Hydralazine in heart failure: a study of the mechanism of action in human blood vessels

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A thesis by

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Submitted in fulfilment of the requirements for the degree of

Doctor of Medicine

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Date of submission January 2015

Abstract

Hydralazine is a vasodilator that has been in clinical use for nearly six decades. Despite this, the mechanism of its action in human blood vessels is uncertain. Understanding how hydralazine works may have importance for the better treatment of heart failure and other cardiovascular diseases. In the first Vasodilator Heart Failure trial, hydralazine was shown, in combination with oral nitrates, to reduce mortality in patients with heart failure, treated at a time when the benefits of ACE inhibitors, beta-blockers and mineralocorticoid receptor antagonists were not known. As the combination of hydralazine and isosorbide dinitrate was subsequently shown to be less effective than an ACE inhibitor in the second Vasodilator Heart Failure trial, it was little used. Recently, however, the same combination was shown to reduce mortality and morbidity in the African-American Heart Failure Trial. Crucially, in this trial, the patients were already treated with the best currently available drug therapy. Though the patients studied were self-designated African-Americans, it is widely believed that the incremental benefits of the combination of hydralazine and isosorbide dinitrate are as likely to be obtained in other patients.

While the vasodilator action of nitrates is well understood, a better understanding of the action of hydralazine (and its interaction with nitrates) could lead to the development of more effective and/or better-tolerated drugs. Nitrate therapy is limited by the development of pharmacological tolerance, possibly secondary to the increased production of reactive oxygen species. Hydralazine co-treatment has been shown to prolong the vasodilator effect of nitrates in animal models and clinical studies, although the mechanism of this protection in humans is uncertain. There are many postulated mechanisms of the vasodilator action of hydralazine, based upon studies carried out - mostly in animals - or animal tissues. Hydralazine reduces contractile responses to a number of vasoconstrictors, and this effect appears greater in arteries than in veins. The most (though not entirely) consistent findings are those suggesting that hydralazine leads to the activation of guanylate cyclase. This action to increase intracellular cGMP, could explain the favourable clinical benefits of its combination with oral nitrates.

Hydralazine may affect a number of other vascular enzymes. These include key regulators of vascular superoxide production such as NAD(P)H oxidases. These systems are regulated *in vivo* and *ex vivo* by angiotensin-II and aldosterone, and are believed to be pivotal in the development of endothelial dysfunction, a key pathophysiological abnormality in heart failure. Renin-angiotensin system activation and oxidative stress are important (and inter-related) pathophysiological processes in heart failure and other cardiovascular problems. There is experimental evidence that hydralazine may inhibit these vascular and mitochondrial oxidases, and may also act as a radical scavenger, thus helping restore the balance between NO and superoxide in endothelial dysfunction. Inhibition of superoxide production may also help prevent nitrate tolerance: this may be critical in permitting therapeutic synergy between hydralazine and nitrates. However, the evidence emanating from different animal species is contradictory. Surprisingly, the antioxidant effect of hydralazine has never been directly characterised in human blood vessels.

In this thesis I investigated the action of hydralazine in human blood vessels. To make this project clinically relevant, I characterised the actions of hydralazine in arteries and veins of various calibre (saphenous vein and internal mammary artery taken at the time of coronary artery bypass surgery and subcutaneous resistance arteries dissected from gluteal biopsies), from patients with low ejection fraction heart failure secondary to coronary artery disease. I also investigated the purported ability of hydralazine to reduce vascular superoxide production. 40 patients undergoing elective coronary artery bypass surgery were recruited for large vessel studies and 20 patients underwent gluteal biopsy, which yielded subcutaneous resistance arteries. Vascular reactivity was assessed using organ bath techniques and wire myography with the construction of cumulative concentration response curves. Production of vascular superoxide was measured using lucigenin chemiluminescence.

Summary of results:

1. There was no direct vasodilator effect of hydralazine at therapeutic concentrations (<1 μmol/L). This suggests that the favourable benefits of hydralazine are not simply dependent on direct vasodilatation.

2. There was a modest – but not statistically significant – interaction between hydralazine and endothelium-dependent vasodilatation using carbachol. This is consistent with a trend of potential biological relevance. There was a similarly modest interaction with organic nitrates. These data are consistent with theories that the therapeutic benefits of hydralazine may be partly explained by improved endothelium-dependent vasodilatation and that the interaction with organic nitrates *in vivo* is not simply dependent on augmented vasodilatation.

3. Hydralazine reduced basal superoxide production in both internal mammary artery [1.09 ± 0.14 nmol/mg/min vs. 0.77 ± 0.16 nmol/mg/min (P=0.026) controls and pre-treated vessels respectively] and saphenous veins [0.77 ± 0.08 nmol/mg/min vs. 0.68 ± 0.08 nmol/mg/min (P=0.018) controls and pre-treated vessels respectively]. A dose-response in superoxide production in saphenous vein (which were more readily available for experimentation) was also evident.

4. Hydralazine significantly inhibited angiotensin-II mediated superoxide production in internal mammary arteries [1.68 ± 0.434 nmol/mg/min vs. 0.843 ± 0.144 nmol/mg/min (P=0.032) controls and pre-treated vessels respectively]. Angiotensin II plays a key role in the pathophysiology of heart failure, with pleotropic effects including increased vascular superoxide production through stimulation of NAD(P)H oxidase. Attenuation of angiotensin-II stimulated superoxide production by hydralazine could mechanistically be through interaction with the NAD(P)H oxidase enzyme group; supporting the best available animal data suggesting that hydralazine prevents nitrate tolerance through modulation of this enzyme group.

Appropriate recognition must be had to the limitations innate in this work and recognise that all protocols were *ex vivo* and, as such, none could accurately reflect the complex phenotype recognised in chronic heart failure. The relatively small sample sizes in the study protocols must also be given recognition; however, my group - and others - have published, scientifically meaningful results utilising similar sample sizes. Future developments ought to include larger scale bench and *in vivo* studies of hydralazine and organic nitrate interaction with particular emphasis on assessing endothelium-dependent vasodilatation. In my studies hydralazine *functionally* reduced vascular superoxide production; future studies will evaluate this *mechanistically* with particular emphasis on the NAD(P)H oxidase system.

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List of publications and presentations related to this work

Publications

Rocchiccioli JP, McMurray JJV

Optimal Therapy for Heart Failure

Supportive Care for the Cardiac Patient (Editors: Sarah Goodlin, James Beattie)

Oxford University Press 2007

Rocchiccioli JP, McMurray JJV

Epidemiology & prevention of heart failure, and treatment of asymptomatic left ventricular systolic dysfunction

Evidence Based Cardiology (Editors: Salim Yusuf, John Cairns, John Camm)

Blackwell Publishing 2009

Rocchiccioli JP, McMurray JJV

Medical management of advanced heart failure

Progress in Palliative Care 2008; 16(5): 1-8

Rocchiccioli JP, McMurray JJV, Dominiczak AF

Biomarkers in heart failure: a clinical review

Heart Failure Reviews 2007;15(4):251-73

Delles C, Dymott J, Rocchiccioli JP et al

Reduced LDL-cholesterol levels in patients with coronary artery disease are paralleled by improved endothelial function: An observational study in patients from 2003 and 2007

Atherosclerosis 2010; 211(1):271-7

Presentations

Current cholesterol lowering therapy improves endothelial function

Dymott J, Rocchiccioli JP, Chow C, Delles C, Hamilton C, Dominiczak AF

Scottish Cardiac Forum 2007

Hydralazine in heart failure: effects beyond vasodilatation

Rocchiccioli JP, Delles C, Hamilton C, Dominiczak AF, McMurray JJV

European Society of Cardiology Heart Failure Congress 2008

Hydralazine in heart failure: effects beyond vasodilatation

Rocchiccioli JP, Delles C, Hamilton C, Dominiczak AF, McMurray JJV

British Cardiovascular Society Scientific Congress 2008

Reduced vascular superoxide production in patients with coronary artery disease and type 2 diabetes

Dymott J, Oswala FO, Hamilton CA, Rocchiccioli JP, Carty D, MacArthur KJ, MacDougall J, Delles C, Dominiczak AF

Diabetic UK Meeting 2008

Diabetic Medicine 2008; 28 (suppl 1):34-162

Oxidative stress does not explain impaired endothelial function in patients with coronary artery disease and type 2 diabetes

Delles C, Dymott J, Moreno MU, Rocchiccioli JP, MacArthur KJ, Hamilton CA, Dominiczak AF

American Heart Association Scientific Sessions 2008

The mechanism of endothelial dysfunction in patients with type 2 diabetes and coronary artery disease

Delles C, Dymott J, Moreno MU, Rocchiccioli JP, MacArthur KJ, Hamilton CA, Dominiczak AF

European Society of Hypertension Meeting 2009

Declaration

The work described in this thesis was performed during my period as a clinical research fellow at the Division of Cardiovascular and Medical Sciences (now Institute of Cardiovascular and Medical Sciences), University of Glasgow.

The experimental design of the work presented in this thesis was devised by me and my supervisors, Professors John McMurray and Anna Dominiczak and Dr Christian Delles. I carried out all participant recruitment, clinical examinations, vessel collection and preparation and gluteal biopsy procedures. All experimental work was performed solely by me apart from vascular superoxide studies, which were performed with the assistance of Dr Carlene Hamilton, and initial wire myography studies, which were performed with the assistance of Ms. Angela Spiers and Ms. Elisabeth Beattie under my supervision. I undertook all statistical analyses and interpretation of results.

I confirm that this thesis has been composed by me solely and that it has not been submitted or accepted in any previous application for a degree. The writing of this thesis is entirely my own work.

All sources of information within this thesis are specifically acknowledged.

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J Paul Rocchiccioli January 2015

Acknowledgments

I would like to thank Professors John McMurray and Anna Dominiczak and Dr Christian Delles for providing me with the opportunity to undertake this research and for their supervision. My research was supported by the British Heart Foundation (BHF), by means of a Clinical Research Training Fellowship.

I would like to thank the staff at the BHF Glasgow Cardiovascular Research Centre (BHF GCRC) for their help and enthusiasm during my research. I am especially grateful to Dr Carlene Hamilton whose expertise and advice proved invaluable. The technical support of Ms. Angela Spiers and Ms. Elisabeth Beattie was crucial to this project and I would like to thank them most sincerely for their collaboration. I must also acknowledge the medical and nursing staff in the department of cardiothoracic surgery at the Western Infirmary and the Golden Jubilee National Hospital, and the nursing staff at the BHF GCRC; notably Sr. Barbara Meyer.

I am indebted to my friends and colleagues Drs Eugene Connolly, Colette Jackson and Jonathon Dalzell for their support throughout the writing of this thesis. I will forever be grateful to my parents for their love and support. I also wish to thank my friends and partner Craig, for their support and patience during this time of research and writing, and providing me with the encouragement to complete this work.

This thesis is dedicated to the staff of the haemato-oncology unit of the Beatson Cancer Centre, without whom I could not have completed this work and to my friend, colleague and mentor, the late Dr Kerry Jane Hogg.

List of Abbreviations, acronyms and symbols

A-HEFT	African-American Heart Failure Trial
ACE	Angiotensin converting enzyme
ALDH-2	Aldehyde dehydrogenase-2
Ang-II	Angiotensin-II
ARB	Angiotensin receptor blocker
ATP	Adenosine triphosphate
ATR-1	Angiotensin receptor type-1
ATR-2	Angiotensin receptor type-2
BH_3	Trihydrobiopterin
BH_4	Tetrahydrobiopterin
BHF	British Heart Foundation
BK_{Ca}	High-conductance Ca^{2+} activated K^+ channels
BP	Blood pressure
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CCRC	Cumulative concentration response curve
cGMP	Cyclic guanosine monophosphate
CICR	Caffeine-sensitive Ca^{2+} activated Ca-release channel
CRP	C-reactive protein
DBP	Diastolic blood pressure
dH_2O	Distilled water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid

EDTA	Ethylenediamine tetra-acetic acid
e-NOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
FDA	United States Federal Food and Drug Administration
GABA	Gamma-aminobutyric acid
GCRC	Glasgow Cardiovascular Research Centre
GDN	Glyceryl-dinitrate
GRACE	Global Risk Assessment of Cardiac Events
GRAHF	Genetic Risk Assessment and Heart Failure
GSH	Reduced glutathione
GSSH	Oxidised glutathione
GTN	Glyceryl-trinitrate
H-ISDN	Hydralazine/Isosorbide-dinitrate combination
H_2O_2	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia inducible factor
ID	Internal diameter
IMA	Internal mammary artery
IP_3	Inositol 1, 4, 5 triphosphate
ISDN	Isosorbide-dinitrate
ISMN	Isosorbide-mononitrate
kg	Kilograms
KPSS	High potassium concentration physiological saline solution
KRH	Krebs-Ringer HEPES buffer
L	Litre

L-NAME	N^G-nitro-L-argininemethyl ester
L-NOARG	N^G-nitro-L-arginine
LVEF	Left ventricular ejection fraction
LVSD	Left ventricular systolic dysfunction
MI	Myocardial infarction
µm	Micrometers
µmol	Micromoles
mm	Millimetres
mmHg	Millimetres of mercury
NA	Noradrenaline
NAD(P)H	nicotinamide adenine (phosphate) dinucleotide
NADH	nicotinamide adenine dinucleotide
nmol	Nanomoles
NNT	Number needed to treat
NO	Nitric oxide
NOS	Nitric oxide synthase
NOS3	Nitric oxide synthase 3 (endothelial nitric oxide synthase)
NOX	nicotinamide adenine dinucleotide phosphate oxidase
NS	Not significant
NT-proBNP	N-terminal pro-B-natriuretic peptide
NYHA	New York Heart Association
O_2-	Superoxide
PE	Phenylephrine
PEDN	Pentaerythrityl-dinitrate
PETN	Pentaerythrityl-tetranitrate

PETriN	Pentaerythrityl-trinitrate
PHD	Prolyl hydroxylase domain
phox	Phagocyte oxidase
REACH	Resource Utilization Among Congestive Heart Failure study
ROS	Reactive oxygen species
RRR	Relative risk reduction
RyR2	Cardiac ryanodine receptor
SBP	Systolic blood pressure
SEM	Standard error of the mean
sGC	Soluble guanylate cyclase
SIN-1	3- morpholino-sydnonimine
SNP	Sodium nitroprusside
SR	Sarcoplasmic reticulum
SRAs	Subcutaneous resistance arteries
SSAO	Semicarbazide-sensitive amine oxidase
SV	Saphenous vein
TEA	Tetraehtylammonium
UK	United Kingdom
US	United States
V-HeFT-1	Vasodilator in heart failure study-1
V-HeFT-2	Vasodilator in heart failure study-2
VASCAB	Vascular Function in Coronary Artery Bypass study
VEGF	Vascular endothelial growth factor
VO_2	Peak oxygen consumption
VSMC	Vascular smooth muscle cell

Chapter 1 - Introduction

1.1 Heart Failure

Heart failure is a progressive, debilitating disorder affecting approximately 2-5% of the adult population of the developed World and is associated with considerable mortality and morbidity(1). Heart failure impacts on the quality and duration of life and places considerable economic burden on our healthcare systems(2). Whilst age-adjusted incidence appears to be stable, prevalence is thought to be increasing, principally as a consequence of an ageing population and improved survival from coronary artery disease. Our greater appreciation of the pathophysiology and natural history of heart failure has allowed development of targeted therapy to achieve symptom control, reduce hospital admissions and prolong life. On the basis of large, randomised controlled trials, drugs are the established mainstay of treatment. The resulting benefits of these developments appear to have been translated to the greater unselected population with observational studies indicating improvements in outcome, which temporally correlate with the emergence of evidenced-based therapy(3).

1.1.2 Definition of heart failure

Heart failure is a term used to describe a commonly observed clinical syndrome resulting from impaired cardiac function. A systemic disease, never occurring in isolation, heart failure is often the terminal manifestation of a legion of cardiovascular and non-cardiovascular conditions, characterised by maladaptive physiological responses including neurohormonal activation, low grade inflammation and molecular adaptations resulting in progressive impaired cardiac performance(4). Contemporary clinical practice guidelines require the presence of symptoms and signs of the heart failure syndrome along with objective evidence of cardiac dysfunction(5). Many of these symptoms and signs are relatively non-specific and do not arise as a direct consequence of the underlying mechanical cardiac disruption, but through secondary dysfunction in other organ systems. Our understanding of the pathophysiology of heart failure has expanded from a simple haemodynamic model to that of a complex multi-system syndrome(6).

1.1.3 Epidemiology of heart failure

Despite the considerable health-economic burden of heart failure, its epidemiology is still poorly defined, especially in primary care(1). In general terms, contemporary analyses can be divided into those examining the prevalence and incidence of *symptomatic* heart failure (some of whom may have preserved left ventricular systolic function) and those investigating the prevalence of left ventricular systolic dysfunction (LVSD) (of which a significant proportion of patients will be asymptomatic). These contrasting approaches explain the epidemiological inconsistencies in observational studies. Ideally, estimates of heart failure epidemiology would emanate from surveys of random samples of the general population, using validated questionnaires and targeted physical examinations, in conjunction with objective measures of LVSD such as imaging, possibly supported by validated biomarkers i.e., B-type natriuretic peptides(7).

1.1.4 Prevalence of heart failure

Epidemiological studies utilising a range of designs suggest that the prevalence of heart failure occurs in around 2-5% of the population in the developed world, increasing considerably with age(1). Prevalence varies widely from 0.4% to 19% in older age groups based on general practice studies in the UK(8, 9). This trend is also observed in landmark population-based cohort studies such as the Framingham study where prevalence of heart failure in 50-59 year olds was 0.8% in contrast to 9.1% in those above 80 years of age and the European Rotterdam study where overall prevalence was 3.7% increasing to 13.0% in those over 85 years(10, 11). In Scotland, a national primary care survey estimated the prevalence of heart failure to be 7.1 per 1000 population, increasing with age to 90.1 per 1000 patients in the very elderly (>85 year of age)(12). All the foregoing data is supported by contemporary studies across the developed world(13-15).

1.1.5 Incidence of heart failure

The incidence of heart failure is more difficult to define, but there are considerable data available, particularly from large population-based studies. In the Framingham Heart study, at 34 years follow-up, incidence was approximately 2/1000 person-years in subjects aged 45-54, increasing to 40/1000 in men aged 85-94(10). Similar patterns were reported in the Olmsted County Study and UK and Finnish population studies(16-18). A prospective cohort study undertaken in the UK identified new cases from a population of 151 000 in London, through the surveillance of hospital admissions and referrals to a rapid-access specialist heart failure clinic(19). Diagnosis of heart failure was confirmed by a panel of cardiologists and supported by echocardiography. Incidence was 1.3/1000 overall for those over the age of 25 years. Incidence increased with age and was higher in men than women. Whilst prevalence is thought to be increasing, age-adjusted incidence is stable. The Resource Utilization Among Congestive Heart Failure (REACH) study retrospectively examined incidence in hospitals in Michigan USA over a 10-year period; the incidence of heart failure in 1999 was 3.7/1000 person-years in men and 4.2/100 person-years in women of all ages with no changes between 1989 and 1999(20). Overall, in Europe and North America, the lifetime risk of developing heart failure is approximately one in five for a 40-year old(21, 22).

1.1.6 Prognosis of heart failure

Heart failure prognosis remains poor, despite considerable therapeutic advances. Population data suggest that heart failure mortality is comparable to that of cancer. In the Framingham Heart study, 5-year mortality was as high as 75% in men(23). Mortality rates in women were slightly more favourable at 46% and 62% respectively. Similarly in a UK study, 1- and 5-year mortality following an index admission was 43% and 73% respectively; risk proportionate to increasing ages(24). In the Rotterdam study, survival rates for *prevalent* heart failure were more favourable, with 1- and 5-year survival rates of 89% and 59% respectively(25). This still represents a threefold increase in the age and gender matched population risk. In the Olmsted County Study, age-adjusted 5-year survival improved from 43% in 1979-84 to 52% in 1990-2000(26). This leads to the conclusion that prognosis does appear to be improving. Recent Scottish data suggest a sustained improvement in age-adjusted survival following first heart failure admission, which temporally correlates to emergence and uptake of effective evidence based therapies **(figures 1-1, 1-2)**(3).

Figure 1.1: Adjusted 30-day (A), 1-year (B) and 5-year (C) mortality according to sex and year of admission (from Jhund et al (3)).

