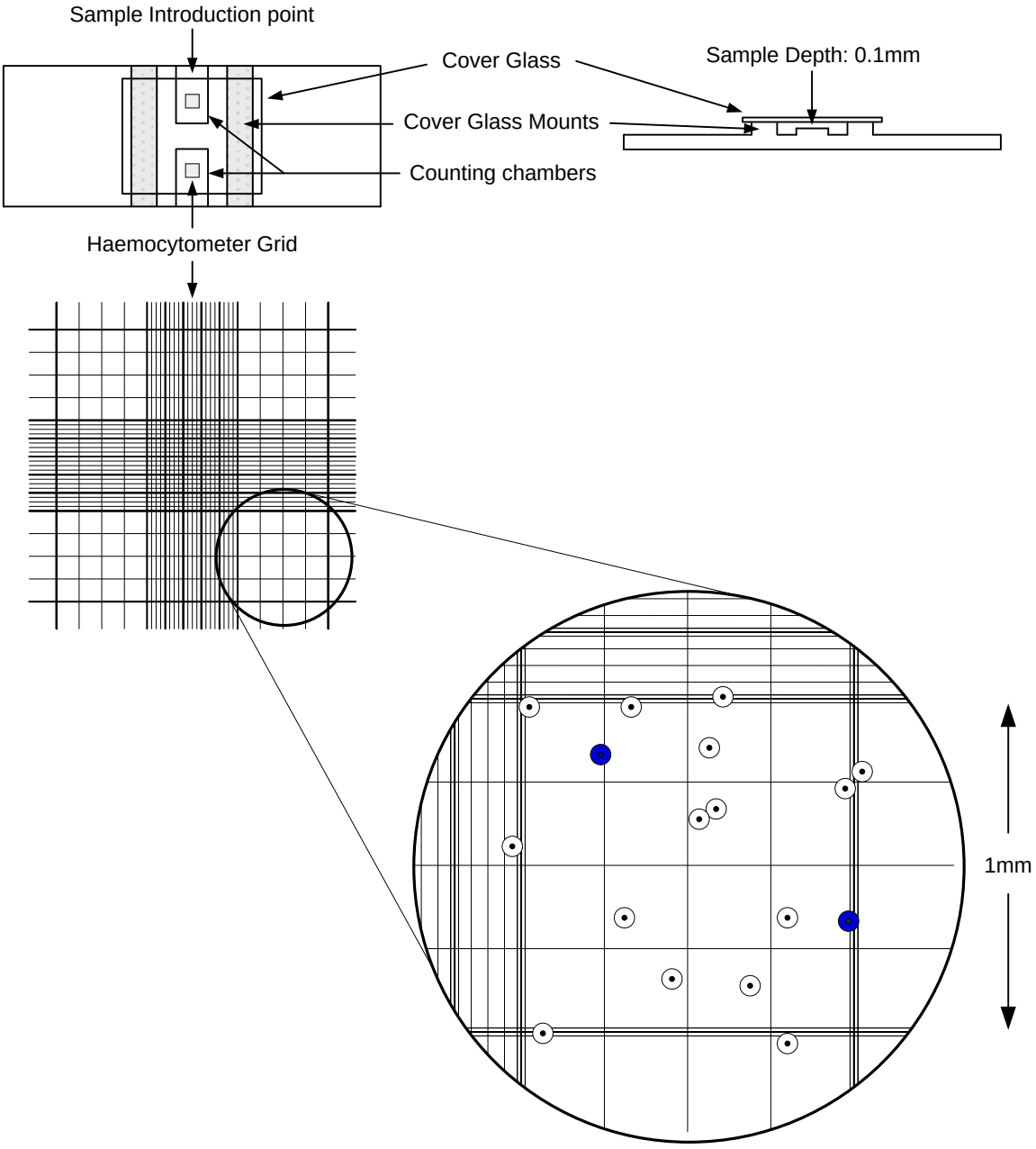

Figure 3.3 Counting Cells Using a Haemocytometer

This diagram shows a schematic representation of a haemocytometer with its grid of 9 quadrants, and a magnified view of one quadrant. For cells overlapping grid boundaries, a cell was counted if it overlapped the top or left grid border, but not if it overlapped the bottom or right grid border. Thus in this example, 11 viable cells would be counted and 1 non-viable cell.

volume in each well made up to 100 µl. Concurrently, some cells were plated out on 6 well plates (BD Falcon^{TM}) with the appropriate drug(s) and the volume in each well made up to 3 ml. These plates were used to examine for changes in gene expression and protein levels. For all experiments cells were incubated in a humidified chamber at 37^{o}C and 5% CO_2 until ready for processing. Further details on the preparation of specific experiments are described in Section 5.3.

3.2.3 Preparation of Drugs for Experiments

Diethylenetriamine-nitric oxide (DETA-NO)

While it would have been preferable to use hypoxic chambers to create the hypoxic conditions to be used in this experimental model, they are difficult to obtain and also significantly expensive. Thus, given its ready availability and in addition its significantly lower cost, a decision was made to use DETA-NO (2,2'-(Hydroxynitrosohydrazono) bis-ethanimine; Sigma) to create a metabolic model of hypoxia in these experiments.

DETA-NO is a slow releasing NO donor, which has been used widely to study the effects of NO on cellular respiration.[55] It allows the controlled release of NO to tissues at physiological levels. Evidence has shown that NO affects mitochondrial function, specifically by inhibiting the electron transport chain by competing with oxygen for binding to cytochrome oxidase at the inner mitochondrial membrane. By binding to cytochrome oxidase and preventing the mitochondria from utilising available oxygen, NO inhibits cellular respiration producing a 'metabolic hypoxia', driving cells into anaerobic metabolism.[6] Pathways associated with hypoxia are also activated, for example the stabilization of HIF-1α is increased.[164]

Doses of DETA-NO ranging from 0.008 mM to 0.5 mM were chosen for these experiments, and are similar to doses used in other studies.[6;16;164] Half mM of DETA-NO can generate 1.5 µM of NO locally for up to 18 hours at 37^{o}C and inhibit respiration by up to 80%.[16;55] For these experiments DETA-NO was solubilised using ultra-pure water (Simplicity 185™; Millipore) as the vehicle. A 50 mM master solution was prepared, from which appropriate dilutions for experiments were made. The final vehicle/water concentration in all experiments was 1%, and all experiments had a control consisting of medium with vehicle, and of medium alone. Further details on the preparation of specific experiments are described in Section 5.3.

Ramiprilat

Ramiprilat (Toronto Research Chemicals Inc.) was chosen as the ACE inhibitor for this experiment. Ramiprilat is the active form of the pro-drug ramipril, which *in* vivo is normally converted to its active form in the liver.

Doses of ramiprilat ranging from 1000 nM/l to 1 nM/l were used in these experiments, being similar doses to those used in other studies examining ACE signalling in endothelial cells.[138;139] For these experiments, ramiprilat was solubilised using dimethyl sulphoxide (DMSO; Sigma) as the drug vehicle. A 20 mM/l master solution of ramiprilat in DMSO was prepared, from which appropriate dilutions for subsequent experiments were made. The final DMSO concentration in all experiments was 0.05%, a level chosen to minimalise its potential effects on cellular metabolism.[210] All experiments involving ramiprilat included controls consisting of medium with vehicle, and of medium alone. Further details on the preparation of specific experiments are described in Section 5.3.

3.2.4 Assessing Cell Viability

At the end of each experiment, cell viability was assessed using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega). This assay uses a novel tetrazolium compound: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) combined with an electron coupling reagent phenazine ethosulfate. This produces a simple one step assay that can be performed in 96-well plates without the need for harvesting the cells. The MTS is reduced by NADPH or NADH present in metabolically active cells into formazan, a blue coloured product which can then be measured.[22] The following protocol was utilised:

The 96-well plates were assessed under the microscope for confluence and signs of infection. Twenty µl of the assay solution was added to each well, and the plate incubated in a humidified atmosphere at 37^{o}C and with 5% CO_2. In addition to the experimental wells, a series of triplicate wells containing culture medium only (no cells) were prepared and used as a 'blank control'. After 2 to 4 hours (see Section 5.3 for details of specific timings), the plates were placed in a plate reader (Dynex Technologies Ltd) and light absorbance at 525 nm recorded. Two separate plate readings were taken and a mean of the 2 readings recorded. The mean reading from the 'blank control' wells was then subtracted from the readings of the experimental wells to correct for background absorbance.

3.2.5 Processing Cells for RNA and Protein Extraction

At the end of each experiment, the 6-well plates were processed for RNA and protein extraction. Prior to harvesting the plates, they were examined microscopically to assess confluence and exclude infection. Rather than preparing individual wells to be harvested

separately for RNA and protein, each experimental well was processed to obtain both protein and RNA. This method allowed a direct comparison between RNA expression and protein levels in a specific population of cells to be made. To achieve this, the cells were lysed with TRI reagent™, from which RNA and protein were later extracted. The culture medium was aspirated from each well and the cells washed with 3 ml HBSS. After discarding the HBSS, 1 ml of TRI reagent™ was added to each well. Lysis was aided by repeat pipetting, and confirmed by microscopy. The products were transferred to 1.5 ml eppendorf tubes and frozen for later processing. Protocols for the extraction of RNA and protein from TRI reagent™, and the subsequent measurement of gene expression and protein levels are described in Sections 3.3 and 3.6.

3.3 DETERMINATION OF GENE EXPRESSION

Gene expression, defined as the amount of messenger RNA (mRNA) produced from the DNA gene template, was measured semi-quantitatively by RT-PCR. In summary, total RNA was extracted from TRI reagent™ and converted to complimentary DNA (cDNA) in a reverse transcriptase (RT) reaction. This cDNA was then amplified by polymerase chain reaction (PCR) using specific primers to target the mRNA sequences of interest. The quantity of product was measured with a scanning densitometer, and the amount expressed relative to the concurrent expression of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH-3), which was used as an internal control.

3.3.1 Nucleic Acid Extraction from TRI reagent™

TRI reagent™ is a solution of guanidine thiocyanate and phenol in a mono-phase solution, which enables the simultaneous extraction of DNA, RNA and protein from cells as described by Chomczynski.[50] The following protocol was used:

Separation of the Mixture into 3 Phases

Samples frozen in TRI reagent™ were thawed by standing at room temperature for 5 minutes. A fifth of a ml of Chloroform (BDH Laboratory Supplies) for each 1 ml of TRI was added to each sample and the tubes vortexed for 15 seconds. After standing at room temperature for 10 minutes, the samples were centrifuged at 12,000 g for 15 minutes. This separated the solution into 3 phases (Figure 3.4): an upper clear inorganic phase (containing RNA), a fine interphase layer (containing DNA) and a lower red organic phase (containing protein).

RNA Extraction

For RNA extraction, the upper aqueous phase was transferred to a fresh Eppendorf tube, with care taken not to disturb the interphase. Half a ml of Isopropanol (BDH Laboratory Supplies) per original 1 ml of TRI reagent™ was added to precipitate the RNA, the solution mixed and left to stand for 10 minutes at room temperature and then centrifuged at 12,000 g for 10 minutes to form an RNA pellet. The supernatant was removed and the RNA pellet washed in 1 ml of 75% ethanol (BDH Laboratory Supplies) per original ml of TRI reagent™. After centrifuging at 7,500 g for 5 minutes, the supernatant was removed and the pellet allowed to partially dry. The pellets from individual samples were combined and dissolved in an appropriate volume of ultrapure water (usually 60 µl), and stored at -40^{o}C.

DNA extraction

The interphase and organic phase were used for DNA and protein extraction. After carefully removing all of the aqueous phase (to reduce RNA contamination), 0.3 ml of 100% ethanol per original 1 ml of TRI reagent™ was added to precipitate the DNA.

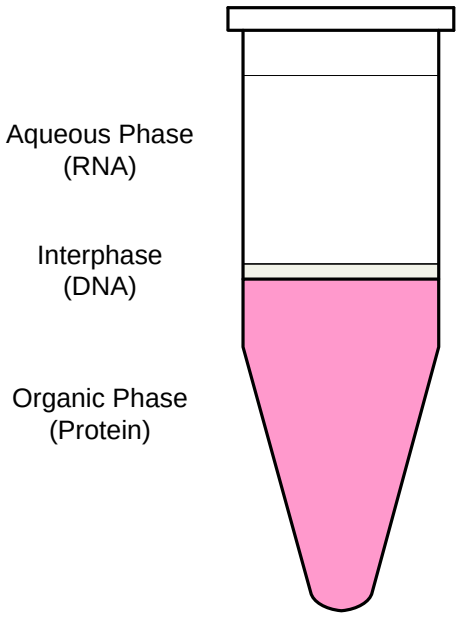

Figure 3.4 Schematic Diagram Showing the 3 Phases after Centrifugation of the TRI reagent™ and Sample Mixture

Following centrifugation the TRI reagent™, chloroform and sample mixture separated into 3 distinct phases: the upper aqueous phase containing dissolved RNA, the interphase precipitated DNA and the lower organic phase dissolved protein.

The solution was gently mixed by inversion, allowed to stand for 3 minutes at room temperature, and centrifuged at 2000 g for 5 minutes to pellet the DNA. The protein containing supernatant was then removed and stored at -40^{o}C (see Section 3.6.3 for protein extraction). The DNA pellet was washed for 40 minutes in a 0.1 M Sodium Citrate/10% Ethanol solution to remove residual phenol. One ml of wash solution was used for each original 1 ml of TRI reagent^{TM}. The pellet was centrifuged at 4000 g for 5 minutes and the wash repeated. The supernatant was then discarded, 1.5 ml of 75% ethanol wash added and the pellet left to stand for 20 minutes. After removing the ethanol, the pellet was then dried out and dissolved in an appropriate volume of ultrapure water (usually 200 µl). To help dissolution of the pellet, the tubes were placed in a water bath at 50^{o}C for 30 minutes. DNA in solution was stored at -20^{o}C.

3.3.2 Quantifying the Amount of Available DNA and RNA

The extraction of nucleic acids from TRI reagent^{TM} produced differing concentrations of DNA and RNA for each sample. It was important to quantify the amount of nucleic acid present so that an equal amount of RNA could be added to subsequent reverse transcriptase (RT) reactions and DNA in polymerase chain reaction (PCR) experiments. Quantification was performed using an ultraviolet (UV) light spectrophotometer (WPA UV1101) set to measure light at a wavelength of 260 nm. RNA and DNA both absorb UV light maximally at this wavelength, with 50 ng/µl of DNA and 40 ng/µl of RNA in a quartz cuvette corresponding to a recorded optical density (OD_{260}) of 1.

Nucleic acid samples were thawed on ice. To ‘zero’ the machine, a ‘blank’ sample containing 150 µl of water in a clean quartz cuvette was used. For test samples, a 50 fold dilution of the DNA/RNA solution (3 µl of DNA/RNA solution and 147 µl water)

was placed in the cuvette and measured. Between samples, the cuvette was cleaned carefully. To calculate the concentration of DNA or RNA in the samples, the following equations were used:

DNA concentration (ng/µl) = (OD_{260} reading)/(1/50) x dilution factor (50)

RNA concentration (ng/µl) = (OD_{260} reading)/(1/40) x dilution factor (50)

(1/50 and 1/40 correspond to the OD_{260} of 1ng/µl DNA and RNA respectively.)

3.3.3 Reverse Transcriptase Reaction

Prior to amplification, it was necessary to convert the relatively unstable single stranded RNA into double stranded cDNA by adding a strand of 'complimentary' bases to the RNA template in an RT reaction. A schematic diagram of an RT reaction is shown in Figure 3.5. cDNA provides a much more stable platform than RNA, allowing it to be used as a template in PCR reactions.

Reagents

The complimentary strand was formed using an RNA-dependent DNA polymerase derived from the Moloney-Murine Leukaemia Virus (M-MLV; Sigma). This was supplied with a buffer (10x M-MLV RT buffer; Sigma) containing 500 mM Tris-HCL, 500 mM KCl, 30 mM $MgCl_2$ and 50 mM DTT to optimise the reaction. To initiate the RT reaction, the RNA template needed to be 'primed' by short strands of nucleotides (primers) bound to the RNA template. Random nonamers (Sigma) were chosen and came as a 50 µM solution. These are random sequences of 9 nucleotides that bind randomly to complimentary segments on the RNA template. The use of random primers allowed total RNA to be converted to cDNA. They have the advantage over oligo-dTs in that they do not require the presence of intact poly-A tails on messenger RNA

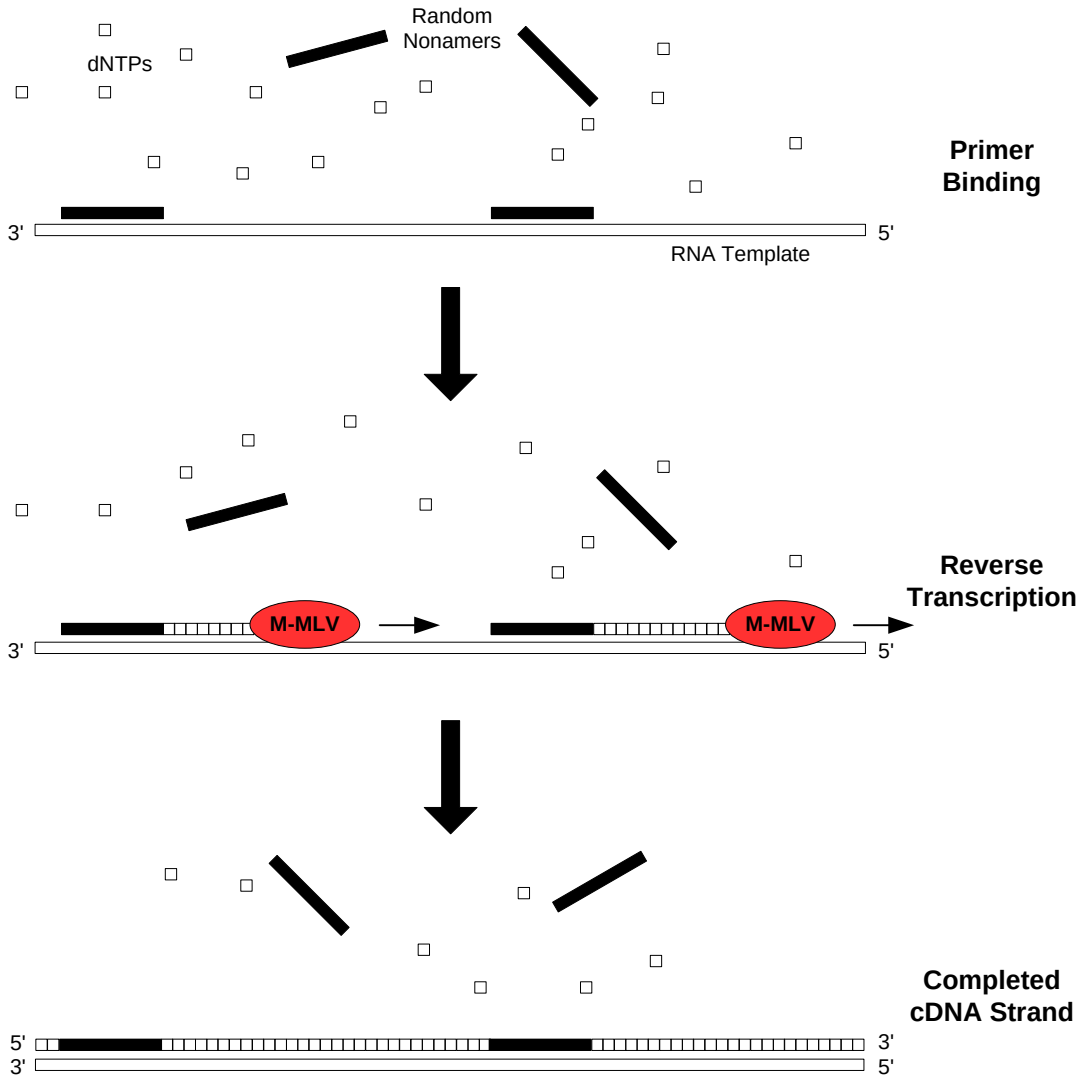

Figure 3.5 Schematic Diagram of a Reverse Transcriptase Reaction

In an RT reaction, unstable single-stranded RNA is converted to stable double-stranded cDNA. Random nonamers (primers) and dNTPs are initially combined with RNA template. After allowing the primers to bind, the enzyme M-MLV is added, which transcribes a complimentary sequence of bases in a 5' to 3' direction, forming a cDNA strand. Primers and dNTPs are added in excess to ensure the complete conversion of RNA into cDNA.

(mRNA) to bind. The poly-A tails can often be damaged during processing, resulting in a decreased yield of cDNA if oligo-dTs are used and hence a false negative result. Finally, a deoxynucleotide-triphosphate mix (dNTPs; Sigma), containing 10 mM of dATP, dCTP, dGTP and dTTP was used. These nucleotides formed the building blocks of the reaction by being incorporated into the new complimentary strands.

Protocol

The following protocol based on that recommended by Sigma® was employed. All reactions were set up on ice. For each reaction, 2 µl of dNTPs and 2 µl of random nonamers were added to a clean, nuclease-free 0.5 ml eppendorf tube. A volume of RNA sample equivalent to 2 µg total RNA was added to each tube, and the volume made up to 30 µl with ultrapure water. After gently mixing, the tubes were centrifuged briefly to get all components to the bottom of the tube. They were then placed in a Progene thermal cycler (Techne Ltd) and heated at 70^{o}C for 10 minutes to eradicate any tertiary RNA structures and subsequently allow efficient primer binding. The tubes were then cooled rapidly on ice to allow the primers to bind, before 10 µl of a 'master-mix' containing 2 µl M-MLV (400 units), 4 µl M-MLV buffer and 4 µl water was added. The tubes were then incubated in the thermal cycler at 20^{o}C for 10 minutes, and then at 37^{o}C for 50 minutes to allow the cDNA stand to be synthesised. Finally, the tubes were heated to 94^{o}C for 10 minutes to denature the M-MLV enzyme and terminate the reaction.

In summary, as the amount of RNA (2 µg) used in every reaction was the same, each reaction produced 2 µg of cDNA in 40 µl total volume, resulting in a cDNA concentration of 50 ng/µl. The cDNA was stored at -20^{o}C.

3.3.4 Polymerase Chain Reaction: Overview

The processes described above produced only a tiny quantity of the target mRNA sequences in the form of stable cDNA. In order to quantify gene expression, these target sequences needed to be amplified until the relevant cDNA products were present in large enough quantities to be measured macroscopically. The technique chosen to amplify the cDNA was PCR. This technique allows millions of copies of a target sequence to be produced in a short length of time. It utilises a cyclical process in which the target cDNA sequence is duplicated during each cycle, resulting in an exponential increase in the amount of product. Thus, a very small amount of starting product can quickly be amplified to a level which can easily be measured.

The PCR Cycle

Each cycle of a PCR reaction has 3 stages: denaturation, annealing and elongation. Denaturation involves heating the cDNA to a high temperature (90-94^{o}C) to break it down into its 2 component strands, and thereby expose the nucleotide bases. The temperature is subsequently reduced during the annealing stage, to provide optimum conditions for the binding of specifically designed oligonucleotide primers to their complimentary nucleotide sequences. The temperature is then raised to the optimum temperature for the action of DNA polymerase which, using the primers as a starting point, adds a strand of nucleotide bases in a 5' to 3' direction to form a double stranded product. As each denatured strand is converted into a new DNA strand, there is effectively a doubling of the target DNA sequence with each cycle. Therefore after 35-40 cycles, the amount of target DNA present will be substantial. A schematic diagram of the first 2 cycles of a PCR reaction is shown in Figure 3.6. A diagram illustrating the exponential increase in target DNA sequences is shown in Figure 3.7. At the end of a

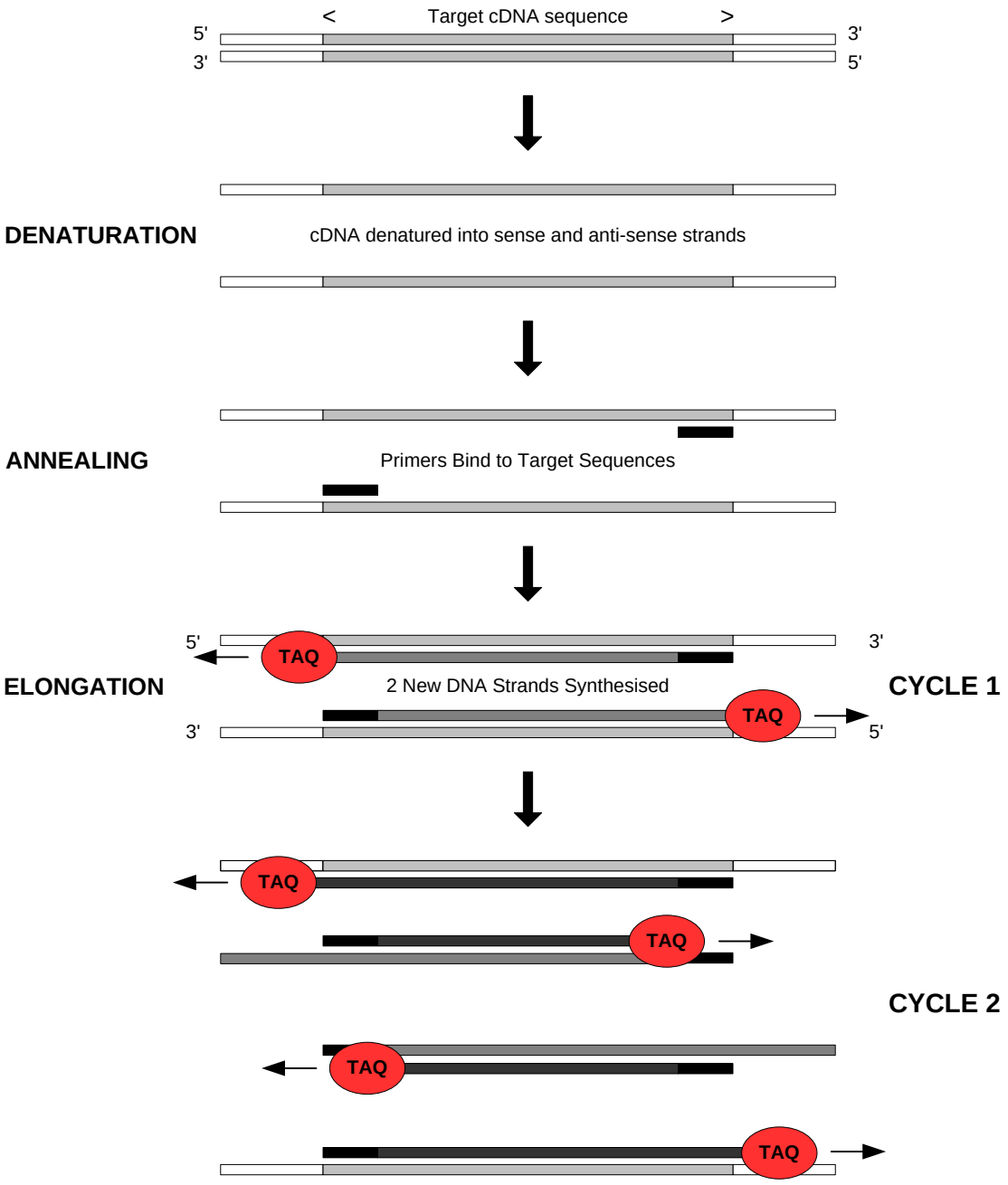

Figure 3.6 Diagram Showing the First 2 Cycles of a PCR Reaction

Heat denaturation separates the cDNA strand into 2 single stranded templates. Lowering the temperature allows oligonucleotide primers specific to the target sequence to anneal. DNA polymerase (TAQ), using the primers as a starting point adds a strand of complimentary dNTPs producing 2 new DNA strands. A further doubling occurs in cycle 2, resulting in 4 DNA strands.

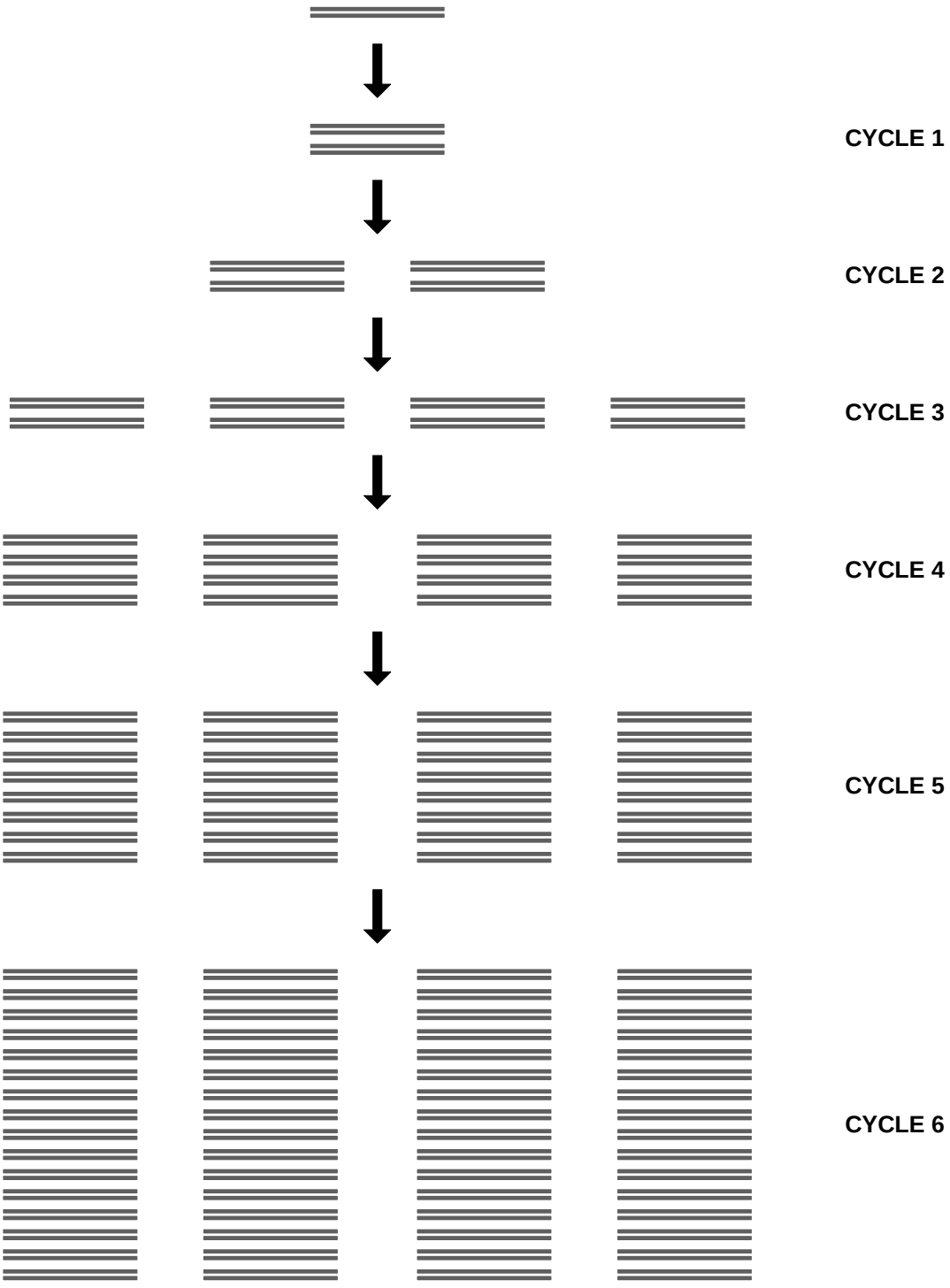

Figure 3.7 Diagram Showing the Doubling of cDNA with Each PCR Cycle

At the end of 6 cycles, there are 64 copies of the original single template. After 35 cycles, depending on the dynamics of the reaction, there is potential for up to 2^{35} copies.

PCR reaction, the amount of target DNA present will be proportionate to the amount of starting template. In these studies, all reactions for a specific target used the same amount of starting template, making it possible to form comparisons between the results from different samples. However, it was also necessary to control for the general activation level of a cell, and also for variation in the efficiency of each reaction. For this purpose, a 'house-keeping' gene or internal control was used.

Internal Control: GAPDH-3

Even very small variations in the efficiency of separate PCR reactions can have a significant effect on the final yield of product, and need to be controlled for. These differences can occur due to disparity in the efficiency of separate RT reactions, loading errors, or even due to slight differences between individual PCR machines. In addition, it is also necessary to control for the general level of cell activation. For example, in proliferative or inflammatory states, gene expression as a whole within cells is increased. If target gene expression alone is measured, it is not possible to determine whether any changes in expression are due to specific changes in the target gene's expression or to a more general change in cell activity. To control for both of these problems, target gene expression was expressed semi-quantitatively as a ratio relative to the expression of the 'house-keeping' gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH-3). GAPDH-3 is a key enzyme in glycolysis and is constitutively expressed at a level reflecting general cell activation.[248] Thus, any change in the ratio of target gene expression relative to GAPDH-3 could only have been due to specific gene activation or suppression, and not to changes in general cell activity. Likewise, any variation in the efficiency of PCR reactions would have affected the final yield of both the target gene and GAPDH-3, but not the ratio between them. Therefore, for each sample and each

target gene, 2 reactions were set up to quantify target gene and concurrent GAPDH-3 expression. To further increase the accuracy of the results, all RT-PCR experiments were performed twice and a mean value of recorded.

Reagents

All reagents were supplied by Sigma® unless stated otherwise. REDTaq, a heat-stable DNA polymerase which can tolerate temperatures of up to 100°C (and thus not be denatured in the PCR reaction) was used to synthesize the DNA strands. It was supplied in a 1 unit DNA polymerase/µl solution with 20 mM Tris-HCL at pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% Igepal CA-630, inert red dye and 50% glycerol. For some reactions, Jumpstart™ REDTaq DNA polymerase was used. It was provided as a 1 unit/µl solution and in an identical buffer to REDTaq. Jumpstart™ REDTaq consists of REDTaq blended with an antibody that binds to the enzyme at low temperatures, inactivating it. During the initial denaturation stage of the PCR reaction, the antibody is displaced, activating the enzyme. This system enables each PCR reaction to be completely assembled in one step, eliminating the need for the 'hot-start' stage required with REDTaq alone, and thus reduces set up time and the potential for contamination. The DNA polymerases were provided with a PCR buffer (10x concentration) containing 100 mM Trizama®-HCL at pH 8.3 and 500 mM KCl, which was diluted 10 fold in the final reaction. A separate 25 mM solution of magnesium chloride was also supplied. Magnesium is an essential co-factor for DNA polymerase, and adjustment of the final magnesium level (1.5-2.5 mM) allowed the stringency or efficacy of each PCR reaction to be optimised. A dNTP mix containing 10 mM of dATP, dCTP, dGTP and dTTP formed the building blocks of the reaction. Finally, oligonucleotide primers specific for each target were obtained.

Primers

The sequences for the primer sets used in these experiments were taken from published work in the literature or from primers previously used in our own laboratory.[269;273] The primer sets all conformed to basic principles of primer design. Each set was designed to amplify a small section of the target cDNA sequence between 100-400 base pairs (bp) in length, with one primer binding to the sense strand and the other to the anti-sense strand of the cDNA target. The primers were all around 20 bp long, to enable a sequence of nucleotides to be designed to allow specific binding to only one location on the genome. This was checked using the Basic Alignment Sequence Homology Tool (BLAST) on the NCBI website. The target sequences chosen also spanned at least one exon-exon boundary on the original mRNA sequence, to prevent the amplification of contaminating genomic DNA. The primer annealing temperature (T_m), defined as the temperature at which half of the primer will anneal to the target sequence, was approximated for each primer using the "Wallace formula":

$$\mathbf{T_m\ (^oC) = [4(No.\ of\ G/C\ bases) + 2(No.\ of\ A/T\ bases)]}$$

The G/C bases contribute more to the Tm as they have stronger hydrogen bonds than A/T bases, conferring greater stability. The estimated T_m for each primer set obtained using this formula was used as a guide when subsequently optimising the PCR reactions (see Section 3.3.5). Details of specific primer sets are shown in Table 3.2.

General PCR Protocols

The PCR protocols used were based on the protocols recommended by Sigma® but were optimised for each primer set. All experiments were performed in a clean environment with sterile technique used throughout, and all plastic wear autoclaved prior to use. Reactions using REDTaq were prepared as follows: Aliquots of sample cDNA were

Table 3.2 Primer Sequences

Forward (F) and reverse (R) primers for each target, primer sequences, calculated annealing temperatures (T_m) and product sizes are shown.