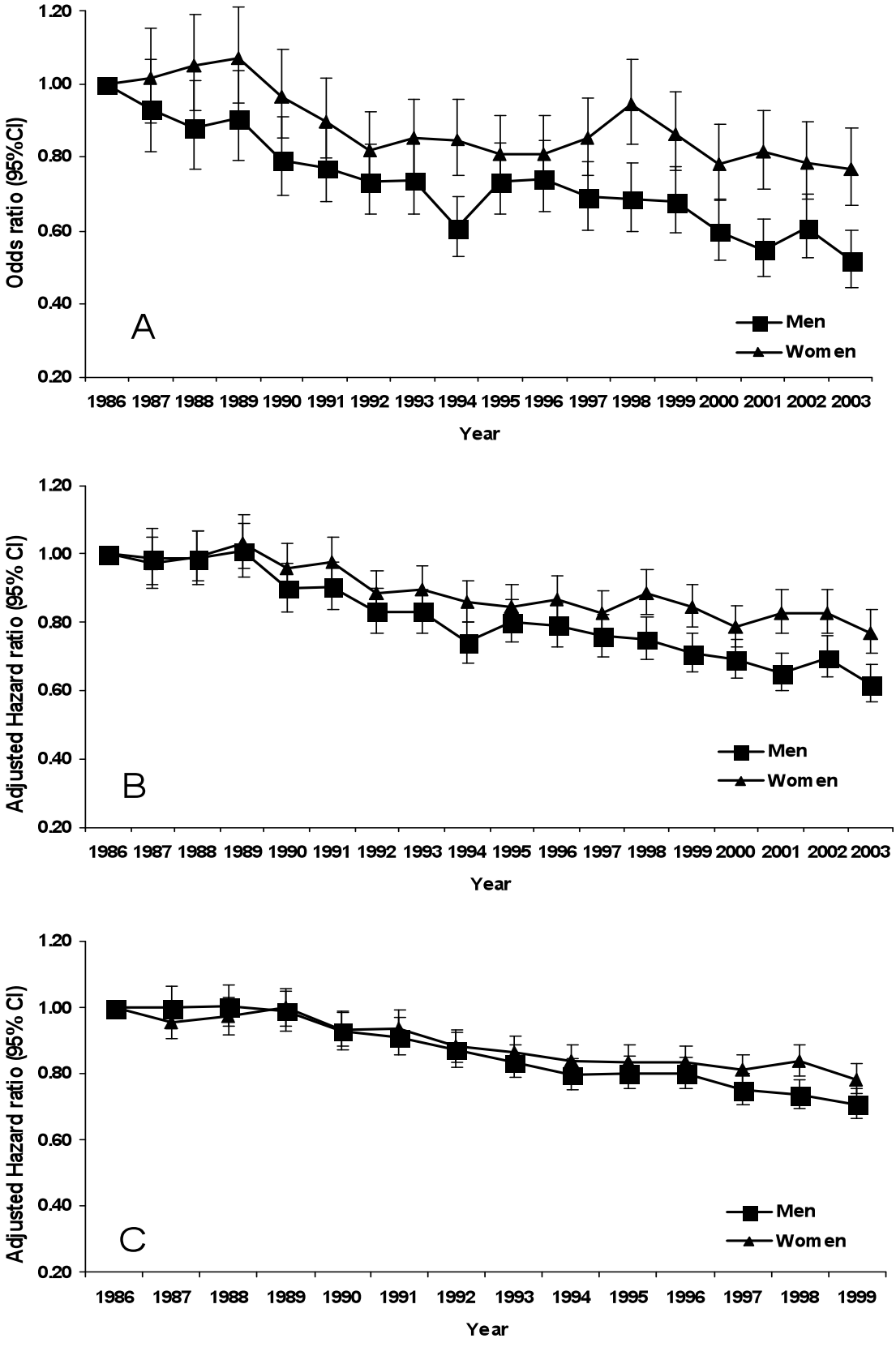
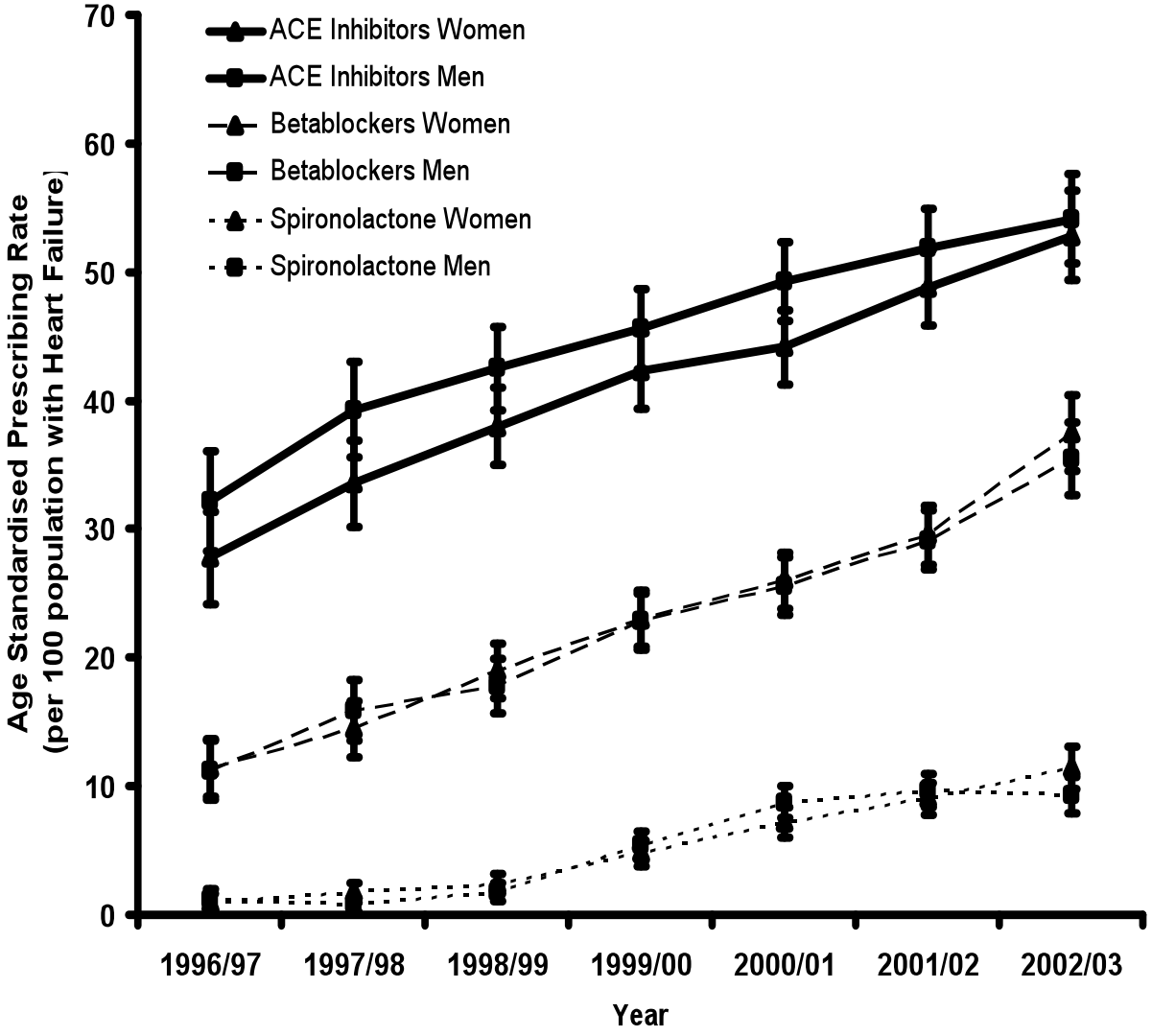


Figure 1-2: Age-adjusted trends in prescribing rates for ACE inhibitors, beta-blockers, and spironolactone in patients with HF in primary care (from Jhund et al)(3).



1.1.6 Aetiology and pathophysiology of heart failure

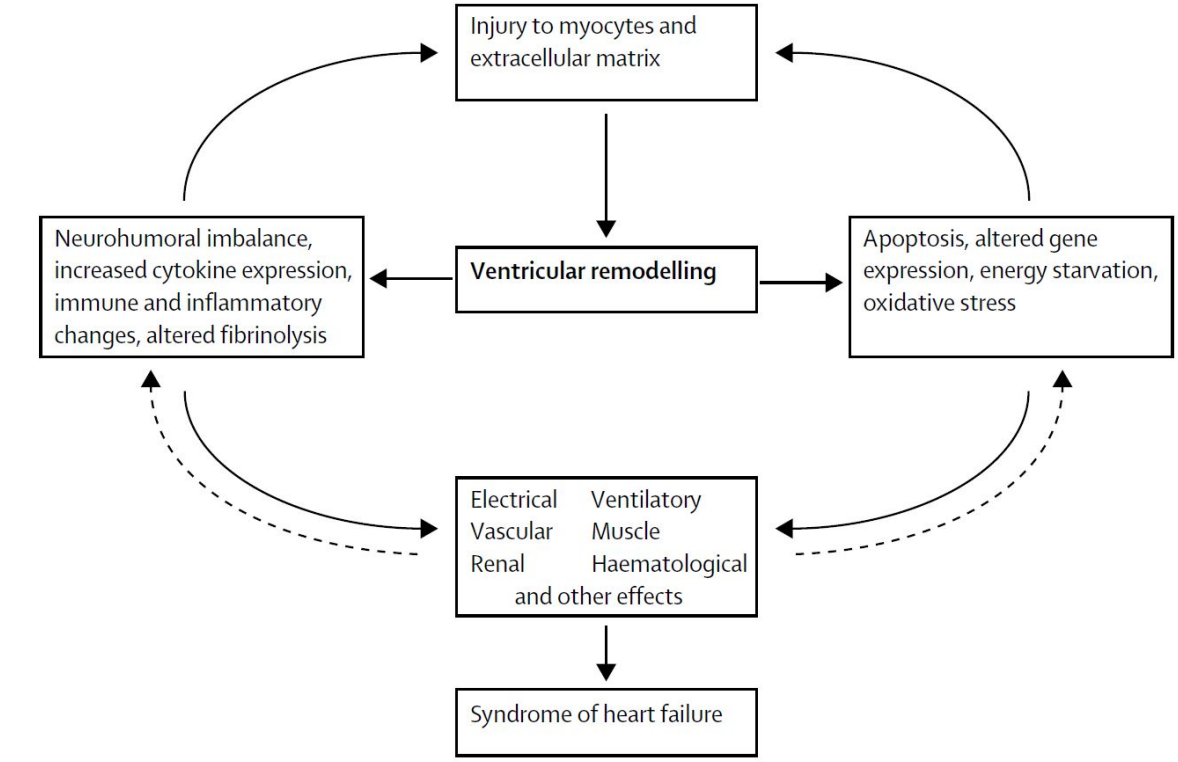
Heart failure never occurs in isolation.

Any structural, mechanical or electrical abnormality of the heart can lead to dysfunction and a cascade of events leading to heart failure syndrome. As such, heart failure is a classic illustration of the cardiovascular disease continuum, whereby multiple, overlapping mechanisms are involved in disease progression(4). LVSD develops gradually, often beginning with an index event - or injury (such as myocardial infarction) - leading to progressive loss of functioning myocytes and consequent maladaptive ventricular remodelling. This remodelling process may persist despite further discrete injury and is accelerated by concomitant risk factors such as hypertension, diabetes mellitus, cigarette smoking and elevated cholesterol levels.

In the developed world, coronary artery disease, either alone or in combination with hypertension appears to be the dominant cause of heart failure(1, 27). In a patient with multiple risk factors the definitive aetiology may not be immediately apparent. The use of adjunctive diagnostic tools including nuclear perfusion imaging or cardiac catheterisation may help determine precise aetiology(27). Heart failure aetiology varies geographically and has varied over time with a shift in relative contribution of hypertension and rheumatic valvular disease towards coronary artery disease. Whilst coronary artery disease is a more dominant individual risk factor than hypertension, the population-attributable risk for the latter is still more influential(28, 29). Data describing the epidemiology of heart failure in the developing world are scarcer; endocardial diseases, trypanosomal infections and rheumatic heart disease are undoubtedly more prevalent. That said, the epidemiological transition to a more “western” lifestyle, atherosclerotic coronary artery disease is rapidly emerging as a dominant player(30).

The progression of LVSD through maladaptive remodelling is a complex multi-system process involving local recurrent injury, autocrine processes and molecular adaptations, enhanced apoptosis and systemic processes, including neurohormonal activation and increased oxidative stress. This myriad of insults leads to progressive structural and functional changes in the heart leading to both electrical and mechanical dysfunction **(Figure 1-3)**(6).

Figure 1-3: Pathophysiology of heart failure as a result of left-ventricular systolic dysfunction. Reproduced with permission from McMurray JJ et al(31).



1.1.7 Abnormalities of haemodynamics and vascular function in heart failure

Symptomatic heart failure with reduced ejection fraction is characterised by the development of progressive cardiac dysfunction with concomitant functional abnormality of other tissues and organs. These processes are a consequence of local - autocrine and molecular adaptation - and systemic events such as neurohormonal activation, sympathetic nervous system activation and inflammation(31). Left ventricular systolic dysfunction progresses gradually, often beginning with an index myocardial injury such as acute myocardial infarction. This leads to a progressive loss of functioning myocytes. Loss of cardiac function occurs as a product of ventricular remodelling, through which ventricular geometry, dimensions and function are altered. Remodelling consists of a multitude of maladaptive pathophysiological processes including myocyte hypertrophy, necrosis and apoptosis and myocardial interstitial fibrosis and is exacerbated by activation of neurohormonal and inflammatory pathways(4, 32). The remodelling process may persist despite any further discrete myocardial injury. Many of these systems act synergistically, reinforcing each other.

The Frank-Starling law describes the intrinsic attempt to maintain stroke volume during acute cardiac injury(33). This adaptative phenomenon is evident early after index injury. Reduction in stroke volume leads to elevated left ventricular end-diastolic pressure and dimension. This in turn leads to increased force of ventricular contraction, thus helping to maintain cardiac output. This is characteristic of the law of heterometric autoregulation – stretch of the cardiac myocytes leads to increased force of contraction. In addition to structural and molecular abnormalities, retention of water and sodium in the vasculature and venous vasoconstriction - through extrinsic neurohormonal activation – increase preload in an attempt to maintain left ventricular filling pressure. The same extrinsic processes promote arterial stiffness leading to increased afterload and progressive ventricular dysfunction(34).

Heart failure is associated with chronic peripheral vasoconstriction – both venous and arterial – through sympathetic nervous system activation, neurohormonal activation and endothelial dysfunction(35-37). This contributes to reduced tissue perfusion, impaired pulmonary vasodilatation and resultant reduction in exercise capacity. Chronic hypo-perfusion promotes skeletal muscle ischaemia leading to inflammation and imbalance of reactive oxygen species. Endothelial dysfunction – characterised by reduced bioavailability of NO and enhanced vasoconstriction in response to exercise – is described in all vascular beds(37, 38). It is proportionate to severity of heart failure and predictive of adverse events(39, 40). Direct *in vivo* measurement of NO bioavailability in humans is difficult; as such vasodilator activity such as flow-mediated dilatation, laser Doppler imaging and quantification of NO-related compounds are measured as surrogates(41, 42). Vascular dysfunction is not restricted to the endothelium; investigators have demonstrated impaired micro-vascular responses to endothelium-dependent and independent vasodilators in patients with heart failure(43, 44).

Few studies have directly addressed regional and organ haemodynamics in chronic heart failure(45, 46). The lack of experimental data reflects technical limitations in the study of regional haemodynamics - particularly the need for invasive procedures. Most studies have been performed by means of indirect techniques such as venous occlusion plethysmography and radioisotope clearance studies(38). Resistance to blood flow in any tissue is directly related to vascular smooth muscle tone and both intrinsic and extrinsic stressors such as vascular remodelling and neurohormonal activation(47). Reduction in cardiac output is accompanied by reduced blood flow to most regions. Renal function tends to fall in direct proportion to cardiac output but at extreme levels appears to plateau through protective autoregulation(46). Conversely, hepatosplanchnic blood flow is strongly correlated to cardiac output but not protected by autoregulatory mechanisms. Skeletal muscle blood flow is similarly proportionate to cardiac output; thus contributing to fatigue and exercise intolerance typical of the heart failure syndrome(48).

Augmenting ventricular systolic function or regional haemodynamics would appear to be an attractive therapeutic strategy but is limited in evidence-base and largely restricted in clinical practice to acute decompensated heart failure with hypoperfusion. Choice of agents includes sympathomimetics (*e.g*. dopamine, dobutamine, and epinephrine), phosphodiesterase inhibitors (*e.g*. milrinone, enoximone) or calcium sensitizers (*e.g*. levosimendan). Although effective in enhancing contractility, these agents (particularly sympathomimetics) have the unwanted effects of increasing myocardial oxygen consumption, promoting myocyte calcium loading and accelerating cell death, with the net effect of inducing maladaptive remodelling and promoting tachyarrhythmia. In fact, there is concern that inotropic therapy (particularly when administered chronically) may increase mortality in heart failure(49-51).

Vasodilator therapy can influence central and regional haemodynamics in heart failure. Hydralazine and the alpha-adrenoreceptor antagonist prazosin significantly augment cardiac index and stroke volume whilst reducing pulmonary artery capillary pressure (a measure of left ventricular filling pressure) in patients with chronic heart failure(52). Both hydralazine and prazosin elicit significant improvements in resting forearm blood flow whereas hydralazine alone reduced renal vascular resistance with concomitant increase in renal blood flow. These and other data suggest sustained haemodynamic effects may be seen with chronic vasodilator – particularly hydralazine – therapy(53, 54). Exercise capacity – measured by peak oxygen consumption (VO_2) is strongly correlated to cardiac output. Nevertheless selectively augmenting regional organ haemodynamics and cardiac output neither improves outcome or symptoms (such as exercise intolerance) In a small study in patients with heart failure, hydralazine increased maximal exercise ± 105 versus 779 ± 82 ml/min) but had no effect on peak VO_2(55). Based on experience from clinical trials there is clear dissociation between haemodynamics and other pathophysiological concepts in heart failure(56). Haemodynamic variables are not adequate surrogate end points for symptoms or outcome(57). Indeed some positively inotropic medications, which result in marked improvements in haemodynamic parameters, are associated with harm.

1.1.8 Oxidative stress in heart failure

There is an increasing body of evidence suggesting that oxidative stress is involved in the pathogenesis of heart failure(4). Heart failure is characterised by the activation of a cascade of processes resulting in an imbalance between bio-available nitric oxide and harmful reactive oxygen species (ROS) (the so-called nitroso-redox imbalance). These pro-oxidant processes include pro-inflammatory cytokine activation, mitochondrial dysfunction, recurrent hypoxia and activation of the renin-angiotensin system associated with increased activity of NAD(P)H oxidase in blood vessels, largely through the effects of angiotensin-II(33). Other sources of enhanced ROS generation include *NOS3* itself and xanthine oxidase(58, 59). The pro-oxidant state contributes to myocyte apoptosis and necrosis, endothelial dysfunction and remodelling.

Several markers of ROS burden are elevated in heart failure including: urinary biopyrrins (derived from the oxidative metabolism of bilirubin); urinary isoprostanes, 8-epi prostaglandin-F-α and plasma malonyldialdehyde (markers of lipid peroxidation), and plasma reduced (GSH) and oxidised glutathione (GSSH) (60-63). Elevated markers of ROS also contribute to the severity of myocardial dysfunction. In addition, urinary byopyrrins have been found to be elevated in proportion to severity of LVSD and NYHA functional class(60). Similarly, other markers of oxidative stress have been shown to be elevated in proportion to the severity of heart failure and positively correlate with markers of neurohormonal activation and inflammation(62, 63). However these are global measurements of oxidative stress, which may not always detect localised changes within the heart. Hyperuricaemia, as a consequence of increased activity of xanthine oxidase (a critical component of nitroso-redox balance) is a biomarker of oxidative stress in heart failure(59). Levels are a marker of deranged oxidative metabolism and influenced by hyperinsulinaemia, inflammatory cytokine activation and endothelial dysfunction; all of which are present in heart failure. It is debatable whether uric acid merely reflects the degree of immune activation (a so called ‘danger signal’) and tissue damage or has itself, a direct pathophysiological role. Nevertheless hyperuricaemia appears to have prognostic relevance(64-66).

Inhibition of xanthine oxidase is an attractive target in addressing nitroso-redox balance in cardiovascular disease. In a small randomised-controlled trial of 66 patients with IHD and left ventricular hypertrophy, high dose (600mg per day) allopurinol regressed LV mass with a parallel improvement in endothelial function assessed by flow-mediated dilatation(67). Allopurinol (at a more conventional dose of 300mg per day) improved endothelial function – assessed by forearm venous occlusion plethysmography in a group of 11 patients with heart failure(68). A subsequent publication by the same group demonstrated a dose-response relationship between allopurinol and its effects on endothelial dysfunction(69). Translation of these surrogate markers to tangible clinical improvements has been less clearly defined. The primary metabolite of allopurinol is oxypurinol. The Oxypurinol Compared With Placebo for Class III to IV New York Heart Association Congestive Heart Failure (OPT-CHF) Trial tested whether oxypurinol produces clinical benefits in patients with moderate-severe heart failure(70). 405 patients treated with optimal medical therapy (including a beta-blocker in 92%) were randomized to receive oxypurinol 600 mg once daily or placebo for 24 weeks. Efficacy was assessed using a composite end point comprising HF morbidity, mortality, and quality of life. The primary endpoint was not met. The absence of clinical efficacy may relate to the low dose. The 600 mg dose of oxypurinol has a relative bioavailability equivalent to just 81 mg of allopurinol(71). In hypothesis-generating sub-group analyses those patients with highest baseline levels of uric acid appeared to receive clinical benefit.

The benefits of neurohormonal blockade with ACE inhibitors, angiotensin-II-receptor blockers and beta-blockers are well described(5). A component of this benefit may be derived from addressing the associated nitroso-redox imbalance. The magnitude of said benefit remains uncertain. In animal models of heart failure, cardiac protection is observed with anti-oxidant treatment(72, 73). However, in human clinical studies, the evidence is less compelling. Whilst short-term treatment with inotropic support improves markers of oxidative stress in parallel with inflammatory indices, this contrasts with a recent study of the optimal combination of an angiotensin-II-receptor blocker with an ACE inhibitor and beta-blocker in chronic heart failure(74, 75). Although this combination significantly decreased validated heart failure biomarkers NT-proBNP (N-terminal pro B-natriuretic peptide) and CRP (C-reactive protein), there was no effect on markers of oxidative stress. This potentially indicates that other mechanisms, independent of the renin-angiotensin system driving the pro-oxidative state, could be applicable therapeutic targets.

1.2 Hydralazine: clinical efficacy

1.2.1 Historical background and medical use

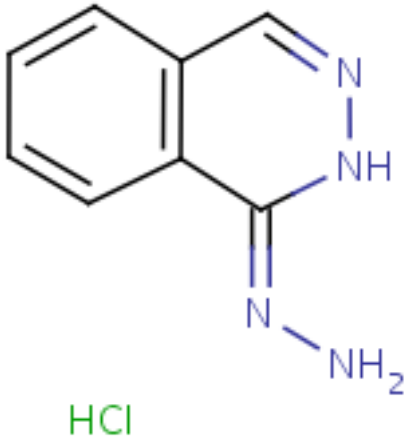
Hydralazine is part of the imadazoline family of compounds, discovered by Alfred Ladenburg in 1888 at the University of Breslau and first purported as an anti-hypertensive and vasodilator agent in 1951 in work by Ciba Laboratories(76). Hydralazine is used to treat severe hypertension and in particular, pregnancy-associated hypertension(77). It is not considered a first line drug for treating hypertension because it causes reflex sympathetic activation - through the baroreceptor reflex – leading to an unfavourable rise in heart rate and cardiac output (which may lead to myocardial ischaemia in patients with coronary artery disease). Treatment with hydralazine may also increase plasma renin concentration, leading to fluid retention. Hydralazine has also been recently used as a treatment for myeloproliferative conditions, including chronic myeloid leukaemia, through its capacity as a DNA methyltransferase inhibitor (which may also potentiate drug-induced lupus)(78).

1.2.2 Biochemistry

Hydrazine [1-hydrazinophthaline] hydrochloride **(figure 1-4)** is a synthetic compound prepared by the action of hydrazine hydrate on 1-chloro or 1-phenoxypthalazine. Its bioavailability is variable ranging from 50-90% of a single oral dose. Depending on the dose, peak plasma levels occur from 0.3-1.0h after administration(79, 80). Hydralazine is well absorbed through the gastrointestinal tract and undergoes first pass metabolism, which is determined by the acetylator phenotype. As such, different bioavailability patterns are expected: most notably greater in slow acetylators than fast acetylators. The prevalence of the slow-acetylator phenotype among American and European Caucasians and African Americans is around 50%(81). Because the acetylated compound is inactive, the dose necessary to produce a systemic effect is higher in fast acetylators. N-acetylation of hydralazine occurs in the bowel and/or the liver. The half-life of hydralazine is 1 hour and its systemic clearance is approximately 50ml/kg/min.

Hydralazine rapidly combines with circulating α-keto-acid to form hydrazones; the major metabolite recovered from plasma is hydralazine-pyruvic-acid-hydrazone. This metabolite possesses a longer half-life than hydralazine but does not appear to be active. Hydralazine's peak concentration in plasma and peak effects occurs within 30-120 minutes following administration. Although its half-life is approximately 1 hour, the duration of its effect can last for 12 hours. After stabilisation with multiple daily doses, a twice-daily dose regimen can be effective. Slow acetylators require a lower dose. For heart failure, the recommended doses are higher(82).

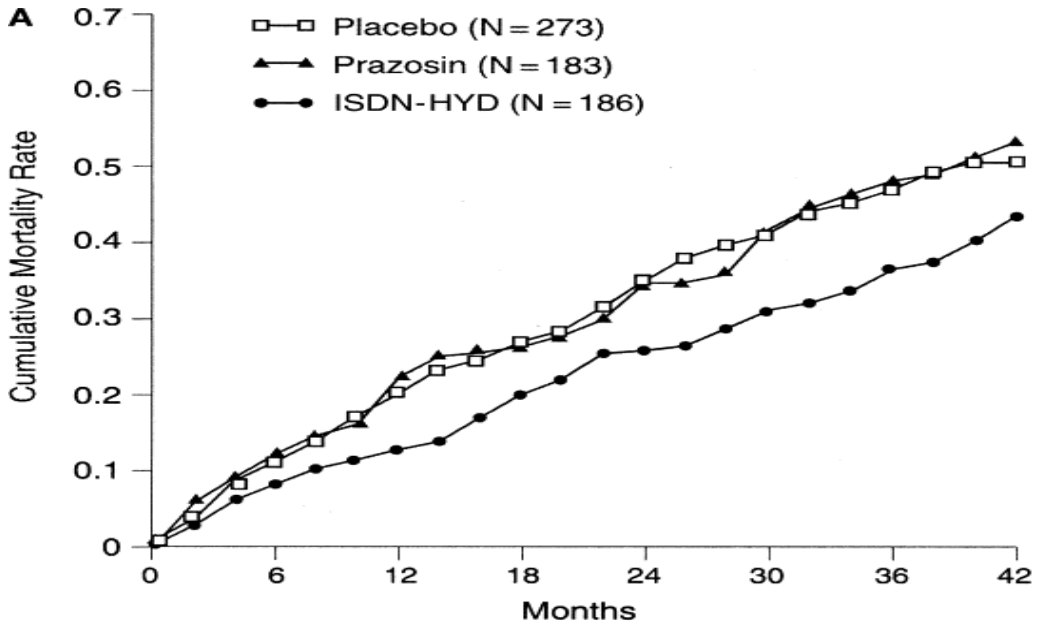
Figure 1-4: chemical structure of hydralazine hydrochloride.



1.2.2 The V-HeFT studies

In 1986 the vasodilator in heart failure (V-HeFT-I) study was the first placebo-controlled clinical trial to study the effect of vasodilator therapy on survival in patients with chronic heart failure and was, in fact, the first therapeutic approach shown to improve heart failure survival(83). This study randomised 642 men with mild to moderate heart failure to placebo, the alpha-adrenoreceptor antagonist prazosin or to the combination of hydralazine and isosorbide dinitrate (ISDN) added to standard therapy (at that time) with a diuretic and digoxin. These patients were treated at a time when the benefits of angiotensin-converting enzyme (ACE) inhibitors, beta-blockers and mineralocorticoid-receptor antagonists were not known. Two years after randomisation, survival in the hydralazine-ISDN group was significantly enhanced than in the placebo group (P =0.028) (**Figure 1-5)**. Hydralazine-ISDN also increased exercise capacity and left ventricular ejection fraction compared to the placebo group. Interestingly, these benefits were despite the fact the prazosin had a greater BP lowering effect and was one of the first indicators that the clinical benefits of hydralazine might exceed simply that of BP reduction. Mortality in the prazosin group was not different from the placebo group.

Figure 1-5: Kaplan-Meier survival curves from Ve-HeFT-I study. Reproduced with permission from Cohn JN et al(83).



The second V-HeFT-II study compared the efficacy of hydralazine-ISDN with that of the angiotensin-converting-enzyme inhibitor enalapril(84). 804 men in NYHA II-III functional status were randomised to hydralazine-ISDN or enalapril in addition to standard therapy with a diuretic and digoxin. Two years after randomisation all-cause mortality was 18% in the enalapril group as compared with 25% in the hydralazine-ISDN group (P=0.016). A clear early difference was observed, with superiority in the enalapril arm, which produced a 27% relative risk reduction in mortality (attributed to a reduction in sudden cardiac death). A similar 2-year mortality in the H-ISDN group (25%) compared with Ve-HeFT-I indicated that the patients involved were just as sick. Because this drug combination was shown to be less effective than an ACE inhibitor it was little used.

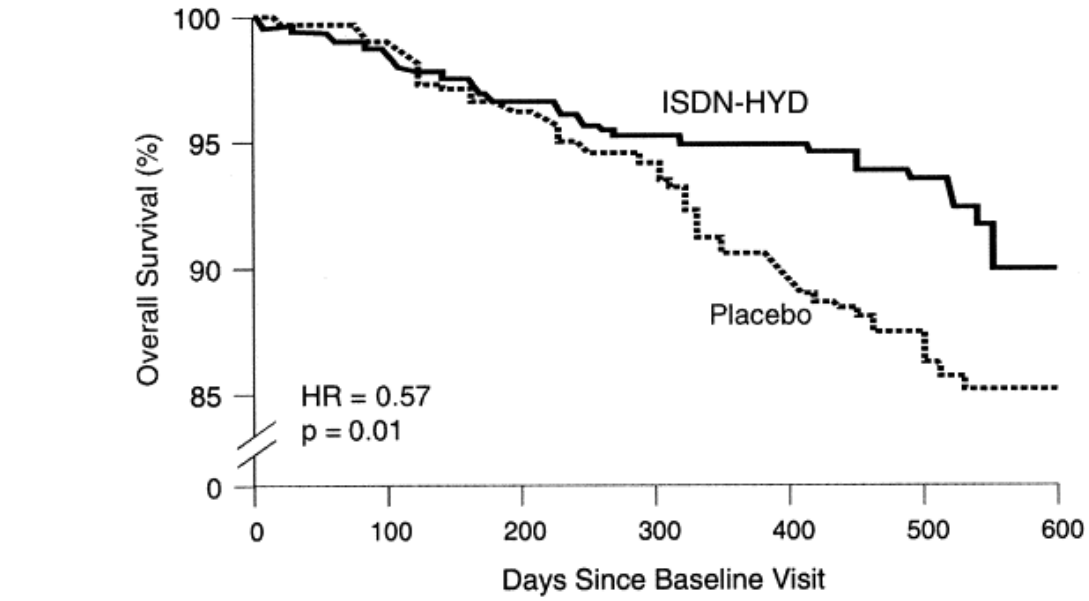
1.2.3 The A-HeFT study

Retrospective analyses of both V-HeFT-I and V-HeFT-II suggested that African-Americans derived benefit from the hydralazine-ISDN combination whereas Caucasians did not(85, 86). Conceptually, this analysis was based on a series of observations that suggested a relative deficiency of nitric oxide in African Americans, though the exact nature of the defect(s) has not been established. The biological plausibility of inter-racial differences in drug efficacy is supported by epidemiological and clinical trial data, particularly in hypertension. Cardiovascular disease is the leading cause of death among African Americans, and the incidence (and mortality) of both IHD and stroke is higher compared to Caucasian Americans(87). African Americans are more likely to develop hypertension earlier in their lifetime and have a more severe phenotype. Complex environmental and epigenetic interactions may explain this phenomenon, particularly socio-economic deprivation. Nevertheless, there are robust data demonstrating variations in the renin-angiotensin system in African American patients and clinical efficacy of anti-hypertensive agents(88-90). In addition to variations in endothelial function and nitric oxide-mediated vascular responses, African American patients have a less significant blood pressure response to renin-angiotensin inhibitors and beta-adrenoreceptor antagonists(90). Contemporary clinical practice guidelines reflect these data and recommend calcium-channel antagonists and thiazide diuretics as first line therapy(91).

The African-American Heart Failure (A-HeFT) trial was undertaken and randomised 1050 patients who were NYHA class III and IV to receive a fixed dose combination of hydralazine-ISDN three times daily(86). Critically, in this trial, the patients were already treated with the optimal currently available drug therapy, including an ACE inhibitor (70%), beta-blocker (74%), and, in many cases, a mineralocorticoid-receptor antagonist (39%). The initial dose of treatment was 20mg ISDN/37.5mg hydralazine administered three times daily, increased to a target of 40mg/75mg. However, the trial was discontinued prematurely at a median follow-up of 10 months following a significant reduction in all-cause mortality (RRR 43%, ARR 4.0% NNT 25, *P*=0.01) (**Figure 1-6)**. A composite endpoint was used, combining mortality, quality of life (as measured on the Minnesota Living with Heart Failure Questionnaire) and time to first hospitalisation; each component was statistically significant in favour of the combination therapy. This fixed-dose combination is approved by the US Food and Drug Administration (FDA) and specifically licensed in this heart failure indication as BiDil©, produced by Arbor Pharmaceuticals Inc(92).

The magnitude of these defects parallels – on in some cases – exceeded almost all other double-blind placebo-controlled trials in heart failure. However, whilst providing an opportunity to advance medical therapy for heart failure, the controversial approval based on race by the US FDA has negatively impacted adoption by practitioners. Similarly, the relatively small study size, the very selected population studied and the trial's early termination have left some uncertainty about the value of this combination therapy, especially in non African-American patients. Nevertheless, it is widely believed that these incremental benefits could be achieved in patients of other ethnicities who remain symptomatic despite optimal neurohormonal therapy. Although the precise mechanism of action is largely unexplained, the combination of hydralazine-ISDN results in vasodilatation through increased production of endothelial nitric oxide and has thus been considered a "nitric oxide enhancing" therapy. This approach may provide incremental benefits in patients already receiving standard neurohormonal therapies by producing further vasodilatation and directly targeting endothelial dysfunction, a hallmark of the heart failure syndrome(93).

Figure 1-6: Kaplan-Meier survival curves comparing isosorbide dinitrate (ISDN)-hydralazine (HYD) combination with placebo in A-HeFT study. Reproduced with permission from Taylor et al(94).



No. at Risk							
Placebo	532	466	401	340	285	232	24
ISDN-HYD	518	463	407	359	313	251	13

1.2.4 Contemporary clinical practice guidelines

Current European guidelines recommend that other than for African Americans, the main indication for hydralazine-ISDN is as a *substitute* in patients with intolerance to an ACE inhibitor or an angiotensin-receptor antagonist (ARB) (Class IIb, level of evidence B)(5). Hydralazine-ISDN should also be considered as an *additional* treatment in African Americans and considered on an empirical basis for other patients who remain symptomatic on other proven therapies (including a mineralocorticoid receptor antagonist) (Class IIb, level of evidence B). Neither drug on its own has been demonstrated to be beneficial in heart failure. The main dose-limiting adverse effects are headache and dizziness. A rare adverse effect of higher doses of hydralazine, especially in slow acetylators, is a systemic lupus erythematosus-like syndrome, which is likely to result from inhibition of DNA methylation(95).

1.3 Mechanism of action of hydralazine: current knowledge

Hydralazine is a potent vasodilator that has been in clinical use for nearly six decades. Despite that, its mechanism of action remains poorly understood. Hydralazine appears to act as a dominant arterial vasodilator with potential mild inotropic properties, which is thought to be due to reflect activation of the sympathetic nervous systems(54, 96). This inotropic action might be responsible for a less favourable effect on myocardial oxygen consumption thereby counteracting the unloading effects of vasodilatation(97, 98). Most evidence suggests an intracellular mechanism, possibly with involvement of altered calcium balance in vascular smooth muscle cells by inhibition of calcium release from the sarcoplasmic reticulum. This may be secondary to inhibition of membrane ATPases, kinases or ion channels or a membrane hyperpolarisation effect(99-101). The scarce functional studies undertaken in human blood vessels indicate a dominant arterial effect but do not provide mechanistic insight(102).

Hydralazine preferentially decreases vascular resistance in the coronary, cerebral and renal circulation with a smaller effect in skin and muscle. It does not appear to utilise established vasodilator mechanisms such as alpha-adrenoceptor antagonism or calcium entry blockade. It appears to have a direct action on vascular smooth muscles, which may not be endothelium dependent. Nevertheless, its action may be potentiated by the presence of endothelium in some models suggesting a further indirect effect on smooth muscle(103). In addition to the vasodilator role of hydralazine, it has been shown to prolong the effects of ISDN in experimental and clinical models(104, 105). Hydralazine appears to be an effective antioxidant and, by reducing antioxidant stress, protects against nitric oxide degradation(106). It thus may have beneficial effects in states where endothelial dysfunction predominates.

Identification of the mechanism of action of hydralazine in human blood vessels may allow the design of drugs with a comparable prognostic benefit which avoiding its documented adverse effects. Studies to date have failed to produce conclusive evidence of the mechanism of action of hydralazine. The variety of animal models used in the literature and the varying techniques used could explain this disparity. There have been no comprehensive investigations of the commonly examines vasodilator systems in human blood vessels, particularly in the contemporary era.

1.3.1 Role of potassium channels

Membrane hyperpolarisation due to activation of K^+ channels is a recognised important mode of action for several vasodilators including synthetic openers of ATP-sensitive K^+ channels such as cromakalim, pinacadil and minoxidil(107). Hydralazine has been reported to produce membrane hyperpolarisation in isolated rabbit femoral arteries(101, 108). In these studies, hydralazine preferentially relaxed contractions induced by moderately raised K^+ (20mM) compared with those induced by highly elevated K^+ (124mM). This effect profile is characteristic of drugs acting by K^+ channel opening and associated membrane hyperpolarisation(109). Conversely, the anti-diabetic drug glibenclamide, which is an effective blocker of ATP-sensitive K^+ channels, has failed to influence hydralazine-induced vasodilatation in rabbit femoral arteries. Likewise, Ba^{2+}, also failed to influence to relaxant effect in this study(108). These results suggest that hydralazine does not exert vasodilatation by activation of ATP-sensitive K^+ channels unlike alternative vasodilators. The membrane hyperpolarisation identified must therefore be explained by the activation of alternative channels.

High-conductance Ca^{2+} activated K^+ channels (BK_{Ca}) serve as a negative feedback mechanism limiting the depolarisation and Ca^{2+} increasing effects of vasoconstrictors. Opening of these channels allows K^+ flux out of the cell leading to a change in membrane potential in a hyperpolarising direction, thus inducing vasodilatation. There are also recent data suggesting that such channels may be activated via the nitric-oxide (NO) cyclic guanosine monophosphate (cGMP) pathway, which may modulate the vasodilator response to both exogenous nitroso-vasodilators and endogenous receptor-mediated release of NO(110, 111). This effect may - in part - be through an effect on endothelial superoxide production elicited by changes in the membrane potential through BK_{Ca} channel activation. The effect of hydralazine on these channels has been investigated in animal studies; both *ex vivo* and *in vivo* studies in isolated porcine coronary arteries and perfused rabbit hearts demonstrated that the blockade of these channels attenuated the vasodilator effect of hydralazine(100). This result can be demonstrated by the use of selective BK_{Ca} channel inhibitors such as tetraethylammonium (TEA) and iberiotoxin. This effect appears to be attenuated by arterial endothelial removal suggesting that hydralazine response may be *partly* mediated through such channels in the endothelium. A similar effect was demonstrated in studies of nitro-

glycerine mediated vasodilatation in human arteries, but not veins(111). Conflicting results were seen in a study of the effects of hydralazine in rabbit aorta and pulmonary arteries(99). Hydralazine failed to alter the potassium currents recorded from isolated smooth muscle cells using a whole-cell patch-clamp technique. There was no apparent effect on membrane potential. The authors of this study acknowledged that hydralazine may act differently in other vascular preparations. They suggested that potassium channel blockade (by iberiotoxin or TEA) influenced the response to hydralazine by promoting membrane depolarisation and enhancing Ca^{2+} influx through voltage-operated calcium channels. This theory is partly supported by the lack of effect of hydralazine on Ca^{2+} influx or contractile responses mediated by Ca^{2+} influx in earlier studies(112).

1.3.2 Sarcoplasmic reticulum - role of calcium

The vascular smooth muscle cell (VSMC) sarcoplasmic reticulum (SR) is an attractive site of action for hydralazine. Hydralazine has been shown to induce a fall in intracellular $Ca^{2=}$ concentration available for contraction, and to inhibit contractions evoked by caffeine (which directly stimulates release of Ca^{2+} from SR)(112, 113). Hydralazine may act by inhibiting the release of Ca^{2+} evoked by inositol 1,4,5 triphosphate (IP_3)(112, 113). The alpha-adrenergic agonist vasoconstrictor phenylephrine is thought to induce tonic tension through sustained and oscillating Ca^{2+} influx through permeable channels in the VSMC membrane. The release of SR Ca^{2+} mediated by intracellular IP_3 is thought to underlie the initial, usual transient phase of tension. Therefore, hydralazine should inhibit this initial phasic response in addition to tonic tension. Hydralazine was equally effective at inhibiting both phasic and tonic contractions evoked by PE and IP_3 in the Ellershaw study of rabbit aorta and pulmonary artery(99). Hydralazine had similar efficacy in reducing caffeine induced contraction and VSMC intracellular Ca^{2+} concentration via the caffeine-sensitive Ca^{2+} activated Ca-release (CICR) channel(99). There was, however, a loss of effectiveness when SR Ca^{2+} stores were pharmacologically depleted with ionomycin, further supporting a role for the SR in hydralazine action. It is unclear whether this disrupted Ca^{2+} balance is due to direct antagonism of IP_3 or CICR mediated Ca^{2+} release or was, in fact, secondary to an event such as membrane hyperpolarisation or elevation in cGMP levels. It does not, however, appear to be endothelium dependent in this rabbit model.

1.3.3 Role of second messengers

Studies in non-vascular smooth muscle suggest that hydralazine may activate guanylate cyclase leading to increased cGMP levels. This hypothesis is yet to be proven as there continues to be disparity between studies(103, 114, 115). There is evidence that hydralazine activates this system in the human placental circulation and in women with pre-eclampsia(116, 117). Another, as yet, unresolved issue is the dependence of hydralazine action on intact vascular endothelium. The issue is complex, with evidence that hydralazine may normalise impaired endothelium-dependent relaxation elicited by acetylcholine in nitric-oxide deficient states. Supportive studies include that of Wei *et al* who investigated endothelium-dependent vasodilatation of hydralazine in porcine coronary arteries(103). Hydralazine-induced relaxation was not significantly affected by the presence of L-NOARG, an inhibitor of NO production, nor indomethacin, an eicosanoid inhibitor. In addition, hydralazine had no effect on cyclic adenosine monophosphate (cAMP) levels; rather it induced a 1.5-fold increase in cGMP levels in endothelium-intact arteries. NO did not appear to contribute to the endothelium-dependent relaxation because neither L-NOARG, nor haemoglobin, a chelator of NO, affected hydralazine-induced endothelium-dependant relaxation. Pre-treatment of arteries with actinomycin D, a transcription inhibitor significantly reduced the hydralazine-induced vasodilatation and increase in cGMP level. This did not affect ionomycin-induced relaxation, which stimulated the NO/cGMP system. This tends to suggest that the endothelium-dependent relaxation could be secondary to the immediate transcription of an unidentified organic molecule in endothelial cells. Basal cytosolic cGMP production was unchanged in the presence of hydralazine although it was increased in presence of phenylhydralazine.