Primer	Sequence (5' to 3')	Tm (^oC)	Product Size (bp)
ACE 216 (F)	CACCAATGACACGGAAAGTG	64	216
ACE 216 (R)	GCATCAAAGTGGGTTTCGTT	64	
VEGF (F)	CATCCTGTGTGCCCCTGATG	64	243 ($VEGF_{121}$)
VEGF (R)	TTCCTCCTGCCCGGCTCAC	64	375 ($VEGF_{165}$)
VEGFR-1 (F)	TCATGAATGTTTCCCTGCAA	56	277
VEGFR-1 (R)	GTGCTGCTTCCTGGTCCTAA	62	
sVEGFR-1 (F)	TCATGAATGTTTCCCTGCAA	56	119
sVEGFR-1 (R)	TTTGTTGCAGTGCTCACCTC	60	
VEGFR-2 (F)	AGACTTTGAGCATGGAAG	52	312
VEGFR-2 (R)	CCATTCCACCAAAAGATG	52	
GAPDH-3 (F)	GAGTCAACGGATTTGGTCGT	64	185
GAPDH-3 (R)	GACAAGCTTCCCGTTCTCAG	64	

added to 500 µl eppendorf tubes. Positive and negative control tubes, containing proven cDNA and ultrapure water respectively, were also set up for each experiment. A pre-prepared master-mix solution containing 10x PCR buffer, magnesium chloride, dNTPs, ultrapure water and primers was then added to each tube to make the volume up to 45 µl. The tubes were then vortexed for 5 seconds to ensure mixing, and centrifuged briefly at 2000 g to collect the tube contents at the base. Each reaction tube was then placed in a Progene thermal cycler (Techne Ltd) and heated to 94ºC for 4 minutes to denature the DNA template. The temperature was reduced to 65ºC and 5 µl of a master-mix containing 0.5 µl REDTaq, 0.5 µl 10x PCR buffer and 4 µl water added. Adding the REDTaq at this stage in a 'hot-start' protocol helped to decrease non-specific amplification. Each tube then underwent 35 cycles of denaturation, annealing and elongation, followed by a final 10 minute elongation period at 72ºC to ensure complete sequence extension. Final protocols for each primer set are described in Section 3.3.5. Jumpstart REDTaq was used for all the RT-PCR reactions in the cell culture experiments, and for the VEGF receptor RT-PCR reactions in the claudication trial. The experiments were prepared as follows: Aliquots of sample cDNA were carefully added to the bottom of the wells on 96-well PCR plates. A pre-prepared master-mix solution containing 10x PCR buffer, magnesium chloride, dNTPs, ultrapure water, primers and jumpstart REDTaq was then added to each well to make a total reaction volume of 50 µl. Each plate also had positive control and negative control wells. The plate was then covered with a PCR film, placed in a Progene thermal cycler and heated to 94ºC for 4 minutes to denature the DNA template and activate the REDTaq. Each plate then underwent 35 cycles of denaturation, annealing and elongation, followed by a final period of 10 minutes of elongation at 72ºC to ensure complete sequence extension. The specific optimised protocols for each primer set are described in Section 3.3.5.

3.3.5 PCR: Optimisation and Final Experimental Conditions

Variations in primer properties, PCR product length and cDNA template availability all alter the dynamics of a PCR reaction. Given these variables, it was necessary to adapt the basic PCR protocols for each primer set to produce consistent, clean and measurable products. This optimisation process was performed by:

Altering Reaction Stringency

The stringency of a reaction describes its efficacy and specificity, and was altered by varying T_m and $MgCl_2$ concentration (see Appendix 2). A stringent reaction will produce clean products but will have lower yields than a less stringent reaction, which will conversely have a less clean product. Lowering T_m increases primer binding to the target site, but it also increases non-specific primer binding, resulting in spurious PCR products. Raising T_m has the opposite effect, increasing the stringency of the reaction. $MgCl_2$ acts as a co-factor for DNA polymerase. High levels of $MgCl_2$ increase the efficacy of DNA polymerase, but allow sequencing errors to occur (low stringency), whilst low magnesium levels have the opposite effect (high stringency). Initially, a T_m slightly lower than that predicted by the Wallace formula was chosen, combined with a high $MgCl_2$ concentration to produce a low stringency reaction. The stringency was then gradually increased (by increasing T_m or lowering $MgCl_2$) until a clean product was produced. For these experiments, a final $MgCl_2$ of between 1.0 mM and 2.5 mM was used, and annealing temperatures varied between 56^oC and 60^oC.

Varying the Amount of Template

If raising the stringency of the reaction to obtain a clean product resulted in low product yields, the amount of starting template was increased to circumvent this problem (see

Appendix 2). For these experiments, between 50 and 400 ng of cDNA per reaction was used. In addition, the concentration of primer could also be altered (0.5 to 1.0 µM used).

Altering PCR Cycle Conditions

Finally, the PCR reactions could be optimised by varying the PCR cycle conditions (see Appendix 2). Annealing time was varied between 30-45 seconds, elongation time between 45-60 seconds, and total number of PCR cycles between 35-40 cycles. Increasing annealing and elongation times allowed greater time for primer binding or DNA synthesis to occur respectively. Increasing the cycle number allowed more amplification to occur.

Therefore, following the optimisation process, a specific protocol was developed for each primer set. The exact conditions used for ACE 216, VEGF, VEGFR-1, sVEGFR-1 and VEGFR-2 primers are described in Tables 3.3 to 3.6. For the GAPDH-3 internal controls, identical PCR conditions to the study primer set being examined were used. The GAPDH-3 controls were prepared concurrently, using the same reagents and run in the same thermal cycler or plate as the study experiments. The only difference was that the GAPDH-3 experiments required only 50 ng (1 µl) of cDNA template, with the volume made up to 50 µl with ultrapure water.

3.3.6 Analysis of PCR Products

The products of the PCR reactions were analysed by gel electrophoresis to visualise the products and separate them by size. A photograph of the gel was scanned, and scanning densitometry performed to quantify the amount of product present.

Table 3.3 Final ACE 216 PCR Conditions

For reactions using Jumpstart REDTaq, the initial master-mix was prepared containing all the constituents of the reaction (using the same quantities shown below), and the PCR cycling conditions were identical except that the hot-start stage was omitted.

Reagent	[Stock]	Volume added (µl)	[Reaction]
cDNA Template	50 ng/µl	6	-
PCR Master-mix			
PCR Buffer	10x	4.5	1x
$MgCl_2$	25 mM	3	1.5 mM
dNTPs	10 mM	1	0.2 mM
Primers (F+R)	50 µM	0.5	0.5 µM
Ultrapure water		30	-
TAQ Master-mix			
PCR Buffer	10x	0.5	1x
REDTaq	1 u/µl	0.5	0.01 u/µl
Ultrapure water		4	-
Total PCR Reaction Volume		50	

	Temperature (ºC)	Time
Denaturation	94	4 minutes
Hot-start	65	
35 Cycles		
Denaturation	94	30 seconds
Annealing	60	45 seconds
Elongation	72	45 seconds
Polyadenylation	72	10 minutes

Table 3.4 Final VEGF PCR Conditions

For reactions using Jumpstart REDTaq, the initial master-mix was prepared containing all the constituents of the reaction (using the same quantities shown below), and the PCR cycling conditions were identical except that the hot-start stage was omitted.

Reagent	[Stock]	Volume added (µl)	[Reaction]
cDNA Template	50 ng/µl	4	-
PCR Master-mix			
PCR Buffer	10x	4.5	1x
$MgCl_2$	25 mM	3	1.5 mM
dNTPs	10 mM	1	0.2 mM
Primers (F+R)	50 µM	1	1 µM
Ultrapure water		31.5	-
TAQ Master-mix			
PCR Buffer	10x	0.5	1x
REDTaq	1 u/µl	0.5	0.01 u/µl
Ultrapure water		4	-
Total PCR Reaction Volume		50	

	Temperature (ºC)	Time
Denaturation	94	4 minutes
Hot-start	65	
35 Cycles		
Denaturation	94	30 seconds
Annealing	58	45 seconds
Elongation	72	60 seconds
Polyadenylation	72	10 minutes

Table 3.5 Final VEGFR-1 and sVEGFR-1 PCR Conditions

VEGFR-1 and sVEGFR-1 PCR conditions were identical. The same forward primer was used for both primer sets, with the distinct reverse primers targeting the specific RNA sequences for each receptor.

Reagent	[Stock]	Volume added (µl)	[Reaction]
cDNA Template	50 ng/µl	6	-
Jumpstart Master-mix			
PCR Buffer	10x	5	1x
$MgCl_2$	25 mM	2	1 mM
dNTPs	10 mM	1	0.2 mM
Primers (F+R)	50 µM	1	1 µM
Jumpstart REDTaq	1 u/µl	0.5	0.01 u/µl
Ultrapure water		34.5	-
Total PCR Reaction Volume		50	

	Temperature (ºC)	Time
Denaturation	94	4 minutes
35 Cycles		
Denaturation	94	45 seconds
Annealing	58	45 seconds
Elongation	72	60 seconds
Polyadenylation	72	10 minutes

Table 3.6 Final VEGFR-2 PCR Conditions

Reagent	[Stock]	Volume added (µl)	[Reaction]
cDNA Template	50 ng/µl	8	-
Jumpstart Master-mix			
PCR Buffer	10x	5	1x
$MgCl_2$	25 mM	4	2 mM
dNTPs	10 mM	1	0.2 mM
Primers (F+R)	50 µM	1	1 µM
Jumpstart REDTaq	1 u/µl	0.5	0.01 u/µl
Ultrapure water		30.5	-
Total PCR Reaction Volume		50	

	Temperature (ºC)	Time
Denaturation	94	4 minutes
40 Cycles		
Denaturation	94	45 seconds
Annealing	56	45 seconds
Elongation	72	60 seconds
Polyadenylation	72	10 minutes

Gel Electrophoresis

Gel electrophoresis was used to separate the PCR products by size, allowing confirmation of the presence of correct sized PCR product, and excluding the presence of spurious products. Gel electrophoresis involves using an electric field to pull the PCR products across a gel matrix, which acts as a sieve separating out the molecules. As DNA is uniformly negatively charged, the products migrate towards the positive electrode at a rate according to their size with smaller molecules migrating faster than large molecules. All reagents were supplied by Sigma® unless stated otherwise. A 2% agarose gel was prepared by adding 2 g of agarose to 100 ml 1x Tris-Borate-EDTA (TBE; 89 mM Tris-borate, 2 mM EDTA, pH 8.3) buffer and heating in a microwave until the agarose had completely dissolved. Ten µl of ethidium bromide was then added which, by later intercalating with DNA bases allowed PCR products to be visualised under ultraviolet (UV) light. The mixture was poured into a gel tray and combs placed to create wells for the loading of PCR products. After setting, the gel was placed in a gel rig and immersed in TBE buffer. Ten µl of PCR product, combined with 2 µl of loading buffer (30% glycerol in water and 0.25% bromophenol blue) was loaded into each well. In each lane, a well was loaded with 3 µl of a 100 bp ladder (fragment sizes 100-1000 bp) to allow product size to be judged. A 120 V, 60 mA current was run across the gel for 30 minutes. The gel was then placed on a UV-light illuminator box and a Polaroid photo taken using standardised exposure settings (F-stop 8, shutter speed 0.5 seconds). A photograph of a typical gel is shown in Figure 3.8.

Scanning Densitometry

Scanning densitometry was used to quantify band intensity in the gel photographs, and hence measure the amount of PCR product present, allowing the level of gene

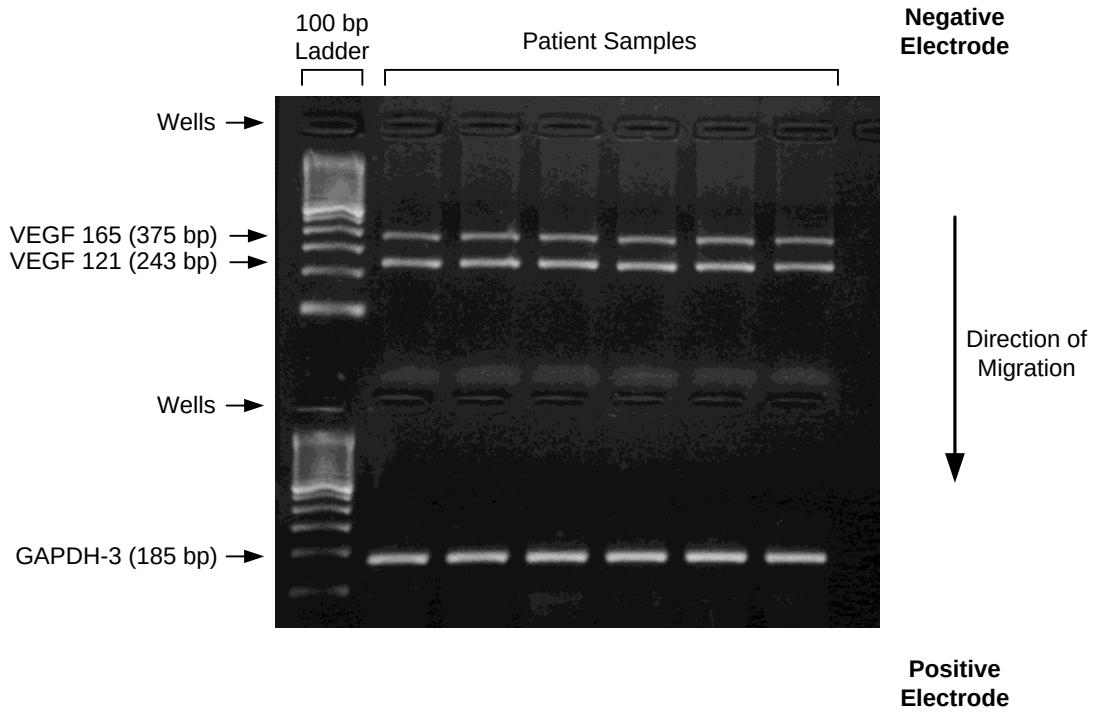

Figure 3.8 Photograph of a Typical Agarose Gel

The products from a VEGF RT-PCR reaction and a concurrently run GAPDH-3 control reaction are shown. The direction of migration (towards the positive electrode) is illustrated, with smaller fragments migrating further through the gel matrix than larger products. The size of the products can be assessed using the 100 bp ladder.

transcription to be determined. The photographs were scanned using an Epson GT-9500 scanner using Epson Twain software. Labworks™ Image Acquisition and Analysis software (Ultra-Violet Products Ltd) was used to identify the bands on the photograph, and the integrated optical density (IOD) of each band recorded. As stated previously, gene transcription was expressed semi-quantitatively, as a ratio of the IOD of the target gene band to the IOD of the GAPDH-3 band.

3.3.7 Alternatives to RT-PCR for Measuring Gene Expression

Although RT-PCR was chosen to quantify RNA and gene transcription in this thesis, other well recognised methods are available. However, for the following reasons these techniques were discounted: Northern blotting analysis and nuclease protection assays were discounted as they are less sensitive than RT-PCR. *In-situ* hybridization was considered inappropriate as it was not necessary to measure gene expression within the tissues, and it is also less sensitive than RT-PCR. Gene arrays were also regarded as inappropriate due to extreme cost, decreased sensitivity and as only a few target genes were being examined.[232] Another more recently developed technique for measuring RNA levels is Real-time RT-PCR. This highly sensitive technique is based on the same principles as RT-PCR, but has the additional advantage of allowing the quantification of PCR products in 'real-time' as they are manufactured in each cycle. This is usually achieved by using a direct fluorescence system. The benefits of this system include the elimination of the electrophoresis and scanning densitometry stages, increased sensitivity and increased accuracy in the quantitation of mRNA.[291] However, disadvantages include the large number of preliminary experiments required to optimise reactions, and the considerable expense of the required equipment. Although RT-PCR has some limitations compared to real-time RT-PCR, it still remains a sensitive and

robust technique for RNA quantification. Therefore, due to its availability and cost advantages, it was chosen to measure gene expression for this thesis.

3.4 ACE GENOTYPE DETERMINATION

ACE genotype was determined by PCR of patient DNA extracted from whole blood. In summary, genomic DNA was extracted from whole blood, as described in Section 3.3.1, amplified using primers specific to the polymorphic region on the ACE gene, and the products examined by gel electrophoresis to determine genotype.

3.4.1 Overview

The method used for establishing ACE genotype was first described by Lindpaintner *et al* in 1995.[154] This technique employs two sets of primers: H ACE 3 primers were designed to flank the polymorphic region in the ACE gene, amplifying both the deletion and insertion alleles, producing two possible products (319 and 597 bp respectively). However, in heterozygous (ID) subjects, the D-allele was preferentially amplified by these primers, to the extent that the product from the I-allele was difficult to detect after gel electrophoresis. This created a potential problem of mistyping ID heterozygotes with the DD genotype. To circumvent this problem, a second pair of primers (H ACE 5), with a binding site specific to the 287 insertion sequence was designed. These 'insertion specific' primers produced a 335 bp product only in the presence of the I-allele, and therefore helped to differentiate between the ID and DD genotypes.

3.4.2 PCR conditions

PCR was performed on patient DNA samples using REDTaq and a 'hot-start' protocol as described in Section 3.3.4. Details of the primers used and possible PCR products are

illustrated in Figure 3.9. Details of the exact conditions used following an optimisation process are shown in Table 3.7. The PCR products were separated by gel electrophoresis as described in Section 3.3.6 and genotype interpreted from the gel photograph. A gel picture illustrating the 3 possible genotypes is shown in Figure 3.10.

3.5 DIFFERENTIAL TRANSCRIPTION OF ACE I/D ALLELES ANALYSIS

The mechanism by which the I/D polymorphism at intron 16 alters ACE gene expression may relate to increased production or stability of mRNA originating from the D-allele, but still remains unclear. However, in healthy I/D heterozygotes, more ACE mRNA has been shown to arise from the D-allele compared to the I-allele.[246] In this study, the transcription of mRNA from each allele in heterozygotes, and also the potential differential effects of training on each allele were examined. In summary, cDNA was obtained from patient PBMNs as described in Sections 3.1.4 and 3.3.1. The cDNA was amplified by RT-PCR using primers targeting a segment of ACE mRNA containing a potential restriction enzyme site specific to mRNA originating from the I-allele. Following a restriction enzyme reaction, the products were examined by gel electrophoresis and scanning densitometry. All experiments were performed twice on separate occasions and the mean result taken.

3.5.1 Overview

The method used for quantifying mRNA expression from the I/D alleles was based on a protocol first described by Suehiro *et al* in 2004.[246] Since the ACE I/D polymorphism is located at intron 16, it is spliced out of the RNA sequence as it is converted to mRNA and thus cannot be used to determine the origin of mRNA strands. However, a single nucleotide polymorphism (SNP) is known to exist in exon 15, which is directly linked

Primer	Sequence (5' to 3')	Tm (^{o}C)	Product Size (bp)	
			D-allele	I- allele
H ACE 3 (F)	GCCCTGCAGGTGTCTGCAGCATGT	64	319	597
H ACE 3 (R)	GGATGGCTCTCCCCGCCTTGTCTC	64		
H ACE 5 (F)	TGGGACCACAGCGCCCGCCACTAC	64	No Product	335
H ACE 5 (R)	TCGCCAGCCCTCCCATGCCCATAA	64		

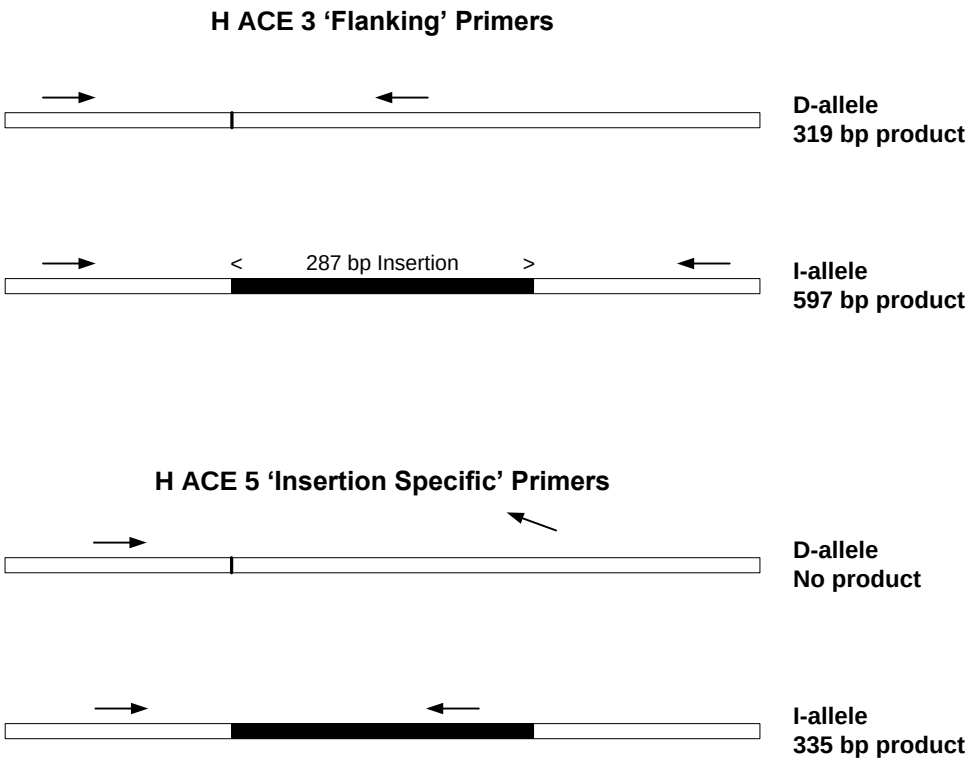

Figure 3.9 ACE Genotyping: Primers and Possible Products

The H ACE 3 primer binding sites flank the insertion site on intron 16, resulting in 2 possible products depending on the presence of the 287 bp insertion. The 'insertion specific' H ACE 5 primers generate only 1 possible product, indicating the presence of the insertion sequence.

Table 3.7 Final ACE Genotype PCR Conditions

Both H ACE 3 and H ACE 5 primer sets used same PCR conditions.

Reagent	[Stock]	Volume added (µl)	[Reaction]
cDNA Template	50 ng/µl	2	-
PCR Master-mix			
PCR Buffer	10x	4.5	1x
$MgCl_2$	25 mM	3	1.5 mM
dNTPs	10 mM	1	0.2 mM
Primers (F+R)	50 µM	1	1 µM
Ultrapure water		33.5	-
TAQ Master-mix			
PCR Buffer	10x	0.5	1x
REDTaq	1 u/µl	0.5	0.01 u/µl
Ultrapure water		4	-
Total PCR Reaction Volume		50	

	Temperature (ºC)	Time
Denaturation	94	4 minutes
Hot-start	65	
35 Cycles		
Denaturation	94	45 seconds
Annealing	64	45 seconds
Elongation	72	60 seconds
Polyadenylation	72	10 minutes

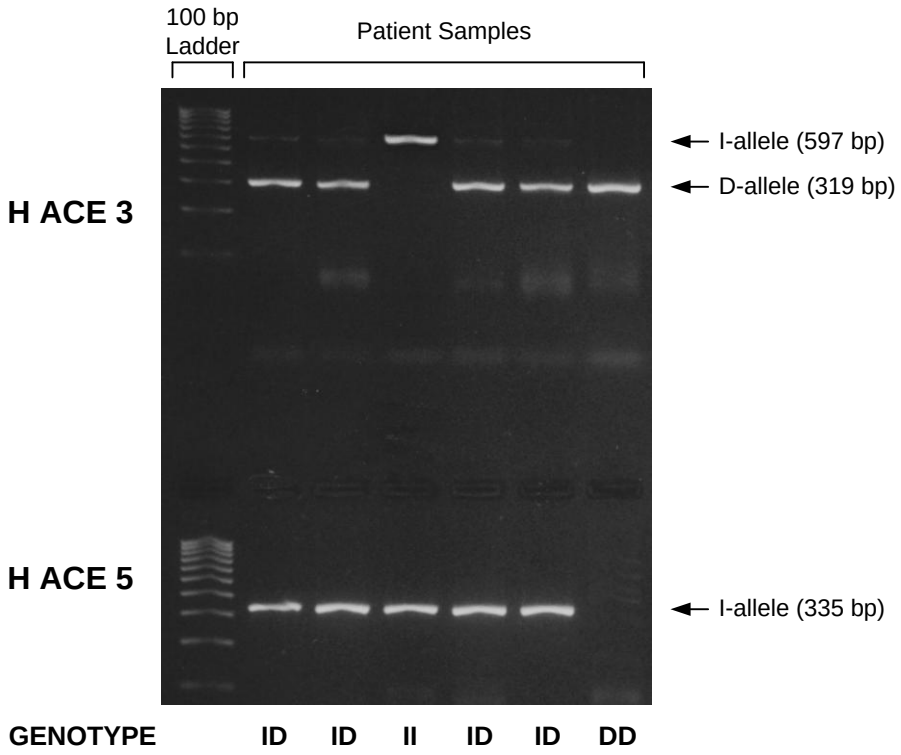

Figure 3.10 Gel Electrophoresis of ACE PCR Products

PCR using H ACE 3 and H ACE 5 primers allowed ACE genotype to be determined. H ACE 3 primers produced 2 possible products depending on the presence or not of the 287 bp insertion. In heterozygotes (ID) the product representing the I-allele was often faint or undetectable. Therefore, H ACE 5 primers specific for the insertion sequence were used to confirm or refute presence of the I-allele.

to the I/D polymorphism. This SNP involves a G for A substitution at position 2215 (G2215A) on the cDNA strand, with the 2215G allele directly linked with the 287bp insertion, and the 2215A allele with the deletion.[134] The G2215A SNP is 'silent' and does not change the amino acid sequence, but as it is exonic and thus present in the final mRNA strand, can be used to distinguish between mRNA originating from each allele. In addition to its direct link to the 287bp insertion, the 2215G allele also creates a restriction site in the cDNA which is recognised by the restriction enzyme *HAE II*. Restriction enzymes were first described in bacteria, where they degrade DNA from invading viruses and thereby 'restrict' viral replication. They act by recognising specific nucleotide sequences (restriction sites), and cleaving the DNA at that site. Thus, the specific properties of the 2215G allele were exploited to measure mRNA transcription from each I/D allele. Flanking primers were used to amplify a 242 bp segment of cDNA containing the G2215A polymorphism by RT-PCR. The PCR products were then incubated with *HAE II*, which cleaved all cDNA strands containing the 2215G allele into 2 smaller fragments (153 and 89 bp), whilst strands with the 2215A allele were unaffected (Figure 3.11). Thus, for II subjects, all the cDNA products were cleaved whilst in DD subjects no cleavage occurred. In ID heterozygotes, the products originating from the I-allele were cleaved but the products from the D-allele were unaffected. Gel electrophoresis was used to separate the products by size, and scanning densitometry used to quantify the relative amounts of each product from each allele.

3.5.2 Protocols

RT-PCR Reaction

RT-PCR was performed on patient cDNA samples using REDTaq and a 'hot-start' protocol as described in Section 3.3.4. Details of the ACE primers (New England

Biolabs) and exact details of the conditions used following optimisation are shown in Table 3.8. Samples from subjects of all ACE I/D genotypes (II,ID,DD) were studied to enable the 100% linkage between the I/D and G2215A polymorphism to be confirmed.

Restriction Enzyme Reaction

The *HAE II* restriction enzyme protocol used was based on the manufacturer's recommendations (New England Biolabs). *HAE II* was supplied as a 20,000 unit/ml solution, with 1 unit of enzyme able to digest 1 µg of DNA in 1 hour in a reaction volume of 50 µl at 37^{o}C. It was supplied with a buffer (10x NEBuffer 4) containing 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate and 1 mM dithiothreiotol when diluted to a 1x solution. Bovine serum albumin (BSA) was also provided in a 100x solution. Twenty µl of a master-mix solution containing 10 units *HAE II*, 1x NEBuffer 4, 1x BSA and ultrapure water was added to each 0.5 ml eppendorf tube on ice. Thirty µl of PCR product from each sample was then added to make the volume up to 50 µl (Figure 3.11). The tubes were vortexed briefly to mix the contents, and pulse centrifuged at 2000 g to collect the tube contents at the base. Finally, the tubes were placed in a Progene thermal cycler and incubated at 37ºC for 2 hours, before the reaction was terminated by denaturing the enzyme at 80ºC for 20 minutes. This protocol enabled a complete digestion of all the products containing the restriction site, with control experiments using 12 hours digestion time providing identical results.

Gel Electrophoresis and Scanning Densitometry

The products from the original PCR reaction and the restriction enzyme reaction were separated by gel electrophoresis and analysed by scanning densitometry, as described in

Table 3.8 ACE 2215 Primer Details and Final PCR Conditions

Primer	Sequence (5' to 3')	T_m (^oC)	Product Size (bp)
ACE 2215 (F)	CACACCCTGAAGTACGGCAC	64	242
ACE 2215 (R)	GTGGCCATCACATTCGTCAG	62	

Reagent	[Stock]	Volume added (µl)	[Reaction]
cDNA Template	50 ng/µl	4	-
PCR Master-mix			
PCR Buffer	10x	4.5	1x
$MgCl_2$	25 mM	4	1.5 mM
dNTPs	10 mM	1	0.2 mM
Primers (F+R)	50 µM	1	1 µM
Ultrapure water		30.5	-
TAQ Master-mix			
PCR Buffer	10x	0.5	1x
REDTaq	1 u/µl	0.5	0.01 u/µl
Ultrapure water		4	-
Total PCR Reaction Volume		50	

	Temperature (^oC)	Time
Denaturation	94	4 minutes
Hot-start	65	
35 Cycles		
Denaturation	94	30 seconds
Annealing	60	30 seconds
Elongation	72	45 seconds
Polyadenylation	72	10 minutes

Reagent	Stock Concentration	Volume/reaction (µl)	Final Concentration
***HAE II* Master-mix**			
HAE II Enzyme	20,000 U/ml	0.5	10 U/reaction
NEBuffer x4	x10	5	x1
BSA	x100	0.5	x1
Ultrapure Water	-	14	-
PCR Product	-	30	-
	Total Reaction Volume	50	

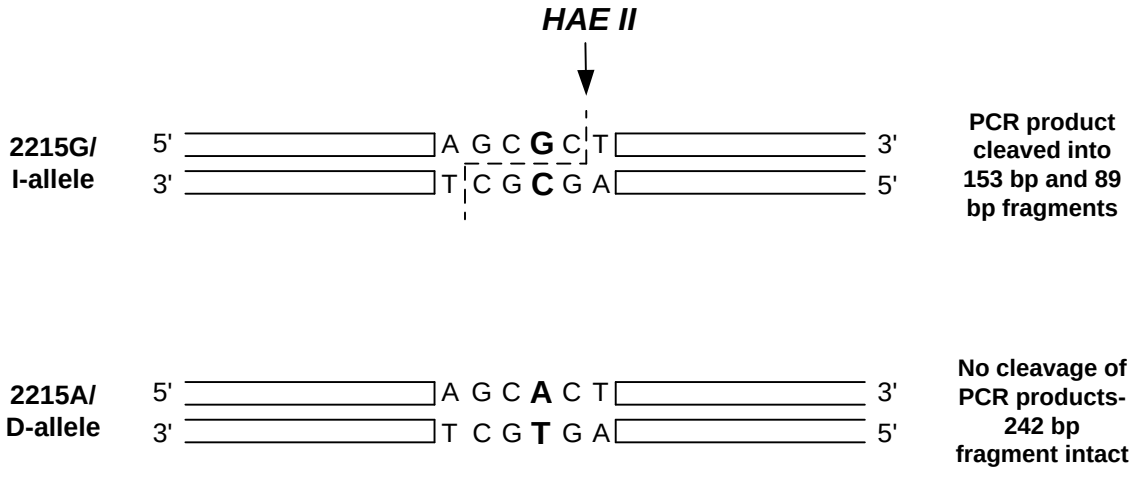

Figure 3.11 ACE mRNA Analysis: *HAE II* Reaction Conditions and Products

The recognition site of *HAE II* on exon 15 of the ACE cDNA and the potential products of the restriction reaction with each allele are shown.

Section 3.3.6. The analysis of the original 242 bp PCR product in all the samples enabled ACE gene expression to be quantified. In the results section, ACE gene transcription in the claudicant trial was taken as the mean of the results obtained from the ACE 216 and ACE 2215 primers combined. Ten µl of each restriction enzyme reaction was loaded onto the agarose gels. In I/D heterozygotes, the IOD of the single band from the uncleaved D-allele (2215A) was compared to the sum of the IOD from the 2 fragments representing the I-allele (2215G), to give a ratio of D/I allele mRNA. An example of a gel photograph with all the potential products is shown in Figure 3.12.

3.6 MEASUREMENT OF PROTEIN LEVELS

Measurement of changes in gene expression provides an indication of cellular activation. However, the end-result of any change in cellular activity or processes is an alteration in protein expression. The level of protein present reflects the entire pathway of gene activation, RNA transcription, post-transcriptional modifications, translation into amino acid chains and finally post-translational modifications. Thus, measuring protein levels is a significant consideration when determining changes resulting from any intervention.

Two main techniques are used to quantify soluble proteins. Western blotting allows target proteins to be identified according to their molecular weight and by the binding of specific antibodies.[270] Enzyme-linked immunosorbant assays (ELISAs) also use highly specific antibodies to identify target proteins, but do not discriminate proteins by size and cannot discriminate between different isoforms. The main advantages of ELISAs are the increased sensitivity and simplicity of the assays.[280] Thus, ELISAs were chosen to measure ACE and VEGF levels in this thesis.

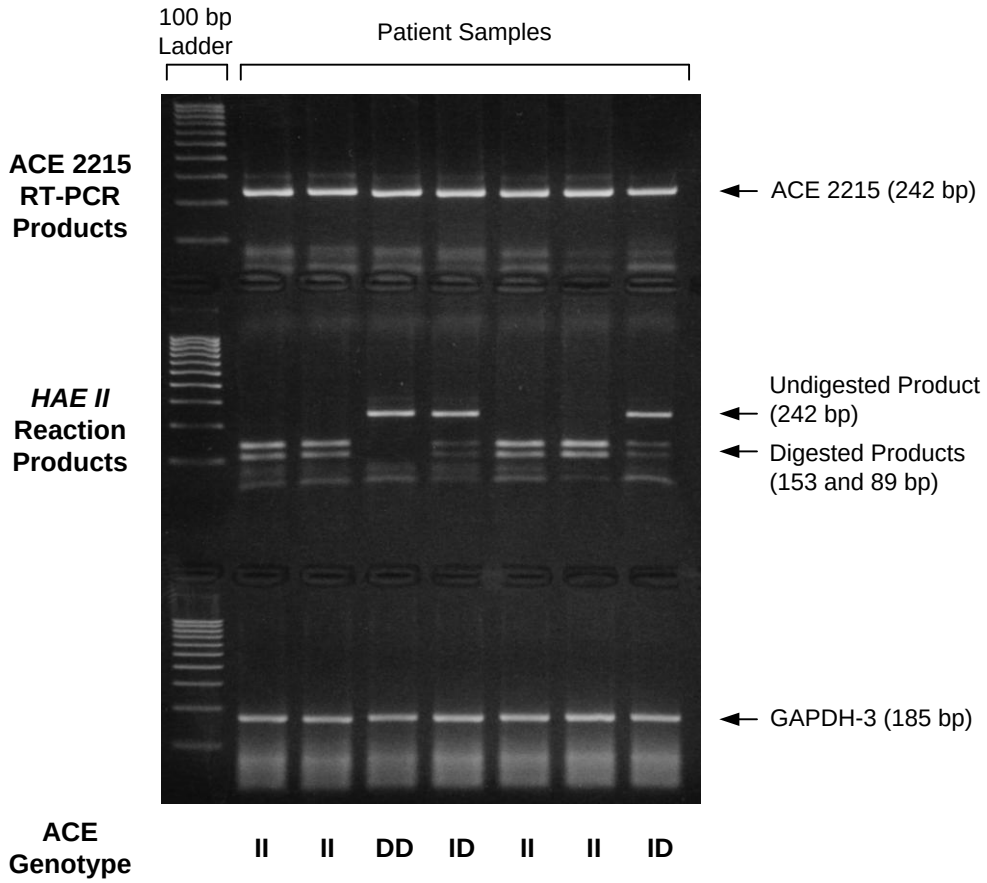

Figure 3.12 Photograph Demonstrating the Products from an ACE 2215 Reaction

PCR products from the original RT-PCR reaction were used to determine ACE expression semi-quantitatively. ACE genotype as established by PCR of genomic DNA is shown. Samples from II subjects (2215G homozygotes) are completely digested following the *HAE II* reaction. Samples from DD subjects (2215A homozygotes) demonstrate no digestion, whilst ID heterozygotes demonstrate partial digestion, allowing the relative expression of I and D-allele derived mRNA to be analysed.