1.3.4 Hypoxia-inducible factor activation

Hydralazine has been known to disturb collagen biosynthesis for some time. It is thought to complex with enzyme-bound Fe^{2+} and thus inhibits enzyme activity - one such target being the protocollagen prolyl hydroxylase, thus preventing the formation of stable collagen fibers. Other members of this family include the prolyl hydroxylase domain enzymes (PHD), which regulate hypoxia inducible factor (HIF). In a novel study, inhibition of the PHD pathways by hydralazine rapidly activated the HIF system and produced vasodilatation through indirect release of NO. Additional potential mediators include endothelin-1 and vascular endothelial growth factor (VEGF), which are likewise known to promote angiogenesis. In this study, the investigators demonstrated that hydralazine transiently activated the HIF system by inhibiting PHD enzyme activity(118). The results of this study therefore identified a potential molecular target for hydralazine activity.

1.3.5 Interaction with reactive oxygen species

The balance of endogenous and exogenously generated nitric oxide (NO) and vascular superoxide production is known to be important in both health and disease. Endothelial dysfunction is a common feature of cardiovascular disease, including heart failure, and has partly been attributed to the generation of increased vascular production of superoxide anions with the resultant inactivation of bioactive NO(119). Hydralazine appears to have the ability to affect a number of enzyme systems both *in vivo* and *ex vivo.* In addition to a high affinity for cations such as Fe^{2+} and Cu^{2+}, hydralazine as a carbonyl reactor has been shown to interact with pyridoxal phosphate and thus interferes with the function of this agent as an enzymatic cofactor(120). In general, however, enzyme inhibition is not considered to perform a role in hydralazine vasodilatation because, *ex vivo,* such high concentrations of hydralazine are required for effective inhibition. However, it is recognised that hydralazine readily accumulates in the vascular smooth muscle cell layer and thus physiologically exerts its effects at proportionally higher local concentrations(121).

Contradictory evidence comes from a study of hydralazine in an animal model of nitric-oxide-deficient hypertension(122). Using Wistar rats, pre-treated with the NO synthase inhibitor L-NAME, investigators examined the effects of hydralazine on superoxide formation and expression of endothelial NO synthase. The vasodilator response to acetylcholine was examined in intact aortic rings. Relaxation was attenuated in L-NAME treated animals; this response was normalised by hydralazine therapy. L-NAME treated animals exhibited increased levels of superoxide production, however, interestingly this was not improved with hydralazine therapy; nor was there a change in NO synthase production. In contrast, soluble guanylate cyclase expression was attenuated in L-NAME treated animals and nearly normalised with concomitant hydralazine therapy. The improvement of acetylcholine-induced relaxation therefore did not appear to involve modulation of NO/superoxide balance but instead increased soluble guanylate cyclase expression. These disparate findings could partly be explained by species difference in the oxidase(s) involved in superoxide formation.

Another enzyme system of recent interest to my study is semicarbazide-sensitive amine oxidase (SSAO). This enzyme group appears to be ubiquitous in biology and is known to act via primary amine substrates to produce a variety of effects(123). It does not appear to be affected by the usual inhibitors of monoamine oxidase and is defined by its sensitivity to inhibition by the hydrazine derivative semicarbazide(124). In humans, SSAO predominates within vascular smooth muscle cells where it assumes a sub-cellular position. It appears to be partially glycosylated and contains a carbonyl group and Cu^{2+}, making potential interaction with hydralazine biologically plausible. The breakdown products of SSAO are active and include hydrogen peroxide and aldehydes. Hydrogen peroxide can act as a powerful oxidant or as a signaling molecule depending on location and concentration. It is recognised that SSAO is associated with pathophysiological processes, in particular vascular endothelial damage. Elevated plasma levels of both enzyme and breakdown products are recognised in diabetes mellitus, heart failure, atherosclerosis and Alzheimer's disease(125). As a hydrazine-containing molecule, hydralazine strongly interacts with SSAO. Several studies have demonstrated that hydralazine-induced hypotension can be potentiated by pre-treatment with other hydrazine groups such as isoniazid. Initially these findings were attributed to central mechanisms of cardiovascular regulation, specifically a reduction in cerebral GABA following high-dose isoniazid therapy. However, lower doses have been demonstrated to potentiate hydralazine effects independently of cerebral GABA(126). This phenomenon has been further

demonstrated using SSAO substrates such as methylamine and benzylamine. Pre-administration appears to enhance hydralazine hypotension and this associated with a reduction in the plasma concentration of the SSAO breakdown product hydrogen peroxide. Interestingly, this effect appears to be prevented by the peroxide scavenger catalase, suggesting that this species is involved in the hypotensive effects of hydralazine(127). The role of hydrogen peroxide in vascular regulation appears to be complex, and in fact it has been demonstrated to be both vasoconstrictor and vasodilator in various models.

The postulated relationship of hydralazine with SSAO finds support from the *in vivo* accumulation of hydralazine in the vascular smooth muscle layer and the cellular location of this enzyme system(121). Other SSAO inhibitors lack this particular distribution and hydralazine is the only known drug for which its vasodilator effect could partly be attributed to vascular SSAO inhibition. This may, in part, explain the disparity between the contractions required to elicit an effect *in vivo* as opposed to *ex vivo* studies. The time lag of effect prior to the onset of hydralazine-induced hypotension *in vivo* may also plausibly reflect the latency of enzymatic interaction. Further supportive evidence of said anti-oxidant effect can be taken from a study investigating the effect of hydralazine on sodium nitroprusside (SNP) induced vasodilatation(128). In that study it was recognised that NAD(P)H oxidase may be involved in the reduction of SNP to nitric oxide in some animal models. More particularly, NAD(P)H oxidase appears to be inhibited by hydralazine and, in fact, co-administration of hydralazine with SNP led to a rightward displacement of CCRCs. This attenuation of vasodilatation was accompanied by a reduction in cGMP production, presumably through inhibition of NO formation. It is interesting to observe that the sensitivity of vessels to hydralazine-SNP-attenuation appeared increased in those without endothelium. Vasodilator response to SNP is considered to be augmented in endothelium-denuded vessels in a manner analogous to denervation. Endothelium-intact vessels appear to be more resistant to the hydralazine-SNP-attenuation thus supports the hypothesis that there is an additional endothelial source of oxidase, which is more resistant to hydralazine therapy. Further supportive evidence for a mechanistic interaction with reactive oxygen species can be gleaned from studies exploring the phenomenon of nitrate tolerance (discussed in **Chapter 1.4.2** below).

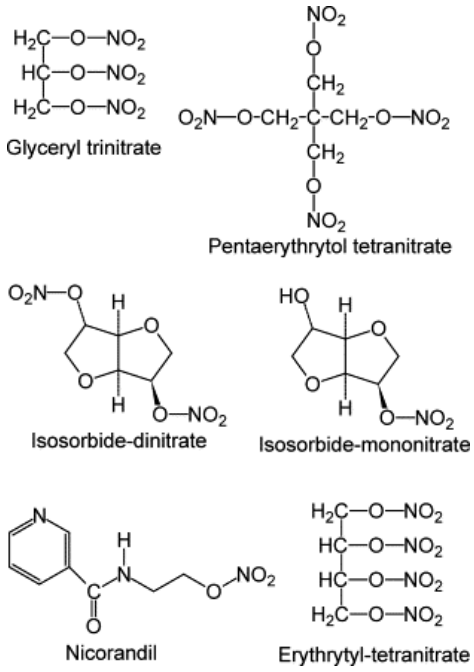
The potential antioxidant effects of hydralazine have – based on my knowledge - never before been examined in human blood vessels.

1.4 Interaction with organic nitrates

1.4.1 Mechanism of action of organic nitrates

Synthesis of the organic nitrate nitro-glycerine was first reported in 1846. 20 years later, its use as an explosive agent became the source of Alfred Nobel's landmark discovery. To quash the medical profession's concerns regarding the ingestion of an explosive drug, it was later renamed glyceryl-trinitrate (GTN) and has been used as a vasodilator drug for the last two centuries. Since then several other clinically relevant organic nitrates have been developed **(Figure 1-7)**. Organic nitrates contain the nitrooxy functional group (-O-NO_2), almost all examples being aliphatic nitrates, owing to the presumed instability of the aromatic nitrate to rearrangement(129).

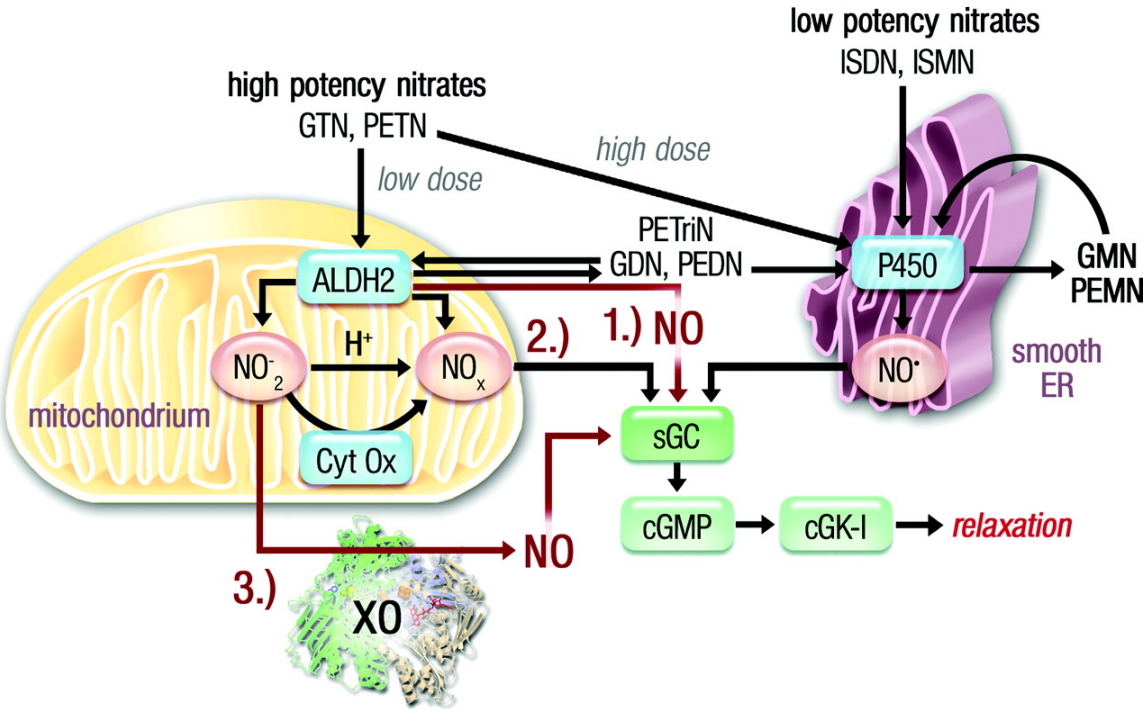
Figure 1-7: Nitrosovasodilators



Organic nitrates must undergo intracellular metabolism in order to promote vasodilatation; a process often referred to as bio-activation. It is widely held that the biologically active product of biotransformation of organic nitrates is NO, and, thereafter, the activation of guanylate cyclase produces cGMP (130). In the past decade, substantial insight into this bio-activation process has been gained. A substantial amount of information has been collated regarding the mechanisms underlying nitrate tolerance. GTN is metabolised by at least two different pathways; first at high doses by a *low-affinity* pathway (via cytochrome P450) and, second at low doses by a *high-affinity* pathway (via aldehyde dehydrogenase (ALDH-2))(131).

High potency nitrates such as GTN, pentaerythrityl-tetranitrate (PETN), and pentaerythrityl-trinitrate (PETriN) are activated by mitochondrial ALDH-2, yielding an NO-containing compound(132). This molecule activates soluble guanylate cyclase (sGC), which decreases cytosolic Ca^{2+} by promoting extracellular currents and increasing Ca^{2+} uptake to intracellular stores such as the sarcoplasmic reticulum. The bio-activation of low potency nitrates such as isosorbide-dinitrate (ISDN), isosorbide-5-mononitrate (ISMN), glyceryl-dinitrate (GDN) and pentaerythrityl-dinitrate (PEDN) are most likely metabolised by P450 enzyme(s) in the endoplasmic reticulum (ER) directly yielding NO. The latter mechanism also metabolises high potency nitrates when they are administered at high concentrations (> 1 μM). **(Figure 1-8)**.

Figure 1-8: Proposed mechanisms underlying bio-activation of organic nitrates. On the left, characterisation of the bio-activation of *high-potency* nitrates; on the right the *low-potency* nitrates. Reproduced with permission from Munzel et al(131).



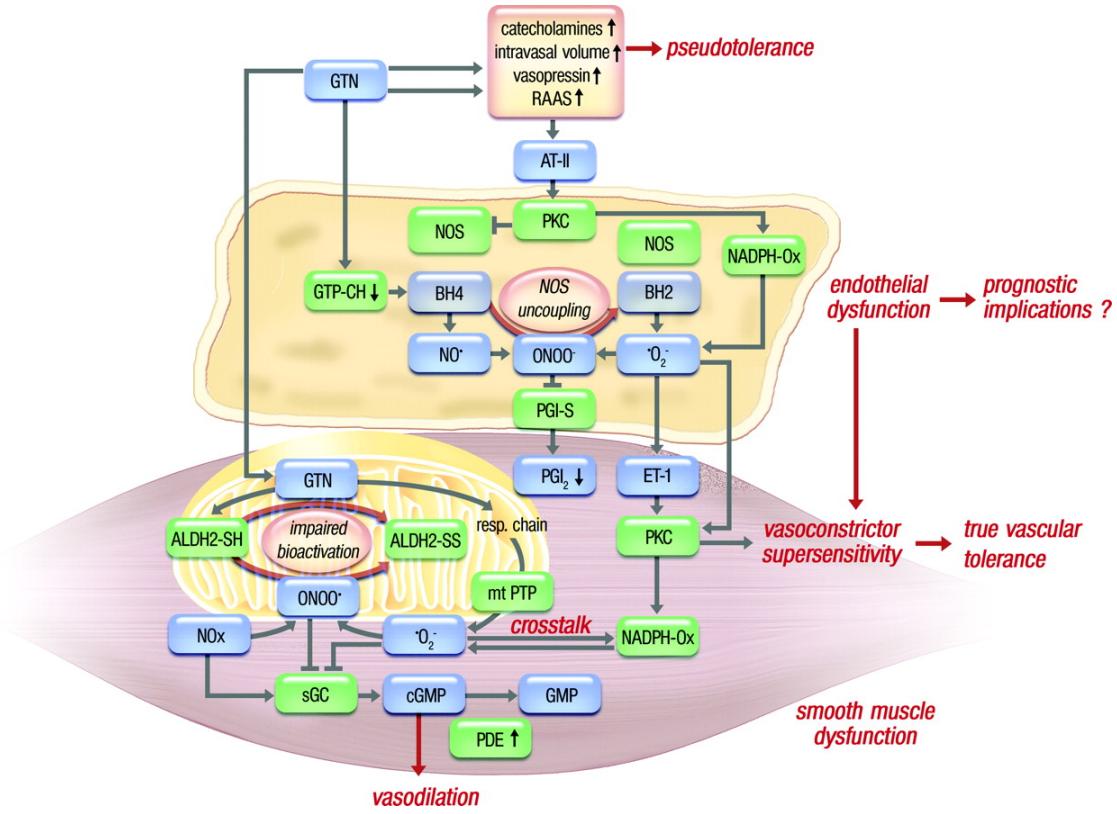
Sodium nitroprusside (SNP) appears to act as a direct NO donor in contrast to nitrates requiring bio-activation. SNP spontaneously releases NO, which is coordinated as a nitrosyl group liganded to iron in a square bipyramidal complex within its structure. NO appears to be released spontaneously at physiological pH from the parent compound(130). Multiple reducing agents, including NAD(P)H oxidase are implicated to catalyse the release of NO from SNP in the vasculature(133, 134). Clinical use of SNP is limited by need for parenteral administration, by means of the development of pharmacological tolerance and the potential development of thiocyanate toxicity with prolonged administration.

1.4.2 Mechanism of nitrate tolerance

The concept of nitrate tolerance is complex and not wholly understood. A number of vascular and extra-vascular phenomena have been observed and shown to compromise the long-term haemodynamic effects of organic nitrates(131). These include the so-called *pseudo-tolerance* arising through neurohormonal activation, intravascular volume expansion, and increased catecholamine and vasopressin production; events which have been recognised in heart failure patients receiving long-term nitrate therapy(135).

The second concept of *vascular tolerance* relates to the intrinsic effects of nitrate therapy on vascular endothelium and vascular smooth muscle cells (VSMCs) and includes impaired nitrate biotransformation, increased vascular superoxide production, the desensitisation of sGC, increased sensitivity to vasoconstrictors and the uncoupling of endothelial nitric oxide synthase (*NOS3*) **(Figure 1-9)**.

Figure 1-9: Molecular mechanisms of nitrate tolerance. Reproduced with permission from Munzel et al(131).



Oxidative stress and redox imbalance is thought to play a critical role in nitrate tolerance and a ROS-dependent interference with NO signalling is compatible with cross-tolerance with other nitrates such as ISDN and ISMN. Sage *et al* demonstrated that nitrate tolerance in patients is directly related to increased superoxide formation and consequent reduced GTN biotransformation in human blood vessels. Rings of saphenous vein and internal mammary artery were harvested at the time of elective coronary artery bypass surgery in patients treated for 24 hours pre-operatively with GTN (10μg/min intravenously)(136). Using lucigenin-enhanced chemiluminescence, the harvested veins demonstrated an increased vascular superoxide formation in GTN-treated patients and a 40% reduction in 1,2-dinitroglycerin (a GTN bio-activation metabolite) measured by gas chromatography. Nitrates stimulate the vascular (particularly endothelial) production of peroxynitrite, a highly reactive intermediate generated from rapid, diffusion-limited reaction of NO with superoxide. Peroxynitrite can oxidise the eNOS cofactor tetrahydrobiopterin (BH_4) to dihydrobiopterin (BH_2) via intermediate formation of trihydrobiopterin (BH_3) radicals(137). This may lead to dysfunctional eNOS activity; the so-called NOS uncoupling.

In 1995, Munzel and colleagues defined a new molecular mechanism accounting for GTN tolerance and cross-tolerance(138). They identified that aortic segments from 3-day GTN-exposed rabbits were tolerant to the vasodilator action of GTN *ex vivo* and exhibited cross-tolerance to acetylcholine and the non-enzymatic stimulator of NO production SIN-1. Removal of the endothelium, however, markedly attenuated tolerance to GTN and cross-tolerance to SIN-1, suggesting a substantial role of the endothelium in mediating tolerance. Other investigators have demonstrated this phenomenon(139). It was postulated that the endothelium was either releasing a vasoconstrictor molecule and/or that NO became chemically inactivated before it could stimulate sGC in VSMCs. In support of this hypothesis, superoxide levels in tolerant vessels were approximately twice that of controls, and were normalised by removal of the endothelium. Because diphenyleneiodonium acutely inhibited superoxide formation, a flavin-containing oxidase was suggested as the likely superoxide source. They subsequently detected an increased activity of membrane-bound NADH/NAD(P)H oxidase in tolerant vascular tissue(106).

Other investigators have implicated increased NAD(P)H oxidase activity in nitrate tolerance, both in animal and human models(140, 141). Thus far, it is not known whether nitrate tolerance increases the expression of subunits critical for NAD(P)H oxidase activity or whether it stimulates an association of cytosolic subunits with the membrane-bound cytochrome b5/p22^{phox} oxidase components.

Other hypotheses exist and the published, literature available in this regard is considerable. My research of these works leads me to conclude that tolerance may not be a class effect, and, as yet a unifying hypothesis has not been established.

1.4.3 Clinical evidence of interaction

Hydralazine co-treatment has been shown in clinical studies to prolong the vasodilator effect of nitrates, though the mechanism of this effect in humans remains uncertain(105). In certain studies, the addition of hydralazine to a nitrate causes a greater effect on the reduction in cardiac filling pressures than can be achieved by hydralazine alone(142, 143). This interaction remains poorly understood.

1.4.4 Experimental evidence of interaction

Enhanced vascular formation of superoxide has been implicated in the development of nitrate tolerance. Nitro-glycerine-induced increase in superoxide production can be inhibited by dipheyleneiodonium; this infers that the anion is flavin-derived(138). A major source of such oxygen radical production is a membrane-bound flavin-containing NADH/NAD(P)H-dependent oxidase which is regulated *in vivo* and *ex vivo* by angiotensin-II(144). This hypothesis has been explored by Munzel and colleagues(106). This study investigated the effect of nitrate therapy on oxidase-system activation and the effect of hydralazine on superoxide production in rabbits. Rates of superoxide production were more than two-fold in animals treated with nitrate. Concomitant *in vivo* treatment with hydralazine significantly reduced superoxide production. This effect was negated by the addition of KCl thereby suggesting that altered membrane potential might modulate production of superoxide.

In vessel homogenates, hydralazine treatment decreased NADH-dependent oxidase in animals treated *in vivo*; however, the same effect was not observed when incubated *ex vivo*. This suggests that hydralazine had no direct scavenging effect. Hydralazine was only effective when administered *in vivo* or to intact vascular rings. Potential explanations include the prevention of assembly of the oxidase rather than inhibition or inhibition by membrane hyperpolarisation.

Hydralazine has also been shown to possess powerful, peroxynitrite-quenching properties, which could explain - in part - its attenuation of experimental nitrate tolerance(145). Daiber and colleagues created in their study an *in vivo* state of nitrate tolerance with prolonged (3 day) subcutaneous administration of GTN to Wistar rats. The antioxidant effects of hydralazine were thereafter examined in cell free systems, cultured VSMCs, isolated cardiac mitochondria, and vascular preparations (aortic rings). Superoxide production was measured using lucigenin-enhanced chemiluminescence and dihydroethidium fluorescence. Their conclusions were that hydralazine appeared to decrease superoxide production in a dose-dependent manner. Additionally, hydralazine inhibited peroxynitrite-mediated nitration of phenols in VSMCs. These data implied that hydralazine was a potent ROS scavenger. Surprisingly, the anti-oxidant effect of hydralazine, and its potential role in nitrate tolerance, has never been directly characterised in human blood vessels.

1.5 Summary and aims of thesis

The conflicting literature available on the effects of hydralazine may be partly explained by the diverse animal models examined, as inter / intra-species variability in enzyme isoforms, channel distribution and second messengers exist. Likewise a novel and, as yet, unidentified mechanism may contribute to the action of hydralazine.

In this thesis my aim was to characterise the actions of therapeutic concentrations of hydralazine in arteries and veins of various calibre, taken from patients with low ejection fraction heart failure secondary to coronary artery disease. From the currently available evidence I postulated that hydralazine would augment the response to vasodilators acting through the cyclic GMP pathways and that this effect would be greater in arteries than veins. I also set out to demonstrate that hydralazine would attenuate both basal and stimulated vascular superoxide production. Such a conclusion would support the existing available data supporting that hydralazine prevents nitrate tolerance through modulation of the nitroso-redox system; moreover, my experiments would be the first such studies conducted using human blood vessels.

Chapter 3 focuses on the direct vasodilator effect of hydralazine in large and small calibre blood vessels. The hypothesis was that hydralazine has a direct vasodilator effect on both arteries and veins taken from patients with LVSD and CAD and that this effect would be greater in arteries.

The aims of **Chapter 3** were therefore:

1. To determine the comparative vasodilator effect of hydralazine on human internal mammary artery and saphenous vein using therapeutically relevant concentrations of hydralazine.
2. To determine the vasodilator effect of hydralazine on human subcutaneous resistance arteries using therapeutically relevant concentrations of hydralazine.

Endothelial dysfunction is understood to be central to the pathophysiology of cardiovascular disease – particularly heart failure. The available literature suggests that hydralazine leads to activation of guanylate cyclase (through endothelial NO production). **Chapter 4** focuses on a series of experiments aimed to determine if *ex vivo* treatment with hydralazine augmented endothelium-dependent vasodilatation (with the stable analogue of acetylcholine, carbachol) in human blood vessels taken from patients with LVSD and CAD.

The aims of **Chapter 4** were therefore:

1. To determine if hydralazine augments the vasodilator response to the endothelium-dependent vasodilator carbachol in large calibre blood vessels.
2. To determine if hydralazine augments the vasodilator response to carbachol in subcutaneous resistance arteries.

Chapter 5 focuses on the interaction between hydralazine and clinically relevant nitrosovasodilators. The therapeutic synergy of ISDN and hydralazine in patients with heart failure has been attributed to favourable haemodynamic effects as well as the purported ability of hydralazine to reduce nitrate tolerance.

The aims of **Chapter 5** were therefore:

1. To determine if hydralazine augments the vasodilator response to clinically relevant nitrosovasodilators (GTN, SNP and ISDN) in human blood vessels taken from patients with LVSD and CAD.

Chapters 6 directs attention to oxidative stress and the production of vascular superoxide – understood to be one of the major mechanisms underlying endothelial dysfunction in heart failure and considered integral to the mechanism of nitrate tolerance in clinical practice. Hydralazine has purported anti-oxidant properties although the direct effect of hydralazine on the production of vascular superoxide has never been investigated before in human blood vessels. The hypothesis is that hydralazine would reduce basal superoxide production in large calibre blood vessels taken from patients with LVSD and CAD.

The aims of **Chapter 6** were therefore:

1. To assess the effect of hydralazine on basal superoxide production in internal mammary arteries and saphenous veins
2. To assess the relative potency of hydralazine on internal mammary arteries and saphenous veins.
3. To assess any apparent dose-response to hydralazine on basal superoxide production.

In **Chapter 7** the interaction between neurohormonal activation – particularly production of angiotensin II – and oxidative stress are discussed. I examined if hydralazine attenuated angiotensin-II mediated superoxide production. Angiotensin-II stimulates superoxide production through activation of NAD(P)H oxidase in the vasculature. This is an important therapeutic target for neurohormonal antagonists but is also a purported enzyme system through which hydralazine may interact.

The aims of **Chapter 7** are therefore:

1. To determine if co-incubation of human IMA vessels with hydralazine attenuated angiotensin-II stimulated superoxide production.

Chapter 2 – General Methods

2.1 Introduction

This thesis was funded by a British Heart Foundation Clinical Research Training Fellowship (FS/06/75).

In this chapter I will describe each of the methods deployed for each of the studies that comprise the thesis.

Laboratory equipment and reagents were of the highest available grades. A laboratory coat and latex powder-free gloves were worn during all procedures. The handling of hazardous reagents was in accordance with the Control of Substances Hazardous to Health Regulations 2002. Laboratory glassware was cleaned in Decon 75 detergent (Decon Laboratories Ltd.), rinsed with distilled water and dried in a 37^{o}C cabinet. Reagents were weighed using a calibrated balance. Volumes from 0.1µl to 1,000µl were dispensed using appropriate Gilson pipettes (Gilson Medical Instruments). Volumes from 1 ml to 25 ml were measured with sterile disposable pipettes (Corning) and a Gilson battery-powered pipetting aid. Distilled water (dH_20) was used to prepare aqueous solutions unless otherwise indicated. A calibrated water bath was utilised for experiments requiring incubations to 37°C

2.2 Patient selection

2.2.1 The VASCAB study

2.2.1.1 Ethics

Ethical approval was obtained for this study from the West of Scotland Ethics Committee. NHS research and development approval was secured from the NHS Greater Glasgow and Clyde health board and the National Waiting Times Centre health board (at the Golden Jubilee National Hospital, Clydebank). Written informed consent was obtained for all study participants in accordance with the Declaration of Helsinki. Ethics approval is detailed in **appendix 1**.

2.2.1.2 Patient Recruitment

The Vascular Function in Coronary Artery Bypass (VASCAB) study was coordinated from the British Heart Foundation Glasgow Cardiovascular Research Centre (BHF GCRC). Volunteers were recruited prospectively at pre-operative assessment clinics in the Western Infirmary, Glasgow between October 2006 and February 2008, and, thereafter from the Cardiothoracic Unit of the West of Scotland Regional Heart and Lung Centre (at the Golden Jubilee National Hospital, Clydebank). Recruits were examined in the Clinical Research Facility of the BHF GCRC on the afternoon prior to admission or at the time of their admission to hospital for surgery (routinely the evening before scheduled surgery). Only patients with objective evidence of left ventricular systolic dysfunction (as defined by left ventricular ejection fraction less than 50% calculated at pre-operative trans-thoracic echocardiogram or ventriculogram or a subjective assessment of impaired systolic function by the operator) were approached as possible candidates.

Moreover, only those patients receiving an individually optimised regimen of neurohormonal antagonists (including ACE inhibitor or ARB and beta-blocker) were included in the study and asked to take their medication as normal. Patients concurrently treated with hydralazine or long-acting nitrates were excluded. Clinical details such as smoking history, past medical history of myocardial infarction or hypertension and current medication were noted.

Routine clinical measurements including height and weight, blood pressure and resting heart rate were recorded. Patients were functionally assessed in accordance with the New York Health Association (NYHA) functional classification (146). All study participants were allocated a unique VASCAB study number, which served as an individual identifier for all clinical data and biological samples. Clinical research files were kept in a secure location in the BHF GCRC.

2.2.2 Gluteal biopsy patients

2.2.2.1 Ethics

Ethical approval was obtained for this study from the West of Scotland Ethics Committee and NHS research and development approval from Greater Glasgow and Clyde Health Board. Written informed consent was obtained for all study participants in accordance with the Declaration of Helsinki. Ethics approval is detailed in **appendix 2**.

2.2.2.2 Patient recruitment

Patients with symptomatic heart failure with reduced left ventricular systolic function secondary to coronary artery disease were prospectively recruited from Cardiology clinics at the Western Infirmary, Glasgow between 2006 and 2009. Patients were provided with written information regarding the gluteal biopsy procedure and contacted subsequently to confirm their participation in the study. Transport was provided to and from the BHF GCRC for participants. Only those on an individually optimised regimen of neurohormonal antagonists (including ACE inhibitor or ARB and beta-blocker) were included in the study. Patients on treatment with warfarin were excluded to exclude the risk of bleeding. Patients currently receiving hydralazine or long acting nitrates were also excluded. All subjects attended the BHF GCRC for the elective biopsy procedure. Detailed past medical and therapeutic history was recorded in addition to NYHA functional class and basic clinical measurements including height and weight, blood pressure and resting heart rate. Participants in this cohort had relatively mild heart failure as indicated by the proportion of those receiving mineralocorticoid receptor antagonists and 28% who were not receiving chronic loop diuretic therapy.

2.3 Organ bath studies: methods for study of effects of hydralazine on human internal mammary arteries and long saphenous veins

2.3.1 Patients

Patient recruitment was undertaken as described above. Patient characteristics and demographics are presented in **Table 2.1.**

All patients were in an NYHA II functional category. Participants in this cohort had relatively mild heart failure as indicated by the proportion of those receiving mineralocorticoid receptor antagonists and 28% who were not receiving chronic loop diuretic therapy. Summary data for age and creatinine were not available at the time of completion of this thesis.

NUMBER OF PATIENTS	40
Sex M/F	29/11
Mean age	62y
Previous MI	32 (80%)
LVEF<50%	40 (100%)
Current smoker	8 (20%)
Atrial fibrillation	7 (17%)
Diabetes Mellitus	12 (30%)
Hypertension	25 (62%)
Mean creatinine μmol/L	105
Drug therapy	
ACE inhibitor/Angiotensin-receptor antagonist	40 (100%)
β-blocker	40 (100%)
Anti-platelet	40 (100%)
Mineralocorticoid receptor antagonist	2 (5%)
HMG CoA reductase inhibitor	35 (87%)
Calcium channel antagonist	12 (30%)
Nicorandil	8 (20%)
Loop diuretic	35 (62%)
Digoxin	6 (1.5%)

2.3.2 Vessel preparation

Distal segments of left internal mammary artery (IMA) and saphenous veins (SV) were harvested at the time of routine coronary artery bypass surgery in the Western Infirmary and Golden Jubilee National Hospital. Only those segments, which were surplus to requirement, were provided by the theatre staff. In some cases no tissue was available in spite of prior consent by patients. The discarded distal end of the IMA (1-2cm) and segments of SV (1-4cm) were immediately taken from theatre to the laboratory in sterile normal saline solution for (NaCl 0.9%) prior to transfer into chilled Krebs-HEPES solution (10mmol) on arrival to the BHF GCRC.

Vessels were carefully cleaned of adherent fat and connective tissue under light microscopy and stored under refrigeration until the following day. Our group has previously shown that storage under these conditions does not impair vascular responses(147). Vessels were then cut into 2-3mm long rings. Rings were then suspended on wires in 10ml organ chambers filled with physiological salt solution [(PSS) 130 mM NaCL, 4.7 mM KCL, 14.9 mM $NaHCO_3$, 1.18 mM KH_2PO_4, 5.5mM glucose, 1.17 mM $MgSO_4.7H_2O$, 1.6mM $CaCl_2.H_20$, 0.03 mM $CaNa_2EDTA$ and 0.02 mM indomethacin dissolved in DMSO (pH 7.49 ± 0.1)], maintained at 37°C and aerated with a mixture of 95% O_2-5% CO_2. The addition of indomethacin to the PSS inhibited prostanoid-mediated vasoactive effects. The rings were connected to a Grass FT03 force transducer and changes in isometric tension recorded using a MacLab dedicated computer.

The rings of human IMA and SV were equilibrated in the organs baths in PSS solutions before study protocols were initiated. After stabilisation at a resting tension for approximately 1 hour, the harvested vessels were activated with the receptor-independent vascular smooth muscle cell depolarising agent potassium chloride (KCl) (100 mmol/L). The vessels were then washed out repeatedly for 30min and activation with KCl (100 mmol/L) repeated. The noradrenaline analogue phenylephrine (PE) (3 μmol/L) was used to constrict vascular rings via α-adrenoceptors. Carbachol (a stable analogue of acetylcholine) (1 μmol/L) was used to relax the rings in an endothelium-dependent manner via muscarinic receptors (resulting in stimulated NO release) confirming endothelial integrity. After activation, vessels were further washed out and allowed to rest for 30 minutes before experimental protocols began.

Resting tension was adjusted to 1g prior to commencement of the cumulative concentration-response curves (CCRCs) incorporated in the study protocol. Vascular rings, which did not contract to either KCl or phenylephrine, were discarded. Only those vessels exhibiting a response to carbachol were included in endothelium-dependent protocols.

2.3.3. Experimental protocols

Following equilibration rings were pre-constricted with PE 3 μmol/L prior to commencement of CCRCs determined in the study protocols.

2.3.3.1 Cumulative concentration response curves to hydralazine alone

The comparative vasodilator effect of hydralazine on human IMA and SVs was studied in a series of CCRCs in vessels taken from 20 patients. Vessels were pre-constricted as described before the addition of cumulative doses of hydralazine (0.01 to 10μmol/L). Plasma concentrations in patients receiving hydralazine for hypertension are 0.1-1.0μmol/L in patients taking therapeutic doses; therefore these CCRCs encompass the therapeutic and supra-therapeutic range (79, 148-151). Maximum relaxation responses of arteries and veins were determined in this series of CCRCs.

2.3.3.2 Interaction between hydralazine and endothelium-dependent vasodilators

This series of experiments sought to determine whether hydralazine augments the vasodilator action of the endothelially active response to the stable analogue of acetylcholine, carbachol. Following the initial equalisation and confirmation of endothelium integrity by response to carbachol (1 μmol/L), vessels were washed out and allowed to equalise for a further 30 minutes. In pairs, vessels were pre-incubated with either hydralazine (1 μmol/L – upper limit of plasma concentration from studies in patients treated with hydralazine for hypertension) or diluent control (PSS) for 30minutes prior to pre-constriction with PE and construction of CCRCs to carbachol (1nmol/L-10μmol/L).

2.3.3.3. Interaction between hydralazine and clinically relevant nitrosovasodilators

This series of experiments aimed to examine the hydralazine-nitrate relationship in human blood vessels and the relationship between nitrosovasodilators known to have differing bio-activation pathways(152). In pairs, vessels were pre-incubated with either hydralazine (1.0 μmol/L – concentration determined as discussed above) or diluent control (PSS) for 30 minutes prior to pre-constriction with PE and construction of CCRCs to the *high-potency* nitrosovasodilators glyceryl-trinitrate (GTN 0.1 nmol/L -0.3μmol/L) and sodium nitroprusside (SNP 1 nmol/L – 30 μmol/L) and the *low potency* nitrosovasodilator isosorbide dinitrate (ISDN 0.1 nmol/L - 0.3μmol/L). Concentration ranges were selected following review of the relevant literature and on the basis of previous vascular reactivity experiments undertaken using these agents by our group(153-156).

2.4 Myography studies: methods for study of effects of hydralazine on human small resistance arteries

2.4.1 Patients

Patient recruitment was undertaken as described above. Patient characteristics and demographics are presented in **Table 2.2**

All patients were in an NYHA II functional category. Participants in this cohort had relatively mild heart failure as indicated by the proportion of those receiving mineralocorticoid receptor antagonists and 15% who were not receiving chronic loop diuretic therapy. Summary data for age and creatinine were not available at the time of completion of this thesis.

NUMBER OF PATIENTS	20
Sex M/F	13/7
Mean age	64y
Previous MI	13 (65%)
LVEF<50%	20 (100%)
Current smoker	5 (25%)
Atrial fibrillation	3 (15%)
Diabetes Mellitus	8 (40%)
Hypertension	10 (50%)
Mean creatinine μmol/L	125
Drug therapy	
ACE inhibitor/Angiotensin-receptor antagonist	20 (100%)
β-blocker	20 (100%)
Anti-platelet	20 (100%)
Mineralocorticoid receptor antagonist	7 (35%)
HMG CoA reductase inhibitor	18 (90%)
Calcium channel antagonist	5 (25%)
Nicorandil	4 (20%)
Loop diuretic	17 (85%)
Digoxin	2 (1%)

2.4.2 Human small resistance arteries

Human small resistance arteries (SRAs) are small arteries (with diameter less than 500 μm) which contribute the greatest resistance to blood flow, and, as such, most involved in regulating blood flow and capillary pressure(157). The importance of SRAs lies in their ability to regulate the distribution of blood to peripheral organs through variation of their diameter and hence resistance to flow. These arteries can be readily obtained from gluteal biopsies in humans. Wire myography is an *ex vivo* technique which allows SRAs with a diameter of 100-500 μm to be studied functionally and morphologically under precise and standardised isometric conditions and is independent of homeostatic mechanisms such as the autonomic nervous system or blood flow(157-159). This technique has been adapted to facilitate the study of a range of animal models (including human) and vascular beds in diverse pathological states including chronic heart failure(160-162).

2.4.3 Gluteal biopsy procedure

The technique of gluteal biopsy has been used extensively in our research group as a source of small resistance arteries (SRA)(163-165). I received training in the technique by Dr Neal Padmanabhan, Senior Lecturer at the University of Glasgow. I performed all the gluteal biopsies in the study under local anaesthetic using sterile surgical instruments. 10-15ml of 1% lignocaine was injected into the upper, outer quadrant of the buttock using an aseptic technique. Typically the left buttock was used unless the patient had undergone gluteal biopsy in a previous study or had a surgical contraindication to a left-sided procedure being undertaken. An elliptical incision was made with a scalpel and a 2cm x 3cm x 2cm biopsy of gluteal skin and subcutaneous fat was taken and immediately placed in chilled PSS. Haemostasis was routinely achieved by manual pressure and wound closure. Three to four non-absorbable (silk) sutures were then used to close the skin using an interrupted mattress technique and a sterile dressing placed on the skin over the sutures.

The biopsy site, volume of local anaesthetic used, number of sutures, serial number of surgical instruments and immediate complications (if any) were recorded in the clinical research file. Patients were encouraged to gently mobilise 1 hour following the procedure and then received transport home. All patients were given written instructions regarding wound care and contact details in event of complications. Patients returned one-week post biopsy for suture removal and wound inspection by a member of the BHF GCRC clinical research facility nursing staff for which patient transport was one again provided. Any post-biopsy complications were recorded in the clinical research file.

2.4.4 Vessel preparation

I was trained to dissect SRAs from the gluteal biopsy specimen by Ms Angela Spiers who kindly performed the first 4 dissections in the study. Dissection of the gluteal biopsy specimen was typically performed on the day of biopsy. Using surgical grade microscopic instruments and with the aid of a high power microscope the specimen was dissected in a Petri dish filled with chilled PSS, regularly changed during the dissection process, which could take several hours. SRAs were isolated from surrounding subcutaneous tissue and placed in a universal container with PSS and refrigerated at 4°C overnight. The routine storage of SRAs in this manner has been demonstrated to have no effect on their vasoactive properties(166). A single biopsy may yield several SRAs (average 2-4). Where possible four SRAs were utilised from each biopsy sample.

2.4.5 The Mulvany-Halpern wire myograph

Wire myography is an *ex vivo* technique to examine the contractile and relaxant isometric properties of small vessels (diameters 100-500 μm). The technique was first described by Mulvany and Halpern in 1977(159). In addition to functional responses, morphological data including internal diameter, normalised for transmural pressure, and wall thickness can be calculated for the vessel under investigation(157, 159). Using this technique, SRAs are dissected into segments of approximately 2mm in length as a ring preparation.

The standard approach for the procedure is for isolated rings to be carefully mounted on two 40-μm-diameter stainless steel wires using a no-touch technique under high-power light microscopy and mounted in the bath of a 4-channel myograph (Danish MyoTechnology, Aarhus, Denmark). This consists of a base-unit on which is mounted 4 myograph blocks in which the wires are attached to a force transducer and micrometer respectively. A schematic diagram of the myograph is shown below.

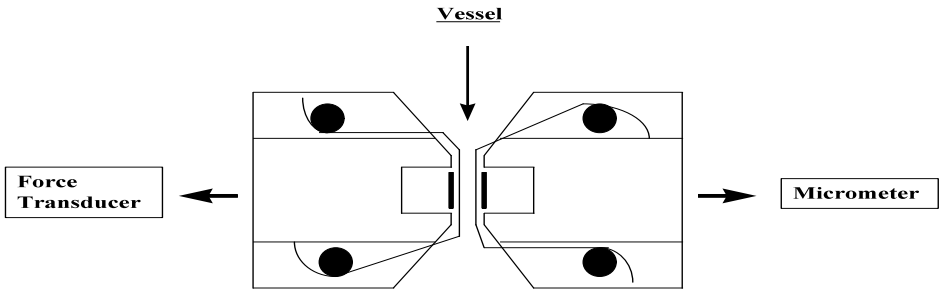


Figure 2.1 The Mulvany-Halpern Myograph (not to scale)

Each bath is kept under physiological conditions with PSS which gives a pH of 7.4 when gassed with a 95% O_2 5% CO_2 mixture and pre-heated to 37°C. These conditions are maintained for the duration of the experiment with an in-built heater and thermostat. The bathing solution can be rapidly exchanged using an internal extraction system and replaced with fresh PSS. The next critical stage in the experimental process is mounting of the vessels. I was trained and assisted in the mounting of vessels by Ms. Angela Spiers and Ms. Elisabeth Beattie. The mounting of vessels is undertaken using high-power light microscopy.