3.6.1 ELISA: Overview

An ELISA is a simple, sensitive and reliable technique for measuring protein levels. A sandwich ELISA was used in these experiments. In a sandwich ELISA, an antibody is used to 'capture' the target protein onto a plate well. A second or 'detection' antibody, which recognises a separate epitope on the protein, is then added. The detection antibodies are conjugated to an enzyme assay, which allows the protein to be quantified.

Reagents

DuoSet ELISA development kits (R&D Systems) for detecting human ACE and VEGF were obtained, which use a Streptavidin-HRP colorimetric detection system to quantify the amount of target protein. These assays work by exploiting the binding properties of biotin and streptavidin. The detection antibodies used are linked to biotin molecules. When streptavidin-HRP conjugates are subsequently added, the streptavidin component binds tightly to the biotin on the detection antibody, and the active HRP component acts on a colour substrate, developing its colour. The intensity of colour produced reflects the amount of bound streptavidin-HRP, which is determined by the quantity of detection antibodies bound to captured target protein, which in turn is determined by the concentration of target protein in the original sample. In addition, each detection antibody is associated with several biotin molecules, allowing a number of streptavidin-HRP conjugates to bind to each antibody, amplifying the signal and increasing the sensitivity of the assay.

The ACE and VEGF kits both utilised the same protocols, with the only differences being the antibodies employed and the diluent used for the samples being measured. Goat anti-human ACE antibody at a working concentration of 0.8 μg/ml in PBS (Sigma)

and 1.0 µg/ml mouse anti-human VEGF antibody in PBS were used as the 'capture' antibodies. The VEGF antibodies recognized both $VEGF_{165}$ and $VEGF_{121}$ isoforms. One percent BSA dissolved in PBS (Sigma) was used to dilute all the other reagents (Reagent diluent), to 'block' the plates, and also used as the sample diluent for VEGF samples. Twenty percent FCS (First Link Ltd) diluted in PBS was used as the sample diluent for ACE samples. Biotinylated goat anti-human ACE and VEGF antibodies diluted in reagent diluent to working concentrations of 200 ng/ml and 50 ng/ml respectively, were used as the 'detection' antibodies. Streptavidin-HRP (1 in 200 in reagent diluent) and a colour substrate (a 1:1 mixture of H_2O_2 and tetramethylbenzidine) comprised the detection system. 2N H_2SO_4 was used to stop the detection assay.

Recombinant human ACE and VEGF in reagent diluent were used to produce standard curves to allow the quantification of protein. Seven-point standard curves were produced using 2-fold serial dilutions of the recombinant protein, with a high standard of 8000 pg/ml used for ACE and 2000 pg/ml for VEGF. Between stages, 0.05% TWEEN® 20 (Sigma) in PBS was used as a wash buffer.

Protocols

The protocols used were based on those recommended by the manufacturer (R&D Systems) and used for both the claudicant and cell culture studies. All experiments were performed at room temperature on 96-well microplates (Nunc™ Products) with the samples and standards prepared in duplicate wells.

One hundred µl of diluted capture antibody was used to coat the plate wells. The plate was sealed and incubated overnight in a fridge to allow the antibodies to attach to the

well surface. The wells were then aspirated, and washed 3 times with wash buffer using a squirt bottle to completely fill the wells, with attention taken to completely remove the wash buffer after each wash by blotting the plate against clean paper towels. Three hundred µl of 1% BSA in PBS was then added to 'block' the plate, by coating the surfaces of the well not already covered in capture antibody with protein. This minimised subsequent non-specific substrate binding. After 1 hour the wells were aspirated and washed 3 times as described above. One hundred µl of sample or standards in diluent (see Sections 3.6.2 and 3.6.3 for details) were added to the wells. After incubating for 1¾ hours, the wells were again aspirated, washed 3 times to remove unbound protein and 100 µl of diluted detection antibody added. The plates were incubated for another 1¾ hours. Following 3 washes to remove unbound detection antibodies, the streptavidin-HRP conjugate was added and allowed to bind to the remaining detection antibodies. The plate was left in a dark room for 15 minutes to prevent HRP activation, and then washed 3 times to remove unbound streptavidin-HRP. One hundred µl of colour substrate (clear) was then added to the wells and left for 15 minutes. This substrate was catalysed by HRP into a blue solution, with the intensity of the solution relating to the amount of bound streptavidin-HRP present. Fifty µl of stop solution was added after 15 minutes, which turned the substrate yellow and halted the HRP reaction. The colour in each well was immediately assessed using a plate-reader (Dynex Technologies Ltd) set to measure optical density at 450 nm (the wavelength of the colour produced). A correction wavelength of 540 nm was used to adjust for non-specific absorbance caused by the plate and reagents. The absorption values obtained from the samples were then compared to the standard curves obtained from the standards of known concentration to calculate the amount of protein present. A summary of the protocol can be seen in Figure 3.13.

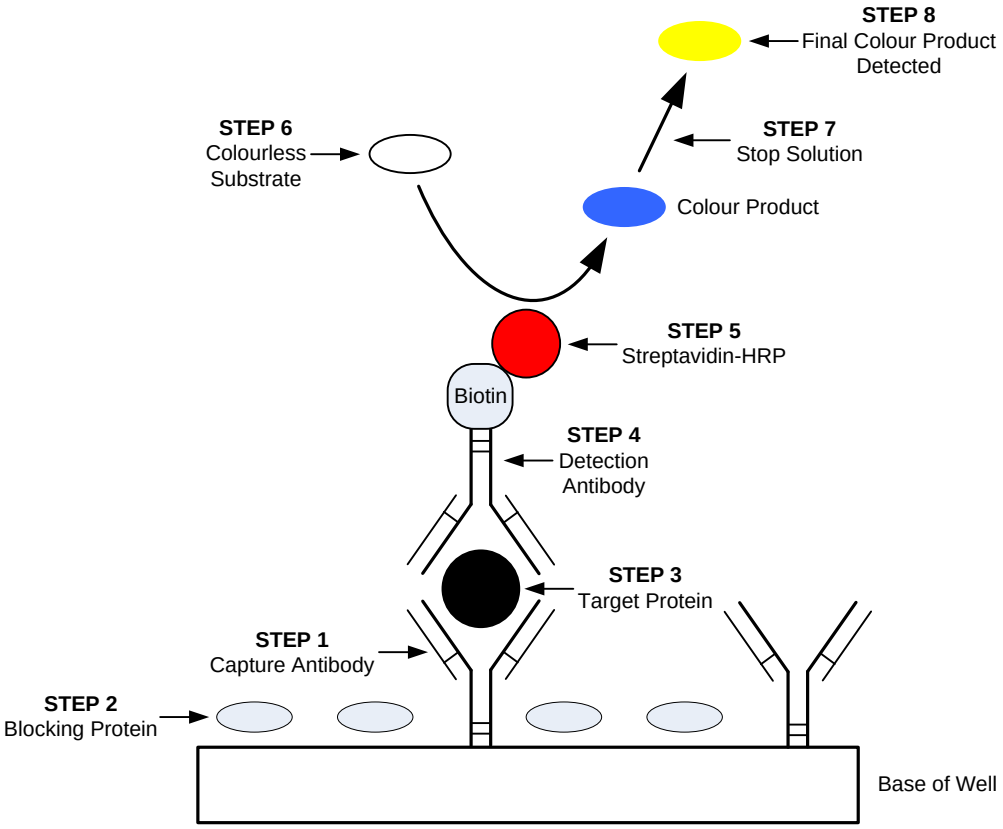

Figure 3.13 Schematic Diagram of an ELISA Reaction

The steps involved in the sandwich ELISA are illustrated. A thorough wash was performed after steps 1-5 to remove excess unbound proteins or antibodies. With all assay conditions being equal, the final colour intensity was determined by the amount of target protein bound by the capture antibody, and hence by the concentration of target protein in the samples.

3.6.2 Clinical Trial Subjects

ACE and VEGF levels were measured in plasma obtained from whole blood as described in Section 3.1.4. Although it would have been preferable to have measured local (muscle) changes in protein levels, this would not have been practical or acceptable for the patients. Thus, plasma was used and should give an indication of changes occurring locally in the tissues.

Optimisation and Final Protocols

The final ELISA protocols were established after the period of optimisation to provide an assay that was sensitive within the range of plasma concentrations encountered. Separate optimisation was required for the ACE and VEGF assays, with each assay requiring a different sample diluent and dilution factor for the patient samples.

For the ACE ELISA, the plasma samples were initially diluted 1 in 2 with 1% BSA. However, the absorbance readings obtained were all above the upper end of the standard curve, and thus unusable. Increasing the dilution factor successively to 1 in 10, 1 in 20, and 1 in 40 still resulted in absorbance values above the upper level of the standard curve. The sample diluent was therefore changed to FCS in PBS to provide a greater degree of block and decrease non-specific substrate binding. Finally, a sample diluent of 20% FCS with a 1 in 40 dilution factor was utilised. These conditions produced absorbance values for the samples consistently within the linear section of the standard curve. For the VEGF ELISA, the initial 1 in 2 sample dilution in 1% BSA provided absorbance readings in the lower part of the standard curve. Decreasing the concentration of BSA to 0.1% produced readings consistently within the linear section of the standard curve.

3.6.3 Cell Culture Experiments

In the cell culture experiments, each experimental well was processed to obtain both protein and RNA as described in Section 3.2.5. This method allowed a direct comparison between RNA expression and protein levels in the same population of cells to be made. Although the original intention was to measure cellular ACE and VEGF levels using Western blotting, the protein yields after extraction from TRI reagent™ were too low to permit this. The total protein yields obtained ranged from approximately 10-20 μg. Thus, as there was insufficient protein to perform Western blots, the more sensitive ELISA technique was employed.

Protein Extraction from TRI reagent™

During the processing of cells lysed in TRI reagent™ for the extraction of nucleic acids, the protein containing organic phase was separated and frozen as described in Section 3.3.1. The following protocol based on manufacturer's guidelines (Sigma) was used to extract and solubilise the protein for later measurement. After thawing, 1.5 ml of isopropanol for each 1 ml of original TRI reagent™ was added to each tube. The tubes were inverted several times, and incubated for 10 minutes at room temperature to precipitate the dissolved proteins. After centrifuging for 4 minutes at 400 g to pellet the protein, the supernatant was discarded and the pellet washed in 1.5 ml 0.3 M guanidine hydrochloride in 95% ethanol per original 1 ml of TRI reagent™ for 20 minutes. The tubes were then centrifuged for 4 minutes at 400 g, the supernatant discarded and the guanidine hydrochloride wash repeated twice for total of 3 washes. Following this, 1.5 ml 100% ethanol was added to each tube, the pellet vortexed and incubated at room temperature for 20 minutes. The pellet was then centrifuged for 4 minutes at 400 g and the ethanol supernatant discarded. Excess ethanol was then removed by placing the

tubes in an oven at low temperature for 2 hours. The dried pellets were then crushed into a powder with a clean spatula, and 100 µl 1% sodium dodecyl sulphate (SDS) added to dissolve the pellet. To aid pellet dissolution, the tubes were incubated in a water bath at 50^{o}C for up to an hour, with repeat pipetting and vortexing used every 15 minutes. Insoluble protein was then removed by centrifuging at 10,000 g for 10 minutes, the supernatant removed and stored at -20^{o}C.

Total Protein Measurement

Although each experimental well originally contained the same number of cells and the same extraction protocol used for each sample, the protein yield obtained from each well was slightly different. Measuring the total protein yield allowed the results of subsequent ELISAs to be corrected for these varying yields. Total protein obtained from each sample was thus measured using a Bradford assay.[26] This is a simple, sensitive assay which utilises the colour change or absorbance shift in Coomassie Brilliant Blue G-250 (CBB) dye when it binds to protein to determine the concentration of protein present in a sample. When unbound, the dye absorbs light at a peak of 465 nm, whereas the protein bound form has an absorbance maximum at 595 nm.

Bio-Rad protein assay dye reagent (Bio-Rad Laboratories) was obtained. A working solution of CBB reagent was prepared immediately prior to use by diluting the dye 1 in 4 with water. The protein samples stored in 1% SDS were diluted to 0.1% SDS using ultrapure water. This step was necessary to prevent the SDS from interfering with the assay. Ten µl of each protein sample then was added in duplicate to the wells of a 96-well plate (Nunc™ Products). A series of protein standards were prepared using BSA containing between 0 and 10 mg/ml. Ten µl of each of these standards was each added

to 1 ml of CBB and mixed thoroughly. Two hundred µl from each standard was then added in duplicate to the plate. Two hundred µl of CBB reagent was added into each sample well. The plate was transferred immediately to a plate reader (Dynex Technologies Ltd), shaken for 10 seconds and absorbance measured at 595 nm. The results from the sample wells were then compared to the standard curve generated by the standards, allowing protein concentration to be calculated. From the results of these assays, the mean protein yield for each experimental series was calculated, and the yield from each well expressed as a ratio of the mean yield. This ratio was used as a corrective factor for subsequent ELISA results, with the measured protein level corrected by dividing the value obtained by the correction factor from the associated well.

ELISA Optimisation and Final Protocols

For the VEGF ELISAs, the protein samples in 0.1% SDS were diluted 1 in 5 with 0.1% BSA in PBS as the sample diluent, and 100 µl added to each well. This maintained the SDS concentration at 0.02%, a level previously shown not to interfere with ELISAs.[147] A 2000 pg/ml top standard was used and a 7-point standard curve produced using serial 2-fold dilutions. The remainder of the ELISA protocol was followed as described above. The VEGF levels measured were corrected according to the total protein yields attained by the Bradford assay, by dividing the measured VEGF level by the ratio of protein yield. For ACE ELISAs, the protein samples were again diluted 1 in 5 with various sample diluents to achieve a final SDS concentration of 0.02%. However, despite varying the protocols and using different sample diluents including BSA and FCS, it was not possible to produce a consistently measurable result with ACE, as the levels present were too low to detect.

3.7 STATISTICAL ANALYSIS

Graphpad Prism 4 was used for statistical analyses and nQuery Advisor 4.0 for power and sample size calculations. Non-parametric data are presented as the median with range in parentheses, while parametric data is presented as the mean ± standard error of the mean (SEM). Two-tailed tests were performed throughout, and p-values of ≤ 0.05 regarded to indicate statistical significance.

Clinical Trial Data Analysis

Due to the small numbers involved, the data were analysed using non-parametric tests as a normal distribution could not be assumed, and tests of normality were of little use with such small sample sizes. All analyses comparing the control and exercise groups were performed using the Mann Whitney U-test. Comparisons between pre- and post-measurements within each group were made using the Wilcoxon matched pairs test. Spearman's rank correlation test was used for all tests analysing relationships between gene expression and protein levels, and between changes in ACE and VEGF. For these comparisons, the data from the control and exercise groups were combined. Power analyses were performed using 1- and 2-sample t-tests.

Cell Culture Data Analysis

Comparisons between Medium and Vehicle control groups were made using unpaired t-tests. Multiple group comparisons were all made by one-way analysis of variance (ANOVA). Dunnett's multiple comparison test was performed to assess differences within each treatment group compared to the experimental control. Bonferroni's Multiple Comparison test was used to identify differences between the 3 treatment groups. Power analyses were performed using a 2-sample t-test.

CHAPTER 4

ACE AND VEGF RESPONSES TO EXERCISE TRAINING IN CLAUDICANTS

CHAPTER 4

4.1 BACKGROUND

Exercise training is well known to improve the symptoms of intermittent claudication, although the precise mechanism by which this occurs is relatively less well recognized.[244;255] Several studies have revealed a role for ACE in exercise performance, with low ACE activity related to increased endurance after training in elite athletes and army recruits.[170;288] Previous work in this laboratory has demonstrated that the I-allele of the ACE I/D polymorphism, which is associated with decreased ACE activity, is related to increased improvements in walking ability in claudicants after an exercise training programme. Furthermore, in other studies reduction in ACE activity has also been linked with increased VEGF-related angiogenesis, with suppression of ACE activity resulting in increased VEGF production.[85;233] This relationship between ACE and VEGF provides a further potential benefit of reduced ACE activity.

The effects of an exercise training programme in claudicants on VEGF and ACE has not yet been reported. A greater understanding of how ACE and VEGF interact and react to an exercise training programme, and how these changes relate to improvements in walking ability will allow potential treatments that modulate ACE and VEGF, such as ACE inhibition, to be targeted effectively.

4.2 AIMS

The aim of this study was to assess the effects of an exercise training programme on ACE mRNA and protein expression, VEGF isoform/VEGFR mRNA expression and VEGF protein levels, and to examine these changes in relation to improvements in walking ability. The link between changes in ACE and VEGF expression was also

examined, as well as differential allele expression and allelic responses in ACE ID heterozygotes.

4.3 METHODS

Patients were recruited to the trial and managed as described in Section 3.1. Determination of ACE, $VEGF_{121}$, $VEGF_{165}$, VEGFR-1, sVEGFR-1 and VEGFR-2 mRNA expression was performed as described in detail in Section 3.3. ACE genotype was recorded as illustrated in Section 3.4, and differential transcription of ACE I/D alleles measured as stated in Section 3.5. ACE and VEGF protein levels were measured as described in Section 3.6. All experiments were run twice and a mean value taken apart from experiments measuring VEGF receptor expression, which were only run once using Jumpstart REDTaq. Overall ACE gene expression was expressed as a mean of the results obtained from the ACE 216 and ACE 2215 primers combined. All ELISA experiments were run twice in duplicate.

4.4 RESULTS

Eleven patients consented to take part in the trial, with 6 subjects (5 female, 1 male) randomised to the supervised exercise group and 5 subjects (2 female, 3 male) to the exercise advice only group. All subjects were Caucasian apart from 1 control of Asian origin. Demographic data and medications for each subject are summarised in Appendix 3. All demographic variables and baseline measurements were independent of treatment group at the start of the trial (Table 4.1). In the exercise group, 1 subject was ACE II genotype, 3 were ID and 2 were DD genotype. In the control group, 3 subjects were ID and 2 were II genotype. All subjects completed all aspects of the trial successfully, with no deaths or adverse effects reported from the exercise training programme.

Table 4.1 Baseline Data and Demographics

All measurements were independent of treatment group at initial assessment (Mann Whitney test). Values shown are medians (range).

	Group		
	Exercise	**Control**	**p-value**
Number	6	5	
Age (years)	77 (56-82)	65 (54-80)	0.33
Height (cm)	165 (162-169)	167 (155-173)	>0.50
Weight (kg)	61.8 (55.5-84.4)	72.3 (51.2-76.0)	0.43
BMI (kg/m^2)	22.3 (21.2-30.6)	24.49 (21.3-31.0)	0.25
Mean Arterial Pressure (mmHg)	107.5 (96.7-108.3)	101.7 (91.7-111.7)	0.33
Resting Heart Rate (bpm)	76 (60-80)	72 (60-84)	>0.50
ABPI	0.82 (0.68-0.92)	0.72 (0.71-1.30)	>0.50
CD (m)	300 (175-482)	124 (74-482)	0.25
MWD (m)	490 (353-718)	366 (134-859)	>0.50
Heel Raises	55 (25-208)	85 (25-139)	>0.50
ACE Genotype	1 II;3 ID;2 DD	2 II; 3 ID	-
ACE mRNA Expression	0.56 (0.35-0.93)	0.54 (0.39-0.99)	>0.50
$VEGF_{121}$ mRNA Expression	0.36 (0.23-0.68)	0.47 (0.40-0.66)	0.25
$VEGF_{165}$ mRNA Expression	0.14 (0.05-0.48)	0.21 (0.11-0.43)	>0.50
VEGFR-1 mRNA Expression	0.36 (0.19-0.46)	0.35 (0.22-0.45)	>0.50
sVEGFR-1 mRNA Expression	0.15 (0.09-0.24)	0.15 (0.09-0.23)	>0.50
VEGFR-2 mRNA Expression	0.11 (0.02-0.25)	0.15 (0.13-0.19)	0.33
ACE Protein Levels	187 (87-229)	186 (107-266)	>0.50
VEGF Protein Levels	16.9 (7.2-82.3)	25.8 (3.1-49.0)	>0.50

4.4.1 Physiological Responses to Exercise Training

General Responses

After the 8 week training period, no changes were noted in weight, resting heart rate, mean arterial pressure and ABPI in either the exercise or the control groups (Table 4.2).

Walking Ability

In the control group, there was no improvement in CD (pre 124 (74-482) metres, post 158 (88-418) metres; $p = 0.44$) or MWD (pre 366 (134-859) metres, post 361 (135-835) metres; $p = 0.625$). However, in the exercise group CD improved significantly from an initial 300 (175-482) metres to 406 (221-705) metres after training ($p < 0.05$), and MWD improved from 490 (353-718) metres to 634 (460-864) metres after training ($p < 0.05$). When comparing the two groups, the increase in MWD in the exercise group was significant ($p < 0.01$) compared to the control group, but no difference ($p = 0.25$) was found between the groups for the change in CD (Figures 4.1 and 4.2). Maximum heart rate and perceived exertion and pain scores at MWD were also unchanged in both groups (Table 4.2).

Heel Raises

In the control group, there was no change in heel raises ($p = 0.06$; pre 85 (25-139), post 54 (18-129)) after 8 weeks. In the exercise group, heel raises increased in 5 of the 6 subjects, although the increase was not significant ($p = 0.06$; pre 55 (25-208), post 136 (42-360); Figure 4.3a). However, the change in heel raises in the exercise group was significant compared to the control group ($P < 0.005$; Figure 4.3b). Perceived exertion and pain scores at the end of the heel raise test were also unchanged in both groups (Table 4.2).

Table 4.2 Changes in General Measurements after 8 weeks.

No significant changes were noted in general measurements after 8 weeks (Wilcoxon signed rank test). Values shown are medians (range).

	Control Group		
	Pre	**Post**	**p-value**
Weight (kg)	72.3 (51.2-76.0)	72.6 (52.5-75.2)	>0.50
Resting Heart Rate (bpm)	72 (60-84)	76 (68-92)	0.25
Mean Arterial Pressure (mmHg)	101.7 (91.7-111.7)	100.0 (85.0-113.3)	>0.50
ABPI	0.72 (0.71-1.30)	0.79 (0.67-1.33)	>0.50
Heart Rate at MWD (bpm)	100 (76-126)	100 (76-116)	>0.50
Perceived Pain (RPP) at MWD	4 (3-6)	5 (3-7)	0.50
Perceived Exertion (RPE) at MWD	15 (13-17)	13 (11-17)	0.50
RPP at maximum heel raises	5 (4-5)	5 (3-5)	>0.50
RPE at maximum heel raises	14 (11-17)	15 (11-17)	>0.50

	Exercise Group		
	Pre	**Post**	**p-value**
Weight (kg)	61.8 (55.5-84.4)	60.0 (54.6-85.2)	0.13
Resting Heart Rate (bpm)	76 (60-80)	76 (64-84)	0.16
Mean Arterial Pressure (mmHg)	107.5 (96.7-108.3)	105 (91.7-113.3)	>0.50
ABPI	0.82 (0.68-0.92)	0.77 (0.64-0.93)	>0.50
Heart Rate at MWD (bpm)	92 (68-114)	90 (80-104)	>0.50
Perceived Pain at MWD (RPP)	4.5 (3-6)	5 (3-7)	>0.50
Perceived Exertion at MWD (RPE)	15 (13-15)	15 (13-15)	>0.50
RPP at maximum heel raises	4.5 (3-7)	5 (3-7)	>0.50
RPE at maximum heel raises	13 (11-17)	14 (13-15)	>0.50

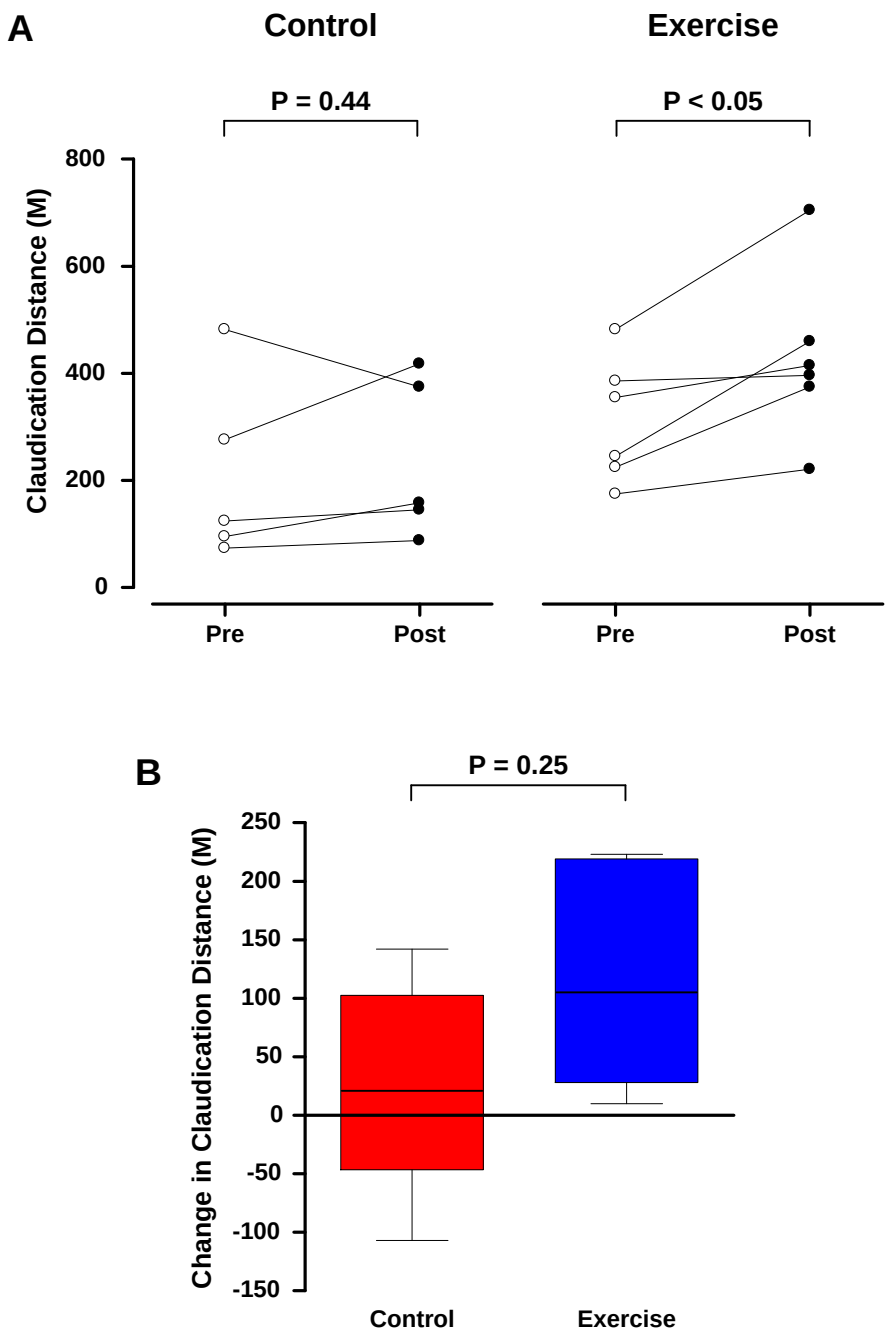

Figure 4.1 Changes in CD after Exercise Training

A Pre and post CD in the control and exercise groups. CD in the exercise group increased significantly, but no change was found in the controls (Wilcoxon signed rank test). **B** The exercise group increased CD by 105 (10-223) metres after training, but this increase was not significant compared to the controls (Mann Whitney test).

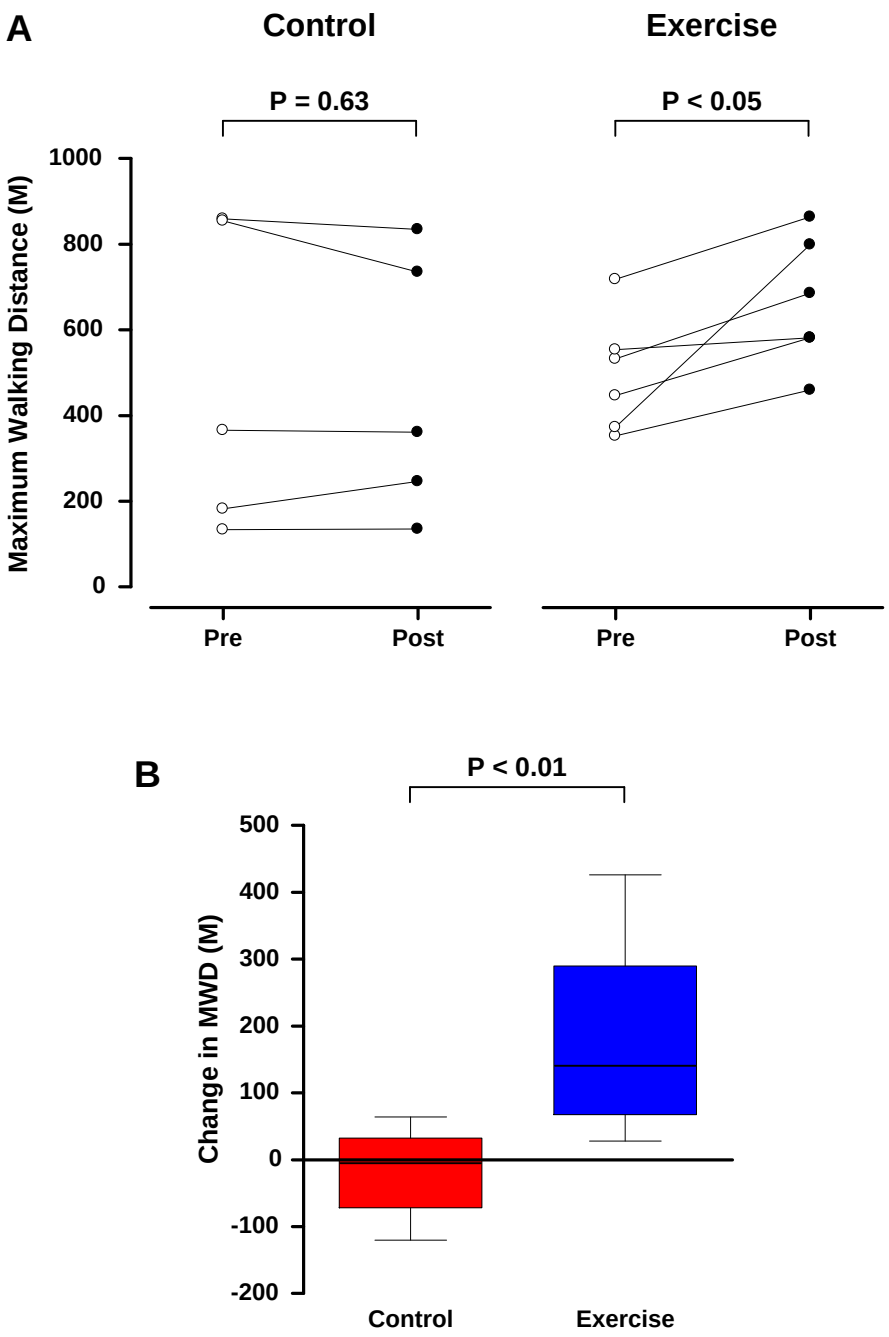

Figure 4.2 Changes in MWD after Exercise Training

A Pre and post MWD in the control and exercise groups. MWD in the exercise group increased significantly, but no change was found in the controls (Wilcoxon signed rank test). **B** The exercise group increased MWD by 141 (28-426) metres after training, which was a significant increase compared to the control group (Mann Whitney test).

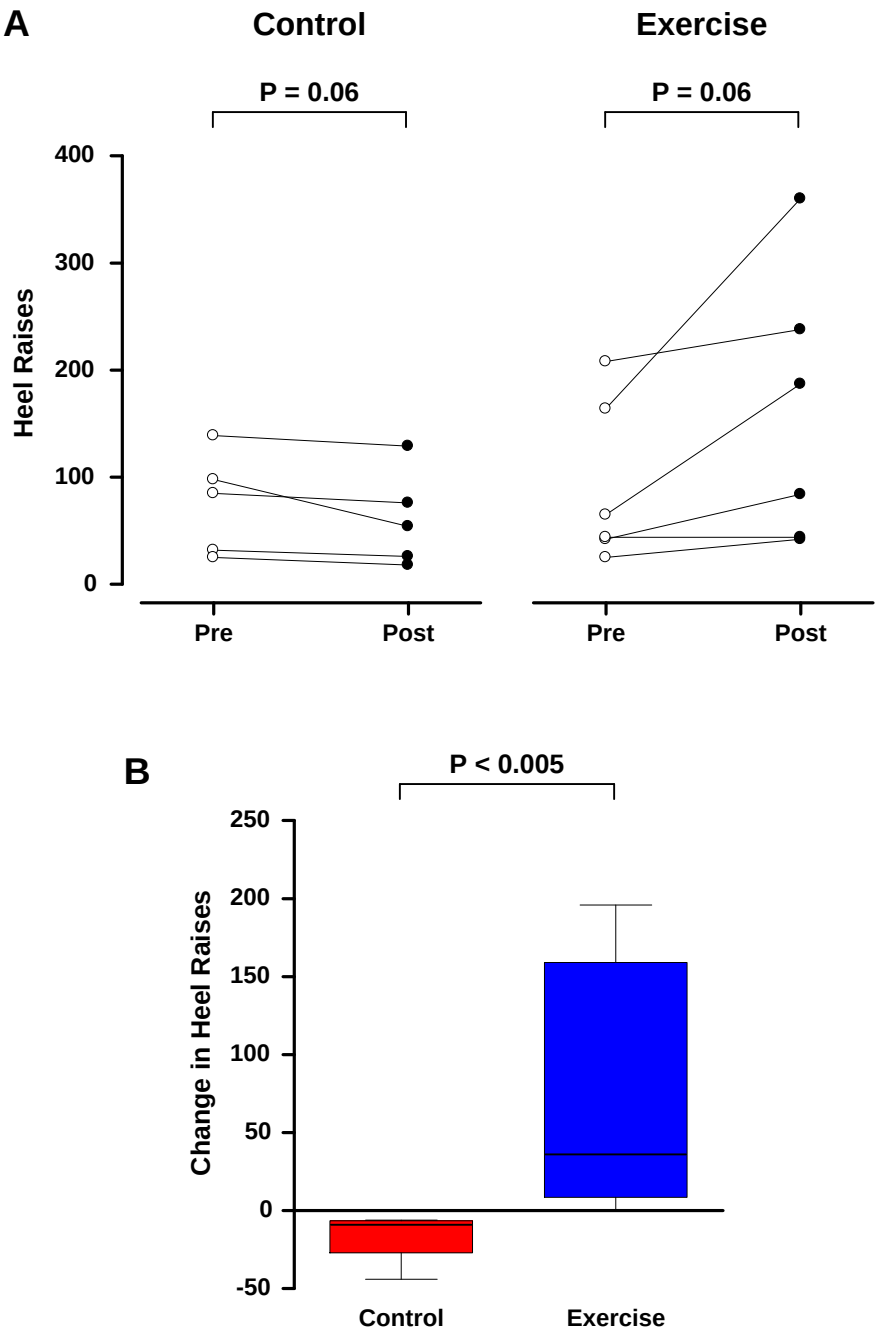

Figure 4.3 Changes in Heel Raises after Exercise Training

A No significant changes in heel raises were noted (Wilcoxon signed rank test) although in the exercise group 5 of the 6 subjects recorded an increase in heel raises. **B** Post training, repetitive heel raises in the control group decreased by 9 (-44 to -6) while the exercise group increased by 36 (0-196). The change in number of repetitive heel raises between the 2 groups was significant (Mann Whitney test).

A correlation between the change in number of repetitive heel raises and change in MWD was noted ($R = 0.81$, $p < 0.005$; Figure 4.4).

4.4.2 Molecular Responses to Exercise Training

ACE Responses to Exercise Training

ACE gene expression was unchanged in the control group, but increased 30% (7-57%) in the exercise group after 8 weeks training ($p < 0.05$). The difference between the two groups was not significant (Figure 4.5). However, the increases in ACE gene expression in the exercise group were not reflected by increases in plasma ACE levels, which were unchanged in both groups (Figure 4.6). Interestingly, changes in ACE gene expression appeared to be genotype influenced, with DD subjects having a median increase of 50%, ID subjects 19% and II subjects 16%. In comparing ACE gene expression with ACE protein level, there was no correlation between baseline ACE gene expression and ACE protein levels ($R = -0.536$; $p < 0.10$), and no correlation was found between the percentage change in gene expression and protein levels ($R = 0.21$; $p>0.50$).