Isolated rings were cannulated with pre-cut stainless steel wires within the PSS filled Petri dish. The wire was then transferred and connected to left myograph head. The second wire is then threaded through the mounted ring taking care not to damage the endothelial surface. Both wires were then secured to their respective myograph heads until taut. Any excess vessel protruding from the jaw of the myograph was cut away to ensure this segment was not able to contract. Vessel length was measured using a calibrated micrometer eyepiece in the dissecting microscope; the eyepiece having been previous calibrated using a graticule. The length of the vessel was then measured to far edge of the myograph jaw (α_1 ocular divisions) then measured to near edge of segment (α_2 ocular divisions) The heads of the myograph were then adjusted until the wires were just touching and the micrometer reading at that point ($X_{0)}$ was recorded. These measurements were recorded onto the normalisation experiment sheet prepared for the purposes of this study. After mounting and measurement of each isolated ring the baths were returned to the base unit to begin the normalisation process.

2.4.6 Normalisation

After a rest period of 30 minutes, a normalisation procedure was followed for each artery to determine the normalised internal diameter (ID), L0, at which contraction is thought to be optimal. This aims to set vessels to standard initial conditions to allow physiological responses to be measured in a reliable fashion. Some studies have indicated that the initial passive condition of an artery (resting tension) may influence its subsequent response to pharmacological agonists and antagonists(167). Similarly, the dissection of a vessel from its adherent connective tissue impacts on its intrinsic pressure-length and pressure-diameter relationship(168). The original technique of normalisation was designed by Mulvany and Halpern in an attempt to overcome these limitations(157). The intrinsic diameter of an elastic tissue such as an SRA is influenced by transmural pressure (and this needs to be defined by the normalisation process). The active response of the vessel is determined by the degree of stretch it is exposed to and finally the sensitivity of the vessel to pharmacological stimulation is also influenced by stretch. That said, even optimal *ex vivo* conditions cannot replicate dynamic in vivo physiological responses.

The normalisation process determines the internal circumference a vessel would have if relaxed and under a transmural pressure of 100mmHg (IC_{100}). First described in rat mesenteric arteries, the size of the vessel that was optimal for contraction was the IC_1 or 0.9 of the IC_{100}(169). Maximum active tension development can be calculated from the passive internal circumference/tension relationship of each vessel. Each vessel is incrementally distended using the micrometer and the passive force measured (*F*) using the chart recorder. Wall tension (*T*) is calculated by dividing the force by twice the segment length (which had been measured using the micrometer eyepiece). Internal circumference could be calculated from the micrometer reading (X_0) and the knowledge that each wire has a diameter of 40 μm. The equivalent increase in pressure can be determined by applying the Laplace equation, which relates effective internal pressure, wall tension and internal circumference. The process is aided with the use of a programmable hand-held calculator, which is able to calculate the actual values from each chart reading, given the relevant calibration factors. This process is repeated in a stepwise sequence at 1-minute intervals to allow for "stress relaxation"; force recordings are taken at the end of each interval until the effective pressure has exceeded 100mmHg. At this point the computer fits an exponential curve to the internal circumference-pressure data allowing calculation of the IC_{100}. The computer can then interpolate the equivalent micrometer readings necessary to set the vessel to IC_1 (i.e. 0.9 of IC_{100}). The micrometer is then set to this point.

2.4.7 Myography experimental protocols

Following normalisation, vessels were washed with fresh PSS and then allowed to equilibrate for 1 hour. Viability of each artery is then assessed using response to a high (123 mmol/L) concentration potassium solution [KPSS (PSS with KCl substituted for NaCl on an equimolar basis)] for a series of 5-minute intervals until reproducible maximal contractions were achieved and then to noradrenaline (NA) (1 μmol/L). When contraction to NA had reached a plateau, the vessels' endothelium-dependent vasodilator response was assessed with the addition of the stable analogue of acetylcholine carbachol (3 μmol/L). Arteries that were unable to contract to KPSS or NA were discarded. Those that failed to relax in response to carbachol were not included in endothelium-dependent protocols. The arteries were then incubated for a further 30 minutes in fresh PSS prior to commencing cumulative concentration-response curves (CCRCs) incorporated in the study protocols. Reponses to vasodilators were expressed as a percentage relaxation following pre-constriction with 1 μmol/L NA.

2.4.7.1. Cumulative concentration response curves to hydralazine alone

This series of experiments aimed to determine the vasodilator effects of hydralazine on SRAs. Following normalisation and a rest period of 30 minutes CCRCs to hydralazine (1 nmol/L-10 μmol/L) were constructed in vessels pre-constricted with 1 μmol/L NA. Plasma concentrations in patients receiving hydralazine for hypertension are 0.1-1.0 μmol/L in patients taking therapeutic doses; therefore these CCRCs encompass the therapeutic and supra-therapeutic range (79, 148-151).

2.4.7.2. Interaction between hydralazine and endothelium-dependent vasodilators

This series of experiments aimed to determine if hydralazine augments the vasodilator action of the, endothelially active response to carbachol. Following normalisation and confirmation of endothelial integrity by response to carbachol, vessels were pre-incubated (for 30 min at 37ºC) in pairs with either hydralazine (1 μmol/L – concentration determined as discussed above) or diluent control (PSS) prior to pre-constriction with 1 μmol/L NA and construction of CCRCs to carbachol (1 nmol/L – 30 μmol/L).

2.4.7.3. Interaction between hydralazine and clinically relevant organic nitrosovasodilators

This series of experiments aimed to examine the hydralazine-nitrate relationship in human blood vessels and the relationship between nitrosovasodilators known to have differing bio-activation pathways(152).

In pairs, vessels were pre-incubated with either hydralazine (1 μmol/L – concentration determined as discussed above) or diluent control (PSS) for 30minutes prior to pre-constriction with 1 μmol/L NA and construction of CCRCs to the *high-potency* nitrosovasodilators glyceryl-trinitrate (GTN 0.1 nmol/L -0.3 μmol/L) and sodium nitroprusside (SNP 1 nmol/L – 30 μmol/L) and the *low potency* nitrosovasodilator isosorbide dinitrate (ISDN 1 nmol/L -0.3 μmol/L). Concentration ranges were selected following review of the relevant literature and on the basis of previous vascular reactivity experiments undertaken using these agents by our group(153-156).

2.5 Vascular superoxide studies: methods for study of effects of hydralazine on superoxide production in human internal mammary arteries and long saphenous veins.

2.5.1 Patients

Patient recruitment was undertaken as described in **Chapter 2.2.1.2** above. Residual segments of saphenous vein (SV) and internal mammary artery (IMA) were obtained during elective CABG surgery in the patient cohort described in **Chapter 2.2.1.2** above. Participant characteristics and demographics for the entire cohort are presented in **table 2.1**. All patients met the inclusion criteria established for the study.

2.5.2 Vessel preparation

In the operating theatre, the vessels were immediately transferred to sterile normal saline solution (NaCl 0.9%) in a universal container prior to transfer to the laboratories at the BHF GCRC. On arrival, the vessels were immediately transferred into chilled Krebs HEPES solution (10mmol/L) and refrigerated at 4°C until experimental protocols were undertaken the following day. The vessels were then carefully dissected from adherent connective tissue under light microscopy and divided into 3-4 mm segments and weighed.
The vessels were then incubated in Krebs-Ringer HEPES (KRH) buffer (119 mM NaCl, 20mM Na-HEPES, pH 7.4, 5mM $NaHCO_3$, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4.7H_2O$, 10 mM glucose, 1mM KH_2PO_4) until experimental protocols were undertaken.

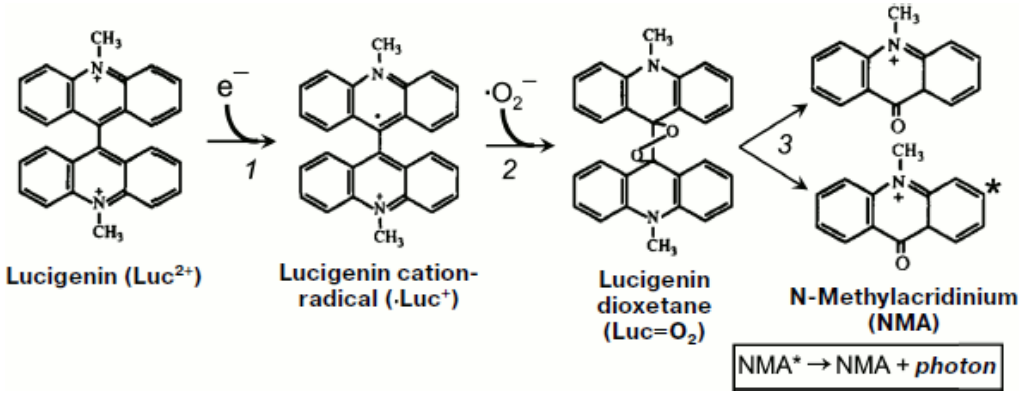
2.5.3 Lucigenin-enhanced chemiluminescence

Superoxide production was measured in vascular rings by chemiluminescence using lucigenin (*bis*-*N*-methylacridicium). This is an established technique used by our research group and is the most commonly used chemiluminescence method for the detection of vascular superoxide(170, 171). Chemiluminescent probes are small molecules capable of crossing the cell plasma membrane and detect intracellular reactive oxygen species (ROS). The interaction of the probe with the selected ROS results in a photon-emitting reaction, which can then be detected (and quantified) by a luminometer or scintillation counter. Lucigenin is relatively specific for the detection of superoxide. Lucigenin is first reduced to produce the lucigenin

cation radical. Superoxide is then capable of reducing this cation to dioxetane, which decomposes to produce two molecules of N-methylacridone, one of which exists in the excited state and emits a photon upon relaxation to the ground state (**figure 2.2)**.

Lucigenin-enhanced chemiluminescence can be used to determine basal, unstimulated production in intact vascular segments; this facilitates superoxide measurements, which more closely resemble physiological conditions. One of the major concerns with this technique is that lucigenin can undergo redox cycling, reacting with oxygen to artificially generate superoxide resulting in overestimation of superoxide production. This phenomenon is well recognised and can be partly overcome by using low doses of lucigenin (less than 20μM)(170, 172).

Figure 2.2 The lucigenin reaction pathway



2.5.4 Experimental protocols

Dr Carlene Hamilton assisted with the chemiluminescence experiments. Ring segments were placed in KRH buffer and allowed to equilibrate at 37°C for 30 minutes. Samples were then added to scintillation vials containing 2ml buffer and low concentration lucigenin as described previously by our group(170). Samples were analysed in a liquid scintillation counter (Hewlett Packard Tricarb 2100TR) in the out-of-coincidence mode with a single active photomultiplier tube. Readings were taken every 10 seconds for 3 minutes and absolute counts quantified with a xanthine/xanthine oxidase calibration curve for superoxide generation and standardised to wet weight of the tissue. Calibration curves were in the range of 28nM to 280 nM xanthine and prepared by adding 20 μl xanthine oxidase (0.1 U/ml), 5μM lucigenin and increasing volumes of 20 μM xanthine to a scintillation vial containing 2ml KRH buffer. Counts were reported as nmol/mg/min. In all experiments undertaken, superoxide production was measured in paired samples.

2.5.4.1 Basal superoxide production

To investigate the effect of hydralazine on basal superoxide production pairs of rings (SV and IMA) were compared and pre-treated for 30min at 37 °C with a clinically relevant range of concentrations of hydralazine (0.01, 0.1, 1 μmol/L) with paired untreated controls. Plasma concentrations in patients receiving hydralazine for hypertension are ≤1.0μmol/L in patients taking therapeutic doses; therefore these protocols encompass the therapeutic range (79, 148-151).

2.5.4.2. Angiotensin-II enhanced superoxide production in human internal mammary arteries

Angiotensin-II (Ang II) increases vascular superoxide production through activation of NAD(P)H oxidase. Ang II production is one of the hallmarks of neurohormonal activation in heart failure with a myriad of adverse effects on blood vessels and the heart. Berry *et al* have previously demonstrated that superoxide production in greater in human IMAs than SVs and that Ang II-mediated superoxide production could be attenuated by drug therapy (ATR1 receptor antagonist losartan). In this series of experiments we sought to determine whether the co-incubation of vessels with hydralazine could attenuate the Ang II-mediated increase in superoxide production and thus partly explain its favourable effects in heart failure (a clinical syndrome characterised by Ang II excess). Paired rings of IMA were incubated at 37°C in the absence (control) and presence of hydralazine (1μmol/L – concentration determined as discussed above) and Ang II (1μmol/L) for 4 hours prior to quantification of superoxide production as described in **Chapter 2.5.4** above.

2.6 Data and statistical analyses

For clinical data and measurements in blood vessels, continuous data are shown as mean ± standard error of the mean (SEM), unless otherwise indicated. For comparisons of a continuous variable between 2 experimental groups, paired and unpaired Student's t-tests were applied as appropriate and, if necessary, *post hoc* analysis of variation with Bonferroni correction to account for multiple comparisons to reduce type-1 error. In vascular response protocols, results are expressed as relative the maximum preconstriction to PE or NE (as a percentage). In the case of CCRCs generated with carbachol and organic nitrates the EC_{50} (concentration of agonist required to effect a 50% response was calculated to determine the additional effects of pre-incubation on the vasodilator action of these agents. A *P-value* of less than 0.05 (two tailed) was considered significant. Statistical analyses and graph generation were performed using Minitab Version 16.1.0 (© Minitab Inc 2010) and Prism 6.0 (© GraphPad Software Inc 2014).

Chapter 3 – Comparative vasodilator effect of hydralazine in human internal mammary arteries long saphenous veins and subcutaneous resistance arteries

3.1 Summary

Hydralazine has been in clinical use as an anti-hypertensive agent for nearly six decades. Notwithstanding that, its mechanism of action has been poorly understood. Most of the literature available on its action arises from studies on animal models(99, 100, 108). Hydralazine appears to reduce the contractile responses to a number of vasoconstrictors, and this affect appears to be greater in arteries compared with veins(102, 173). Differential effects on arterial and venous smooth muscle may be therapeutically relevant, particularly when considering use in combination with other vasodilator drugs such as organic nitrates. Historically *in vivo* studies in humans suggested a preferential effect on arterial vessels(174). The cardinal *ex vivo* studies used a post mortem preparation of human metacarpal veins and digital arteries and examined the effect of hydralazine pre-treatment on contractile responses to various potent agonists. Hydralazine significantly shifted the contractile curves to the right (i.e. evincing antagonised contractility), more so in arteries than in with veins. There has hitherto never been a comprehensive assessment of the *direct* vasodilator effects of hydralazine on large and small calibre blood vessels taken from patients with chronic heart failure.

3.2 Aims

The hypothesis was that hydralazine would have a vasodilator effect on arteries and veins taken from patients with LVSD and CAD and have a greater effect on arteries than veins.

The aims of this study were:

1. To determine the comparative vasodilator effect of hydralazine on human internal mammary artery (IMA) and saphenous vein (SV) using therapeutically relevant concentrations of hydralazine.

2. To determine the vasodilator effect of hydralazine on human subcutaneous resistance arteries using therapeutically relevant concentrations of hydralazine.

3.3 Patients

Organ bath studies were performed in saphenous veins (SVs) and internal mammary arteries (IMAs) taken from patients undergoing elective CABG. All patients were recruited as part of the VASCAB study as described in **Chapter 2.2.1.2** above.

Wire myography was performed using subcutaneous resistance arteries dissected from gluteal biopsies from patients in chronic heart failure secondary to coronary artery disease. Detailed recruitment is described in **Chapter 2.2.2.2** above.

Participant characteristics and demographics for the entire cohort are presented in **tables 2.1 and 2.2** above respectively.

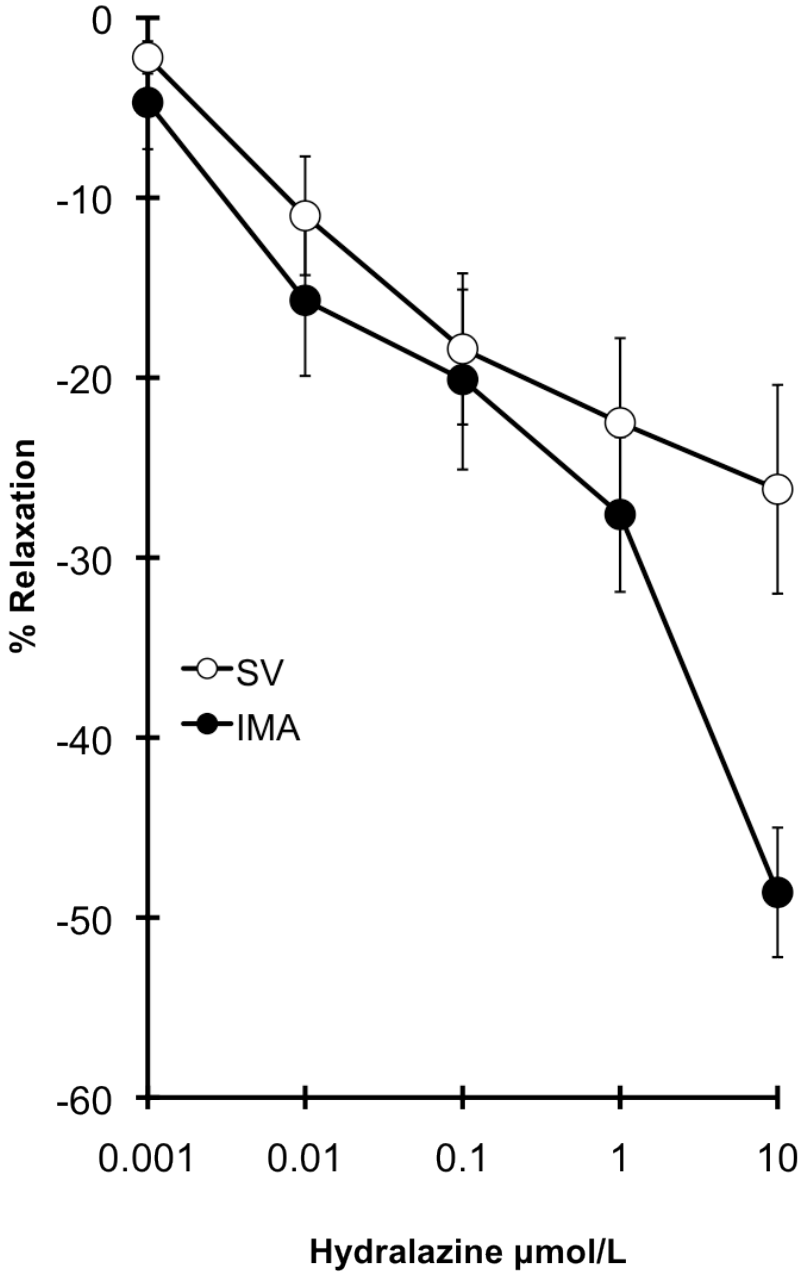
3.4 Organ bath technique

Rings of IMA and SV were prepared, mounted in organ baths and underwent standard start-up protocols as described in **Chapter 2.3.2** above. All vessels were pre-constricted with the noradrenaline analogue phenylephrine (3 μmol/L) after which CCRCs were constructed for hydralazine (0.01 to 10 μmol/L). Vasodilator responses (mean+/-SEM) are expressed as percentage relaxation from maximally pre-constricted values.

3.4.1 Hydralazine cumulative concentration response curves in human internal mammary arteries and saphenous veins

Maximum relaxation achieved to hydralazine was 26.2±5.81% in SVs compared to 45.35 ±4.25% in IMAs (P=0.032). There was only slightly less venous dilation than arterial at "therapeutic" concentrations (0.1-1μmol/L). Hydralazine no significant vasodilator action at "therapeutic" concentrations – this effect was observed in both veins and arteries to a similar degree (**Figure 3-1**).

Figure 3.1 Cumulative concentration response curves showing vasodilatation in saphenous vein (SV) and internal mammary artery (IMA) rings (n=10 for each) in response to hydralazine 0.01 to 10 µmol/L. Results shown as mean ± SEM



3.5 Systemic resistance artery studies

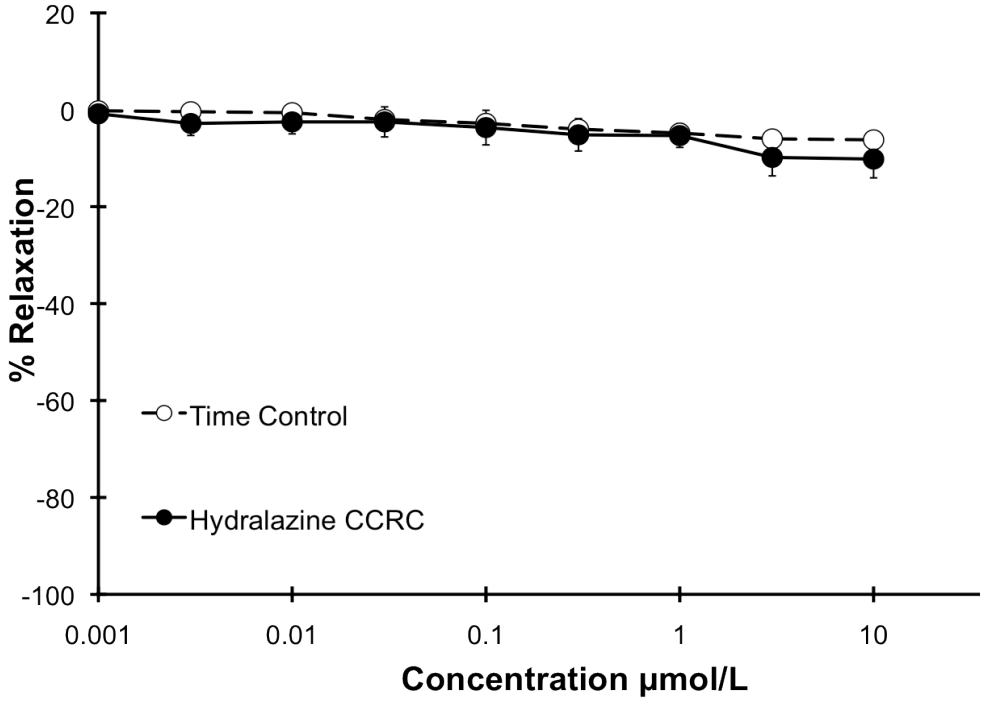
3.5.1 Gluteal biopsy procedure and artery preparation

Gluteal biopsies were obtained under local anaesthesia (1% lignocaine), as previously described in **Chapter 2.4.3** above. Resistance arteries (diameter < 500 μm, length approximately 2mm) were dissected and mounted in the 4-channel myograph. The bath was gassed and heated for the duration of the experiment. Start-up and normalisation protocols were undertaken as previously described in **Chapter 2.4.6** above. Vessel viability was determined by intact contractile response to KPSS (123 mmol/L) and noradrenaline (1 μmol/L). Endothelial integrity was determined by establishing intact vasodilator response to the stable acetylcholine analogue carbachol (3 μmol/L). The mean internal diameter (ID) of the systemic resistance arteries was 344.8 ± 62.6 (SD) μm.