Analysis of the ACE 2215 G/A polymorphism revealed that all patients genotyped (by standard PCR of genomic DNA) as DD expressed only the 2215A allele. Likewise, all subjects genotyped as II expressed only the 2215G allele, and all ID heterozygotes expressed both the 2215A and 2215G alleles. Thus, the direct linkage between the 2215A and the ACE D-alleles, and between the 2215G and ACE I-alleles, as shown previously by Suehiro *et al* was confirmed.[246] When examining I/D (G/A) heterozygote subjects at initial assessment, in all bar one subject expression of the D-allele was greater than the I-allele (Figure 4.7a). Furthermore, in the 3 subjects from the exercise group, the D:I allele ratio increased further after the training period, in conjunction with

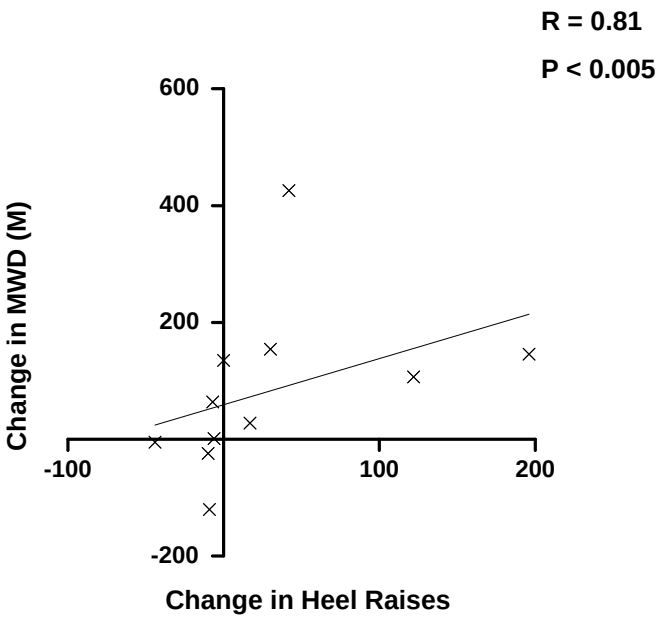

Figure 4.4 Correlation Between Changes in Heel Raises and MWD

The changes in the number of heel raises correlated to the changes in MWD (Spearman's correlation test).

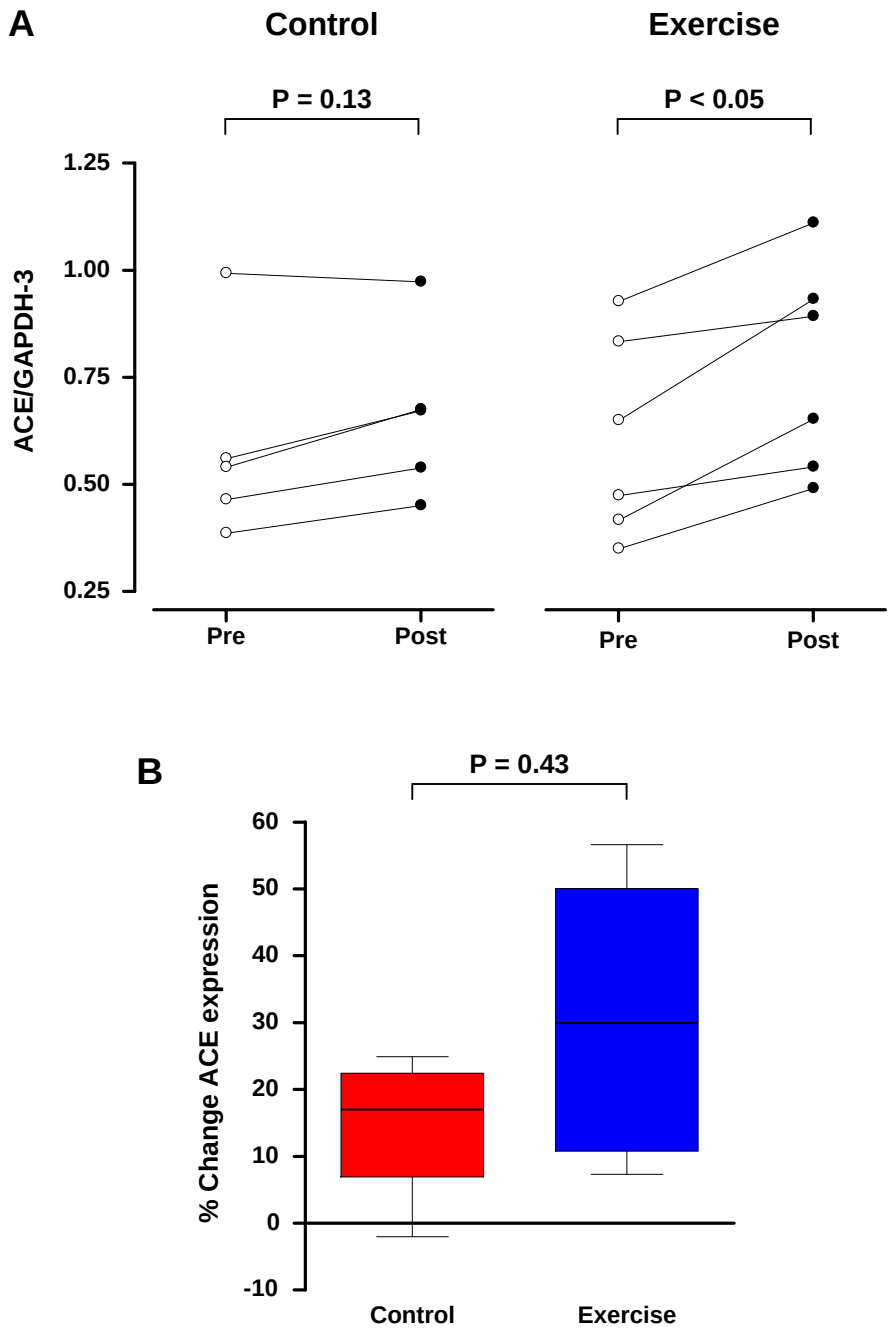

Figure 4.5 Changes in ACE Expression after Exercise Training

A Pre and post ACE expression in the control and exercise groups. In the exercise group, ACE expression increased significantly but no change was found in the controls (Wilcoxon signed rank test). **B** In the exercise group, ACE expression increased by 30% (7-57%) following the training period, but this increase was not significant compared to the controls (Mann Whitney test).

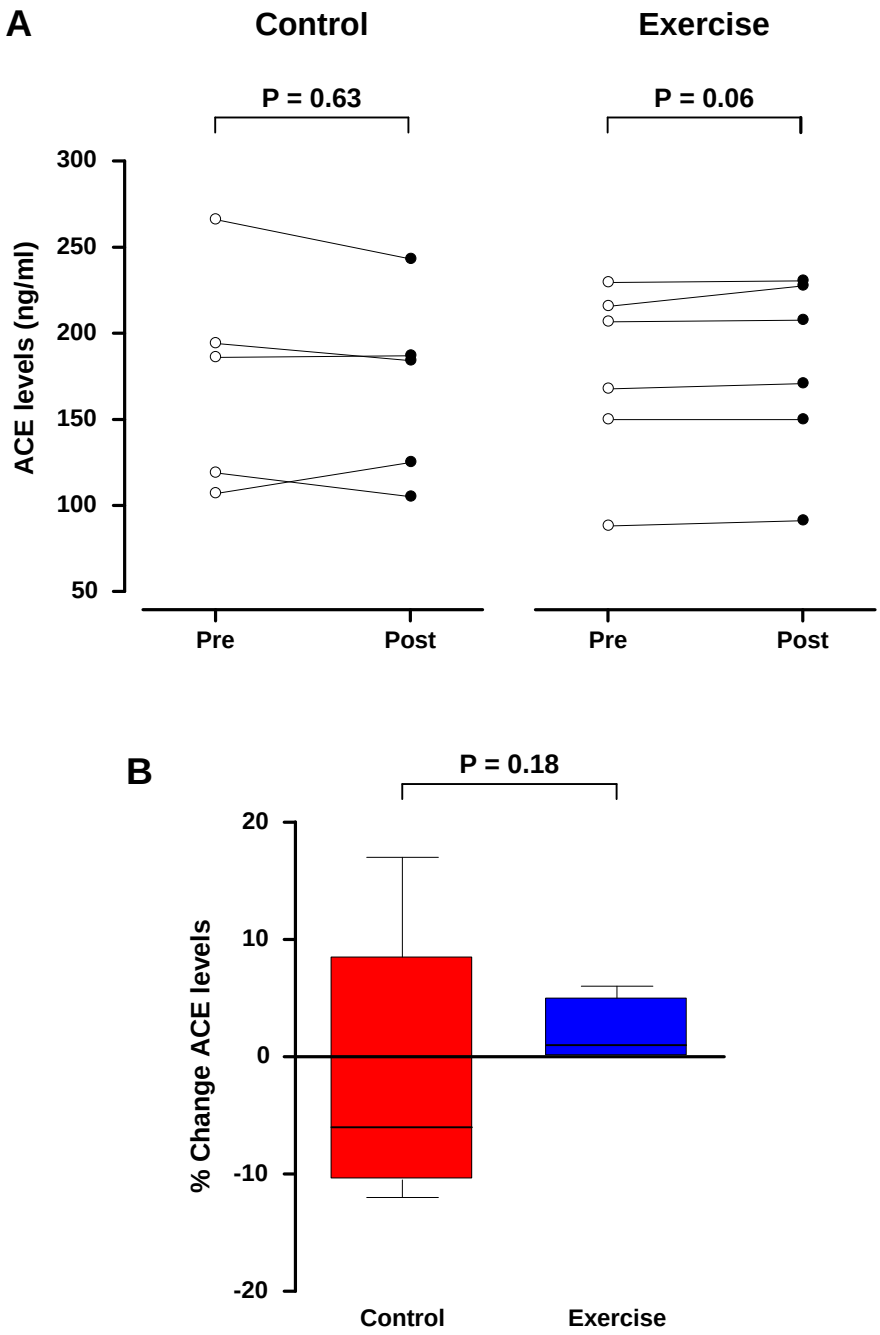

Figure 4.6 Changes in Plasma ACE Levels after Exercise Training

A Pre and post ACE levels in the control and exercise groups. No significant change was noted in plasma ACE levels for the exercise group or the control group (Wilcoxon signed rank test). **B** No significant difference was noted between the groups (Mann Whitney test).

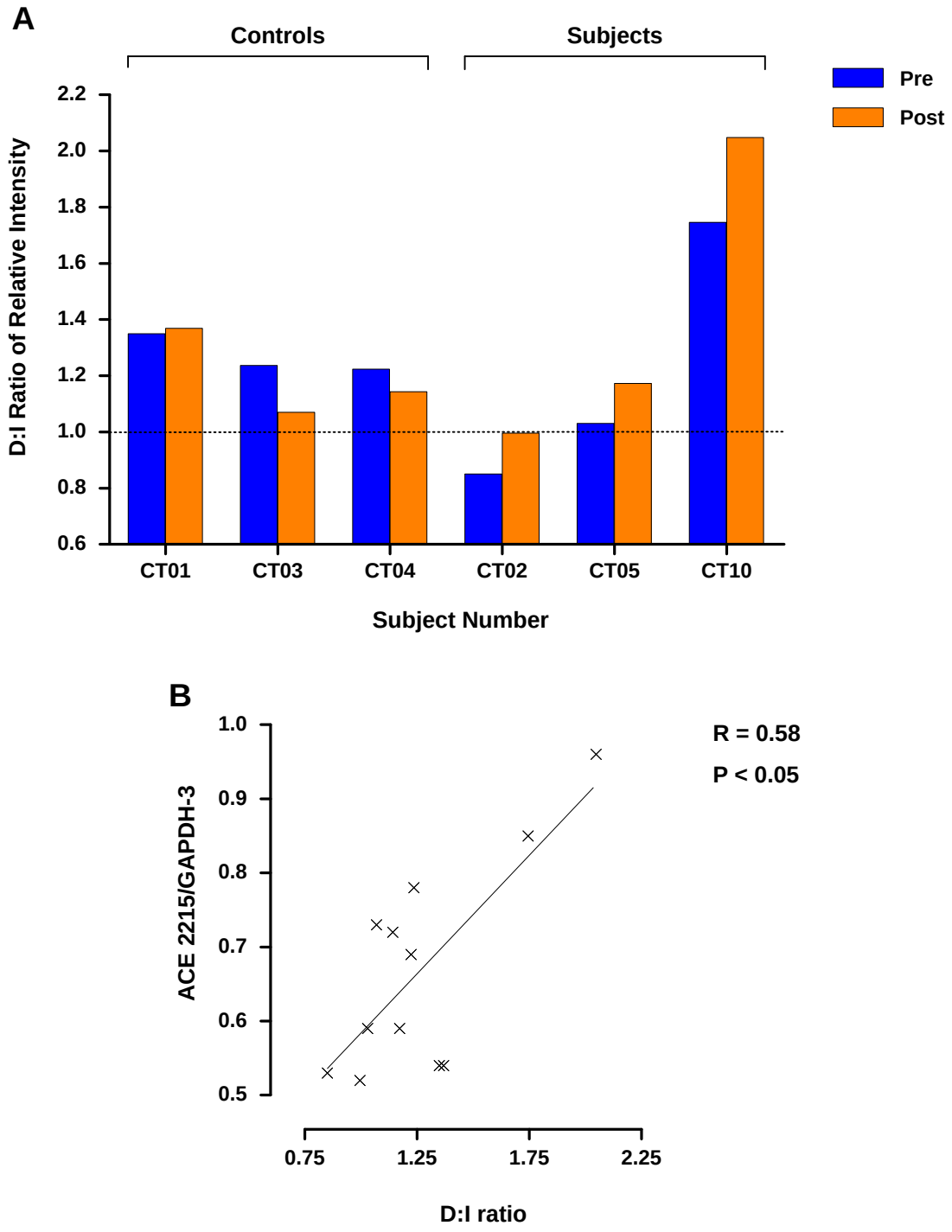

Figure 4.7 ACE D:I Allele Ratio and ACE Expression

A Among the ID heterozygotes, all bar one subject had a D:I ratio greater than 1 at initial assessment. All 3 subjects in the exercise group increased D:I ratio after 8 weeks training. **B** A significant correlation between ACE expression and D:I ratio was noted (Spearman's correlation test).

an overall increase in ACE gene expression, indicating preferential activation of the D allele in these subjects. In the control group, 2 out of 3 subjects decreased the D:I ratio after 8 weeks, with 1 subject showing a slight increase (Figure 4.7a).

When examining the link between ACE expression and D:I allele ratio, the results from both groups and assessments were combined. Analysis showed a significant correlation between ACE expression and the D:I ratio ($R = 0.58$; $p < 0.05$, Figure 4.7b). However, the correlation between ACE protein level and D:I ratio was not statistically significant ($R = -0.50$; $p < 0.10$).

VEGF Responses to Exercise Training

In the exercise group, $VEGF_{121}$ expression increased by 43% (6-158%, $p < 0.05$) whilst no change was found in the control group (Figure 4.8a). The increase in $VEGF_{121}$ expression in the exercise group was significant compared to the controls (Figure 4.8b). Similarly, $VEGF_{165}$ expression also significantly increased by 70% (3-255%; $p < 0.05$) in the exercise group compared to the controls, which did not change (Figure 4.9). Although VEGF protein levels increased in 5 of the 6 subjects in the exercise group, no significant change was found (94% (-7-359%); $p < 0.10$), and no change detected in the control group (Figure 4.10).

No correlation was noted between initial $VEGF_{121}$ expression and baseline VEGF protein levels ($R = 0.16$; $p > 0.50$), or between initial $VEGF_{165}$ expression and VEGF levels ($R = -0.08$; $p > 0.50$). In addition, there was no correlation between the percentage change in $VEGF_{121}$ expression ($R = 0.16$; $p > 0.50$) or $VEGF_{165}$ expression ($R = 0.06$; $p > 0.50$) compared to the percentage change in VEGF protein levels.

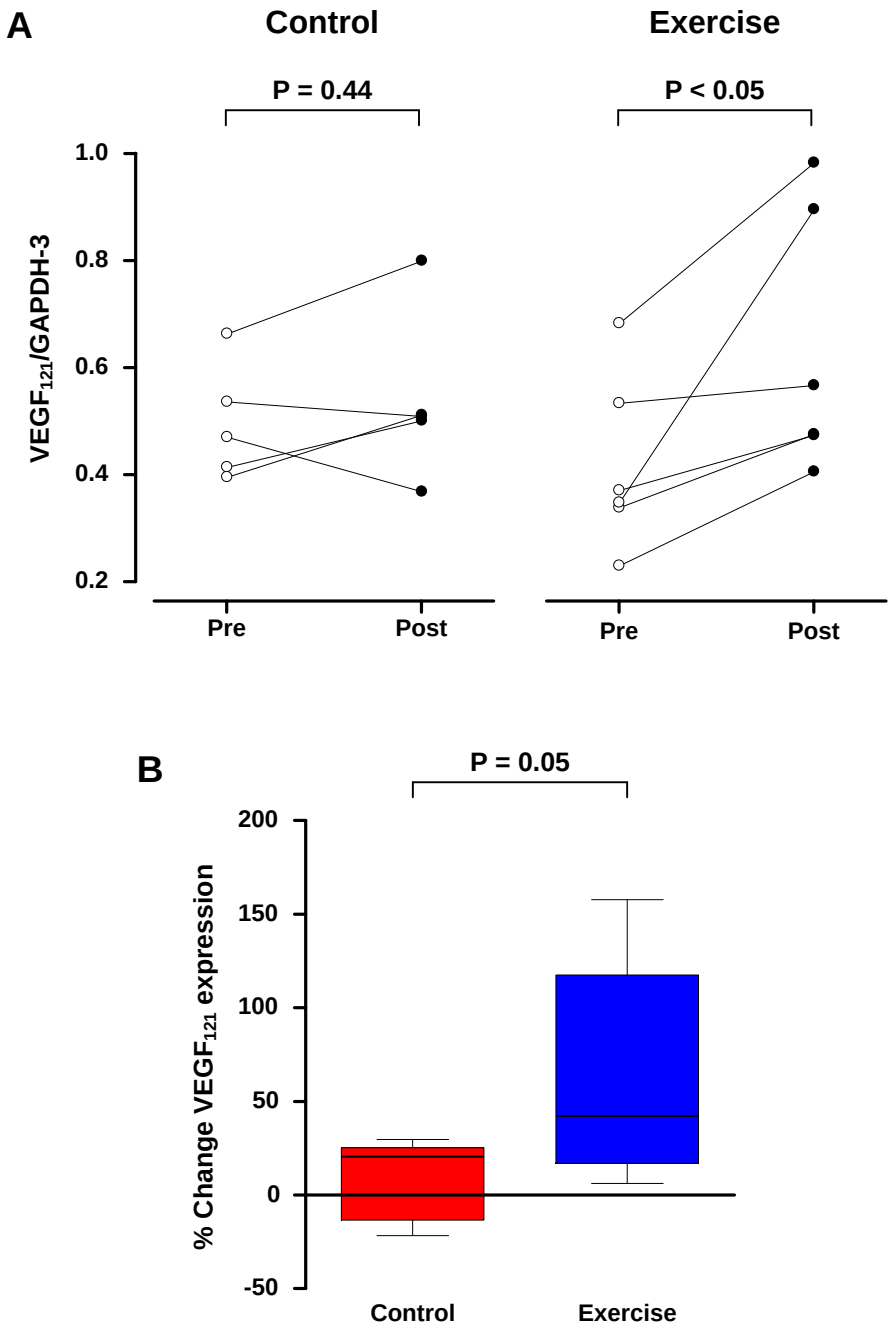

Figure 4.8 Changes in $VEGF_{121}$ Expression after Exercise Training

A Pre and post $VEGF_{121}$ expression in the control and exercise groups. $VEGF_{121}$ expression increased significantly in the exercise group, with no change in the controls (Wilcoxon signed rank test). **B** $VEGF_{121}$ expression increased by 43% (6-158%) in the exercise group, which was a significant increase compared to the control group (Mann Whitney test).

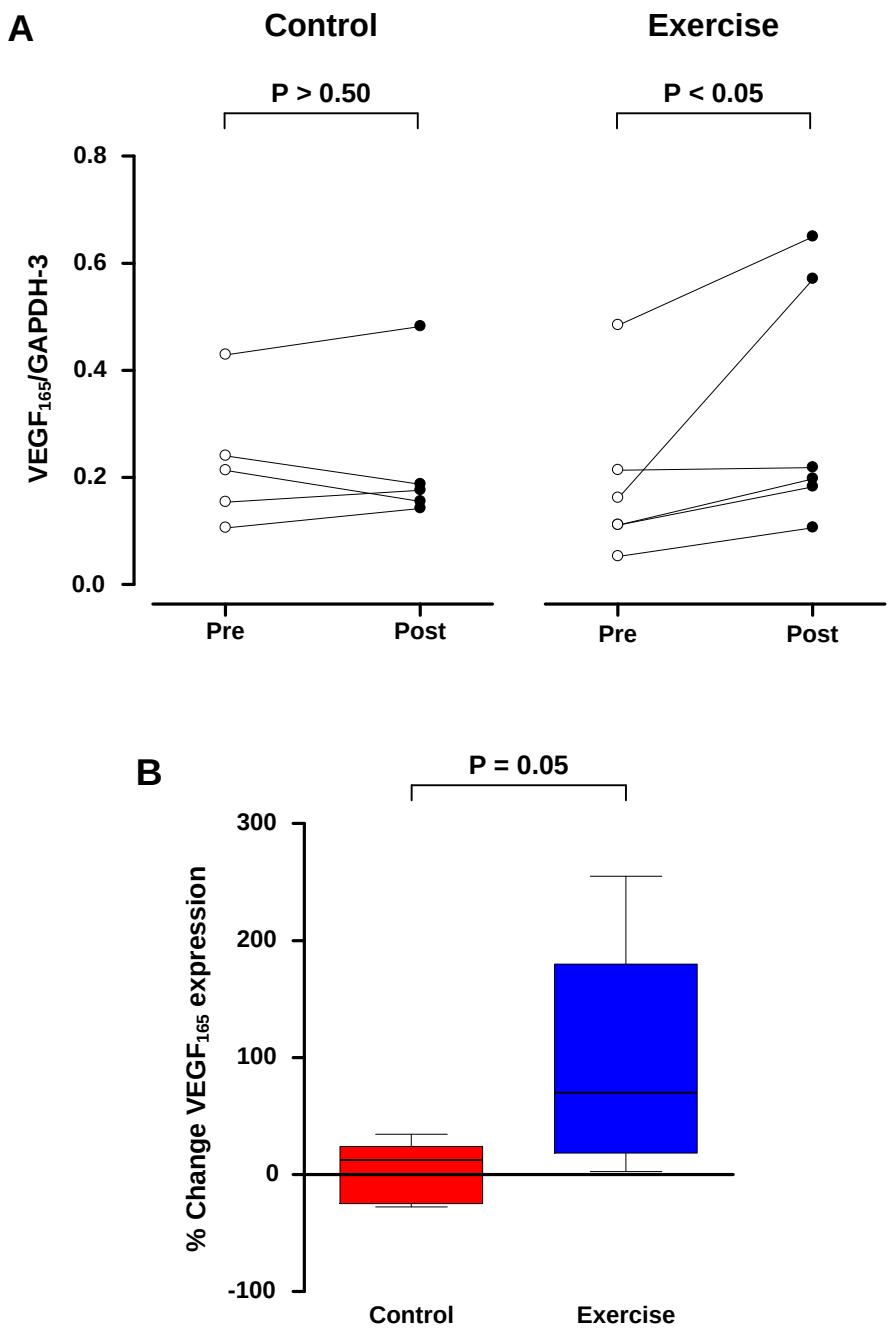

Figure 4.9 Changes in $VEGF_{165}$ Expression after Exercise Training

A Pre and post $VEGF_{165}$ expression in the control and exercise groups. $VEGF_{165}$ expression increased significantly in the exercise group, but not in the controls (Wilcoxon signed rank test). **B** In the exercise group, $VEGF_{165}$ expression increased by 70% (3-255%), which was significant compared to the controls (Mann Whitney test).

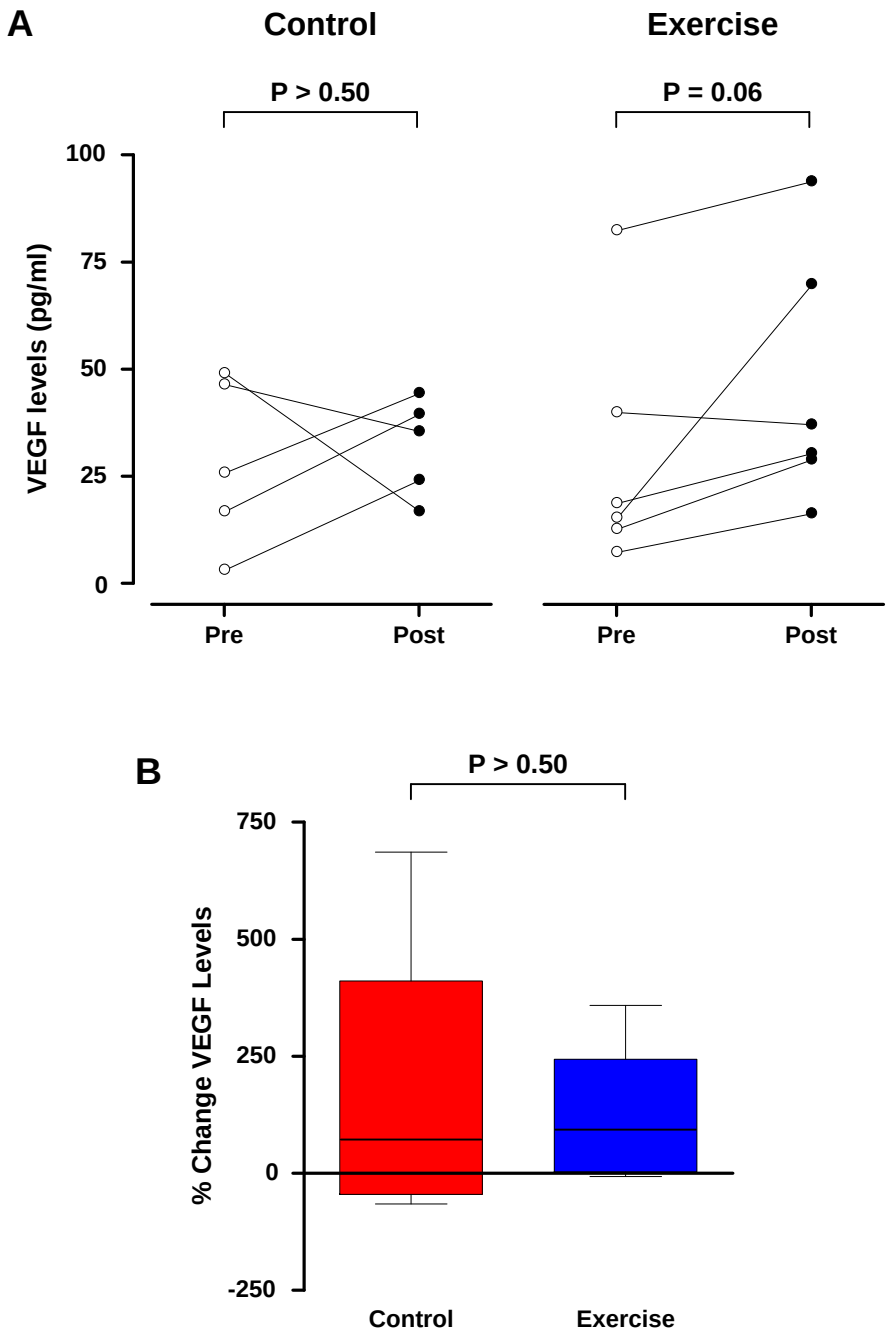

Figure 4.10 Changes in Plasma VEGF Levels after Exercise Training

A Pre and post VEGF levels in the control and exercise groups. VEGF levels in the control group were unchanged, while VEGF increased in 5 of 6 of the exercise group although this was not significant (Wilcoxon signed rank test). **B** An increase of 94% (-7-359%) in VEGF levels in the exercise group was found, but this was not significant when compared to the controls (Mann Whitney test).

VEGF Receptor Responses to Exercise Training

No significant increase in VEGFR-1 receptor expression was noted in the exercise group, the control group or between the 2 groups (Figure 4.11). For sVEGFR-1 receptors, expression increased by 63% (2-149%) in the exercise group ($P < 0.05$), but was unchanged in the control group (Figure 4.12a). However, the increase in expression in the exercise group was not significant compared to the controls (Figure 4.12b). Finally, for VEGFR-2 the exercise group again showed a significant ($p < 0.05$) increase in gene expression, with a rise of 72% (22-633%). No change was noted in the controls. The increase in VEGFR-2 expression in the exercise group was significant compared to the control group (Figure 4.13).

Relationship between ACE and VEGF

A correlation was found between baseline ACE gene expression and both $VEGF_{121}$ expression ($R = 0.65$, $p < 0.05$) and $VEGF_{165}$ expression ($R = 0.64$, $P < 0.05$; Figure 4.14). However, no correlation was noted between percentage change in ACE and $VEGF_{121}$ ($R = -0.136$, $p>0.50$) or $VEGF_{165}$ ($R = -0.06$, $p>0.50$) expression. For protein levels, there was no correlation between initial ACE and VEGF levels ($R = -0.09$, $p>0.50$), or in percentage change in ACE and VEGF levels ($R = -0.33$, $p = 0.31$).

4.4.3 Correlation between Physiological and Molecular Responses

No relationship was found between any physiological measurements (CD, MWD, heel raises) and initial or changes in ACE, VEGF, or VEGF receptor gene expression and protein levels. None of the measured physiological parameters appeared to be influenced by ACE genotype, original ACE concentration or changes in ACE mRNA or protein levels.

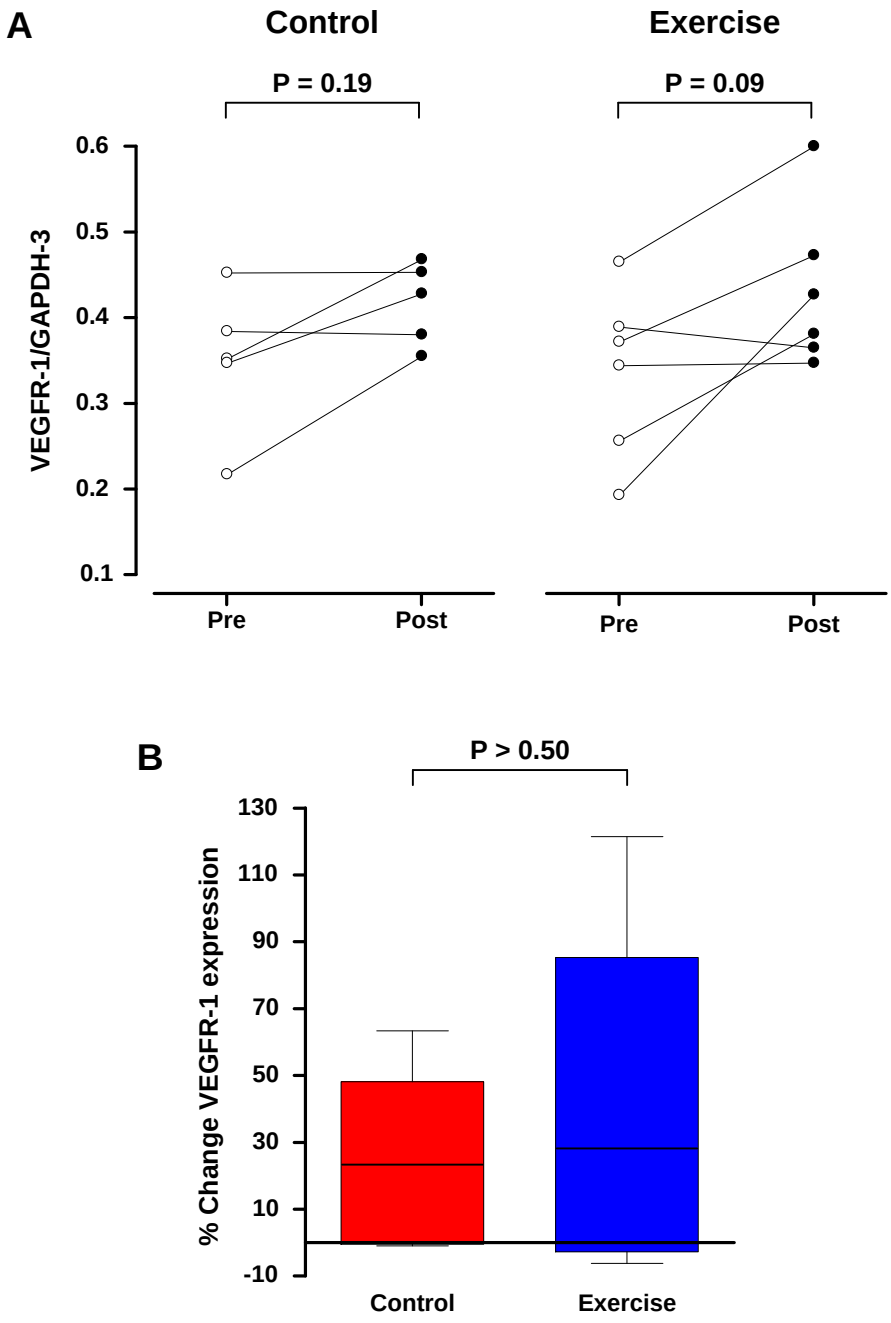

Figure 4.11 Changes in VEGFR-1 Expression after Exercise Training

A Pre and post VEGFR-1 expression in the control and exercise groups. No change in VEGFR-1 expression was found in the exercise or control groups (Wilcoxon signed rank test). **B** An increase of 28% (-6-122%) in VEGFR-1 receptor expression was found in the exercise group, but this was not significant compared to the controls (Mann Whitney test).

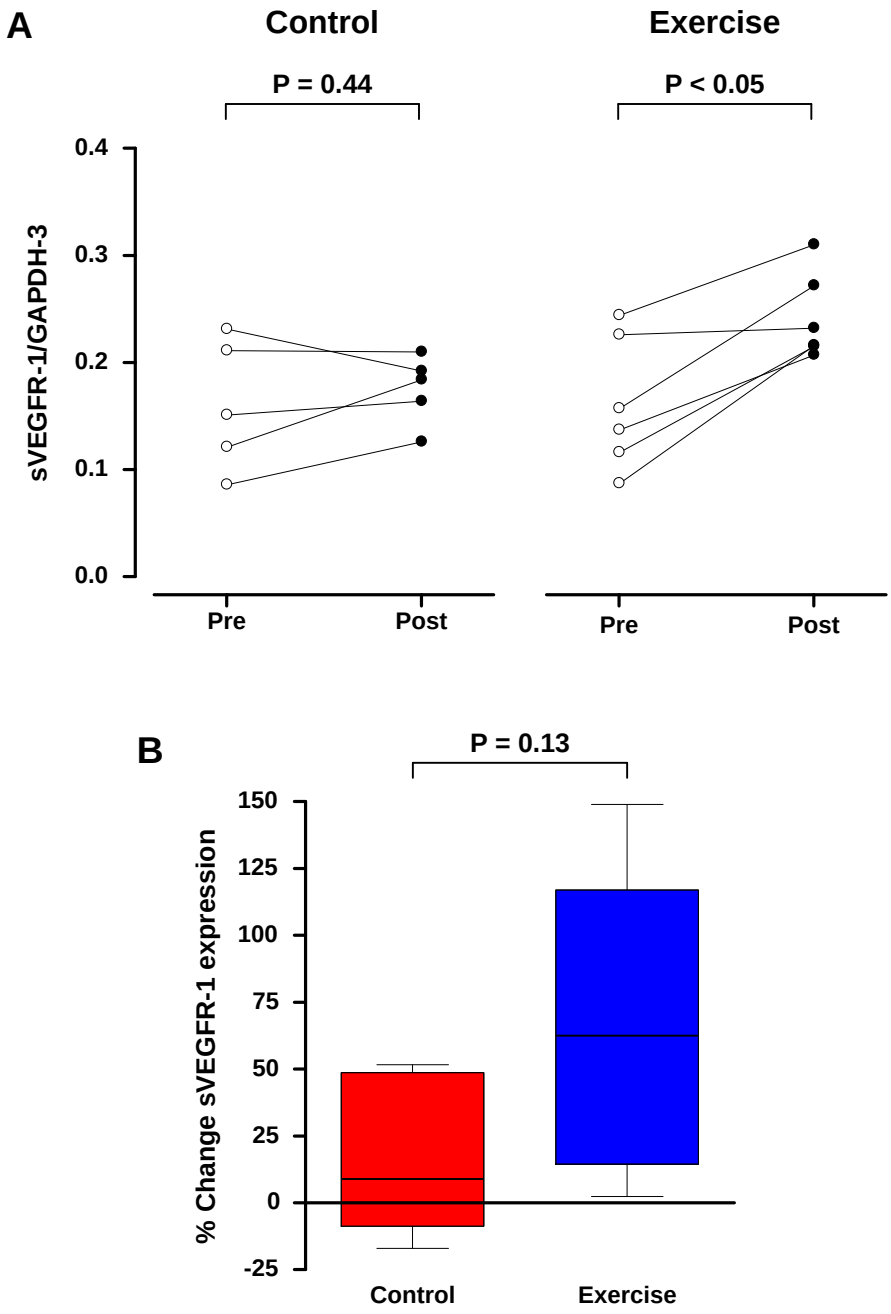

Figure 4.12 Changes in sVEGFR-1 Expression after Exercise Training

A Pre and post sVEGFR-1 expression in the control and exercise groups. In the exercise group, sVEGFR-1 expression increased significantly while the control group was unchanged (Wilcoxon signed rank test). **B** sVEGFR-1 expression increased by 63% (2-149%) in the exercise group, but this was not significant compared to the controls (Mann Whitney test).

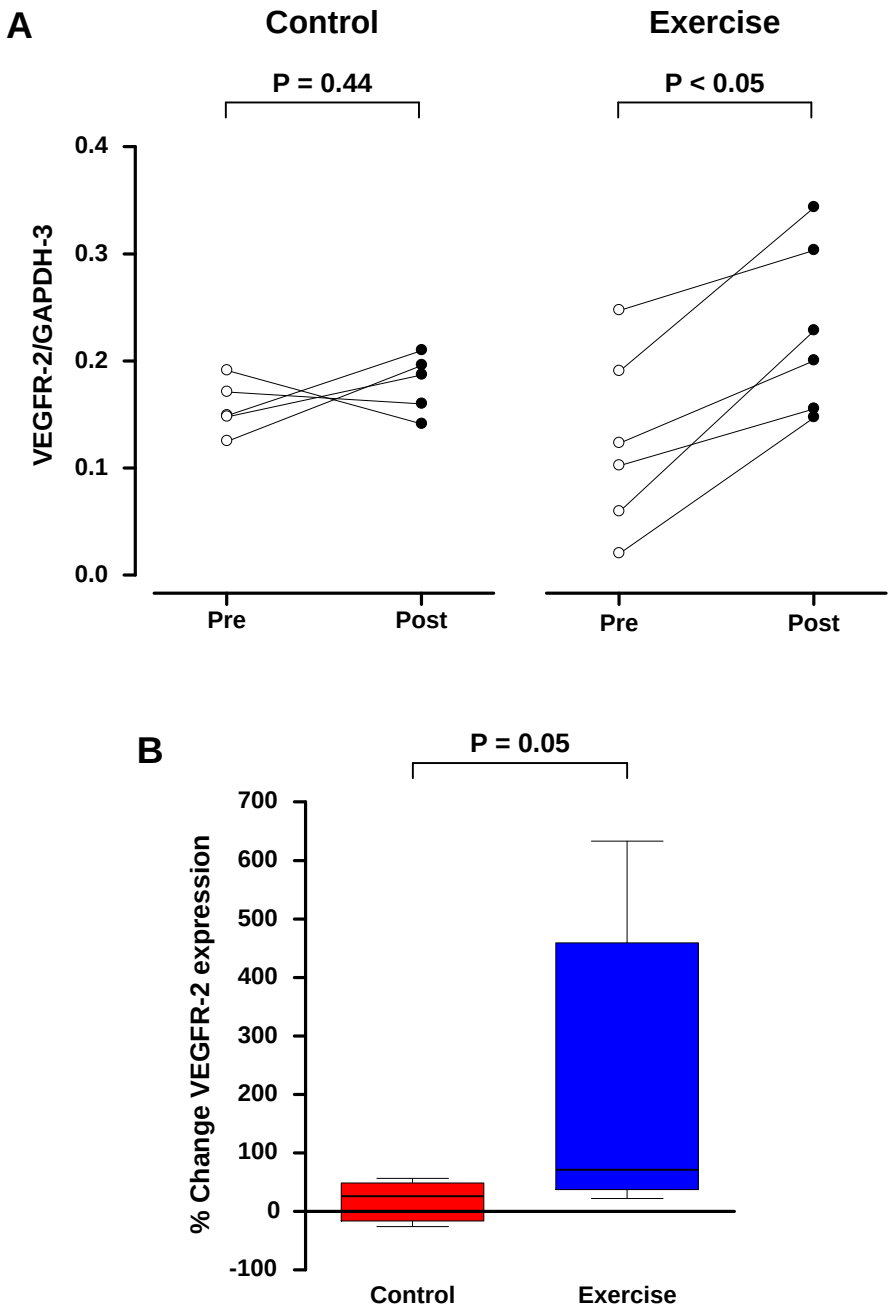

Figure 4.13 Changes in VEGFR-2 Expression after Exercise Training

A Pre and post VEGFR-2 expression in the control and exercise groups. VEGFR-2 expression increased significantly in the exercise group but not in the control group (Wilcoxon signed rank test). **B** VEGFR-2 expression increased by 72% (22-633%) in the exercise group, which was significant when compared to the controls (Mann Whitney test).

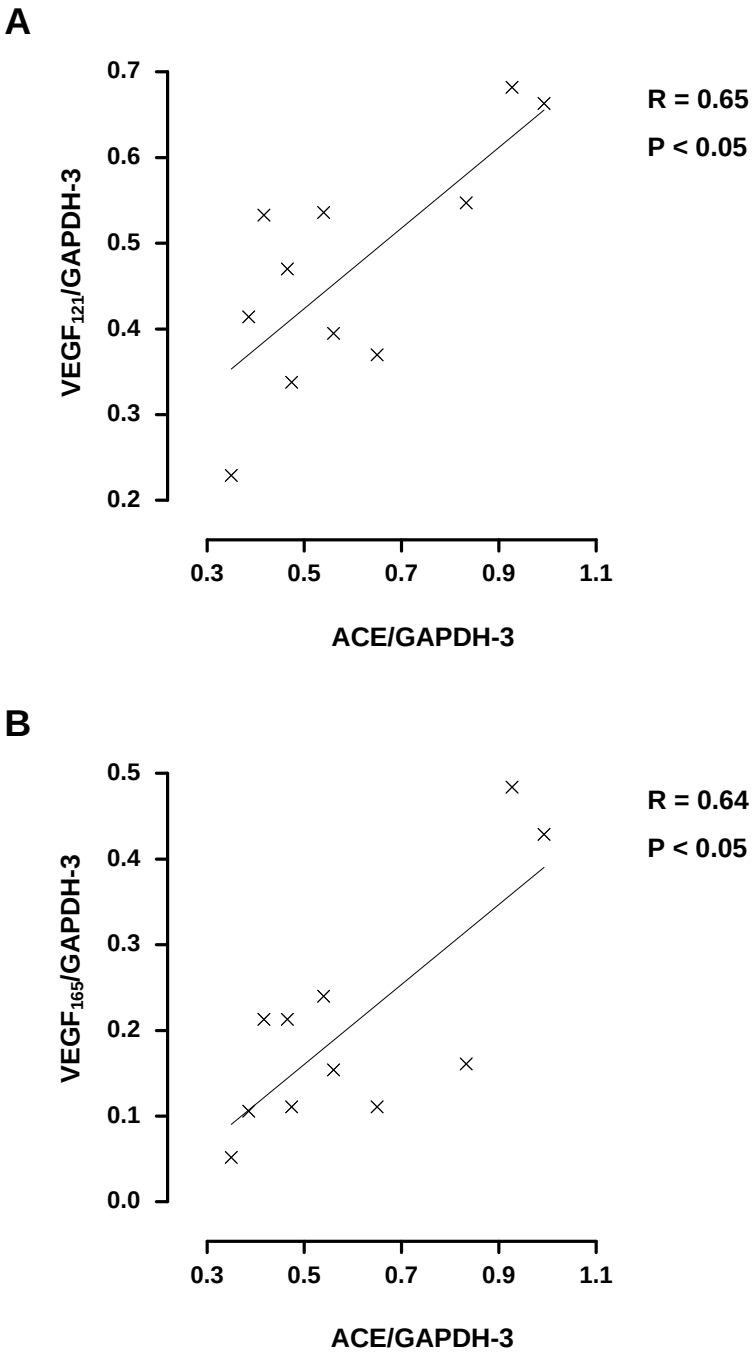

Figure 4.14 Correlation Between ACE and VEGF Gene Expression

A correlation was found between ACE gene expression with **A** $VEGF_{121}$ expression and **B** $VEGF_{165}$ expression (Spearman's correlation test).

4.5 DISCUSSION

Evidence from gene-association studies and gene-environment interaction studies have demonstrated that low ACE activity (related to the I-allele of the ACE I/D polymorphism) is linked to improved endurance performance in athletes and army recruits.[123;170;173] Patients with intermittent claudication represent a unique group quite different from elite athletes and army recruits. Nevertheless, a pilot study performed in this laboratory also suggested that the I-allele was beneficial to training responses in claudicants. To date, studies examining the effects of ACE on endurance performance have only investigated the link between ACE genotype and endurance. Of potentially more relevance is the association between ACE phenotype (gene expression and protein activity) and endurance, as a connection between ACE gene activity and endurance would provide stronger evidence of a link. Moreover, ACE phenotype can be readily altered using ACE inhibitors. However, before considering the potential benefits from modulating ACE, it is important to assess how exercise training alone affects ACE gene activity. Thus, in this study the association between changes in walking ability in claudicants after training and responses in ACE gene activity were studied.

A further possible benefit from reduced ACE activity in claudicants is an increase in angiogenesis. Animal models of ischaemia have revealed an increase in VEGF-mediated angiogenesis with the use of ACE inhibitors.[77;85;233] Indeed, the regulation of ACE and VEGF appears to be linked, with variations in ACE affecting VEGF and *vice-versa*.[141;219;233] Thus, this study also examined the links between the effects of exercise training and changes in VEGF, and the links between changes in VEGF and ACE. To appropriately study the effects of the training programme on VEGF, it was necessary to examine changes in both VEGF isoform and receptor expression, as these all have

contrasting roles in VEGF biology.[39;46;90] Thus, this study examined the relationship between changes in walking ability with changes in ACE and VEGF. It was hypothesised that exercise training would lead to increased walking ability, with an associated suppression of ACE gene expression and protein levels (already elevated by ischaemia), and that there would also be an associated increase in VEGF/VEGFR gene expression and protein levels.

Patient Recruitment

Prior to discussing the results, it is important to consider that the study sample sizes were small, with only 6 subjects in the 'exercise' group and 5 subjects in the 'control' group. Thus, statistical analysis of the results often gave non-significant results. Unfortunately, poor recruitment to exercise programmes and clinical trials among claudicants is not uncommon and has been well documented in the past.[69;119] Our experience in this trial was that many patients had contraindications to entry, such as severe heart disease, respiratory disease, and osteoarthritis. Furthermore, a significant proportion of potential recruits were already prescribed ACE inhibitors and were thus unsuitable for the trial. Many were also reluctant to participate due to having long distances to travel, or were unwilling to commit the necessary time to training sessions. As a result, it is questionable as to whether the findings from this trial can be extended to cover the claudicant population as a whole. The recruited subjects are likely to reflect a select group of well motivated claudicants with limited co-morbidities, a group that probably represents a minority of the general claudicant population.

There was also marked heterogeneity between the exercise and control groups (Table 4.1). Most of the exercise group (5 of 6) were female, whereas the control group was

predominantly male (3 of 5). The exercise group also tended to be older (median ages: subjects 77, controls 65), and lighter (median BMIs: subjects 22.3, controls 24.5). Furthermore, CD and MWD were respectively 142% and 33% greater in the exercise group at baseline. However, although the subjects were lighter, they performed fewer heel raises (median heel raises: subjects 55, controls 85) at baseline. VEGF protein levels at baseline were also notably different between the groups (median VEGF level: subjects 17, controls 26). This heterogeneity between the groups could have affected the responses in the trial, making comparisons between the groups potentially unreliable.

Trial Limitations

When considering the results, other limitations of the study must also be addressed. The results from this trial among claudicants cannot be extended to relate to other groups of individuals such as athletes. One must also take into consideration the varied medications the participants were prescribed, such as Aspirin (all participants bar 1 control), Beta-blockers (1 control, 1 subject) and Statins (4 controls, 3 subjects). Aspirin and statins would have had potential anti-inflammatory effects that could have affected the responses to training. Likewise, Beta-blockers would have affected the responses to exercise by limiting cardiovascular responses, and also had effects on renin release and glucose metabolism. However, to design a trial to control for the potential effects of all these medications would not have been feasible.

Another major limitation of this study was that ACE/VEGF mRNA expression were determined from PBMNs isolated from peripheral blood and ACE/VEGF protein levels measured from plasma. Although baseline expression and changes in expression measured in this study may reflect changes occurring locally in the leg muscles, there is

no rationale for this and in this study can only be presumed. It is entirely possible that direct measurement of gene activity and protein levels from muscle tissue could have produced different results, and experience from previous studies has often shown a differential effect of a stimulus on ACE depending upon the tissue involved.[77] However, in designing this study it was decided that taking muscle biopsies from subjects would have been too invasive and thus unacceptable.

Physiological Responses to Exercise Training

This study confirmed the already well described finding that supervised exercise training improves walking ability among claudicants. In the training group, significant increases in CD (105 metres) and MWD (141 metres) were recorded without any significant change in perceived pain or exertion scores, demonstrating decreased symptoms rather than an increased ability to walk through pain. These results are comparable to other studies of supervised exercise programmes, and add to the weight of evidence supporting the widespread introduction of these programmes.[18;99;243] In addition, this trial also suggests that 8 weeks of training is sufficient to produce significant increases in walking distance, in contrast to the 6 month period that has been previously recommended.[71] Furthermore, no changes were noted in ABPI or maximum heart rate, indicating no major changes in gross blood flow or cardiovascular fitness underlying these improvements.

Calf muscle endurance, as measured by repetitive heel raises, was also improved in the exercise group. Interestingly, as described previously, improvements in heel raises were closely correlated to increases in MWD.[178] If confirmed in larger-scale studies, assessment of the number of repetitive heel raises could function as a simple and fast

method to objectively gauge a patient's claudication symptoms without the need for more extensive testing procedures, and so be of use in an outpatient setting. Using a heel raise test as an endpoint in studies of claudication may also have benefits compared to treadmill testing. A heel raise test is unaffected by changes in walking gait that contribute to improvements in treadmill walking after training.[142] In addition, a heel raise test is independent of the training mode, and gains produced by familiarity with the testing apparatus are negated.

Responses of ACE to Exercise Training

No previous studies have examined the effects of endurance training on ACE mRNA expression or ACE levels. In contrary to expectations, this study revealed an increase of 30% in ACE mRNA expression after exercise training. However, when examining plasma ACE levels, the increase in gene expression was not reflected by a significant increase in ACE protein levels. Protein levels are more likely to be reflective of the physiological relevance of ACE, as they are directly related to ACE activity.[28] Thus, these results suggest that a decrease in ACE activity is not an important mechanism underlying improvements in walking ability in claudicants.

The small numbers of subjects also prevented any statistical analysis of ACE genotype data. However, when combining the data from both study groups, changes in ACE gene expression appeared to be genotype influenced, with DD subjects having a median increase of 50%, ID subjects 19% and II subjects 16%. It is possible to speculate that subjects with different genotypes could have responded in a different way to the training programme. For example, those with II genotype could have increased ACE expression less than those with DD genotype, still conferring a theoretical advantage. However,

significantly more numbers of subjects would have been needed to perform such an analysis. Finally, this study only assessed the impact of training on ACE expression and levels. To get a full picture of how exercise training affects the RAS, the whole system including Ang-II levels and AT receptor levels would need to be assessed.

Analysis of baseline ACE D- and I-allele expression in ID heterozygotes revealed that in 5 out of 6 subjects, more ACE mRNA was produced from the D-allele than the I-allele. This substantiates the findings of Suehiro *et al*., who performed their original experiment in healthy individuals.[246] In addition, in the 3 ID subjects who underwent exercise training, the D:I ratio increased further in conjunction with an overall increase in ACE expression, suggesting preferential activation of the D-allele. Furthermore, there was a correlation between overall ACE gene expression and D:I ratio, also suggesting a link between D-allele activation and overall ACE gene activity. The mechanism underlying the predominance of mRNA originating from the D-allele is not understood. However, it has been speculated that it may relate to selective enhancement of transcription of the D-allele or increased stability of mRNA produced from the D-allele.[246] Further studies would be required to elucidate this mechanism.

Responses of VEGF to Exercise Training

No previous studies have examined the effects of training in claudicants on VEGF isoform mRNA expression in conjunction with VEGF levels and VEGF receptor expression. Analysis of transcriptional data revealed a substantial increase in the $VEGF_{121}$ isoform (43%) and an even greater increase in the more biologically active $VEGF_{165}$ isoform (70%) in the exercise group. This increase was significant compared to the control group. On examining VEGF protein levels, baseline levels were

comparable to those recorded in previous studies.[190;292] After exercise training, no significant increase in VEGF protein was recorded, although 5 of the 6 subjects studied had an increase in VEGF protein level after training, giving a median increase of 94%. These findings are in line with the only previous study of VEGF responses to exercise training in claudicants, which showed that exercise training had no acute effect on plasma VEGF levels or on baseline VEGF levels after 6 weeks training.[292] However, the sample sizes in both studies were small (n=6 and n=7), and larger scale studies are required to fully clarify the situation.

The results obtained from VEGF receptor expression analysis also point towards an overall increase in VEGF signalling capacity. Although the expression of sVEGFR-1, an inhibitory 'decoy' receptor was increased (63%), the expression of the signal transducing VEGFR-1 and VEGFR-2 receptors was also increased. Indeed, the transcription of VEGFR-2, which has the strongest signalling properties and is most important VEGF receptor in producing the signal for angiogenesis, showed the greatest increase of all (72%).[90] The recorded increases in VEGF receptors may also have masked the true rise in VEGF protein levels, due to increased binding of plasma VEGF to endothelial bound VEGF receptors and sVEGFR-1.[129] However, in order to confirm the increase in VEGF signalling potential indicated by these mRNA expression studies, future studies would also need to assess tissue and plasma VEGF receptor protein levels. When considering these results, it is again important to consider the limitations of the trial. As discussed above, these results relate to mRNA expression in PBMNs and plasma levels of VEGF, and cannot be guaranteed to reflect changes occurring locally within the tissues. This issue could help explain the lack of correlation between mRNA and protein levels both ACE and VEGF in this study. Overall, the results reveal that

exercise training leads to an overall increase in potential VEGF signalling activity, as determined by $VEGF_{121}/VEGF_{165}$ expression, plasma VEGF levels and VEGF receptor expression. Given that these increases in VEGF signalling capacity occur in conjunction with increases in walking ability, it is possible to speculate that VEGF and its receptors could be a viable target for treatment of intermittent claudication.

Relationship Between ACE and VEGF

On examining the relationship between VEGF and ACE, a strong positive correlation was found between baseline $VEGF_{121}/VEGF_{165}$ mRNA expression and ACE mRNA expression. However, no relationship was noted between changes in ACE and VEGF expression after training, or between ACE and VEGF protein levels. The simultaneous increase in both ACE and VEGF expression is comparable to that described by Saijonmaa *et al.*, who noted that VEGF up-regulated ACE expression in endothelial cells, suggesting a synergistic relationship between ACE and VEGF.[219] Conversely, the results do not corroborate with evidence from animal models of ischaemia, in which reduction of ACE activity increased VEGF expression.[77;233;250] The differences between these studies could be related to the use of ACE inhibitors in the animal models. ACE inhibitors, as previously described in Section 1.5, can alter gene expression directly via their own cell signalling properties, independent from their actions in reducing in ACE activity.[93;94] Furthermore, although ACE and VEGF expression both increased in this trial, this does not indicate a direct link, and it is possible that the increases occurred independently from each other. The lack of a relationship between protein levels also counts against a link, although the results for protein could have been affected by issues such as the level of receptor binding.[129] Issues relating to the interpretation of mRNA and protein data are discussed below.

Relationship Between mRNA Expression and Protein Levels

In this study, no strong correlation between mRNA expression and protein levels was found for VEGF or ACE. In addition, no link was found between changes in mRNA expression and protein levels. These findings could be explained by the complexity of the process in the progression from genotype to phenotype. The central dogma of genetics states that genetic information progresses from DNA to mRNA to protein. However, in practice this is a hugely complex system and at each stage in this hierarchy variation can be introduced, for example by differential transcription of mRNA and differential translation of protein.[200] Thus, to an extent the findings in this study are not surprising, and many studies have also been published regarding the poor correlation between mRNA expression and protein.[104] The discrepancies between mRNA and protein expression could have been caused by post-transcriptional regulation, as well as variation in mRNA/protein stability and turnover.[59;106] However, the measurement of both mRNA and protein, as well as the determination of gene polymorphisms, allows a more meaningful understanding of complex phenotypes to be established, and is set to become a standard method for studying complex human diseases.[200]

Relationship Between Physiological Changes and Molecular Changes

Although the results of this study reveal increases in walking distance, ACE gene expression, and an increase in VEGF signalling potential, no correlation was found between any of the physiological and molecular changes. There are several reasons to explain this. Firstly, the number of subjects was small, and thus statistical tests would have been unlikely to detect any correlation. In addition, the mechanism for improvement with exercise in intermittent claudication is multi-factorial (as described in Section 1.2.3), and therefore the effects of a single factor would be difficult to detect

amongst the others.[244;255] The patient group was also quite heterogeneous in terms of co-morbidities, disease distribution, and baseline gene activity, making it more difficult to link the physiological improvements with specific gene targets.

Nevertheless, despite the absence of correlation described above, the findings of this study were that exercise training improved measurements of walking ability, and these improvements were noted in association with increases in VEGF mRNA expression, VEGF receptor expression, VEGF protein levels and ACE expression. Whether these molecular changes underlie some of the improvements, and the extent to which they do, is uncertain. However, an increase in VEGF signalling and hence angiogenesis is likely to be of benefit to subjects with intermittent claudication.

Study Power and Sample Size

In analysing the data, several results for changes observed in this study were found to be statistically insignificant. It is likely that this was at least in part related to the small sample sizes attained by this study, which resulted in statistical analyses that were often underpowered. In this trial, power for insignificant results typically varied from between 30-40%. Post-hoc power studies reveal that if sample sizes were increased to 18 in each group (control and exercise), then this trial would have had at least an 80% power to detect differences of the sizes measured for: CD (exercise vs. control groups), heel raises (exercise and control groups- pre vs. post), ACE mRNA expression (exercise vs. control groups), VEGF protein levels (exercise group- pre vs. post), VEGFR-1 (exercise group- pre vs. post; exercise vs. control groups) and sVEGFR-1 (exercise vs. control groups). Thus, increased sample sizes would have probably produced more statistically significant results.

Future studies

Larger scale studies in claudicants are required to confirm the findings of this study, although problems with recruitment will still remain an issue. Furthermore, additional studies could, if admissible, use leg muscle biopsies to assess local changes in mRNA and protein expression. Muscle biopsies would also give the additional benefit of allowing assessment of changes in capillary density with exercise training. An increase in capillary density after training in relation to increased VEGF would provide stronger evidence for the role of VEGF in walking improvements. Future studies could also examine the effects of exercise training on the products downstream of ACE (Ang-II and BK) and on Ang-II receptors.

4.6 SUMMARY

The results of this study relate to a small number of subjects, and so few firm conclusions can be drawn. However, the exercise training programme produced significant increases in treadmill walking ability and calf muscle endurance. Exercise training was associated with an increase in ACE, $VEGF_{121}$, $VEGF_{165}$, sVEGFR-1 and VEGFR-2 mRNA expression. In ACE I/D heterozygotes, more mRNA appeared to originate from the D-allele than from the I-allele. These results suggest that increased VEGF signalling may play a role in exercise associated improvements in walking ability and VEGF may thus be a viable target for modulation in the treatment of claudicants.

CHAPTER 5

EFFECTS OF ACE INHIBITION ON ACE AND VEGF RESPONSES TO HYPOXIA

CHAPTER 5

5.1 BACKGROUND

Several studies have revealed the potential role of ACE in exercise performance. Low ACE activity allied to the I-allele of the ACE I/D polymorphism is associated with improved endurance performance.[170;288] Furthermore, pharmacological reduction of ACE activity using ACE inhibitors has also been linked with increased muscular efficiency and endurance, reduced inflammation, ischaemic preconditioning and enhanced endothelial function.[74;166;256;288;300] Thus, the use of ACE inhibitors in claudicants could be of therapeutic benefit by improving endurance performance and hence walking ability. Another potential gain from ACE inhibition is an increase in angiogenesis. Several animal models of ischaemia have confirmed that ACE inhibitors augment angiogenesis by up-regulating VEGF expression.[77;233] Thus, ACE inhibitors may have dual benefits in ischaemia, by increasing VEGF activity while also maintaining low ACE activity. However, the results of clinical trials of ACE inhibitors in claudicants have shown conflicting results, and the possible benefit from using ACE inhibitors to treat intermittent claudication remains unclear.[3;155] One way to better understand the potential role of ACE inhibition in claudicants would be to assess how ACE inhibition in a human model of hypoxia affects ACE and VEGF biology, as determined by mRNA expression and protein levels.

5.2 AIMS

The first aim of this study was to develop a human endothelial cell culture model of hypoxia in which the cells were stable and viable, and in which ACE and VEGF were reliably expressed. This subsequently allowed the effects of ACE inhibition on ACE and VEGF in a human model of hypoxia to be assessed.

5.3 METHODS

Genomic DNA was harvested from ECV 304 cells and ACE genotype determined as described in Section 3.4. ECV 304 cells possessed the DD genotype (linked to intrinsically high ACE activity). This high ACE activity was considered an advantage when studying the effects of ACE inhibition. However, the differential expression of I- and D-alleles was not able to be assessed. ACE, $VEGF_{121}$ and $VEGF_{165}$ mRNA expression was determined by RT-PCR as described in Section 3.3. ACE and VEGF protein levels were measured by ELISA as stated in Section 3.6.

5.3.1 Work up Experiments

A series of work-up experiments were initially performed in order to determine the optimal cell culture conditions and drug doses for use in the final experiments. Control experiments were performed using medium supplemented with 1% or 10% FCS. This allowed the viability and stability of cells in low nutrient conditions (1% FCS), and also the potential stimulatory and proliferative effects of a growth factor rich medium (10% FCS) to be assessed.

Cell Viability Control Experiment

The first control experiment examined ECV 304 cell viability over time for different nutrient concentrations and cell densities. The aim was to find a cell number and medium concentration where cell viability was stable over time, with the cells quiescent but viable and not proliferating.

ECV cells at passage number 178 were prepared as described in Section 3.2.2. On 96-well plates 1500, 3000, 15,000 and 30,000 cells were seeded in triplicate for both FCS

concentrations. Four identical plates were set up, with viability assessed immediately in one plate (as described in Section 3.2.4) and subsequently at 24, 48 and 72 hours. The medium was refreshed every day for unprocessed plates. When assessing cell viability, the plates were read 4 hours after incubation with the assay solution. From the results of this experiment (Appendix 5) a density of 15,000 cells per well was chosen for future experiments. However, to help with time restrictions all experiments were subsequently processed at 18 hours and viability assessed 3 hours after adding the assay solution.

Effects of DETA-NO or Ramiprilat alone

These experiments examined the individual effects of DETA-NO and ramiprilat on ECV 304 cell viability and gene expression. Again, the effect of low nutrient (1% FCS) and growth factor rich conditions (10% FCS) was examined. The aim was to examine the dose-response curves for both drugs and thus choose the drug doses and medium concentration for the final experiment.

Cells at passage 183 were prepared as described in Section 3.2.2 in both 1% and 10% FCS. Fifteen thousand cells were seeded per well for the cell viability experiments and $5x10^5$ cells seeded per well on 6-well plates for examination of gene expression. The cell numbers were chosen so that the cell density was the same in both the 6- and 96-well plates. Doubling doses of DETA-NO from 0.008 to 0.5 mM and ten-fold increases of ramiprilat from 1 to 1000 nM/l were used in these experiments. 'Medium control' experiments (containing cells and medium), and 'vehicle control' experiments (containing cells, medium and drug vehicle) were also set up. All cell viability experiments were run in quadruplicate, while gene expression experiments were run in single wells, with one RT-PCR run used to assess gene expression. The plates were

processed after 18 hours for cell viability and ACE/VEGF gene expression as described in Sections 3.2.4, 3.2.5 and 3.3. The results are shown in Appendix 5.

5.3.2 Final Experiment

The conditions for the final experiment were decided from the results of the work up experiments (Appendix 5). In all the work-up experiments, no major differences in viability or gene expression were noted between the 1% and 10% FCS experiments. However, as medium with 1% FCS contains less growth factors and has less stimulatory properties that could interfere with the results, 1% FCS was chosen for the final experiments. Doubling doses of DETA-NO from 0.008 to 0.250 mM were used to create a 'metabolic hypoxia', and the effects of 2 doses of ramiprilat (100 and 1000 nM/l) on these responses to hypoxia studied.

The final experiment was run twice on 2 separate occasions using identical conditions. For the first run, ECV cells at passage 188 were used, and for the second ECV cells at passage 190. For each run, the effects of DETA-NO alone, DETA-NO with 100 nM/l ramiprilat, and DETA-NO with 1000 nM/l ramiprilat were examined.

ECV 304 cells were harvested and the experiments were set up as described in Sections 3.2.2 and 3.2.3. Specifically, the wells used as the overall experimental controls contained ECV cells, medium and both drug vehicles. In the DETA-NO only experiment, all wells also contained 0.05% DMSO, the drug vehicle used for ramiprilat. In the DETA-NO with ramiprilat experiments, all wells contained either 100 or 1000 nM/l ramiprilat. The 'medium control' wells in these experiments contained ECV cells in medium and ramiprilat, while the 'vehicle control' wells contained ECV cells in

medium, ramiprilat and the drug vehicle for DETA-NO (water). These control wells allowed the effects of ramiprilat alone to be studied. The experiments were all run for 18 hours. A summary of experimental well contents is described in Appendix 6.

Cell viability was assessed as described in Section 3.2.4. Cells from 6 well plates were harvested for RNA and protein as described in Section 3.2.5. ACE and VEGF gene expression was determined as described in Section 3.3, and VEGF protein levels measured as described in Section 3.6. The viability studies for both experimental runs were run in quadruplicate. Due to a lack of time and resources only a single RT-PCR run was used to assess gene expression from each experiment, and thus no statistical tests were able to be performed on the gene expression results. RT-PCR results for ACE expression were obtained using the ACE 216 primer set. For ELISA measurements, each experimental run was measured once in triplicate. The raw data obtained is illustrated in Appendix 7.

5.4 RESULTS

All experimental wells were examined microscopically to assess confluence and infection at the end of the 18 hour experiment period. In all experiments, the wells were found to be free of infection and the cells confluent. Wells containing ECV cells in medium and both drug vehicles were used as the experimental controls throughout.

5.4.1 Effects of DETA-NO Alone

Cell Viability

In the cell viability experiments, there was no difference in viability between the medium and the vehicle controls (Medium 1.99 ± 0.07, Vehicle 2.04 ± 0.06; unpaired t-

test: $p>0.50$). On examining the effects of DETA-NO, doses ranging from 0.008 to 0.25 mM did not have significant effects on ECV 304 cell viability (Figure 5.1).

ACE and VEGF Gene Expression

As only 2 experimental runs were performed statistical testing was not possible on the gene expression data, and thus broad observations only could be made. DETA-NO generally produced an increase in ACE expression, with a 42% increase in expression observed at 0.06 mM (Figure 5.2). DETA-NO did not appear to elicit any clear effect on $VEGF_{121}$ and $VEGF_{165}$ expression (Figure 5.3).

VEGF Protein Levels

Unfortunately, due to low protein yields ACE levels were not consistently detectable using our ELISA kit. However, VEGF levels increased significantly at the highest DETA-NO dose, with a 53 ± 11% increase in VEGF protein noted at 0.25 mM DETA-NO (Figure 5.4). There was no difference in VEGF levels between the medium and vehicle controls (Medium 132.2 ± 10.6, Vehicle 118.2 ± 12.9; unpaired t-test: $p = 0.42$).

5.4.2 Effects of Ramiprilat Alone

To assess the effects of ramiprilat alone the gene expression results from the two final experiment runs were combined with the results from the ramiprilat control experiment, which used identical conditions.

Cell Viability

There was no difference in viability between the medium and the vehicle controls in either the 100 nM/l ramiprilat (Medium 2.0 ± 0.1, Vehicle 2.1 ± 0.1; unpaired t-test: p =

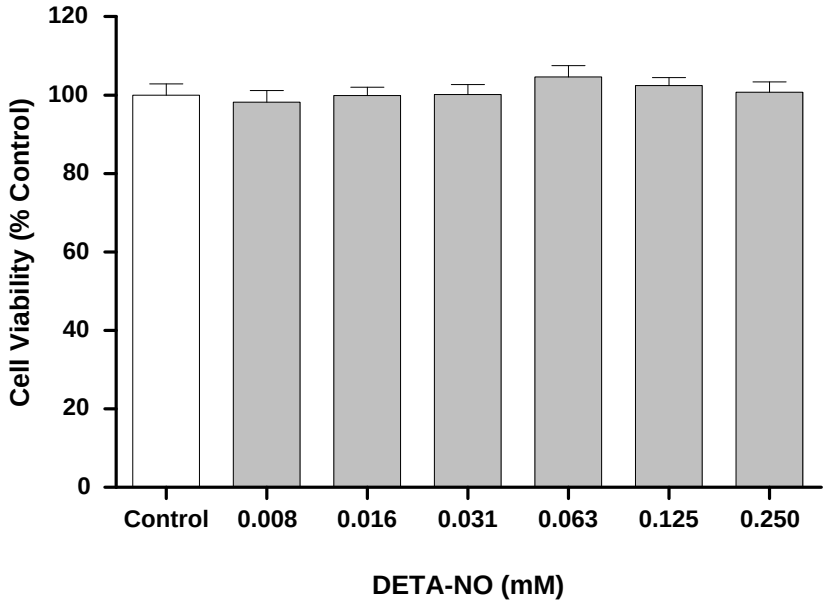

Figure 5.1 Effects of DETA-NO on Cell Viability

Increasing doses of DETA-NO from 0.008 to 0.250 mM did not have a significant effect on ECV 304 cell viability (1-way ANOVA, $p>0.50$). The bars represent the mean values, and error bars SEM, of 2 repeat experiments each run in quadruplicate wells.

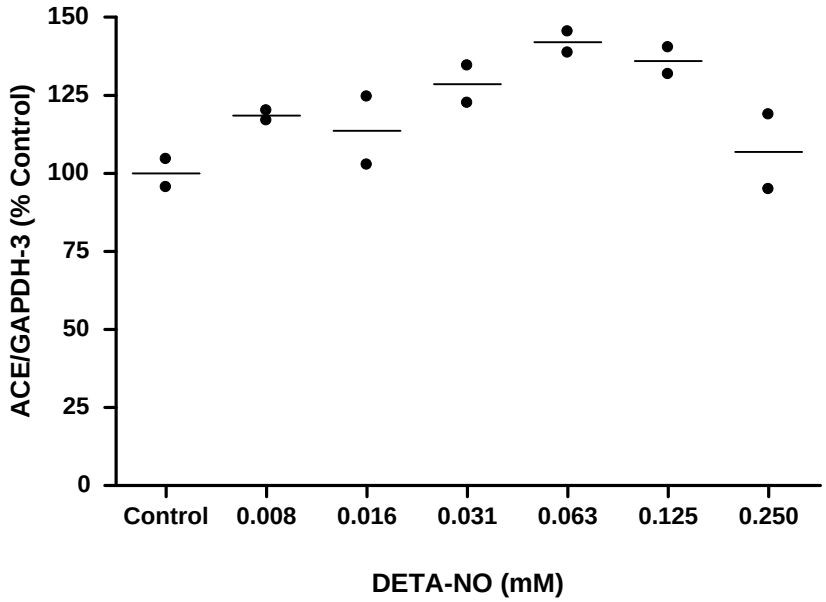

Figure 5.2 Effects of DETA-NO on ACE Expression

With increasing DETA-NO, ACE gene expression also generally appeared to increase, with a maximal increase of 42% noted at 0.063 mM DETA-NO. The points represent individual values and the horizontal bars the mean from 2 repeat experiments.