3.5.2 Cumulative concentration response curves in human resistance arteries

CCRCs were constructed to a range of therapeutically relevant concentrations of hydralazine (1nmol/L - 10μmol/L) or diluent control in pairs of vessels taken from 6 gluteal biopsy samples. All vessels were established to be viable as *per* start up protocols. 8 pairs of vessels had intact endothelium and were used in the protocol. Hydralazine had no effect compared to diluent control (**Figure 3-2**). To confirm the integrity of vasodilator responses a terminal addition of carbachol (3 μmol/L) was added after completion of hydralazine CCRC. This confirmed intact vasodilator responses in all vessels studied (data not shown).

Figure 3.2 Cumulative concentration response curves to hydralazine (1 nmol/L – 10 µmol/L) or diluent control in paired endothelium-intact subcutaneous resistance arteries (n=8). Results are expressed as mean percentage relaxation ± SEM.



3.6 Summary of chapter results

This is the first *ex vivo* assessment of the direct vasodilator effects of hydralazine on human blood vessels taken from patients with chronic heart failure secondary to coronary artery disease. In this study hydralazine had no significant vasodilator effect on subcutaneous resistance arteries. This was a somewhat surprising finding given our understanding that these vessels contribute the greatest resistance to blood flow and thus capillary pressure(157). That said, in the early clinical trials proving efficacy of the hydralazine-ISDN combination, clinical efficacy was *independent* of blood pressure lowering(175). In large calibre vessels, maximal relaxation achieved to hydralazine was significantly greater in arteries compared to veins at supra-maximal drug concentration. At "therapeutic concentrations" (0.1-1 μmol/L) there was no significant vasodilator effect. These data suggest that the therapeutic effects of hydralazine may not simply be dependent on arterial vasodilatation and direct vasodilator activity and that the observed clinical benefits of combination therapy with isosorbide dinitrate may be partly explained by favourable effects elsewhere; perhaps large artery stiffness.

Chapter 4 – Interaction between hydralazine and endothelium-dependent vasodilators

4.1 Summary

Endothelial dysfunction plays a pivotal role in the development of cardiovascular disease, notably heart failure(176, 177). It may be a feature of heart failure of any aetiology, but is best characterised in heart failure secondary to coronary artery disease, where multiple contributory factors such as atherosclerosis, diabetes mellitus and hypertension contribute to endothelial impairment(178). Endothelial dysfunction is considered to be a systemic process. It may involve arterial, venous and microcirculatory vascular beds(179, 180). Multiple aspects of endothelial function can be deregulated, including vasomotor, haemostatic, anti-oxidant and inflammatory pathways.

There are various methods available to assess endothelial function(181). The dominant *ex vivo* approach is to measure endothelium-dependent vasodilatation. This may be impaired either secondary to reduced NO bioavailability, or decreased NO production (arising as a consequence of a legion of inter-dependent factors such as reduced NO synthase activity, reduced cofactor availability and impaired cellular signalling mechanisms). The neurotransmitter acetylcholine is a potent endothelium-dependent vasodilator, predominantly acting via stimulation of NO release and cGMP activation. Vascular reactivity studies can be undertaken in organ bath experiments and wire myography to explore acetylcholine-mediated vasodilatation. Clearly one of the major limitations of *ex vivo* assessment of vascular function is the availability of vessels. Although atherosclerotic lesions do not affect veins to the same extent as arteries, endothelial dysfunction has been demonstrated in both veins and arteries taken from patients with coronary artery disease and heart failure(147, 179). Given the limited availability of arterial samples the use of veins is therefore considered an appropriate surrogate.

The most (though not entirely) consistent literature suggests that hydralazine leads to activation of guanylate cyclase. Clearly, this action to increase cGMP, if true, could explain the favourable clinical benefits of its combination with oral nitrates. It would therefore be crucial to determine if hydralazine augments endothelium-mediated vasodilatation (and thus NO-cGMP activity) in endothelium-intact vessels.

4.2 Aims

This series of experiments was aimed to determine if hydralazine augments the vasodilator response to the endothelially-active agent carbachol.

The aims of this study were:

1. To determine if hydralazine augments the vasodilator response to carbachol in human large calibre blood vessels.

2. To determine if hydralazine augments the vasodilator response to carbachol in human subcutaneous resistance arteries.

4.3 Patients

Organ bath studies were performed in saphenous veins (SVs) from patients undergoing elective CABG. All patients were recruited as part of the VASCAB study as described in **Chapter 2.2.1.2**. Internal mammary arteries were studied but there were insufficient data for inclusion in this chapter owing to lower yield at time of surgery and damage to endothelium during dissection and/or mounting. Whilst atherosclerotic lesions do not affect veins to the same extent as arteries, endothelial dysfunction has been demonstrated in both veins and arteries taken from patients with coronary artery disease and heart failure(147, 179).

Wire myography was performed using subcutaneous resistance arteries dissected from gluteal biopsies from patients in chronic heart failure secondary to coronary artery disease. Detailed recruitment is described in **Chapter 2.2.2.2** above.

Participant characteristics and demographics for the entire cohort are presented in **tables 2.1 and 2.2** respectively.

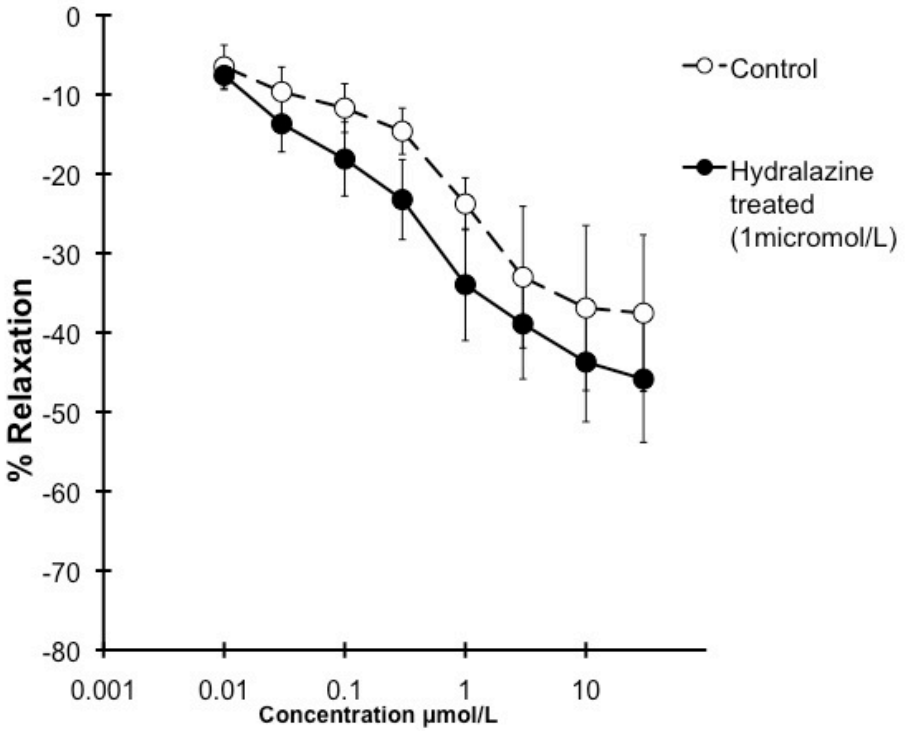
4.4 Organ bath technique

Rings of SV were prepared, mounted in organ baths and underwent standard start-up protocols as described in **Chapter 2.3.2**. All vessels were pre-constricted with the noradrenaline analogue phenylephrine (3 μmol/L) following which cumulative concentration response curves (CCRCs) were constructed. Vasodilator responses (mean+/-SEM) are expressed as percentage relaxation from maximally pre-constricted values. Only SV was used in this series of experiments because of the availability of tissue.

4.4.1 Hydralazine cumulative concentration response curves in human long saphenous veins

CCRCs were constructed with carbachol (a stable analogue of acetylcholine) 1 nmol/l - 10 μmol/l in the presence or absence of hydralazine (1 μmol/L) in SVs from 6 patients. Carbachol produced concentration-dependent relaxation in control SVs with a maximal relaxation of 37.55% (SEM 9.86). Maximal vasodilator action to carbachol was not significantly affected by pre-treatment with hydralazine, with maximal relaxation of 45.86% (SEM 7.96) (P=0.239). Hydralazine pre-treatment did however, lead to an apparent leftward shift in the CCRC suggesting augmented response to carbachol although EC_{50} was not significantly different [control EC_{50} 0.618 μmol/L hydralazine-treated EC_{50} of 0.288 μmol/L (P=0.87)]. **(Figure 4.1)**

Figure 4.1 Cumulative concentration response curves to carbachol (1 nmol/L - 10 μmol/L) in pairs of human saphenous veins (n=6) in the presence (closed symbols) or absence (open symbols) of hydralazine (1 μmol/L). Results are expressed as mean percentage relaxation ± SEM.



4.5 Systemic resistance artery studies

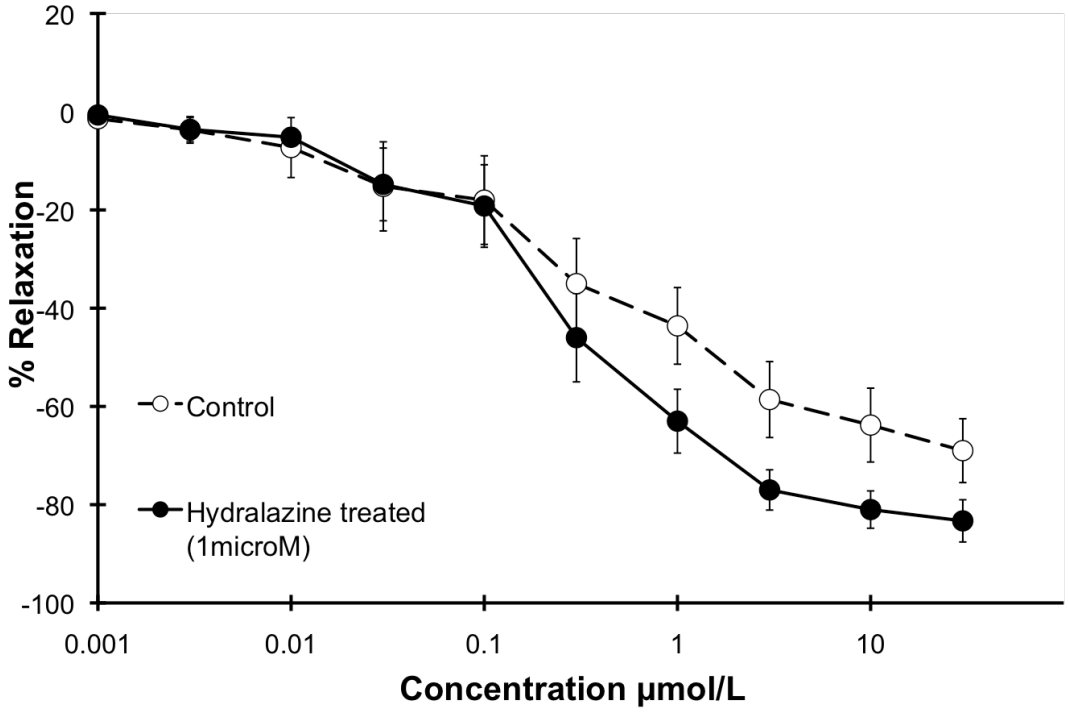
4.5.1 Gluteal biopsy procedure and artery preparation

Gluteal biopsies were obtained under local anaesthesia (1% lignocaine), as previously described (**Chapter 2.4.3**). Resistance arteries (diameter < 500 μm, length approximately 2mm) were dissected and mounted in the 4-channel myograph as previously described. The bath was gassed and heated for the duration of the experiment. Start-up protocols were performed as described in **Chapter 2.4.6**. Following determination of viability with KPSS (123 mmol/L) and NA (1 μmol/L) endothelial-integrity was confirmed by vasodilator response to carbachol (3μmol/L). Those that failed to relax in response to carbachol were not included in the protocol. The mean internal diameter (ID) of the systemic resistance arteries was 303.1± 61(SD) μm.

4.5.2 Cumulative concentration response curves in human resistance arteries

CCRCs were constructed with carbachol (1nmol/L - 30 μmol/L) in presence or absence (diluent control) of hydralazine (1μmol/L) in 8 pairs of arteries (8 patients). Carbachol produced concentration-dependent relaxation in control arteries with a maximal relaxation of 70.38 % (SEM 7.21). Maximal vasodilator action of carbachol was numerically affected by pre-treatment with hydralazine with a maximal relaxation of 83.00% (SEM 4.87) vs 70.38% (SEM 4.87). However, with two-way ANOVA for repeated measures, treatment interactions were not statistically significant, even at maximal concentration (P=0.0806 ANOVA). Hydralazine pre-treatment led to an apparent leftward shift of the CCRC with differences but EC_{50} were not statistically significant [control EC_{50} 0.294 μmol/L and hydralazine-treated EC_{50} of 0.268 μmol/L (*P*=0.1)]. **(Figure 4.2).**

Figure 4.2 Cumulative concentration response curves to carbachol (1nmol/L - 30 µmol/L) in pairs of human subcutaneous resistance arteries (n=9) in the presence (closed symbols) or absence (open symbols) of hydralazine (1µmol/L). Results are expressed as mean percentage relaxation ± SEM. * indicates statistically significant difference between pairs with comparison between respective groups by ANOVA.



4.6 Summary of chapter results

These experiments demonstrate a non-significant trend towards augmented vasodilatation with carbachol in the presence of hydralazine in large and small calibre blood vessels taken from patients with chronic heart failure. Although not reaching statistical significance, there were numerically different maximal responses in vessels treated with hydralazine; suggesting a trend of potential biological relevance. Interaction of hydralazine with endothelium-dependent vasodilatation may contribute to the established favourable effects of hydralazine in combination with ISDN in patients with chronic heart failure, particularly in those known to have excessive degrees of endothelial dysfunction(182). The present study was undertaken exclusively in patients of European Caucasian origin. This study was limited by small numbers of vessels examined. Larger studies are needed to determine if this effect is significant and in other vascular preparations. It would be valuable to examine the effects of hydralazine *in vivo* using techniques to assess endothelial function in patients with heart failure.

With respect to *ex vivo* functional investigation, endothelial dysfunction generally relates to impaired maximal vasodilator response and/or an impaired sensitivity to endothelium-dependent vasodilators such as acetylcholine (and its stable analogue carbachol), bradykinin and calcium ionophore, with preserved response to endothelium-independent dilators such as sodium nitroprusside(183, 184). We only used one endothelium-dependent agonist carbachol, selected on the basis of published work from our group and others(185, 186). Comparison of the effects of hydralazine treatment on vessels with and without endothelium would allow a more complete assessment of the role of eNOS in the observed vasodilator activity. The absence of such control protocols weakens the observations of the present data. However, destruction of vascular wall integrity during the process of endothelial denudation destroys myo-endothelial gap junction communication in VSMC(187). This injury process also promotes oxidative stress signalling which impairs vasodilator responses(188, 189). Nevertheless, inclusion of such control protocols would allow a more confident attribution of the observed differences to endothelial mechanisms.

Chapter 5 – *Ex vivo* interaction of hydralazine with organic nitrates

5.1 Summary

In combination with hydralazine, the organic nitrate ISDN has favourable effects on morbidity and mortality in patients with heart failure. The efficacy of this combination had originally been attributed to the favourable interaction of the different haemodynamic actions exerted by each vasodilator on the arterial and venous vasculature (hydralazine thought by many to be a dominant arterio-vasodilator and nitrates venodilator)(142). Neither drug on its own has mortality benefits in heart failure. Most now believe the therapeutic synergy is unlikely to be simply explained by balanced haemodynamic effects.

The organic nitrates used in clinical practice are believed to vasodilate both arteries and veins through the release of NO and subsequent activation of guanylate cyclase in vascular smooth muscle. They have beneficial effects in reducing cardiac preload and afterload. Most organic nitrates (including GTN and ISDN) require vascular biotransformation to exert their pharmacological effect.

In contrast, SNP is thought to spontaneously release NO and thus act as a direct (endothelium-independent) NO donor. This process may be catalysed by vascular enzyme-systems including NAD(P)H oxidase(190). Long-term nitrate therapy is limited by the rapid development of pharmacological tolerance, possibly secondary to increased production of vascular superoxide(191).

Hydralazine co-treatment prolongs the vasodilator effect of nitrates in animal models and clinical studies, though the mechanism of this protection in humans is uncertain(104, 105). Paradoxically, hydralazine has been shown to attenuate the vasodilator effect of SNP (through inhibition of NAD(P)H oxidase) in one animal model(128). We sought to explore the direct interaction between hydralazine and organic nitrates in human blood vessels from patients with chronic heart failure.

5.2 Aims

This series of experiments was aimed to determine if hydralazine augments the vasodilator response to a range of clinically relevant organic nitrates.

The aims of this study were:

1. To determine if hydralazine augments the vasodilator response to the *high-potency* organic nitrates GTN and SNP and the *low-potency* organic nitrate ISDN in human blood vessels.

5.3 Patients

Organ bath studies were performed in saphenous veins (SVs) taken from patients undergoing elective CABG. All patients were recruited as part of the VASCAB study as described in **Chapter 2.2.1.2**.

Wire myography was performed using subcutaneous resistance arteries dissected from gluteal biopsies from patients in chronic heart failure secondary to coronary artery disease. Detailed recruitment is described in **Chapter 2.2.2.2**.

Participant characteristics and demographics for the entire cohort are presented in **tables 2.1 and 2.2** respectively.

5.4 Organ bath studies

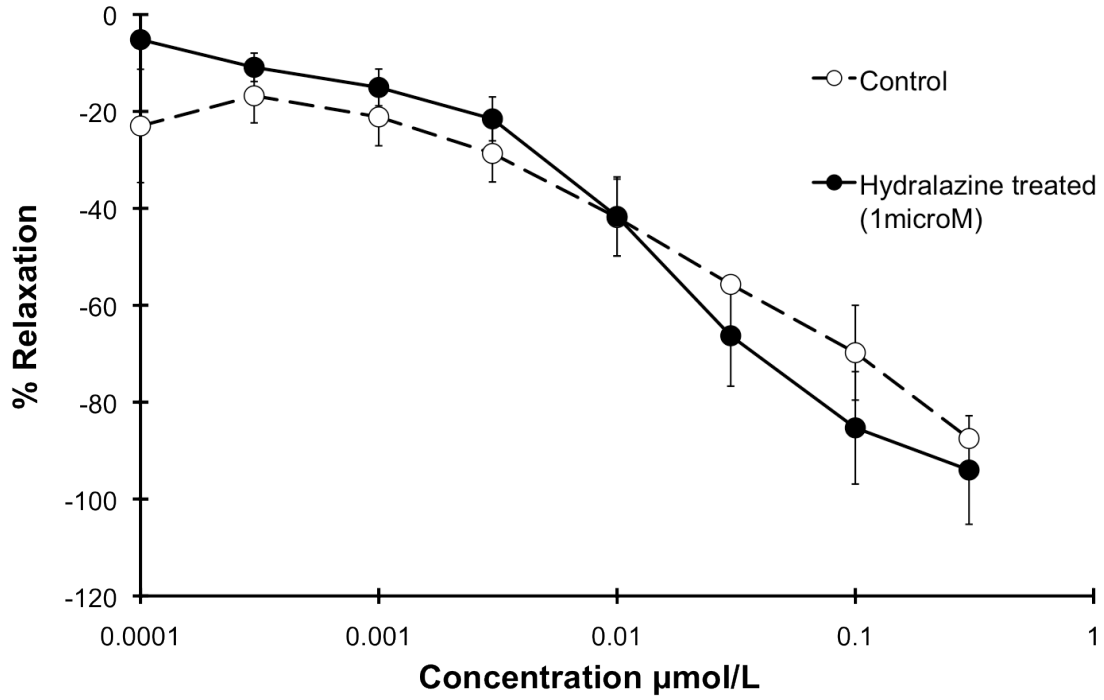
Rings of SV were prepared, mounted in organ baths and underwent standard start-up protocols as described in **Chapter 2.3.2**. All vessels were pre-constricted with phenylephrine (3 μmol/L) after which cumulative concentration response curves (CCRCs) were constructed for the specific nitrate (in a clinically relevant range) in the presence or absence (diluent control) of hydralazine (1μmol/L). Vasodilator responses (mean+/-SEM) are expressed as percentage relaxation from maximally pre-constricted values.