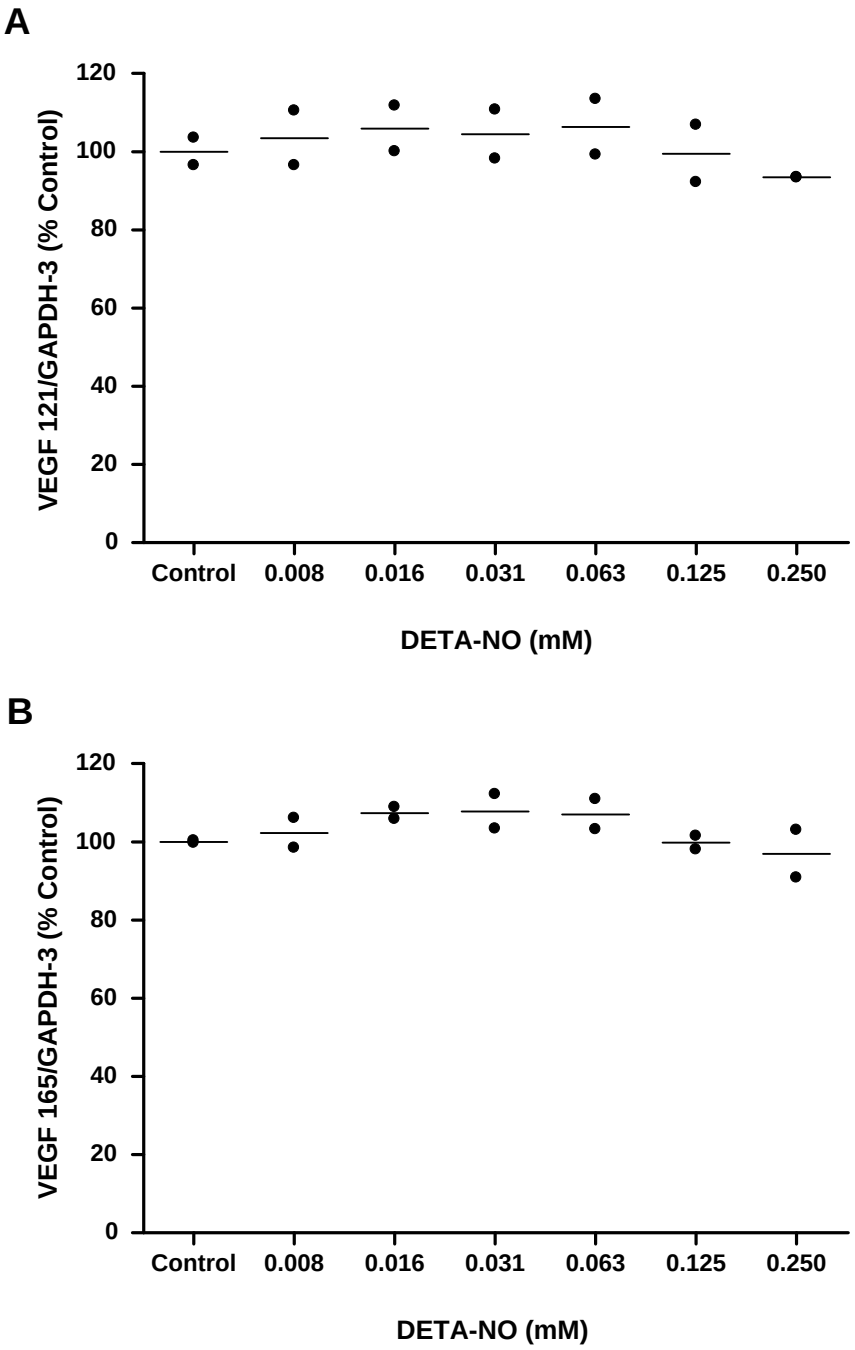

Figure 5.3 Effects of DETA-NO on VEGF Gene Expression

The effects of DETA-NO on **A** $VEGF_{121}$ and **B** $VEGF_{165}$ expression are shown. DETA-NO did not appear to have any noticeable effects on $VEGF_{121}$ and $VEGF_{165}$ expression. The points represent individual values and the horizontal bars the mean from 2 repeat experiments.

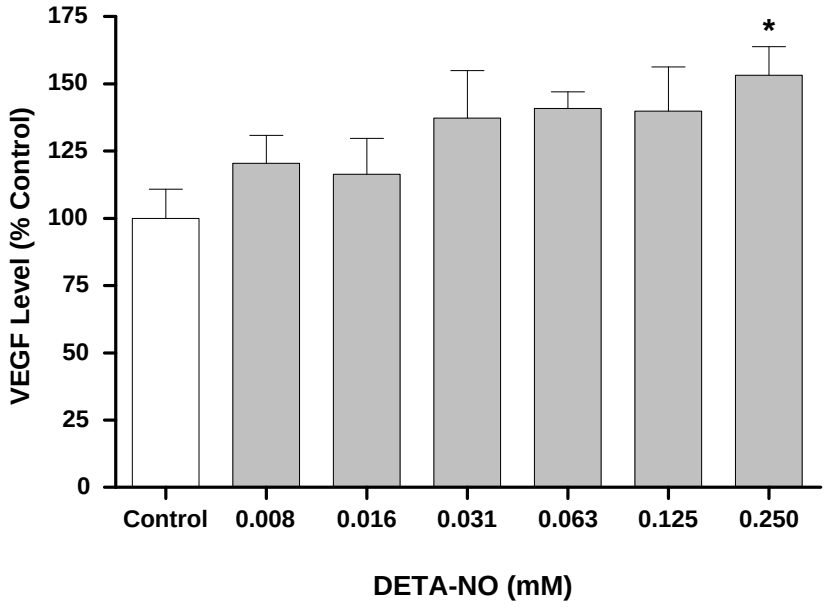

Figure 5.4 Effects of DETA-NO on VEGF Protein Levels

There was a 53 ± 11% increase in VEGF protein level recorded at 0.250 mM DETA-NO (1-way ANOVA with Dunnett's multiple comparison test; * $p < 0.05$ vs Control). The bars represent the mean values, and error bars SEM, of 2 repeat experiments each run in triplicate wells.

0.37) or 1000 nM/l ramiprilat (Medium 1.9 ± 0.0, Vehicle 2.0 ± 0.1; p = 0.43) experiments. In addition, neither dose of ramiprilat had any significant effect on cell viability (Figure 5.5).

ACE and VEGF Gene Expression

Ramiprilat augmented ACE gene expression, with a 40 ± 10% increase recorded with 1000 nM/l ramiprilat (Figure 5.6). In contrast, ramiprilat lowered VEGF expression. $VEGF_{165}$ expression was reduced 16 ± 0.4% and 22 ± 2% by 100 nM/l and 1000 nM/l ramiprilat respectively (Figure 5.7). Ramiprilat did not significantly decrease the expression of $VEGF_{121}$ (Figure 5.7).

VEGF Protein Levels

There was no difference in VEGF levels between the medium and the vehicle controls in either the 100 nM/l ramiprilat (Medium 180 ± 16, Vehicle 186 ± 3; unpaired t-test: p>0.50) or 1000 nM/l ramiprilat (Medium 199 ± 5, Vehicle 211 ± 23; p>0.50) experiments. While ramiprilat decreased $VEGF_{165}$ gene expression at 18 hours, the amount of VEGF protein increased. Increasing doses of ramiprilat progressively augmented VEGF levels, with a 58 ± 3% increase with 100 nM/l ramiprilat and a 79 ± 19% increase with 1000 nM/l ramiprilat (Figure 5.8).

5.4.3 Effects of Ramiprilat on Responses to Metabolic Hypoxia

Cell Viability

Ramiprilat combined with increasing doses of DETA-NO did not have any significant effect on cell viability compared to the experimental control (1-way ANOVA: 100 nM/l ramiprilat/DETA p = 0.82, 1000 nM/l ramiprilat/DETA p = 0.98). In addition, at each

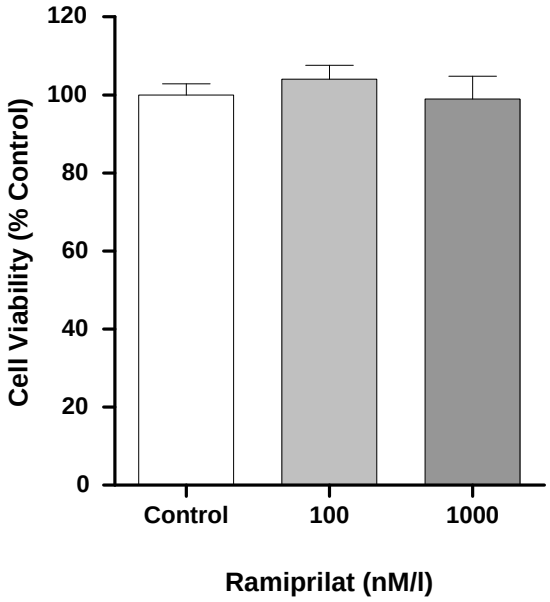

Figure 5.5 Effects of Ramiprilat on Cell Viability

Ramiprilat at 100 and 1000 nM/l did not have an effect on cell viability (1-way ANOVA, $p = 0.69$). The bars represent the mean values, and error bars SEM, of 2 repeat experiments each run in quadruplicate wells.

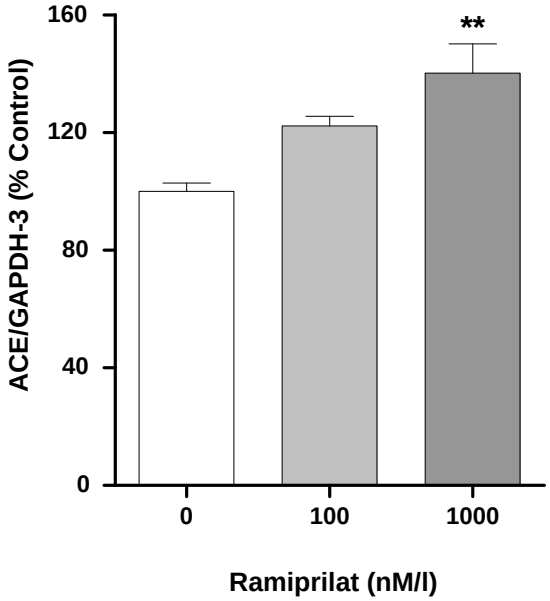

Figure 5.6 Effects of Ramiprilat on ACE Gene Expression

Ramiprilat stimulated ACE gene expression at the highest dose, with a 40 ± 10% increase in expression noted with 1000 nM/l ramiprilat (1-way ANOVA with Dunnett's multiple comparison test; ** $p < 0.01$ vs Control). The bars represent the mean values, and error bars SEM, of 3 repeat experiments.

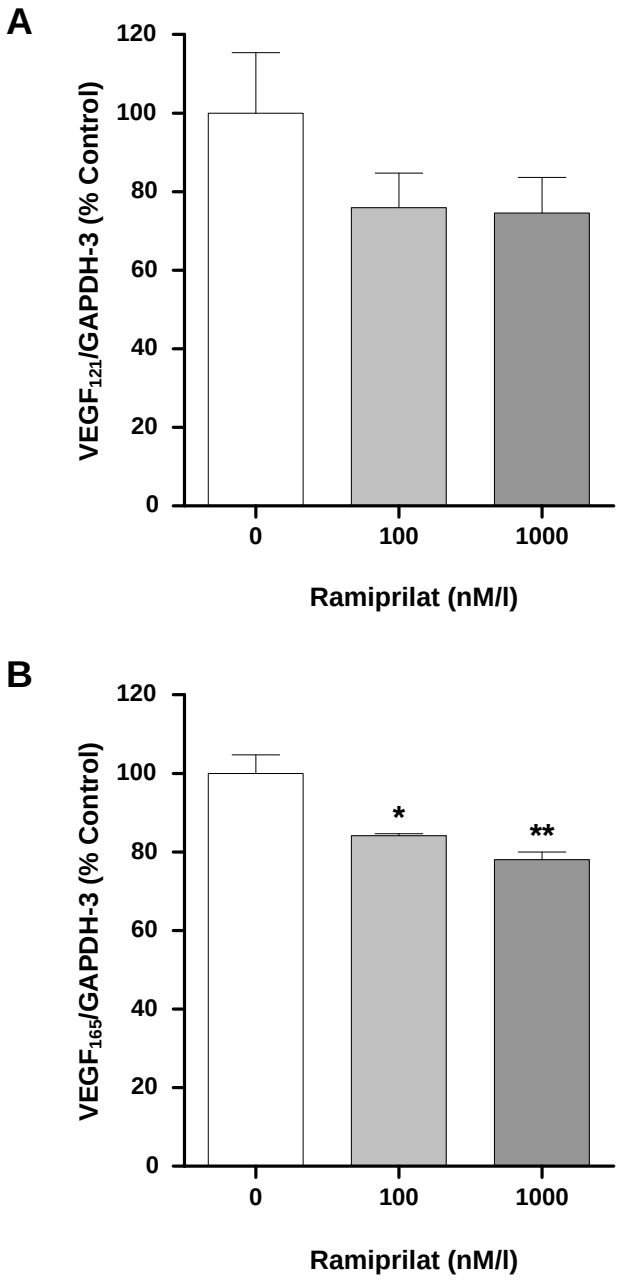

Figure 5.7 Effects of Ramiprilat on VEGF Gene Expression

The effects of ramiprilat on **A** $VEGF_{121}$ and **B** $VEGF_{165}$ expression are shown. Ramiprilat did not reduce $VEGF_{121}$ expression. However, a significant decrease in $VEGF_{165}$ expression was recorded with 1000 nM/l ramiprilat causing a 22 ± 2% reduction (1-way ANOVA with Dunnett's multiple comparison test; * $p < 0.05$ vs Control, ** $p < 0.01$ vs Control). The bars represent the mean values, and error bars SEM, of 3 repeat experiments.

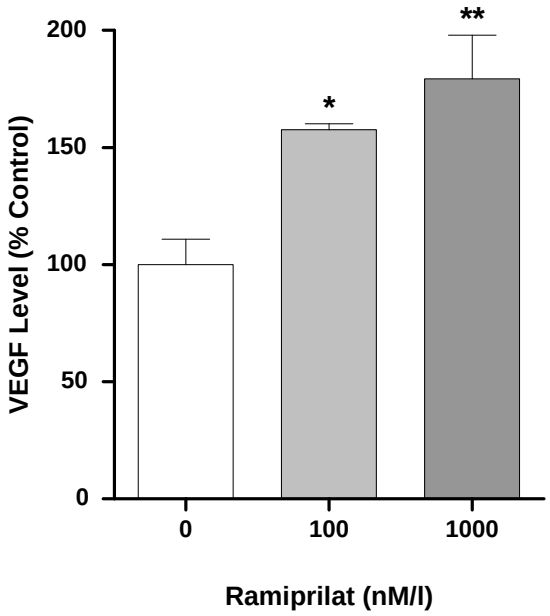

Figure 5.8 Effects of Ramiprilat on VEGF Protein Levels

Increasing doses of ramiprilat significantly augmented VEGF levels, with a 58 ± 3% increase noted with 100 nM/l ramiprilat and a 79 ± 19% increase with 1000 nM/l ramiprilat (1-way ANOVA with Dunnett's multiple comparison test; * $p < 0.05$ vs Control, ** $p < 0.01$ vs Control). The bars represent the mean values, and error bars SEM, of 2 repeat experiments each run in triplicate wells.

DETA-NO dose there was no significant change in cell viability with either 100 or 1000 nM/l ramiprilat compared to DETA-NO alone (Figure 5.9).

ACE and VEGF Gene Expression

To compare the medium (M) and vehicle (V) controls for ACE and $VEGF_{121}$/$VEGF_{165}$ expression, gene expression results for the DETA only, DETA/100 nM/l ramiprilat and DETA/1000 nM/l ramiprilat control groups were combined. The results showed no difference between the control experiments for ACE expression (M 0.28 ± 0.02, V 2.53 ± 0.02; unpaired t-test $p = 0.32$), $VEGF_{121}$ expression (M 0.56 ± 0.02, V 0.55 ± 0.03; $p > 0.50$) or $VEGF_{165}$ expression (M 0.44 ± 0.02, V 0.44 ± 0.02; $p > 0.50$).

As stated previously, only 2 experimental runs were performed, not allowing statistical tests to be performed. Thus, only broad observations can be made. On examining ACE gene expression, there was an apparent increase in ACE expression with the ramiprilat groups compared to DETA-NO alone. At every DETA-NO dose, ACE gene expression was greater (by 12-71%) with both 100 and 1000 nM/l ramiprilat than for DETA-NO alone (Figure 5.10). On studying $VEGF_{121}$ gene expression, there was an apparent decrease in $VEGF_{121}$ expression with both 100 nM/l and 1000 nM/l ramiprilat compared to DETA-NO alone. At every DETA-NO dose, $VEGF_{121}$ gene expression was reduced by both ramiprilat doses (by 8-27%) than with DETA-NO alone (Figure 5.11). For $VEGF_{165}$ gene expression, the tendency again appeared to be for ramiprilat to decrease $VEGF_{165}$ expression in hypoxia (Figure 5.12). $VEGF_{165}$ gene expression was lower with both ramiprilat doses (by 6-21%) at all DETA-NO doses compared to DETA-NO alone. There was no obvious difference between the 2 ramiprilat groups for ACE, $VEGF_{121}$ or $VEGF_{165}$.

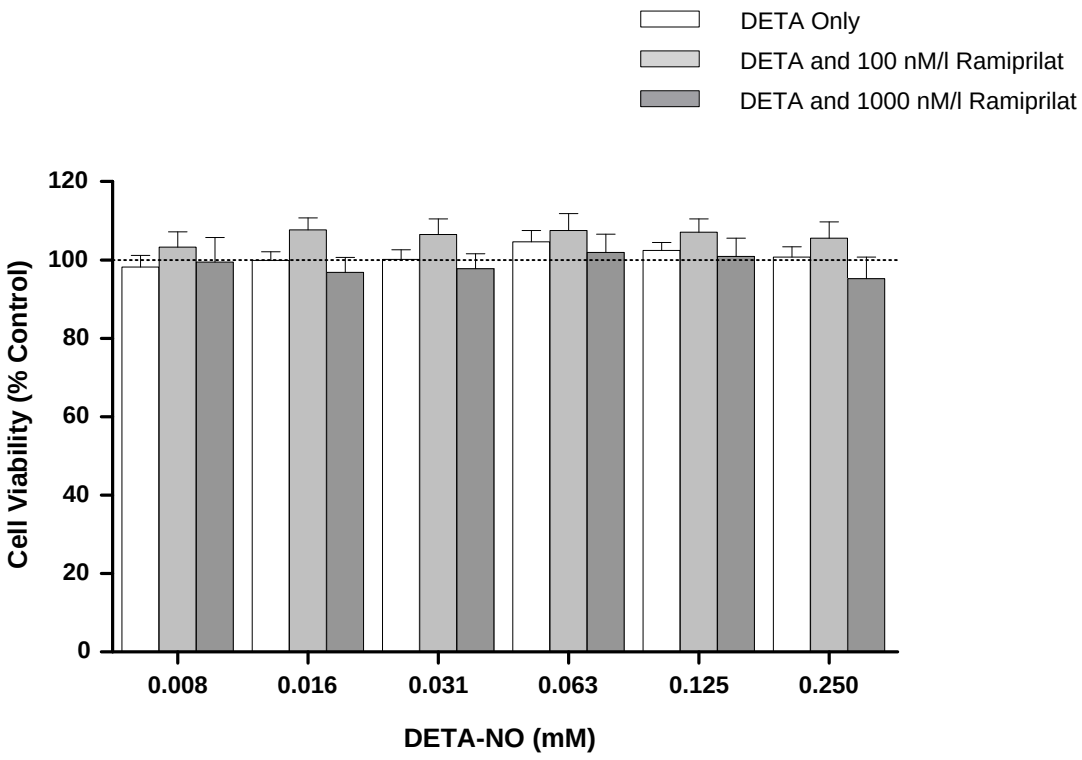

Figure 5.9 Effects of Ramiprilat and Metabolic Hypoxia on Cell Viability

At each dose of DETA-NO there was no significant change in cell viability with ramiprilat (1-way ANOVA). Each bar represents the mean, and the error bars SEM, of 2 repeat experiments each run in quadruplicate wells.

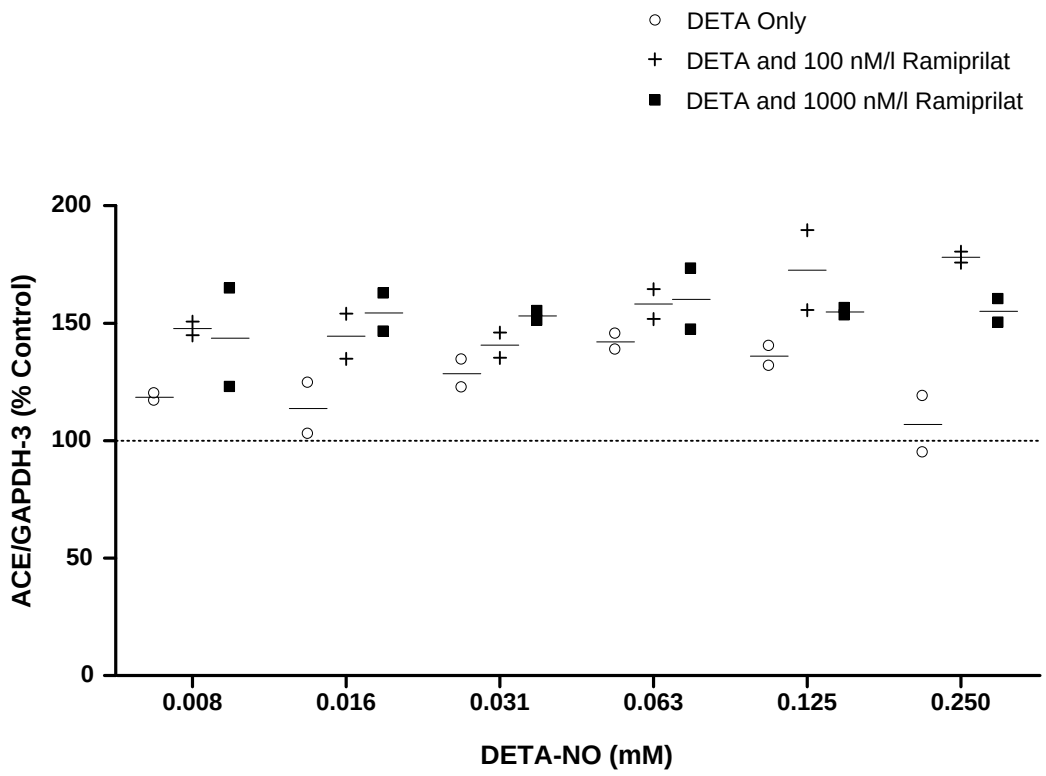

Figure 5.10 Effects of Ramiprilat on ACE Expression in Response to Hypoxia

100 nM/l and 1000 nM/l ramiprilat appear to increase ACE gene expression compared to DETA-NO alone. At every DETA-NO dose, both 100 and 1000 nM/l ramiprilat produced an increase in ACE expression. Each point represents the value obtained from 2 repeat experiments, and the horizontal bars the mean.

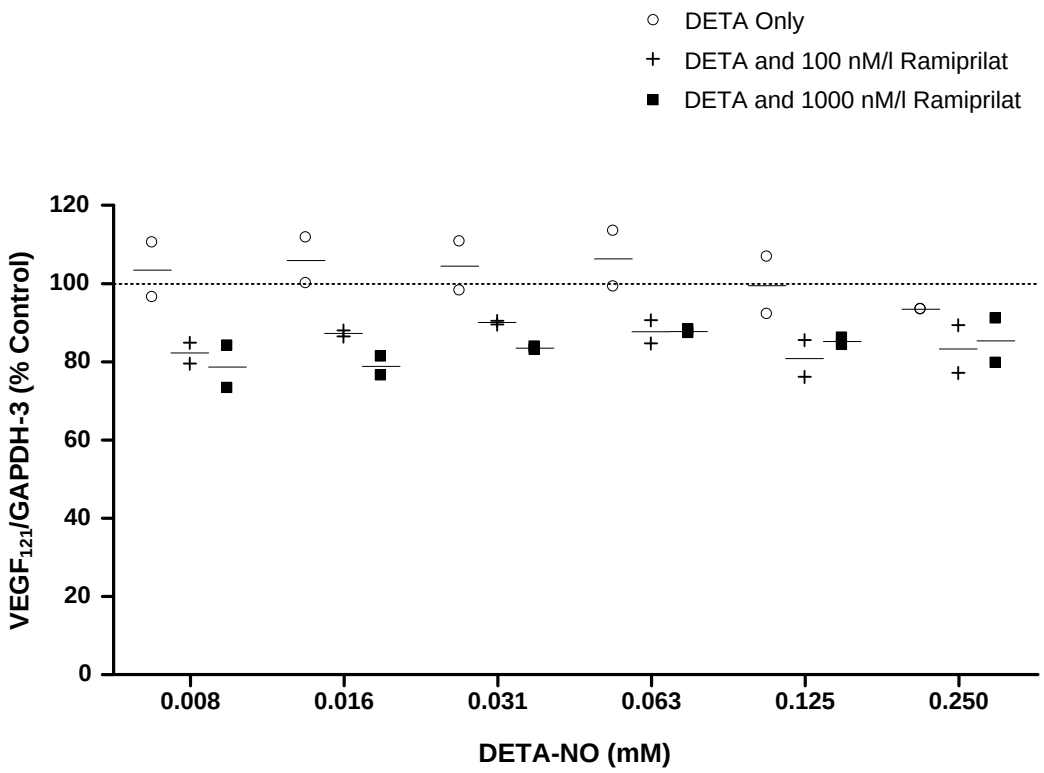

Figure 5.11 Effects of Ramiprilat on $VEGF_{121}$ Expression in Response to Hypoxia

An apparent decrease in $VEGF_{121}$ expression with both 100 nM/l and 1000 nM/l ramiprilat compared to DETA-NO alone is noted. At every dose of DETA-NO, 100 and 1000 nM/l ramiprilat decreased $VEGF_{121}$ mRNA expression compared to DETA-NO alone. Each point represents the mean, and the error bars SEM, of 2 repeat experiments.

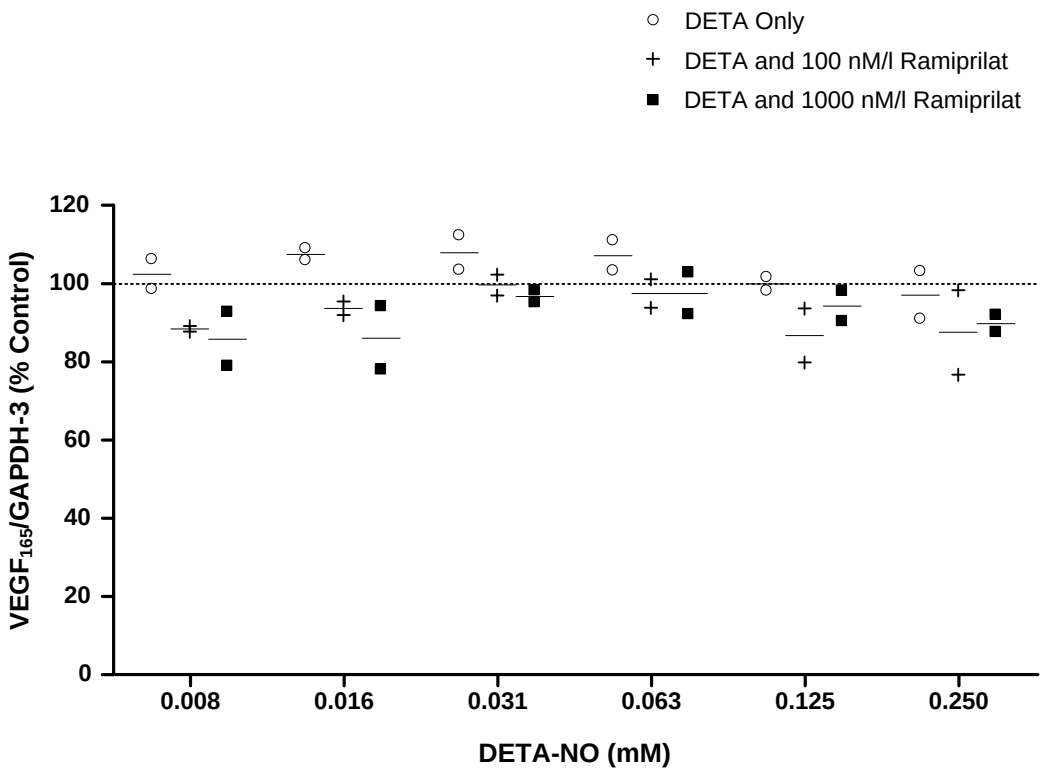

Figure 5.12 Effects of Ramiprilat on $VEGF_{165}$ Expression in Response to Hypoxia

$VEGF_{165}$ expression appears to be suppressed with 100 nM/l and 1000 nM/l ramiprilat compared to DETA-NO alone. Both doses of ramiprilat consistently decreased $VEGF_{165}$ expression compared to DETA-NO alone, although these differences are small. Each point represents the mean, and the error bars SEM, of 2 repeat experiments.

VEGF Protein Levels

Ramiprilat augmented increases in VEGF levels in response to hypoxia (Figure 5.13). 100 nM/l ramiprilat significantly increased VEGF levels over the experimental control at all DETA-NO doses (1-way ANOVA with Dunnett's test: 0.008 to 0.125 mM $p < 0.05$, 0.250 mM $p < 0.01$). 1000 nM/l ramiprilat also significantly increased VEGF levels compared to the experimental control at all DETA-NO doses (1-way ANOVA with Dunnett's test: 0.008 to 0.250 mM $p < 0.01$). There was no significant change in VEGF levels with either dose of ramiprilat compared to DETA alone, apart from at 0.016 mM DETA-NO, where 100 nM/l and 1000 nM/l ramiprilat increased VEGF levels by 54 ± 26% ($p < 0.05$) and 60 ± 7% ($p < 0.01$) respectively (1-way ANOVA with Bonferroni's multiple comparison test). However, at each DETA-NO dose VEGF levels were higher (but not significantly) in the 2 ramiprilat groups than for DETA-NO alone. There was no significant difference between the 2 ramiprilat groups (Figure 5.13).

5.5 DISCUSSION

Studies of ACE inhibition in patients with heart failure and in animal models of ischaemia suggest that ACE inhibitors could produce several benefits in patients with intermittent claudication. Heart failure, like peripheral arterial disease, is characterised by poor tissue perfusion.[53] The use of ACE inhibitors in patients with heart failure has been linked with benefits including increased oxygen extraction and exercise performance.[73;74] Interestingly, these effects are appear to be separate from the effects of ACE inhibitors on cardiac output and blood pressure, and have been suggested to result from increased metabolic efficiency.[167] Low ACE activity related to the I-allele of the ACE I/D polymorphism has also been related to enhanced metabolic efficiency and

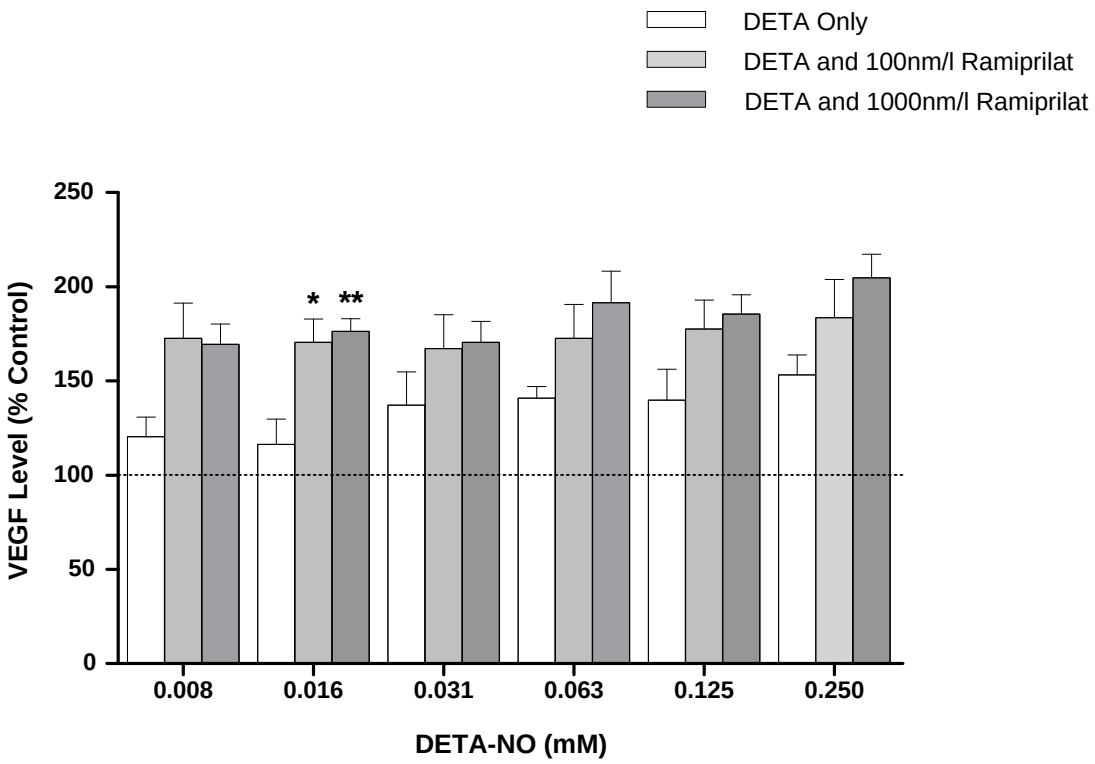

Figure 5.13 Effects of Ramiprilat on VEGF Protein Levels in Response to Hypoxia

There was no significant change in VEGF levels with either dose of ramiprilat compared to DETA alone, apart from at 0.016 mM DETA-NO where both ramiprilat doses increased levels significantly (1-way ANOVA with Bonferroni's multiple comparison test: * $p < 0.05$ ** $p < 0.01$). Each bar represents the mean, and the error bars SEM, of 2 separate repeat experiments with each ELISA run twice in triplicate wells.

endurance performance.[170;288] In addition, animal models of ischaemia have revealed that ACE inhibitors increase VEGF expression and subsequently increase angiogenesis, an effect related to the accumulation of BK.[85;233] Moreover, ACE inhibitors also possess cell-signalling properties, directly increasing ACE, COX-2 and potentially VEGF expression, while maintaining low ACE activity.[10;23;93] Thus, ACE inhibitors have potential dual benefits for improving walking ability in claudicants, by increasing metabolic efficiency and by potentiating angiogenesis. Small scale clinical trials have to date produced conflicting results regarding the benefits of ACE inhibitors in claudication, although the largest study to date showed a marked improvement.[3] This study was designed to assess the effects of ACE inhibition on VEGF and ACE in a human model of hypoxia. A cell model of hypoxia was developed, and the effects of the ACE inhibitor ramiprilat on ACE and VEGF expression examined. The hypothesis was that ramiprilat would increase both ACE and VEGF expression in hypoxia.

Effects of Hypoxia on ACE and VEGF

DETA-NO, a nitric oxide donor, was used in ECV 304 cells to produce a 'metabolic hypoxia', as described previously by Moncada *et al.*[164] No significant effect of DETA-NO on cell viability was noted. Increasing doses of DETA-NO mediated an apparent increase in ACE mRNA levels, although more experimental runs would be required to confirm this statistically. However, previous studies that have shown hypoxia to up-regulate ACE.[136;171;306] The mechanism for this increase has been demonstrated to occur via HIF-1, with Ang-II and HIF-1 acting in a positive feedback loop.[141] DETA-NO did not appear to have any noticeable effect on $VEGF_{121}$ and $VEGF_{165}$ mRNA expression. However, in contrast DETA-NO produced an increase in VEGF protein levels of 53% at 0.250 mM. Of note, the changes ACE and VEGF recorded in this cell culture model

of hypoxia are comparable to those found after exercise training in claudicants, as described in Chapter 4. In these subjects, repetitive ischaemia caused by regular exercise training resulted in increased ACE gene expression, and raised VEGF levels, suggesting a similar response to hypoxia in both models. The increases in VEGF expression with hypoxia in this study conform with previous reports of raised VEGF levels with hypoxia.[29;174] A HIF-1 mediated mechanism is likely to underlie these increases in VEGF transcription.[95;226] Hypoxia also stabilises VEGF mRNA, which is normally intrinsically unstable due to destabilising elements in its 3' and 5' UTR/coding regions, and thereby increases the half-life of VEGF mRNA.[70;242]

In this study, hypoxia did not appear to affect $VEGF_{121}$ or $VEGF_{165}$ mRNA expression but did increase VEGF protein levels. This disparity has been noted previously, with VEGF mRNA and protein not always adapting in parallel. Indeed, low VEGF mRNA levels with conversely high VEGF protein levels has been linked with angiogenesis.[161] Increased VEGF protein in a time of low mRNA expression could partly be explained by post-transcriptional events such as translation via an alternative ribosome entry site.[241] Finally, it is possible that the high VEGF protein levels could downregulate transcription in a negative feedback loop, although thus far there is no evidence to support this. In summary, the evidence in this study supports the notion that hypoxia stimulates VEGF production, creating a favourable environment for angiogenesis.

Effects of Ramiprilat on ACE and VEGF

The doses of ramiprilat used did not have a significant effect on cell viability. However, ACE inhibition in normoxic conditions did influence ACE and VEGF expression. Ramiprilat produced an increase in ACE mRNA expression, with 1000 nM/l ramiprilat

inducing a 40% rise in ACE mRNA levels. This stimulation of ACE transcription as a result of ACE inhibition is in keeping with previous studies.[58;138] Increases in ACE expression during ACE inhibition have been attributed to a lack of negative feedback by Ang-II.[223] In addition, the cell signalling properties of ACE mediated by ACE inhibitors, as described by Kohlstedt *et al.* could also contribute to the increases in gene expression.[138;139] However, even with these increases in ACE levels, overall ACE activity would still be likely to be suppressed due to the continued presence of the ACE inhibitor.[23]

Ramiprilat had an inhibitory effect on VEGF mRNA levels, reducing levels by up to 22% in the case of the more biologically active $VEGF_{165}$. However, in contrast to these decreases in VEGF gene transcription, significant increases in VEGF protein levels of up to 79% were noted. This paradox is similar to that seen with DETA-NO, and possible explanations for this have been discussed above. The increases in VEGF protein levels with ACE inhibition are also similar to those previously described in animal studies on the effects of ACE inhibitors in angiogenesis.[77;233] Given the measured increases in VEGF protein, these results suggest that ACE inhibition amplifies VEGF production in an endothelial cell model, thereby increasing the potential for angiogenesis.

Effects of Ramiprilat on Responses to Hypoxia

Lastly, the effect of ramiprilat on ACE and VEGF expression in the cell culture model of hypoxia was studied. No effect on cell viability was recorded. Given that cell viability remained stable and confluent cultures were used in all experiments, changes were likely to reflect changes in gene expression rather than cell proliferation or death.

Ramiprilat appeared to potentiated ACE gene expression compared with hypoxia alone, although more experimental runs are needed to confirm this statistically. However, ramiprilat significantly increased VEGF protein levels in hypoxic conditions. The increases in VEGF promoted by ACE inhibition in hypoxia indicate a potential role for the use of ACE inhibitors to augment angiogenesis in claudicants. This effect is similar to that previously described in animal models of ischaemia.[77;85;233;234] The mechanism for this increase has been suggested to involve the induction of VEGF expression secondary to the accumulation of BK during ACE inhibition.[85;233] Another possible mechanism could relate to the 'outside-in' signalling pathway triggered by the binding of an ACE inhibitor to membrane-bound ACE.[93;138] This pathway has been demonstrated to up-regulate COX-2 expression, which itself can increase VEGF expression.[10;139] This same pathway could also partially account for the potential increases in ACE gene expression observed in this study. Although an increase in ACE expression would not appear to be beneficial in hypoxia, the continued presence of an ACE inhibitor would negate these increases, maintaining low overall ACE activity.[23] Thus, in this model of hypoxia, ACE inhibition produced 2 effects that could be of benefit if repeated in claudicants: an increase VEGF expression (thereby enhancing the potential for angiogenesis), and secondly an inhibition of ACE activity (thereby providing metabolic advantages and improved endothelial function).

Limitations of the Experimental Protocol

It is important to discuss several limitations associated with the experimental protocol. Unfortunately, ACE levels were not reliably detected in our experimental protocol. One possible reason for this was the processing of the same experimental wells to harvest both RNA and protein, resulting in low yields. The use of separate wells for protein and

RNA could have increased yields and allowed better protein analysis, using techniques such as Western blotting. Furthermore an assumption, based on previous studies, was also made in the discussion that ACE activity remained decreased during ACE inhibition despite increases in ACE expression.[23] However, to confirm this it would be preferable to measure ACE activity in addition to ACE expression and levels.

The cell culture model used in this study also had several limitations. It consisted of a simple experiment created in an endothelial cell model using DETA-NO to create a 'metabolic hypoxia'. This model could not expect to replicate the very complex environment and processes occurring in a claudicant, with the repeated episodes of ischaemia and reperfusion, or the complex interactions between different cell types. For example, peri-endothelial cells, VSMCs and the extracellular matrix play a large role in angiogenesis by interacting with the endothelium, which could not be replicated in our model.[57] Moreover, ECV 304 cells are not proven to express bradykinin, a vital link in both the RAS and angiogenesis. However, our model did give an insight into how endothelial cells react to a hypoxic environment, and the effects of an ACE inhibitor on these responses. Another limitation of the experimental protocol was the use of DETA-NO to produce the hypoxic conditions in our model. DETA-NO acts as a slow releasing NO-donor, and has been shown to inhibit mitochondrial function by binding cytochrome oxidase, interrupting the electron transport chain and preventing aerobic metabolism.[7] However, although the effects of NO in this study could be attributed to 'metabolic hypoxia', other effects of NO cannot be excluded as a cause. The majority of the physiological actions of NO have been attributed to its ability to bind to soluble guanylate cyclase (sGC), an enzyme that acts to produce the second messenger cyclic GMP (cGMP) from GTP. cGMP acts on cGMP-dependent protein kinases or cGMP-

gated ion channels to mediate many physiological actions.[5;165] To exclude this pathway as a possible mechanism for the changes noted in this study, further experiments using a sGC inhibitor such as oxadiazolo-quinoxalin are required to differentiate the cGMP-mediated effects from the cytochrome oxidase effects of NO. Alternatively, hypoxic chambers could be used to generate hypoxia, although these too have their own limitations.

Study Power and Sample Size

A major consideration in this study is the restricted number of experimental runs performed due to the limited time and resources available. For the final experiment, only 2 runs were conducted (each associated with a single RT-PCR or multiple-well ELISA run). As a result, only a few statistically significant results were obtained when comparing the three treatment groups. As only 2 RT-PCR experiments were performed in total, no statistical testing was allowable for mRNA expression, and only general impressions from the data could be made. The small sample sizes also resulted in statistical analyses that were often underpowered, with power typically between 15-40%. Post-hoc power studies reveal that if the number of experimental runs (sample size) was increased to 4, the power of the analyses in the study would have increased substantially. With this sample size, at least an 80% power would have been attained to detect differences of the sizes measured for the effects of 100nM ramiprilat alone on ACE expression and 1000 nM/l ramiprilat on VEGF protein levels.

Future Studies

As described above, a simple experimental protocol of hypoxia was used in this study, which provided a only a limited amount of information. Further experiments using the

same experimental model could use intermittent episodes of hypoxia (ischaemia) followed by re-oxygenation (reperfusion) to create a more representative model of claudication. In addition, other experiments could examine the time course for changes in gene expression and protein levels. It would also be important to study the effects of ACE inhibition on VEGF receptor expression, as changes in the various VEGF receptor types could potentiate or diminish the effects of the increases in VEGF protein levels. Another study could examine changes in AT_1 and AT_2 receptors, as these have opposing effects in angiogenesis.

To further elucidate the mechanism underlying the increases in VEGF expression with ACE inhibition, other studies could be performed to assess the role of each potential pathway. The role of BK could be assessed by using a BK-antagonist such as HOE-140.[120] The contribution of the ACE 'outside-in' signalling pathway could be studied using an endothelial cell line expressing a non-phosphorylatable ACE mutant (Ser1270), which would interrupt this pathway.[138;139] Finally, the involvement of COX-2 on ACE inhibitor mediated increases in VEGF could be assessed using COX-2 inhibitors.

Although in-vitro studies can provide information about how ACE inhibitors effect cellular responses to ischaemia, the only way to ascertain the potential benefits of ACE inhibition in claudicants would be to perform a large scale clinical trial. This would need to be a randomised double blind trial of an ACE inhibitor against placebo in humans enrolled on an exercise training programme, measuring changes in walking ability as the key endpoint. However, given the difficulty in recruiting suitable patients, this study would be difficult to achieve. The other possible beneficial effects of ACE

inhibition, namely decreased inflammation, improved endothelial function, and enhanced metabolic efficiency, could also be studied in these patients.

5.6 SUMMARY

This study suggests that in ECV 304 cells, hypoxia stimulates VEGF expression. ACE inhibition with ramiprilat appears to potentiate this increase. These results concur with previous reports showing that ACE inhibitors are pro-angiogenic in animal models of ischaemia. Thus, ACE inhibitors could be of benefit in the treatment of intermittent claudication, by reducing ACE activity and enhancing angiogenesis.

CHAPTER 6

DISCUSSION AND CONCLUSIONS

CHAPTER 6

Although exercise training has long been associated with improvements in walking ability in claudicants, the precise mechanism by which exercise results in these improvements is not completely appreciated. The process is likely to involve several facets, which include improvements in blood flow or distribution, and peripheral changes in the muscle physiology.[244] The RAS, and low ACE activity in particular, has been linked with improved endurance performance, enhanced metabolic efficiency, improved endothelial function, and reduced inflammation; all associations that could improve walking ability in claudicants.[166;288] Similarly, VEGF is known to play a crucial role in angiogenesis, another phenomenon that could aid claudicants.[253] Moreover, interactions are known to exist between the RAS and VEGF, with low ACE activity linked to increased VEGF levels. Studies of ACE inhibitors in animal models of ischaemia have demonstrated both increased VEGF and enhanced angiogenesis in animal models of ischaemia.[233] The aim of this thesis was to investigate the role of ACE and VEGF in exercise associated improvements in walking ability. The potential benefits of ACE inhibition with regards ACE and VEGF were also studied. The main findings of the thesis are as follows:

6.1 EFFECTS OF EXERCISE TRAINING ON WALKING ABILITY, ACE AND VEGF

The first part of this thesis examined the effect of repetitive supervised exercise training in claudicants on ACE and VEGF mRNA and protein levels, and related these to changes in walking ability. In accordance with the accepted literature, supervised exercise training produced significant increases measures of walking ability and calf muscle endurance.[178;198;244] However, contrary to predictions, exercise training did not

lead to a suppression of ACE, with increases in ACE mRNA expression recorded, although there was no increase in measured ACE protein levels. Given the concurrent improvements in walking ability noted in this study, it is unlikely that suppression of ACE is a mechanism that contributes to improvements in walking ability after exercise training in claudicants. Nevertheless, these findings do not exclude suppression of ACE as a potential means to improve claudication, but only exclude the likelihood of ACE being important in exercise induced improvements. On examining differential I/D allele expression in ACE ID heterozygotes, more mRNA was shown to be produced from the D-allele than the I-allele, in corroboration with previous reports.[246] Exercise training also appeared to preferentially activate the D-allele, further increasing the D:I ratio. The mechanism underlying this predominance of mRNA from the D-allele is not understood and will need further studies to elucidate.

Although an increase in ACE expression could be associated with potentially negative effects in claudicants (such as decreased metabolic efficiency), the clear gains associated with exercise training suggest other beneficial mechanisms outweigh these potential disadvantages. In this study, substantial increases in VEGF mRNA expression, greatest in the most biologically active isoform $VEGF_{165}$, and protein levels were found. In addition, expression of VEGF receptors was also increased, with the greatest increases noted in the transcription of VEGFR-2, which has the strongest signalling properties. Thus, exercise training had an overall effect of increasing the potential for VEGF signalling. Likewise, in the cell culture model of hypoxia, VEGF protein levels were also increased, as shown previously.[95;174] Given that ACE expression had increased, it can be concluded that following exercise training in claudicants, VEGF expression is not stimulated by a mechanism related to decreased ACE activity. The

lack of correlation between changes in ACE and VEGF expression in our subjects also supports this notion. Hence, increases in VEGF are more likely to be related primarily to stimulation by the well described HIF-1 pathway.[226]

No correlation was found between physiological improvements and molecular adaptations, primarily because of small numbers of subjects involved. Although a link between improvements in walking ability and increases in VEGF cannot be demonstrated by this study, the increases in VEGF were associated with improved walking ability in our subjects. An increase in VEGF mediated angiogenesis is a reasonable explanation as part of the mechanism underlying these improvements.

6.2 EFFECTS OF ACE INHIBITION ON ACE AND VEGF RESPONSES TO HYPOXIA

Although the results from the claudicant study do not associate increased walking ability with ACE suppression, ACE inhibitors could still play a role in increasing walking ability, by further potentiating VEGF increases and secondarily by reducing ACE activity. The second part of this thesis examined how ACE inhibitors modulate ACE and VEGF in hypoxia. In accordance with previous reports, ramiprilat in normoxic conditions increased ACE expression.[58;138] Under hypoxic conditions, ACE expression appeared to further increase beyond that observed in response to hypoxia alone. Although this may seem to be a unfavourable response, in the continued presence of an ACE inhibitor, ACE activity would be likely to remain suppressed.[23] Ramiprilat also stimulated VEGF production, increasing VEGF levels in normoxic conditions and potentiating VEGF increases in response to hypoxia. In other words, ACE inhibition and hypoxia would seem to act synergistically to enhance VEGF production. The

mechanism underlying this increase could be related to the accumulation of BK during ACE inhibition, or to a signalling pathway triggered by the binding of the ACE inhibitor to membrane-bound ACE.[10;139;233] The comparative contribution of these pathways could be determined by future experiments in which each pathway could be separately antagonised.

The findings of this study are comparable to previous reports in animal models of ischaemia which demonstrate upregulation of VEGF in response to ACE inhibition. In these studies, increases in VEGF were also related to improved angiogenesis and blood flow.[85;233;250] Thus, there could be a role for the use of ACE inhibitors in claudicants to stimulate angiogenesis in ischaemic leg muscles, and thereby improve walking ability. An additional benefit may also come from a reduction in ACE activity. Indeed, one small scale study has already demonstrated a beneficial effect of ramipril on pain-free and maximum walking time in claudicants.[3] However, in contrast a previous pilot study performed in this laboratory showed no benefit from ramipril and a large randomised double blind placebo controlled trial is required to definitively address this issue.

6.3 CONCLUSIONS

Exercise training in claudicants improves walking ability, and is associated with notable increases in VEGF protein and receptor mRNA expression. In a cell culture model of hypoxia, ACE inhibitors also increase VEGF expression. Thus, the use of ACE inhibitors could be advantageous to claudicants, by potentiating VEGF and facilitating angiogenesis, and secondarily by reducing ACE activity, producing metabolic benefits and increasing endurance performance.

LIST OF PUBLICATIONS

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Ng PWK, Hollingsworth SJ, Luery H, Kumana TJ and Chaloner EJ. Intermittent Claudication: Exercise-increased walking distance is not related to improved cardiopulmonary fitness. *Eur J Vasc Endovasc Surg* 2005; **30(4)**: 391-4

LIST OF PRESENTATIONS

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PWK Ng, JTC Lee, SJ Hollingsworth. Increased mRNA expression from the D-allele compared to the I-allele of the angiotensin-converting enzyme (ACE) ID polymorphism in claudicants: preferential activation of the D-allele in ID heterozygotes after exercise training in claudicants. *ASGBI International Surgical Congress*. May 2008. (Poster Presentation)

PWK Ng, JTC Lee, SJ Hollingsworth. Repetitive heel raises: a simple, reliable and objective method to assess changes in the symptoms of patients with intermittent claudication. *ASGBI International Surgical Congress*. May 2008. (Oral Presentation)

PWK Ng, JTC Lee, SJ Hollingsworth, TJ Kumana, H Luery, EJ Chaloner. The Effects Of The Angiotensin Converting Enzyme (ACE) I/D Polymorphism On Responses To Exercise Training In Patients With Intermittent Claudication. *7th International Congress of the Asian Society for Vascular Surgery*. Kuala Lumpur. August 2006. (Poster Presentation)

PWK Ng, JTC Lee, SJ Hollingsworth, TJ Kumana, H Luery, EJ Chaloner. The Effects Of Low-Dose Ramipril On Exercise Training Responses In Patients With Intermittent Claudication: A Randomised, Double-Blind Cross-Over Trial. *7th International Congress of the Asian Society for Vascular Surgery*. Kuala Lumpur. August 2006. (Oral Presentation)

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APPENDICES

APPENDIX 1: BORG SCALES

0	Nothing at all	"No P"
0.3		
0.5	Extremely weak	Just noticeable
1	Very weak	
1.5		
2	Weak	Light
2.5		
3	Moderate	
4		
5	Strong	Heavy
6		
7	Very strong	
8		
9		
10	**Extremely strong "Max P"**	
11		
↯		
●	Absolute maximum	Highest possible

Borg CR10 scale

6	No exertion at all
7	Extremely light
8	
9	Very light
10	
11	Light
12	
13	Somewhat hard
14	
15	Hard (heavy)
16	
17	Very hard
18	
19	Extremely hard
20	Maximal exertion

Borg RPE scale

APPENDIX 2: RT-PCR OPTIMISATION

EXAMPLES

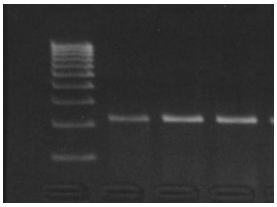
The optimisation process of the PCR conditions for ACE 216 primers is illustrated below. The following parameters were adjusted until consistent clean PCR products were obtained. Representative gel pictures showing the results of the optimisation process are shown:

Annealing Temperature

The annealing temperature was increased in 2°C increments from 58°C to 62°C, gradually increasing the stringency of the PCR reaction. A final annealing temperature of 60°C was chosen.

Annealing Temperature (°C)

62 60 58

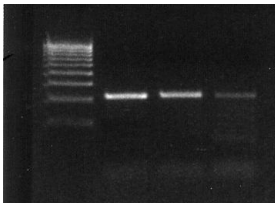


Magnesium Concentration

The reaction magnesium concentrations were increased from 1.0 and 2.0 mM, gradually decreasing the stringency of the PCR reaction. A final concentration of 1.5 mM was chosen.

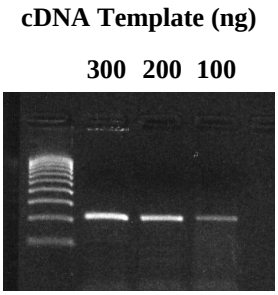
Magnesium (mM)

2.0 1.5 1.0



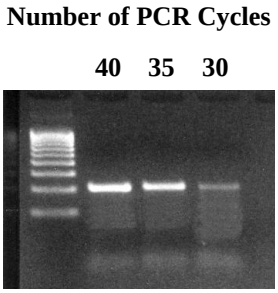
cDNA Template Levels

The initial cDNA template level put into each reaction was varied between 100 and 300ng. A final template level of 300ng was chosen.



PCR Cycle Conditions

The number of PCR cycles was increased from 30 to 40 cycles, increasing the final product yield. At 40 cycles, the PCR products became saturated, so 35 cycles was chosen for the final reactions.



APPENDIX 3: PATIENT DEMOGRAPHICS

Patient	Group	Sex	Age	BMI	Race	Smoking (no/day)	FH	DM	↑ Chol	↑ BP	Affected Leg(s)	Est. Walking Distance	Disease Distribution	Past Intervention
CT01	control	M	65	23.0	Asian	0	Yes	Type II	Yes	No	Bilateral	700	crural	None
CT02	exercise	F	79	21.2	Caucasian	Ex	Yes	No	Yes	Yes	Bilateral	450	crural	None
CT03	control	M	63	25.9	Caucasian	Ex	No	No	No	No	Left	200	L iliac/crural	R Iliac Angioplasty 2004
CT04	control	F	80	31.0	Caucasian	Ex	No	No	Yes	Yes	Bilateral	450	bilateral SFA occlusions	None
CT05	exercise	F	67	24.4	Caucasian	30	No	No	No	No	Bilateral	250	bilateral SFA/crural	None
CT06	exercise	F	75	30.6	Caucasian	Ex	No	No	Yes	Yes	Left	400	L SFA occlusion	None
CT07	exercise	M	56	23.4	Caucasian	10	No	No	No	No	Left	350	L SFA stenosis	None
CT08	control	F	72	21.3	Caucasian	Ex	No	No	Yes	No	Bilateral	250	bilateral SFA occlusions	R fem-popliteal bypass 1997 occluded
CT09	exercise	F	82	21.3	Caucasian	Ex	No	No	Yes	No	Bilateral	200	bilateral SFA occlusions	Failed Left SFA angioplasty 2004
CT10	exercise	F	79	21.2	Caucasian	Ex	Yes	No	Yes	Yes	Bilateral	400	Left iliac stenosis/Right SFA occlusion	Right patch CFA and iliac stent 2005
CT11	control	M	54	24.5	Caucasian	Ex	Yes	No	Yes	No	Bilateral	500	R iliac/femoral stenoses. L femoral occlusion	R iliac angioplasty 1999 R SFA angioplasty 2000 L fem-pop bypass 1998

Patient	Medications
CT01	Aspirin 75mg od, Carvedilol 12.5mg bd, Omeprazole 10mg od, Gliclazide 80mg bd, Metformin 500mg tds, Simvastatin 10mg od
CT02	Aspirin 75mg od, Co-amilozide 5/50 od, Atorvastatin 20mg od
CT03	Aspirin 75mg od, Chlorpromazine 25mg bd, Trifluoperazine 1mg bd
CT04	Aspirin 75mg od, Bendromethafluazide 2.5mg od, Calcichew 1 tds, Simvastatin 20mg od
CT05	Aspirin 75mg od, Lithium 600mg od, Mirtazapine 15mg od, Zoplicone 3.75mg od
CT06	Aspirin 75mg od, Bendromethafluazide 2.5mg od
CT07	Aspirin 75mg od, Diazepam 10mg od, Iron sulphate 200mg bd
CT08	Paracetamol 1g qds, Calcichew 1 tds, Propylthiouracil 10mg od, Iron Sulphate 200mg bd, Atorvastatin 20mg od, Fosamax 70mg once weekly
CT09	Aspirin 75mg od, Simvastatin 40mg od, Calcichew 1 tds
CT10	Aspirin 75mg od, Co-dydramol 2 qds prn, Bisoprolol 5mg od, Atorvastatin 20mg od, Bendromethafluazide 2.5mg od, Omeprazole 20mg od, Diclofenac 75mg tds prn
CT11	Simvastatin 20mg od, Aspirin 75mg od

APPENDIX 4: PATIENT DATA

Patient	Height	Weight (Kg)		Blood Pressure						Resting HR		Pre					Post				
	(cm)	Pre	Post	Pre			Post			(bpm)		Right			Left		Right			Left	
				SBP	DBP	MAP	SBP	DBP	MAP	Pre	Post	Br	AP	ABPI	AP	ABPI	Br	AP	ABPI	AP	ABPI
CT01	171	67.2	65.3	145	80	101.7	150	75	100.0	68	76	150	190	1.27	200	1.33	150	200	1.33	200	1.33
CT02	163	56.2	54.6	145	90	108.3	150	95	113.3	80	84	145	120	0.83	115	0.79	150	100	0.67	110	0.73
CT03	167	72.3	72.6	130	85	100.0	150	90	110.0	82	80	135	130	0.96	110	0.81	145	120	0.83	110	0.76
CT04	156.5	76	75.2	145	80	101.7	135	70	91.7	72	72	178	136	0.76	122	0.69	135	110	0.81	105	0.78
CT05	163.5	65.3	64.1	140	85	103.3	150	85	106.7	80	84	135	120	0.89	75	0.56	150	125	0.83	100	0.67
CT06	166	84.4	85.2	155	85	108.3	150	80	103.3	60	72	144	152	1.06	90	0.63	150	150	1.00	85	0.57
CT07	169	66.8	66	160	80	106.7	135	70	91.7	76	72	147	140	0.95	105	0.71	140	135	0.96	125	0.89
CT08	155	51.2	52.5	125	75	91.7	115	70	85.0	84	92	122	90	0.74	84	0.69	120	90	0.75	85	0.71
CT09	161.5	55.5	55.5	165	80	108.3	155	75	101.7	76	80	165	130	0.79	95	0.58	145	105	0.72	80	0.55
CT10	166	58.3	55.8	150	70	96.7	155	85	108.3	60	64	148	144	0.97	128	0.86	170	160	0.94	150	0.88
CT11	173	73.3	74	155	90	111.7	160	90	113.3	60	68	145	110	0.76	100	0.69	150	110	0.73	90	0.60

SBP- Systolic blood pressure, **DBP-** Diastolic blood pressure, **MAP-** Mean Arterial Pressure, **Br-** Brachial blood pressure (highest), **AP-** Ankle blood pressure (highest)

Patient	Walking Ability: Treadmill Test										Heel Raises Test					
	CD (M)		MWD (M)		Max HR (bpm)		RPP		RPE		Heel Raises		RPP		RPE	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
CT01	482	375	859	835	100	116	6	7	15	17	139	129	5	5	14	15
CT02	175	221	353	460	114	100	5	6	15	15	65	187	4	3	13	15
CT03	124	145	366	361	126	112	4	4	13	13	98	54	4	5	13	13
CT04	74	88	134	135	76	76	5	5	17	13	32	26	5	5	17	15
CT05	225	375	373	799	104	104	5	5	15	15	42	84	5	5	13	13
CT06	355	415	532	686	84	92	4	3	15	15	208	238	7	3	17	15
CT07	245	460	447	582	88	80	6	7	15	15	44	44	5	7	15	15
CT08	95	158	182	246	100	100	3	3	13	11	25	18	5	3	11	11
CT09	386	396	554	582	96	88	4	3	13	13	25	42	3	5	13	13
CT10	482	705	718	864	68	88	3	5	13	15	164	360	4	5	11	13
CT11	276	418	855	735	80	84	3	5	17	17	85	76	5	5	17	17

CD- Claudication distance, **MWD-** Maximum walking distance, **Max HR-** Maximum Heart Rate, **RPP-** Rated Perceived Pain, **RPE-** Rated Perceived Exertion

Patient	ACE Genotype	Ratio ACE 216/GAPDH-3 mRNA Expression						Ratio ACE 2215/GAPDH-3 mRNA Expression						Mean Ratio ACE 216 and 2215		
		Pre			Post			Pre			Post					
		Run 1	Run 2	Mean	Run 1	Run 2	Mean	Run 1	Run 2	Mean	Run 1	Run 2	Mean	Pre	Post	% Change
CT01	ID	0.236	0.217	0.227	0.349	0.368	0.359	0.566	0.522	0.544	0.302	0.785	0.543	0.386	0.451	17.0
CT02	ID	0.258	0.081	0.170	0.555	0.375	0.465	0.402	0.660	0.531	0.429	0.604	0.516	0.350	0.491	40.0
CT03	ID	0.360	0.318	0.339	0.724	0.496	0.610	0.834	0.726	0.780	0.513	0.953	0.733	0.560	0.672	20.0
CT04	ID	0.448	0.343	0.396	0.647	0.604	0.626	0.756	0.613	0.685	0.778	0.670	0.724	0.540	0.675	24.9
CT05	ID	0.426	0.285	0.356	0.498	0.495	0.497	0.537	0.648	0.593	0.654	0.518	0.586	0.474	0.541	14.2
CT06	DD	0.186	0.174	0.180	0.535	0.570	0.553	0.735	0.572	0.654	0.633	0.874	0.753	0.417	0.653	56.6
CT07	DD	0.465	0.276	0.371	0.685	0.876	0.780	0.808	1.050	0.929	0.937	1.233	1.085	0.650	0.933	43.5
CT08	II	0.561	0.463	0.512	0.645	0.435	0.540	0.619	0.219	0.419	0.571	0.504	0.537	0.465	0.539	15.8
CT09	II	0.821	0.761	0.791	1.022	0.751	0.886	1.222	0.902	1.062	1.295	1.376	1.336	0.927	1.111	19.9
CT10	ID	0.787	0.848	0.817	0.868	0.790	0.829	0.805	0.891	0.848	0.924	0.990	0.957	0.833	0.893	7.3
CT11	II	0.847	0.946	0.896	0.848	0.801	0.824	1.046	1.135	1.090	1.055	1.187	1.121	0.993	0.973	-2.0

Patient	Ratio ACE D:I alleles						%	ACE levels (ng/ml)						
	Pre			Post			Change	Pre			Post			
	Run 1	Run 2	Mean	Run 1	Run 2	Mean	D:I ratio	Run 1	Run 2	Mean	Run 1	Run 2	Mean	% Change
CT01	1.444	1.256	1.350	1.172	1.565	1.369	1.4	193.6	194.9	194.3	190.0	177.1	183.5	-5.5
CT02	0.891	0.811	0.851	0.973	1.020	0.996	17.0	215.5	213.6	214.6	225.0	229.9	227.4	6.0
CT03	1.434	1.039	1.237	0.997	1.142	1.070	-13.5	186.3	185.4	185.9	189.6	183.2	186.4	0.3
CT04	1.323	1.124	1.224	1.001	1.284	1.143	-6.6	251.3	279.6	265.5	226.0	259.0	242.5	-8.7
CT05	1.008	1.054	1.031	1.233	1.114	1.173	13.7	170.8	162.5	166.7	173.9	165.7	169.8	1.9
CT06	n/a	n/a	n/a	n/a	n/a	n/a	n/a	221.5	236.9	229.2	219.8	240.0	229.9	0.3
CT07	n/a	n/a	n/a	n/a	n/a	n/a	n/a	203.5	209.1	206.3	204.5	209.0	206.8	0.2
CT08	n/a	n/a	n/a	n/a	n/a	n/a	n/a	94.1	119.9	107.0	113.4	137.4	125.4	17.2
CT09	n/a	n/a	n/a	n/a	n/a	n/a	n/a	75.3	98.7	87.0	79.3	100.8	90.0	3.5
CT10	1.930	1.561	1.746	2.355	1.742	2.048	17.3	141.9	156.9	149.4	136.2	161.9	149.0	-0.2
CT11	n/a	n/a	n/a	n/a	n/a	n/a	n/a	104.3	134.0	119.2	96.7	113.7	105.2	-11.7

Patient	Ratio $VEGF_{121}$/GAPDH-3 mRNA Expression						Ratio $VEGF_{165}$/GAPDH-3 mRNA Expression						% Change VEGF	
	Pre			Post			Pre			Post			$VEGF_{121}$	$VEGF_{165}$
	Run 1	Run 2	Mean	Run 1	Run 2	Mean	Run 1	Run 2	Mean	Run 1	Run 2	Mean		
CT01	0.349	0.480	0.414	0.508	0.493	0.501	0.086	0.125	0.106	0.115	0.170	0.142	20.9	34.6
CT02	0.095	0.362	0.229	0.452	0.358	0.405	0.028	0.075	0.052	0.156	0.055	0.106	77.1	104.8
CT03	0.387	0.402	0.395	0.524	0.499	0.511	0.148	0.161	0.154	0.165	0.186	0.176	29.6	13.7
CT04	0.494	0.579	0.536	0.555	0.463	0.509	0.241	0.239	0.240	0.189	0.185	0.187	-5.1	-22.0
CT05	0.264	0.413	0.338	0.397	0.554	0.475	0.090	0.133	0.111	0.134	0.230	0.182	40.4	63.1
CT06	0.442	0.624	0.533	0.571	0.561	0.566	0.173	0.253	0.213	0.230	0.207	0.218	6.2	2.6
CT07	0.434	0.306	0.370	0.448	0.498	0.473	0.123	0.099	0.111	0.193	0.201	0.197	27.8	77.3
CT08	0.487	0.453	0.470	0.433	0.303	0.368	0.234	0.192	0.213	0.200	0.109	0.155	-21.7	-27.6
CT09	1.109	0.254	0.682	1.202	0.762	0.982	0.708	0.261	0.484	0.862	0.436	0.649	44.1	34.0
CT10	0.501	0.193	0.347	1.214	0.576	0.895	0.213	0.109	0.161	0.861	0.279	0.570	157.8	254.9
CT11	1.064	0.262	0.663	1.048	0.550	0.799	0.769	0.088	0.429	0.749	0.216	0.482	20.5	12.5

Patient	Ratio VEGF Receptor/GAPDH-3 mRNA Expression									VEGF Levels (pg/ml) ELISA						
	VEGFR-1		sVEGFR-1		VEGFR-2		% Change			Pre			Post			%
	Pre	Post	Pre	Post	Pre	Post	VEGFR-1	sVEGFR-1	VEGFR-2	Run 1	Run 2	Mean	Run 1	Run 2	Mean	Change
CT01	0.217	0.355	0.086	0.126	0.125	0.196	63.4	45.8	56.5	54.3	43.6	49.0	17.7	15.9	16.8	-65.8
CT02	0.192	0.426	0.087	0.216	0.020	0.147	121.5	148.9	633.3	41.0	38.7	39.8	33.6	40.4	37.0	-7.1
CT03	0.352	0.468	0.121	0.184	0.191	0.141	32.9	51.6	-25.8	17.3	16.2	16.8	42.1	36.9	39.5	135.8
CT04	0.347	0.428	0.151	0.164	0.171	0.160	23.3	8.9	-6.1	4.0	2.2	3.1	24.3	23.9	24.1	686.2
CT05	0.255	0.380	0.116	0.215	0.190	0.343	49.0	85.0	80.6	18.0	12.4	15.2	75.8	63.6	69.7	358.7
CT06	0.371	0.472	0.137	0.207	0.059	0.228	27.2	51.4	285.2	95.1	69.5	82.3	99.9	87.4	93.7	13.8
CT07	0.343	0.346	0.157	0.272	0.102	0.155	0.7	73.5	52.8	8.8	5.7	7.2	15.8	16.6	16.2	123.9
CT08	0.384	0.380	0.231	0.192	0.149	0.210	-1.0	-17.0	41.3	42.1	50.6	46.4	40.6	30.2	35.4	-23.5
CT09	0.464	0.599	0.244	0.310	0.123	0.200	29.1	26.6	62.5	21.0	16.2	18.6	32.9	27.8	30.3	63.0
CT10	0.388	0.364	0.226	0.232	0.247	0.303	-6.2	2.3	22.4	12.5	12.7	12.6	24.0	33.6	28.8	128.5
CT11	0.452	0.453	0.211	0.210	0.148	0.187	0.0	-0.5	26.1	26.2	25.3	25.8	47.3	41.5	44.4	72.3

APPENDIX 5: CELL CULTURE WORK-UP

EXPERIMENT RESULTS

Cell Viability Control Experiments

For both medium concentrations, the wells seeded with 1,500 and 3,000 cells showed evidence of changes in cell viability over time. This was especially obvious in the wells seeded with 3,000 cells in 10% FCS, which proliferated markedly over the experimental period. More stability in cell viability was shown in the wells seeded with 15,000 and 30,000 cells, which were confluent throughout the experiment, although in wells seeded with 30,000 cells in 10% FCS there was a significant rise in absorbance (Figure A). From these experiments, a decision was made to use 15,000 cells/well in future experiments, as at this density the cells were always confluent and stably viable over 72 hours. In this quiescent state, the cells were more likely to have steady gene and protein expression.

DETA-NO Only Experiment

In the cell viability experiments, there was no difference in viability between the medium and the vehicle controls in both the 1% FCS experiment (Medium only 1.53 ± 0.08, Vehicle 1.77 ± 0.20; $p = 0.32$) and the 10% FCS experiment (Medium only 1.80 ± 0.07, Vehicle 1.93 ± 0.09; $p = 0.28$). The vehicle control was used as the experimental control. With increasing doses of DETA-NO, there was no significant change in viability from the controls (Figure B) although the viability appeared to drop slightly at higher DETA-NO doses. Changes in ACE and VEGF gene expression are shown in Figures C and D respectively. From the results of these experiments, DETA-NO doses between 0.008 to 0.25 mM were used in the final experiments.

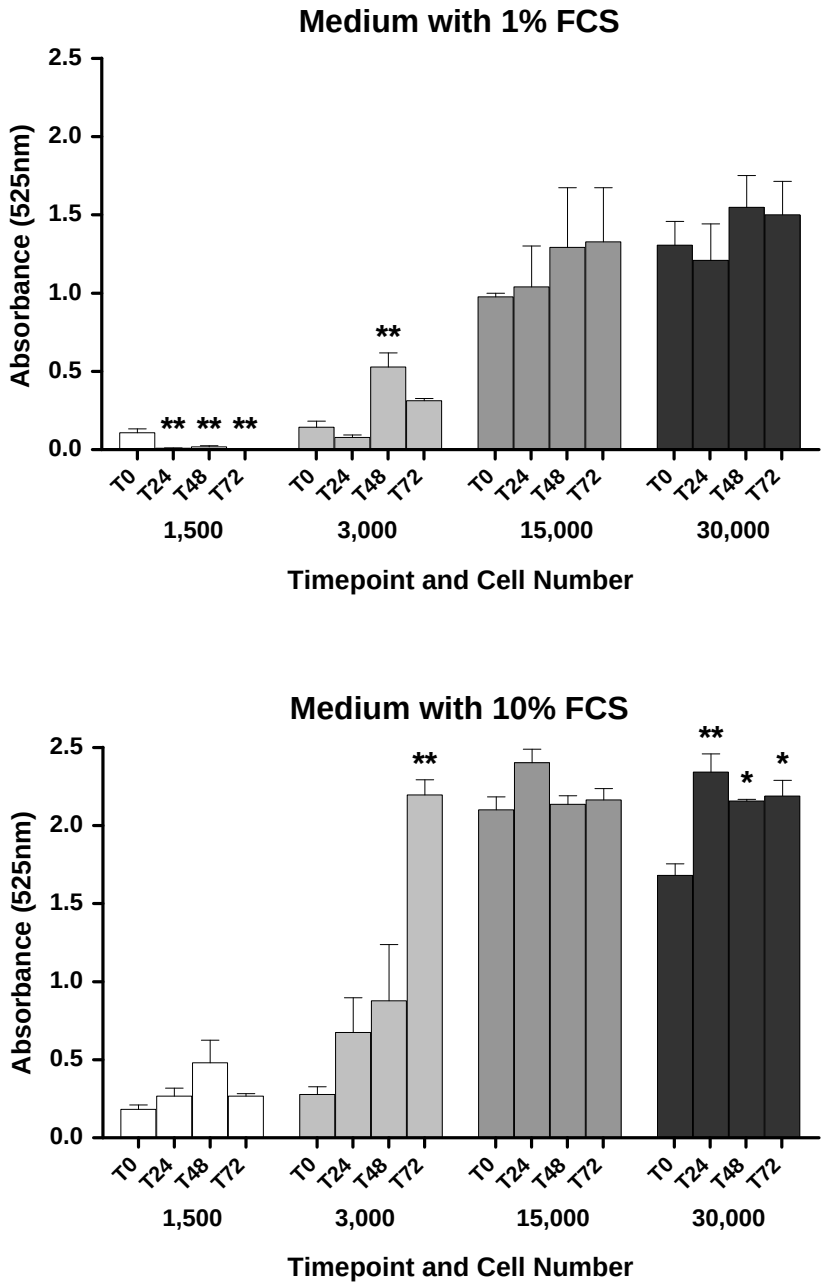
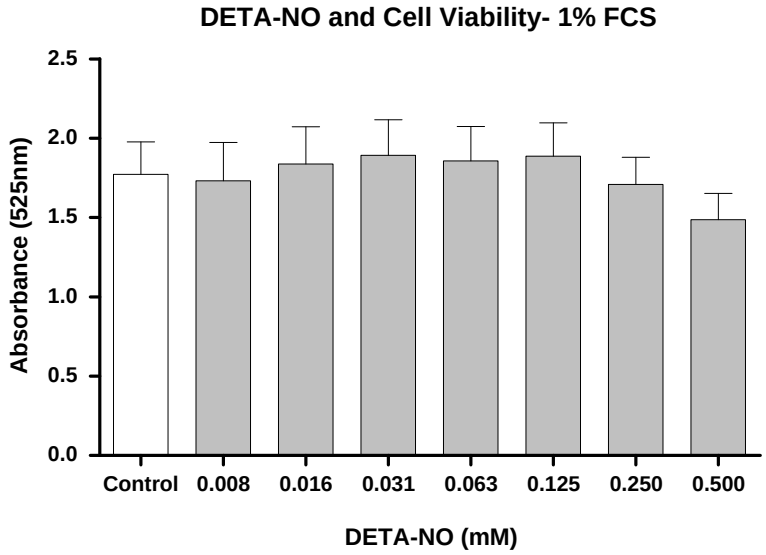


Figure A Graphs of Cell Viability for Varying Cell Numbers in 1% and 10% FCS over Time

Higher absorbance readings indicate increased cell viability or proliferation. The bars represent means (of triplicate readings) and the error bars SEM. T_0 represents the start of the experiment, with further readings taken every 24 hours. The differences between time points were analysed by 1-way ANOVA with Bonferroni's multiple comparison test. * $p < 0.05$ vs T_0, ** $p < 0.01$ vs T_0.



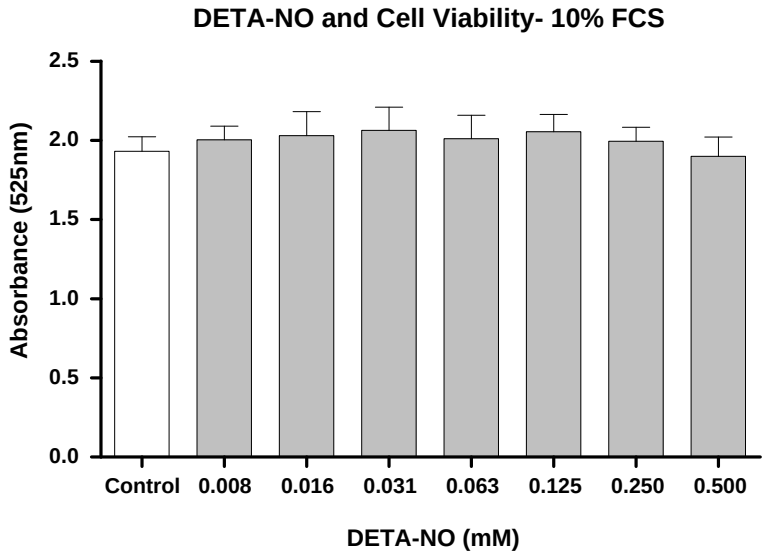


Figure B Cell Viability with Increasing Doses of DETA-NO with 1% and 10% FCS

The bars represent means (of quadruplicate readings) and the error bars SEM. The control experiments consisted of cells, medium and drug vehicle. No significant difference in viability from the controls was found (1-way ANOVA: 1% FCS $p = 0.89$; 10% FCS $p = 0.98$).

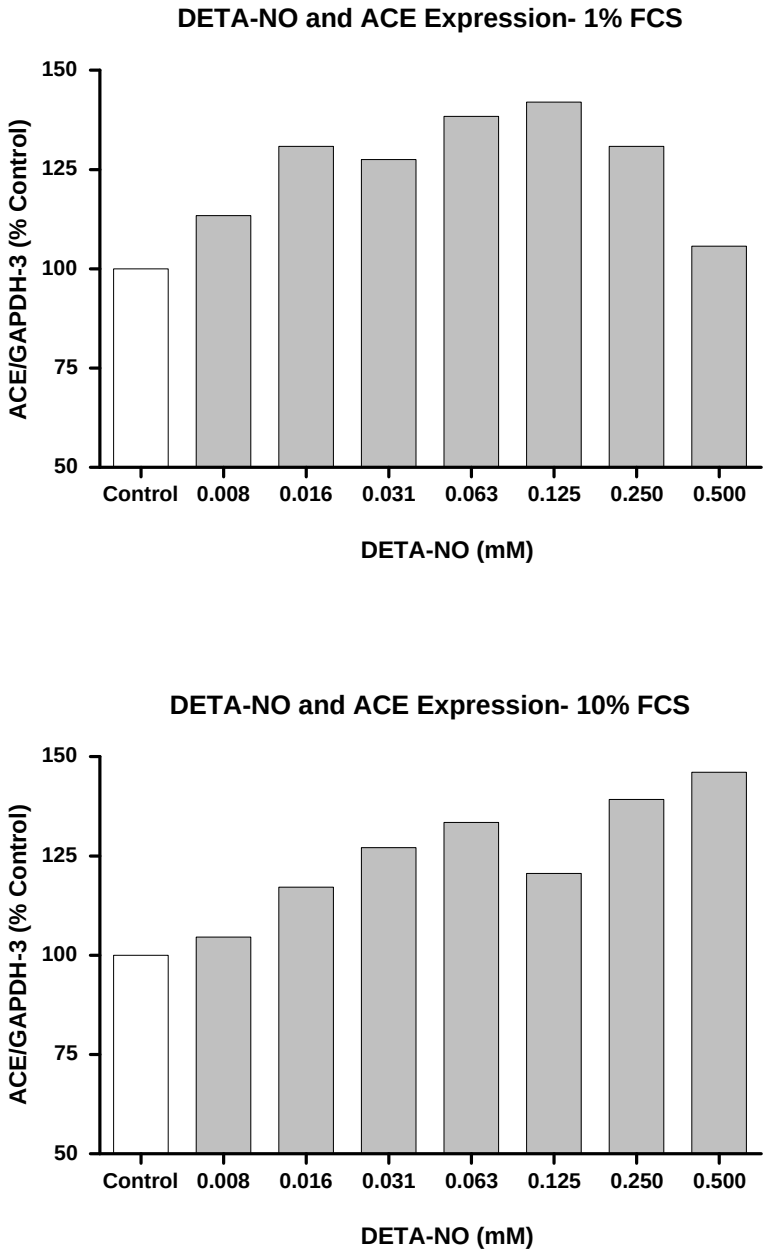


Figure C Changes in ACE Expression with Increasing Doses of DETA-NO with 1% and 10% FCS

With both serum concentrations, increasing doses of DETA-NO resulted in an increase in ACE expression over the controls. However, at higher DETA-NO doses with 1% FCS this increase was attenuated. The bars represent the results of a single RT-PCR run.

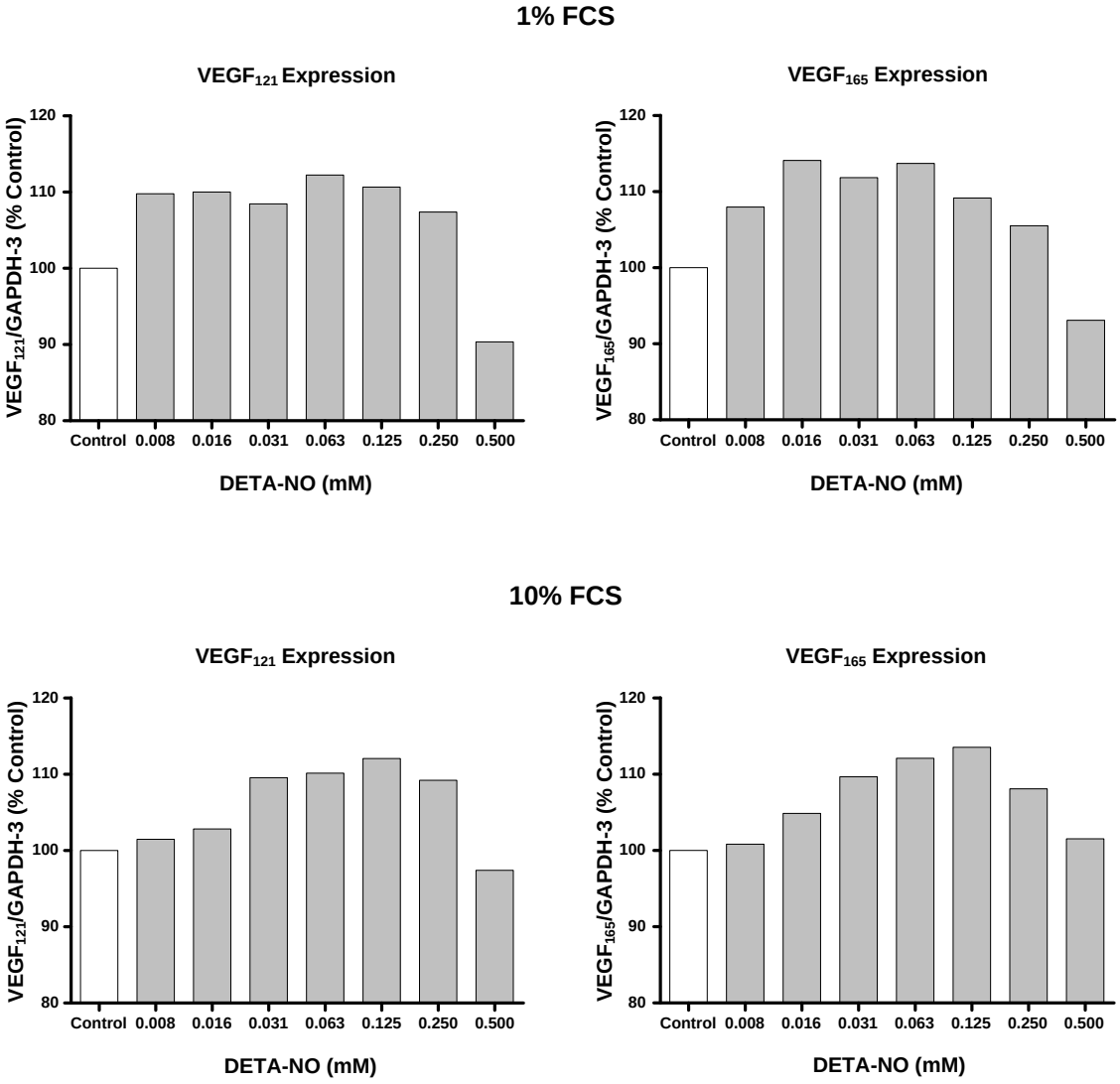


Figure D Changes in $VEGF_{121}$ and $VEGF_{165}$ Expression with Increasing doses of DETA-NO with 1% and 10% FCS

With both serum concentrations, increasing doses of DETA-NO resulted in an increase in $VEGF_{121}$ and $VEGF_{165}$ expression over the controls. However, at the highest DETA-NO dose, VEGF expression was reduced. The bars represent the results of a single RT-PCR run.

Ramiprilat Only Experiment

In the cell viability experiments, there was no difference in viability between the medium and vehicle controls in both the 1% FCS experiment (Medium only 1.67 ± 0.15, Vehicle 1.96 ± 0.15; $p = 0.22$) and the 10% FCS experiment (Medium only 2.13 ± 0.11, Vehicle 2.17 ± 0.11; $p = 0.77$). The vehicle control was used as the experimental control. With increasing doses of ramiprilat, there was no significant change in viability from the controls (Figure E). Changes in ACE and VEGF gene expression are shown in Figures E and F respectively. From the results of these experiments, the ramiprilat doses 100 and 1000 nM/l were used in the final experiments.

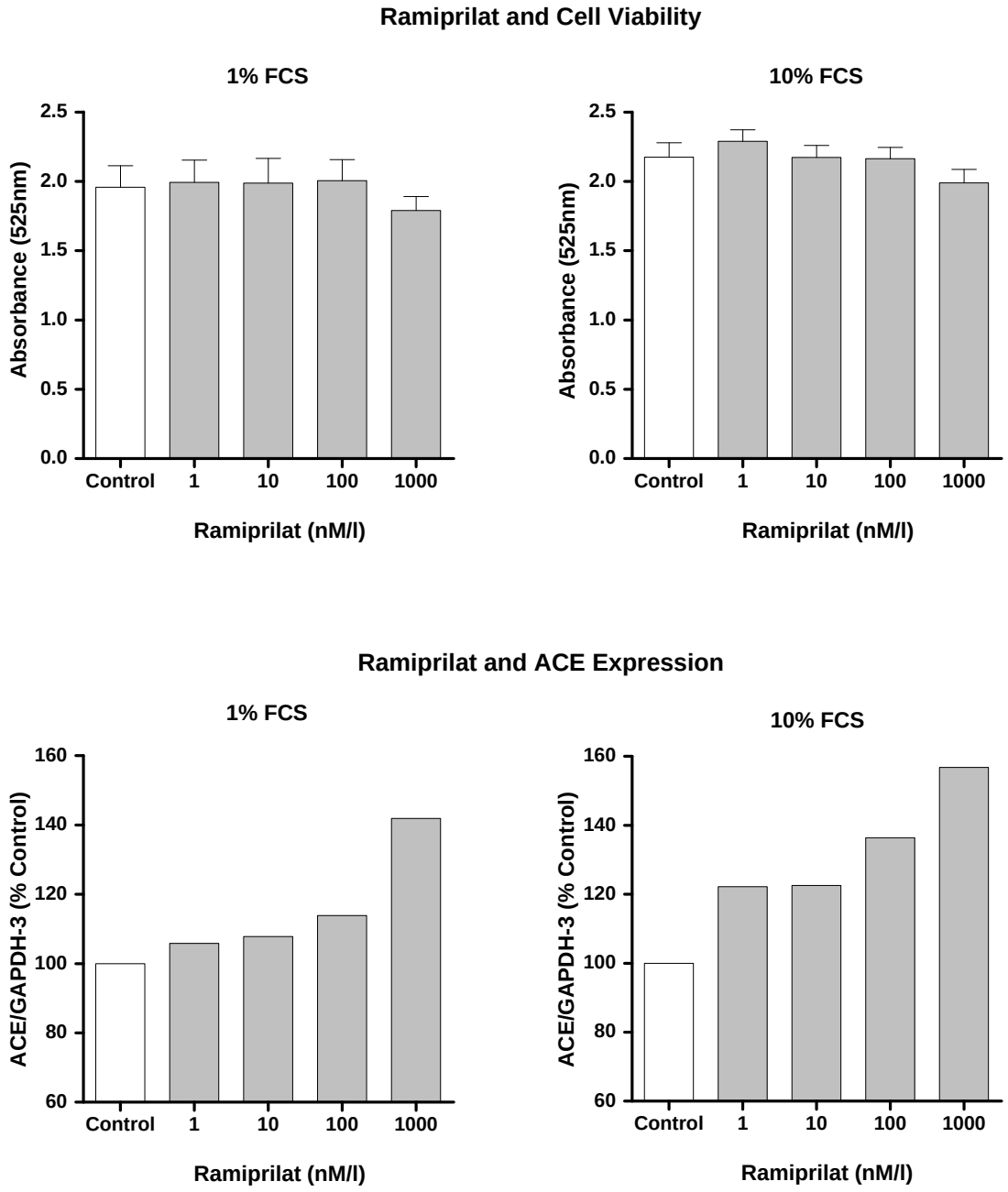


Figure E Cell Viability and ACE Expression with Increasing doses of Ramiprilat with 1% and 10% FCS

In the viability graphs, the bars represent means (of quadruplicate readings) and the error bars SEM. No significant difference in viability from the controls was found (1-way ANOVA: 1% FCS p = 0.84; 10% FCS p = 0.28). Increasing doses of ramiprilat augmented ACE gene expression at both FCS concentrations.

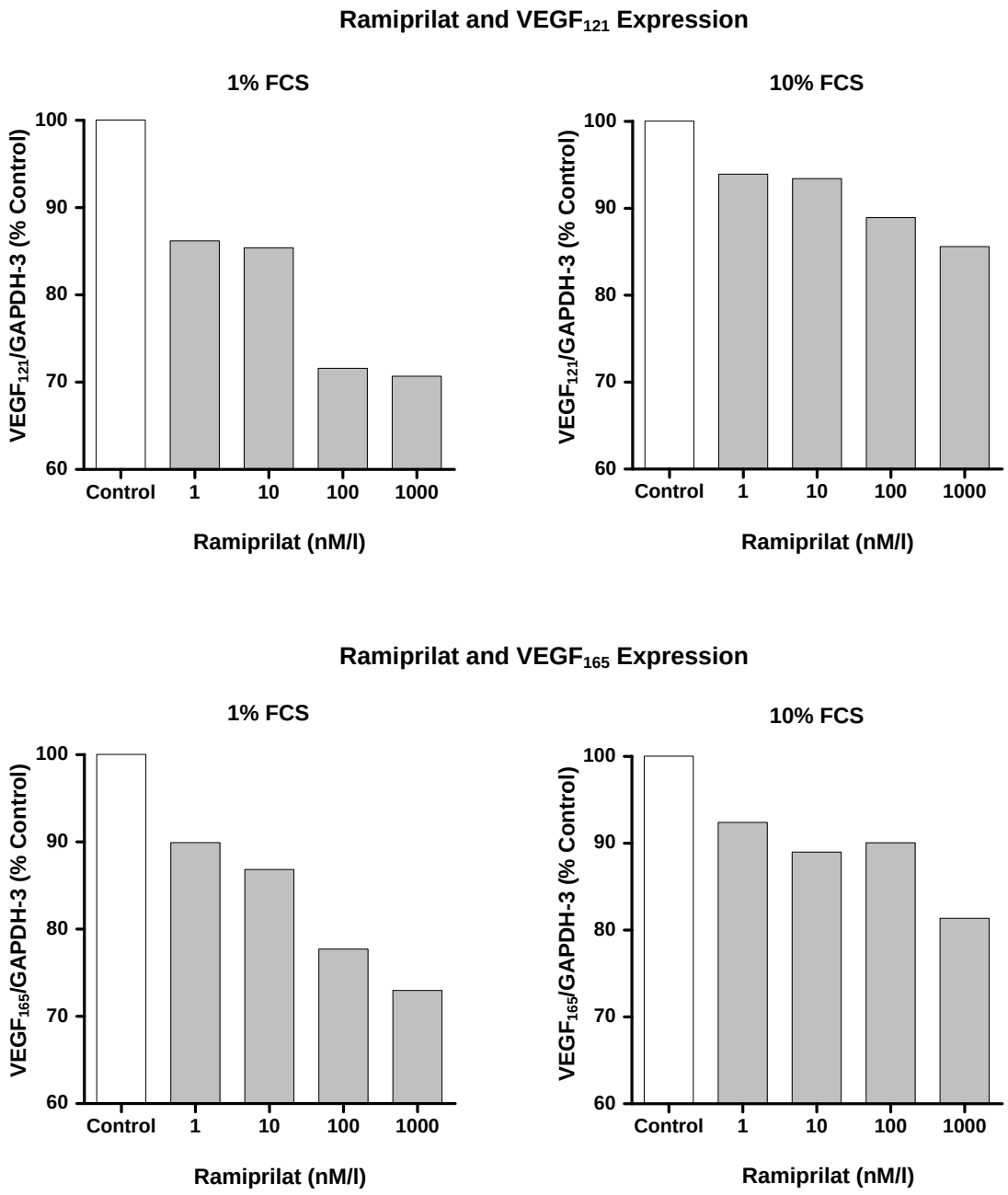


Figure F Changes in $VEGF_{121}$ and $VEGF_{165}$ Expression with Increasing doses of Ramiprilat with 1% and 10% FCS

In both 1% and 10% FCS, increasing doses of ramiprilat progressively suppressed $VEGF_{121}$ and $VEGF_{165}$ expression compared to the controls, with a more marked effect seen with 1% FCS.

APPENDIX 6: FINAL CELL CULTURE EXPERIMENTS- WELL CONTENTS

DETA-NO Only Experiments

All wells contained 15,000 ECV 304 cells in 1% FCS and 0.05% DMSO with:

Well	**Additional Contents**
DO-M 'Medium Control'	-
DO-V 'Vehicle Control'	1% water
DO-0.008	0.008 mM DETA-NO in water (1% of total volume)
DO-0.016	0.016 mM DETA-NO in water (1% of total volume)
DO-0.031	0.031 mM DETA-NO in water (1% of total volume)
DO-0.063	0.063 mM DETA-NO in water (1% of total volume)
DO-0.125	0.125 mM DETA-NO in water (1% of total volume)
DO-0.250	0.250 mM DETA-NO in water (1% of total volume)

The '**DO-V**' experimental wells (containing cells, medium, and both drug vehicles) were used as the overall **experimental controls**.

Ramiprilat 100 nM/l Experiments

All wells contained 15,000 ECV 304 cells in 1% FCS, 100 nM/l ramiprilat and 0.05% DMSO with:

Well	**Additional Contents**
R100-M 'Medium Control'	-
R100-V 'Vehicle Control'	1% water
R100-0.008	0.008 mM DETA-NO in water (1% of total volume)
R100-0.016	0.016 mM DETA-NO in water (1% of total volume)
R100-0.031	0.031 mM DETA-NO in water (1% of total volume)
R100-0.063	0.063 mM DETA-NO in water (1% of total volume)
R100-0.125	0.125 mM DETA-NO in water (1% of total volume)
R100-0.250	0.250 mM DETA-NO in water (1% of total volume)

Ramiprilat 1000 nM/l Experiments

All wells contained 15,000 ECV 304 cells in 1% FCS, 1000 nM/l ramiprilat and 0.05% DMSO with:

Well	**Additional Contents**
R1000-M 'Medium Control'	-
R1000-V 'Vehicle Control'	1% water
R1000-0.008	0.008 mM DETA-NO in water (1% of total volume)
R1000-0.016	0.016 mM DETA-NO in water (1% of total volume)
R1000-0.031	0.031 mM DETA-NO in water (1% of total volume)
R1000-0.063	0.063 mM DETA-NO in water (1% of total volume)
R1000-0.125	0.125 mM DETA-NO in water (1% of total volume)
R1000-0.250	0.250 mM DETA-NO in water (1% of total volume)

The DO-M, R100-M and R1000-M wells, combined with the previous ramiprilat only work-up experiment results, were used to assess the effects of ramiprilat alone.

APPENDIX 7: CELL CULTURE EXPERIMENT DATA

	ECV 304 Cell Viability										Viability	
	Experiment 1				Experiment 2						(% Control)	
	1	2	3	4	1	2	3	4	Mean	SEM	Mean	SEM
DO-M	2.137	2.288	2.181	1.838	1.949	1.886	1.941	1.717	1.992	0.068	-	-
DO-V	2.098	2.328	1.842	1.869	2.038	1.999	1.948	2.194	2.039	0.058	100.0	2.848
DO-0.008	2.329	2.037	1.936	2.073	1.994	2.061	1.815	1.779	2.003	0.061	98.2	2.971
DO-0.016	2.254	2.012	2.047	2.026	1.954	2.059	2.122	1.823	2.037	0.044	99.9	2.156
DO-0.031	1.841	2.074	2.167	2.079	2.143	1.857	2.233	1.941	2.042	0.052	100.1	2.533
DO-0.063	2.028	2.159	2.261	2.214	2.172	2.187	2.286	1.759	2.133	0.060	104.6	2.946
DO-0.125	1.914	2.166	2.194	1.937	2.183	2.205	2.075	2.035	2.089	0.041	102.4	2.032
DO-0.250	2.308	2.062	2.204	1.993	1.807	1.995	2.094	1.974	2.055	0.054	100.7	2.647
R100-M	2.016	2.129	1.996	1.882	2.365	2.202	1.790	1.840	2.027	0.069	-	-
R100-V	2.175	2.047	2.450	1.730	2.006	2.269	2.120	2.172	2.121	0.074	104.0	3.633
R100-0.008	2.404	2.223	2.092	1.774	2.227	2.186	2.184	1.773	2.108	0.079	103.3	3.880
R100-0.016	2.192	2.472	2.102	2.146	2.309	2.290	2.194	1.867	2.196	0.062	107.7	3.053
R100-0.031	2.254	2.319	2.013	1.753	2.347	2.298	2.407	1.993	2.173	0.080	106.5	3.946
R100-0.063	2.411	2.581	2.028	1.845	2.146	2.266	2.322	1.946	2.193	0.088	107.5	4.306
R100-0.125	2.204	2.391	2.062	1.896	2.378	2.351	2.259	1.941	2.185	0.070	107.1	3.413
R100-0.250	2.430	2.422	2.042	2.074	2.255	2.161	2.158	1.687	2.153	0.084	105.6	4.121
R1000-M	2.005	2.005	1.987	1.813	1.856	1.903	1.899	1.901	1.921	0.025	-	-
R1000-V	2.249	2.441	2.530	1.674	1.789	1.768	1.812	1.887	2.019	0.119	99.0	5.818
R1000-0.008	2.244	2.603	2.454	1.629	1.819	1.693	1.854	1.937	2.029	0.128	99.5	6.264
R1000-0.016	2.280	2.285	2.048	1.707	1.831	1.748	1.896	1.993	1.973	0.079	96.8	3.851
R1000-0.031	2.263	2.173	2.173	1.581	1.893	2.011	1.993	1.863	1.994	0.077	97.8	3.792
R1000-0.063	2.304	2.262	2.443	1.583	1.909	2.027	2.123	1.969	2.077	0.095	101.8	4.672
R1000-0.125	2.270	2.362	2.219	1.528	2.055	1.856	2.160	2.010	2.057	0.094	100.9	4.624
R1000-0.250	2.337	2.400	2.148	1.480	1.770	1.728	1.834	1.843	1.942	0.113	95.2	5.556

DO: DETA-NO alone, **R100**: DETA-NO with 100 nM/l Ramiprilat, **R1000**: DETA-NO with 1000 nM/l Ramiprilat, **M**: Medium Control, **V**: Vehicle Control, **0.008-0.250**: represent doses of DETA-NO.

	ACE Expression					
	Ratio ACE/GAPDH-3				ACE (% Control)	
	Exp 1	Exp 2	Mean	SEM	Mean	SEM
DO-M	0.180	0.273	0.226	0.047	-	-
DO-V	0.198	0.217	0.207	0.009	100.0	4.51
DO-0.008	0.249	0.242	0.246	0.003	118.5	1.62
DO-0.016	0.213	0.258	0.236	0.023	113.7	10.90
DO-0.031	0.254	0.279	0.266	0.012	128.5	5.97
DO-0.063	0.288	0.302	0.295	0.007	142.1	3.39
DO-0.125	0.291	0.273	0.282	0.009	136.0	4.25
DO-0.250	0.246	0.197	0.222	0.025	106.9	11.99
R100-M	0.300	0.306	0.303	0.003	-	-
R100-V	0.269	0.256	0.262	0.007	126.5	3.20
R100-0.008	0.300	0.312	0.306	0.006	147.6	2.93
R100-0.016	0.319	0.279	0.299	0.020	144.3	9.64
R100-0.031	0.280	0.302	0.291	0.011	140.5	5.32
R100-0.063	0.341	0.314	0.328	0.013	158.0	6.32
R100-0.125	0.393	0.322	0.358	0.035	172.5	16.98
R100-0.250	0.374	0.364	0.369	0.005	178.0	2.35
R1000-M	0.288	0.359	0.323	0.036	-	-
R1000-V	0.254	0.324	0.289	0.035	139.2	16.85
R1000-0.008	0.254	0.341	0.298	0.043	143.5	20.97
R1000-0.016	0.303	0.337	0.320	0.017	154.3	8.09
R1000-0.031	0.313	0.321	0.317	0.004	152.9	2.00
R1000-0.063	0.305	0.359	0.332	0.027	160.0	12.96
R1000-0.125	0.317	0.324	0.321	0.003	154.6	1.55
R1000-0.250	0.311	0.332	0.321	0.010	155.0	4.98

	VEGF$_{121}$ Expression					
	Ratio VEGF$_{121}$/GAPDH-3				VEGF$_{121}$ (% Control)	
	Exp 1	Exp 2	Mean	SEM	Mean	SEM
DO-M	0.614	0.618	0.616	0.002	-	-
DO-V	0.621	0.666	0.643	0.023	100.0	3.54
DO-0.008	0.621	0.711	0.666	0.045	103.5	6.98
DO-0.016	0.644	0.719	0.681	0.038	105.9	5.84
DO-0.031	0.632	0.713	0.672	0.040	104.5	6.29
DO-0.063	0.638	0.730	0.684	0.046	106.3	7.13
DO-0.125	0.593	0.687	0.640	0.047	99.5	7.33
DO-0.250	0.601	0.601	0.601	0.000	93.4	0.04
R100-M	0.540	0.510	0.525	0.015	-	-
R100-V	0.518	0.502	0.510	0.008	79.3	1.21
R100-0.008	0.546	0.512	0.529	0.017	82.2	2.63
R100-0.016	0.566	0.557	0.562	0.005	87.3	0.74
R100-0.031	0.576	0.582	0.579	0.003	90.0	0.47
R100-0.063	0.583	0.545	0.564	0.019	87.7	2.96
R100-0.125	0.550	0.490	0.520	0.030	80.9	4.68
R100-0.250	0.575	0.497	0.536	0.039	83.3	6.11
R1000-M	0.521	0.539	0.530	0.009	-	-
R1000-V	0.473	0.524	0.499	0.025	77.5	3.95
R1000-0.008	0.471	0.540	0.505	0.035	78.5	5.38
R1000-0.016	0.491	0.522	0.507	0.016	78.8	2.45
R1000-0.031	0.538	0.534	0.536	0.002	83.3	0.36
R1000-0.063	0.561	0.567	0.564	0.003	87.6	0.43
R1000-0.125	0.554	0.542	0.548	0.006	85.1	0.93
R1000-0.250	0.585	0.512	0.548	0.037	85.2	5.68

	VEGF$_{165}$ Expression					
	Ratio VEGF$_{165}$/GAPDH-3				VEGF$_{165}$ (% Control)	
	Exp 1	Exp 2	Mean	SEM	Mean	SEM
DO-M	0.458	0.482	0.470	0.012	-	-
DO-V	0.486	0.488	0.487	0.001	100.0	0.29
DO-0.008	0.479	0.517	0.498	0.019	102.3	3.84
DO-0.016	0.515	0.530	0.523	0.008	107.3	1.54
DO-0.031	0.503	0.546	0.525	0.022	107.8	4.42
DO-0.063	0.503	0.540	0.521	0.019	107.0	3.84
DO-0.125	0.494	0.478	0.486	0.008	99.8	1.74
DO-0.250	0.502	0.443	0.472	0.030	96.9	6.06
R100-M	0.412	0.422	0.417	0.005	-	-
R100-V	0.427	0.428	0.428	0.001	87.9	0.11
R100-0.008	0.434	0.427	0.430	0.004	88.3	0.73
R100-0.016	0.447	0.464	0.456	0.008	93.6	1.70
R100-0.031	0.498	0.472	0.485	0.013	99.5	2.65
R100-0.063	0.492	0.456	0.474	0.018	97.4	3.62
R100-0.125	0.456	0.388	0.422	0.034	86.6	6.91
R100-0.250	0.478	0.373	0.426	0.053	87.4	10.82
R1000-M	0.383	0.458	0.421	0.037	-	-
R1000-V	0.409	0.379	0.394	0.015	80.9	3.11
R1000-0.008	0.384	0.451	0.417	0.034	85.7	6.93
R1000-0.016	0.379	0.458	0.418	0.039	85.9	8.11
R1000-0.031	0.478	0.463	0.470	0.007	96.6	1.51
R1000-0.063	0.448	0.500	0.474	0.026	97.3	5.35
R1000-0.125	0.477	0.439	0.458	0.019	94.1	3.90
R1000-0.250	0.447	0.426	0.436	0.011	89.6	2.21

	VEGF Level (pg/ml)								VEGF Level (% Control)	
	Experiment 1			Experiment 2						
	VEGF Level	Corr. Factor	Corr. VEGF	VEGF Level	Corr. Factor	Corr. VEGF	Mean	SEM	Mean	SEM
DO-M	126.7	0.89	142.8	109.6	0.90	121.6	132.2	10.59	-	-
DO-V	143.6	1.10	131.0	98.9	0.94	105.3	118.2	12.86	100.0	10.9
DO-0.008	158.4	1.02	154.6	126.3	0.97	130.0	142.3	12.32	120.4	10.4
DO-0.016	151.7	0.99	153.2	118.3	0.97	121.7	137.5	15.74	116.4	13.3
DO-0.031	174.9	0.96	183.0	152.3	1.08	141.3	162.2	20.87	137.2	17.7
DO-0.063	192.7	1.11	173.7	135.0	0.85	159.2	166.4	7.25	140.9	6.1
DO-0.125	195.4	1.06	184.6	160.0	1.10	146.0	165.3	19.31	139.9	16.3
DO-0.250	181.8	0.94	193.6	181.7	1.08	168.5	181.1	12.58	153.2	10.6
R100-M	196.0	1.20	164.0	214.4	1.10	195.7	179.8	15.83	-	-
R100-V	155.4	0.85	183.3	177.7	0.94	189.3	186.3	3.00	157.6	2.5
R100-0.008	167.7	0.92	181.9	203.8	0.90	226.1	204.0	22.09	172.7	18.7
R100-0.016	178.8	0.96	187.0	206.3	0.95	216.1	201.6	14.55	170.6	12.3
R100-0.031	186.6	1.06	176.3	220.4	1.01	218.8	197.5	21.27	167.2	18.0
R100-0.063	165.4	0.90	182.9	242.9	1.08	225.3	204.1	21.20	172.7	17.9
R100-0.125	189.9	0.99	191.8	205.4	0.90	227.9	209.9	18.07	177.6	15.3
R100-0.250	197.7	1.02	193.0	221.3	0.92	240.8	216.9	23.90	183.6	20.2
R1000-M	201.5	0.99	203.5	233.8	1.20	194.4	199.0	4.52	-	-
R1000-V	215.5	0.92	233.9	202.6	1.08	187.9	210.9	22.99	178.5	19.5
R1000-0.008	207.3	0.97	213.0	192.1	1.03	187.4	200.2	12.83	169.4	10.9
R1000-0.016	221.5	1.02	216.3	230.4	1.15	200.5	208.4	7.88	176.4	6.7
R1000-0.031	234.5	1.09	214.6	192.9	1.03	188.2	201.4	13.21	170.5	11.2
R1000-0.063	239.7	1.16	206.4	247.9	1.01	246.1	226.3	19.83	191.5	16.8
R1000-0.125	225.1	0.97	231.3	197.9	0.95	207.4	219.4	11.96	185.6	10.1
R1000-0.250	213.2	0.94	227.1	222.3	0.87	256.7	241.9	14.80	204.7	12.5

Corr. Factor: Corrective factor based on protein yields as measured by Bradford assay.