5.4.2 Cumulative concentration response curves with organic nitrates

5.4.2.1 Glyceryl-trinitrate

CCRCs were constructed with GTN (0.1 nmol/L – 0.3 μmol/L) in the presence or absence of hydralazine (1 μmol/L) in paired rings of SVs from 8 patients from the cohort. GTN had marked vasodilator effect on both pairs of vessels but there was no significant difference at maximal relaxation with 87.5% ± 10.3 compared with 94% ± 11.2 (P=0.411). There was no significant shift in the CCRC with hydralazine pre-treatment [control EC_{50} 0.0127 μmol/L hydralazine-treated EC_{50} of 0.0143 μmol/L (P=0.993)] **(Figure 5.1).**

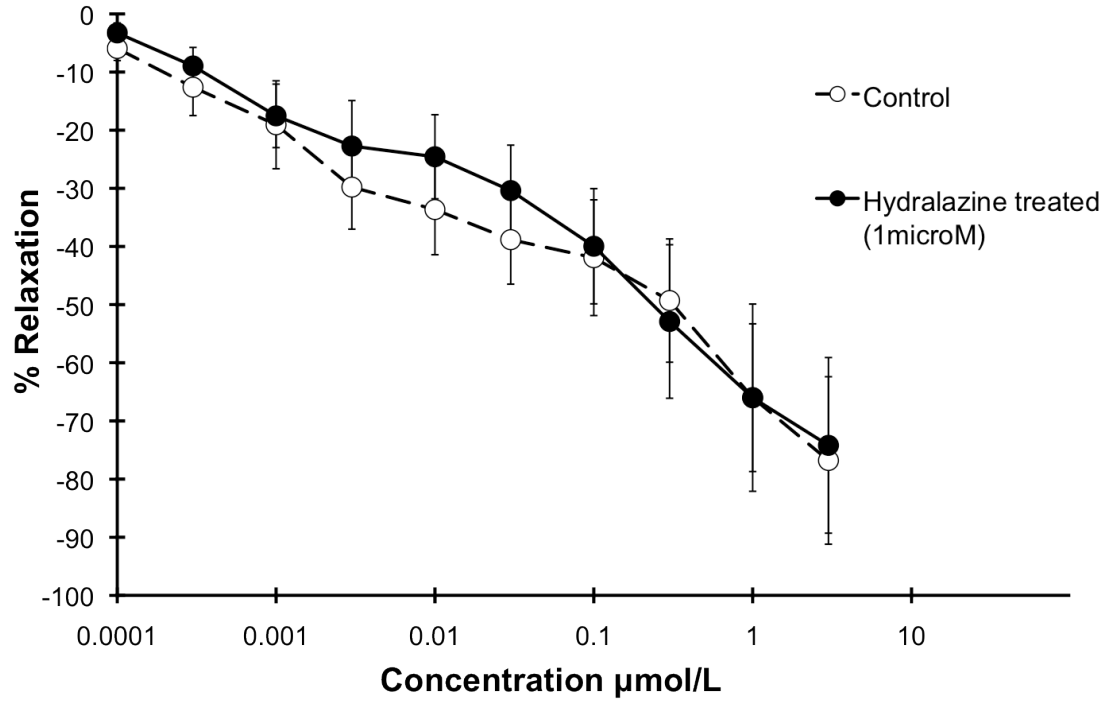
Figure 5.1 Cumulative concentration response curves to GTN (0.1 nmol/L-0.3 µmol/L) in pairs of human saphenous veins (n=8) in the presence (closed symbols) or absence (open symbols) of hydralazine (1 µmol/L). Results are expressed as mean percentage relaxation ± SEM



5.4.2.2 Isosorbide dinitrate

CCRCs were constructed with ISDN (0.1nmol/L - 3μmol/L) in the presence or absence of hydralazine (1μmol/L) in paired rings of SVs from 5 patients from the cohort. Maximal vasodilator response was 76.8% ± 14.14 in control vessels versus 74.2% ± 15.1 in hydralazine-treated (p=0.7). There was also no significant shift in the CCRC with hydralazine pre-treatment [control EC_{50} 2.04 nmol/L hydralazine-treated EC_{50} 0.0165 μmol/L (p=0.1)] **(Figure 5.2).**

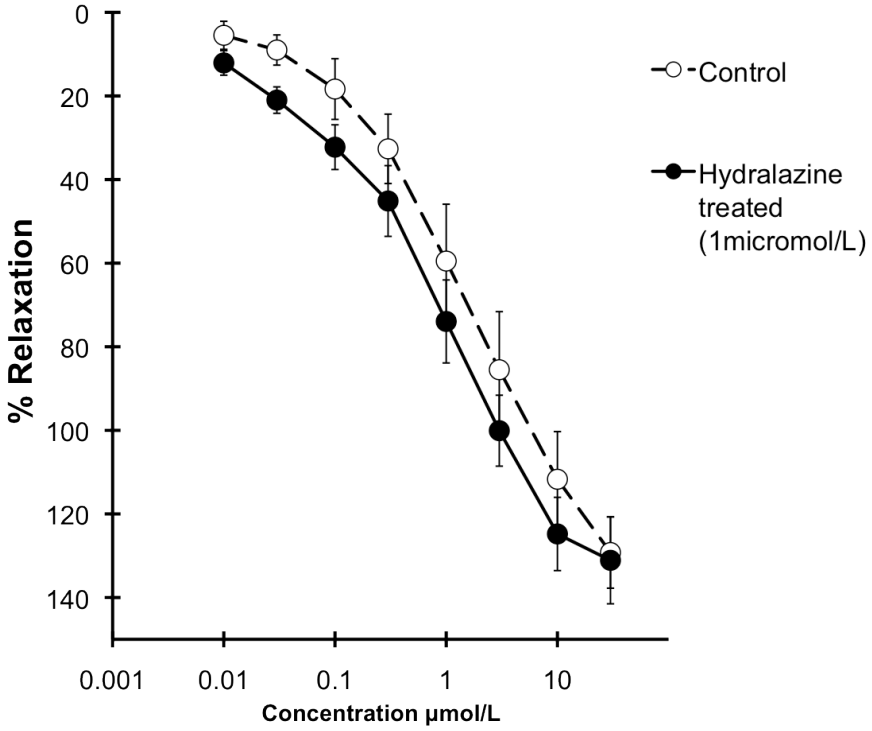
Figure 5.1 Cumulative concentration response curves to ISDN (0.1nmol/L-3µmol/L) in pairs of human saphenous veins (n=5) in the presence (closed symbols) or absence (open symbols) of hydralazine (1µmol/L). Results are expressed as mean percentage relaxation ± SEM



5.4.2.3 Sodium nitroprusside

CCRCs were constructed with SNP (1.0nmol/l - 30μmol/L) in the presence or absence of hydralazine (1μmol/L) in paired rings of SVs from 8 patients from the cohort. Maximal vasodilator response was 129.23% ± 8.53 in controls versus 131.1% ± 10.4 in hydralazine-treated (*P*=0.8). Hydralazine pre-treatment appeared to produce a leftward shift in the SNP CCRC curve although this was not statistically significant [control EC_{50} 1.39 μmol/L hydralazine-treated EC_{50} of 0.796 μmol/L (p=0.262)] **(Figure 5.3)**.

Figure 5.3 Cumulative concentration response curves to SNP (1 nmol/L - 30 µmol/L) in pairs of human saphenous veins (n=8) in presence (closed symbols) or absence (open symbols) of hydralazine (1µmol/L). Results are expressed as mean percentage relaxation ± SEM



5.5 Systemic resistance artery studies

5.5.1 Vessel preparation and myography procedure

Gluteal biopsies were obtained under local anaesthesia (1% lignocaine), as previously described (Chapter 2.4.3). Resistance arteries (diameter < 500 μm, length approximately 2mm) were dissected and mounted in the 4-channel myograph as previously described. The bath was gassed and heated for the duration of the experiment.

The mean internal diameter (ID) of the systemic resistance arteries was 283 + 21 μm.

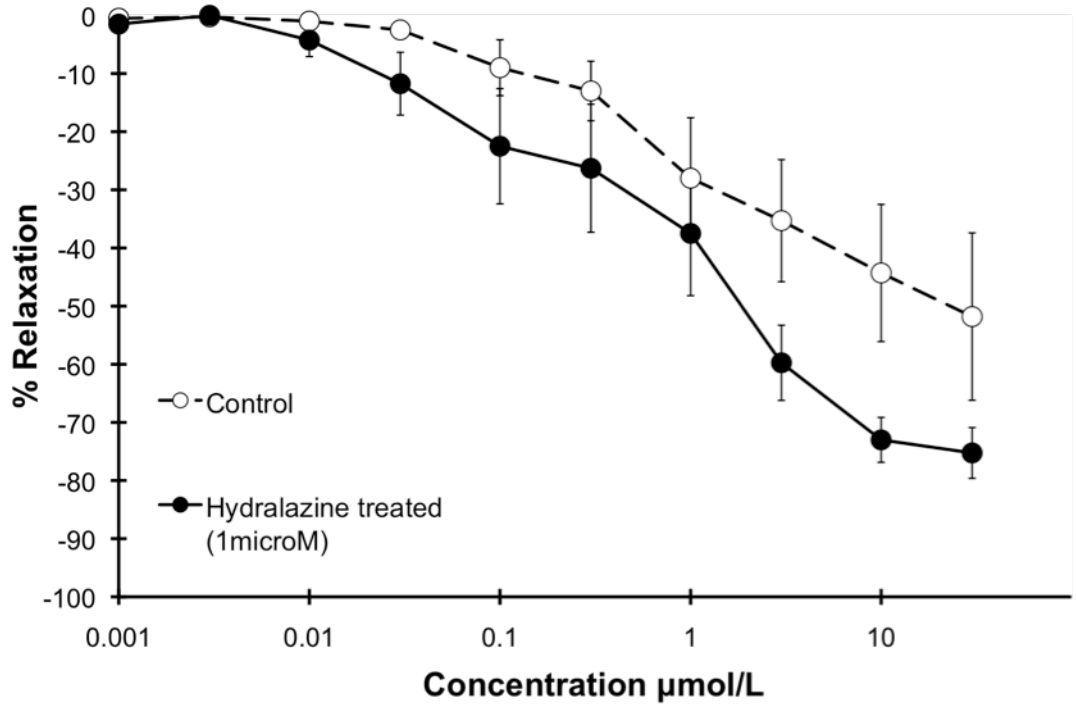
5.5.3 Cumulative concentration response curves with organic nitrates

Start-up protocols were performed as described in **Chapter 2.4.6**. Following determination of viability with KPSS (123 mmol/L) and NA (1 μmol/L) vessels were allowed to equilibrate. Due to time constraints for experimentation and the limited availability of human vessels to work with, the protocol was limited to SNP (to which hydralazine had had an apparent effect to shift the CCRC leftward in the organ bath protocols). Vessels were pre-treated with hydralazine (1 μmol/L) or diluent control for 30 minutes prior to pre-constriction with NA (100 μmol/L) and construction of SNP CCRC (1 nmol/L – 30 μmol/L).

5.5.3.1 Sodium nitroprusside

CCRCs were constructed as described in 6 pairs of vessels (control/hydralazine-treated) from 6 patients in the cohort. Vasodilator action of SNP appeared affected by pre-treatment with hydralazine although at maximal relaxation this was not statistically significant: 56.7% ± (SEM 9.9) in control vessels versus 81% ± (SEM 4.57) (P= 0.05). Hydralazine pre-treatment shifted the CCRC to the left although the EC_{50} was not significantly different [control EC_{50} 0.727 μmol/L hydralazine-treated EC_{50} of 1.01 μmol/L (P=0.177)].

Figure 5.4 Cumulative concentration response curves to SNP (1 nmol/L-30 µmol/L) in pairs of human subcutaneous resistance arteries (n=6) in the presence (closed symbols) or absence (open symbols) of hydralazine (1µmol/L). Results are expressed as mean percentage relaxation ± SEM. * indicates statistically significant difference between pairs at given concentration.



5.6 Chapter summary

The *ex vivo* interaction between hydralazine and organic nitrates appears to be very modest in blood vessels taken from patients with heart failure. This would tend to suggest that the beneficial interaction *in vivo* is not simply explained by direct vasodilator activity. The effects of the direct NO donor SNP appeared to be modestly augmented (although not reaching statistical significance). There is conflict in the literature about this interaction; some groups suggesting that hydralazine attenuate SNP-mediated vasodilatation (and subsequent cGMP production) whilst others found no such effect(128, 192, 193). These disparities may be partly explained by the diverse animal models used. In human blood vessels from patients with heart, this interaction has not been previously characterised.

Chapter 6 – Effects of hydralazine on *ex vivo* basal superoxide production in human internal mammary arteries and long saphenous veins.

6.1 Summary

Heart failure is characterised by a legion of pathophysiological processes, which are thought to include oxidative stress(4). The term oxidative stress refers to complex interactions between reactive oxygen species (ROS) and anti-oxidant systems. Levels of ROS can be measured directly or assessed using indirect markers. The biomarkers of ROS burden have been shown to be elevated in heart failure and correlate with the severity of the clinical syndrome(7). The principal source of ROS *in vivo* is superoxide (O_2-). This can be directly measured in vascular tissue or other cellular systems using a variety of techniques(172). There is a wealth of data directly implicating increased O_2- as a major underlying mechanism in the pathophysiology of cardiovascular disease(58). Strategies to reduce oxidative stress (or improve nitroso-redox balance) are an attractive therapeutic goal.

Within the heart and blood vessels there are a number of enzymatic sources of O_2-. These include NAD(P)H oxidase, xanthine oxidase, endothelial nitric oxide synthase (*NOS3*) and the mitochondrial electron transport system(194). Of these NADH/NAD(P)H dependent oxidases are understood to be some of the principal sources(195). These enzyme systems are regulated *in vivo* and *ex vivo* by angiotensin-II and aldosterone, and are believed to play a pivotal role in the development of endothelial dysfunction, a key pathophysiological abnormality in heart failure and other cardiovascular diseases(144, 196, 197). Studies from our group have demonstrated elevated O_2^- generation in saphenous vein and internal mammary artery from patients with advanced coronary artery disease (CAD) undergoing CABG compared with vascular tissue taken from healthy controls(198). Importantly, our group has also previously established that angiotensin-II increases superoxide production in human internal mammary artery through enhanced NAD(P)H oxidase activity(170).

Traditional methods for the detection of O_2^- in vascular tissue include lucigenin-enhanced chemiluminescence and fluorescence techniques involving the use of probes such as dihydroethidium (DHE)(172). Recent applications of these techniques have succeeded in demonstrating increased levels of O_2^- in vascular tissue from patients with advanced CAD as compared to individuals with no documented vascular disease(198, 199). Lucigenin-enhanced chemiluminescence is an established and well-validated technique used by our research group and is the most commonly used chemiluminescence method for the detection of vascular superoxide(171, 197). One of the concerns of this technique is redox cycling, where lucigenin itself acts as a source of O_2^- resulting in overestimation. This can be overcome by using low doses of lucigenin (less than 20μM)(170, 172).

As discussed previously (Chapter 1.3.2) there is an increasing body of evidence suggesting a favourable effect of hydralazine on nitroso-redox balance. These data have been exclusively restricted to animal models. Mechanistically a number of enzyme systems and processes have been implicated including increased soluble guanylate cyclase expression, inhibition of semicarbazide-sensitive amine oxidase and NAD(P)H oxidase(122, 126). The latter enzyme system has also been implicated in the development of vascular nitrate tolerance and is an attractive theoretical target for hydralazine(106). The potential anti-oxidant effects of hydralazine have never before been directly characterised in human blood vessels.

6.2 Aims

The principal hypothesis was that hydralazine would reduce basal vascular O_2^- production in internal mammary arteries (IMAs) and saphenous veins (SVs) taken from patients with established CAD and LVSD.

The aims of this study were:

1. To assess the effect of hydralazine on basal O_2^- production in IMAs and SVs
2. To assess the relative potency of hydralazine on IMAs vs. SVs
3. To assess any apparent dose-response to hydralazine on basal O_2^- production.

6.3 Patients

Vascular O_2^- measurements were performed in SVs and IMAs from patients undergoing elective CABG. All patients were recruited as part of the VASCAB study as described in **Chapter 2.2.1.2**.

Participant characteristics and demographics for the entire cohort are presented in **table 2.1**.

6.4 Lucigenin-enhanced chemiluminescence

6.4.1 Vessel preparation

O_2^-production was measured in 3-4 mm rings by chemiluminescence using lucigenin. Samples were analysed in a liquid scintillation counter (Hewlett Packard Tricarb 2100TR) in the out-of-coincidence mode. Readings were taken every 10 seconds for 3 minutes and absolute counts quantified with a xanthine / xanthine oxidase calibration curve for O_2^- generation and standardised to wet weight of the tissue. Detailed methods are described in **Chapter 2.5.4**.

To investigate the effect of hydralazine on basal O_2^-production we studied IMA and SV rings which had been pre-treated for 30min at 37°C with a range of concentrations of hydralazine (0.01, 0.1, 1 μmol/L). Each ring was paired with a control ring from the same subject incubated with buffer.

6.4.2 Basal superoxide production IMAs and SVs from patients with heart failure

We compared basal O_2^- production in arteries and veins from patients with heart failure. Basal $O_2^-$was significantly higher in IMA (n=12) compared with SV (n=12) 1.08 ± 0.14 nmol/mg/min vs. 0.74 ± 0.08 nmol/mg/min (*P*=0.006) **(Figure 6.1).**

6.4.3 Basal superoxide production in hydralazine treated vessels

Co-administration of vessels with hydralazine (1 μmol/L) reduced basal O_2^- production significantly in both IMAs 1.09 ± 0.14 vs. 0.77 ±0 .16 nmol/mg/min (P=0.026) **(Figure 6.2**) and SVs 0.77 ± 0.08 vs. 0.68 ± 0.08 nmol/mg/min (*P*=0.018) **(Figure 6.3)**.

Figure 6.1 Basal O_2^-production in SV and IMA rings from patients with heart failure. Results expressed as nmol/mg/min and are shown as mean± SEM. Red column represents IMA (n=12) and blue column SV (n=12)

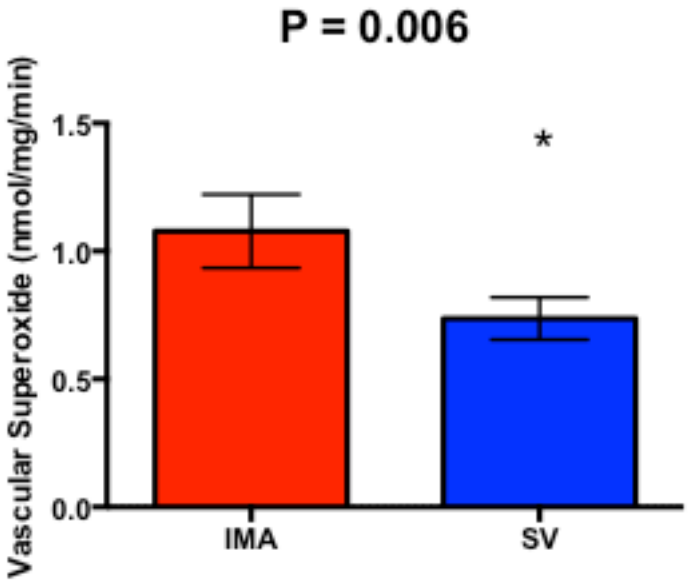


Figure 6.2 Effect of hydralazine on O_2^- production in human IMA. Blood vessels were incubated in the presence or absence of hydralazine (1 μmol/L) for 30 minutes prior to quantification of O_2^-. Results are expressed as nmol/mg/min and are shown as mean ± SEM. Red column represents control IMAs (n=12) and shaded column hydralazine-treated (n=12).

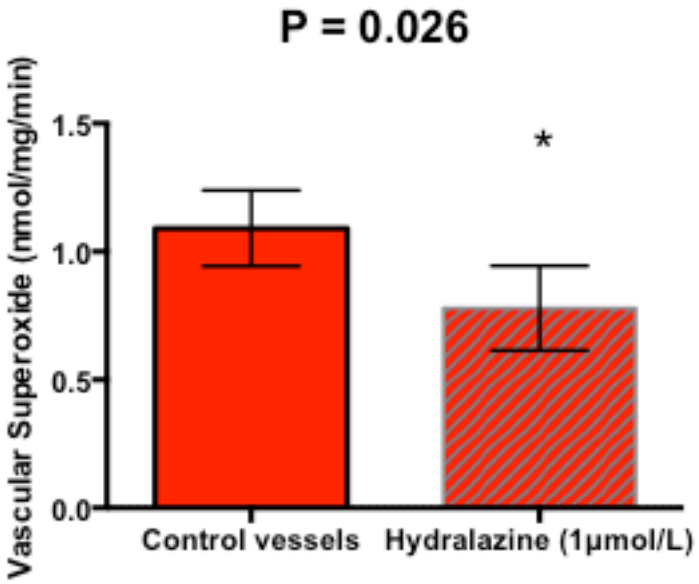
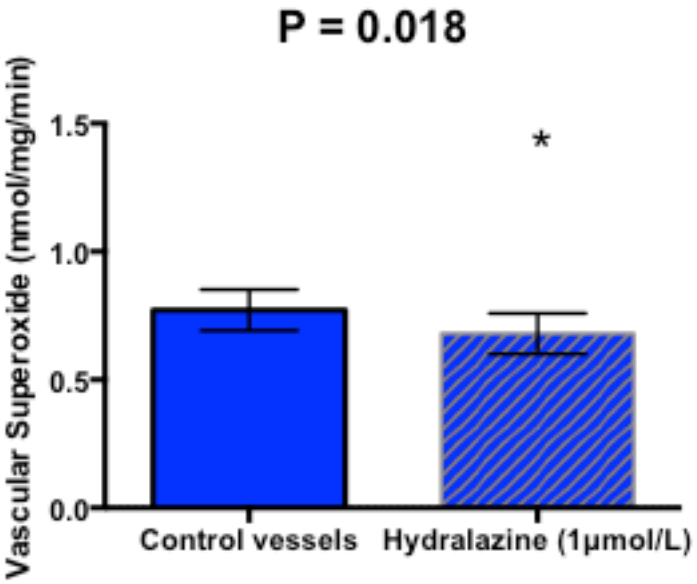


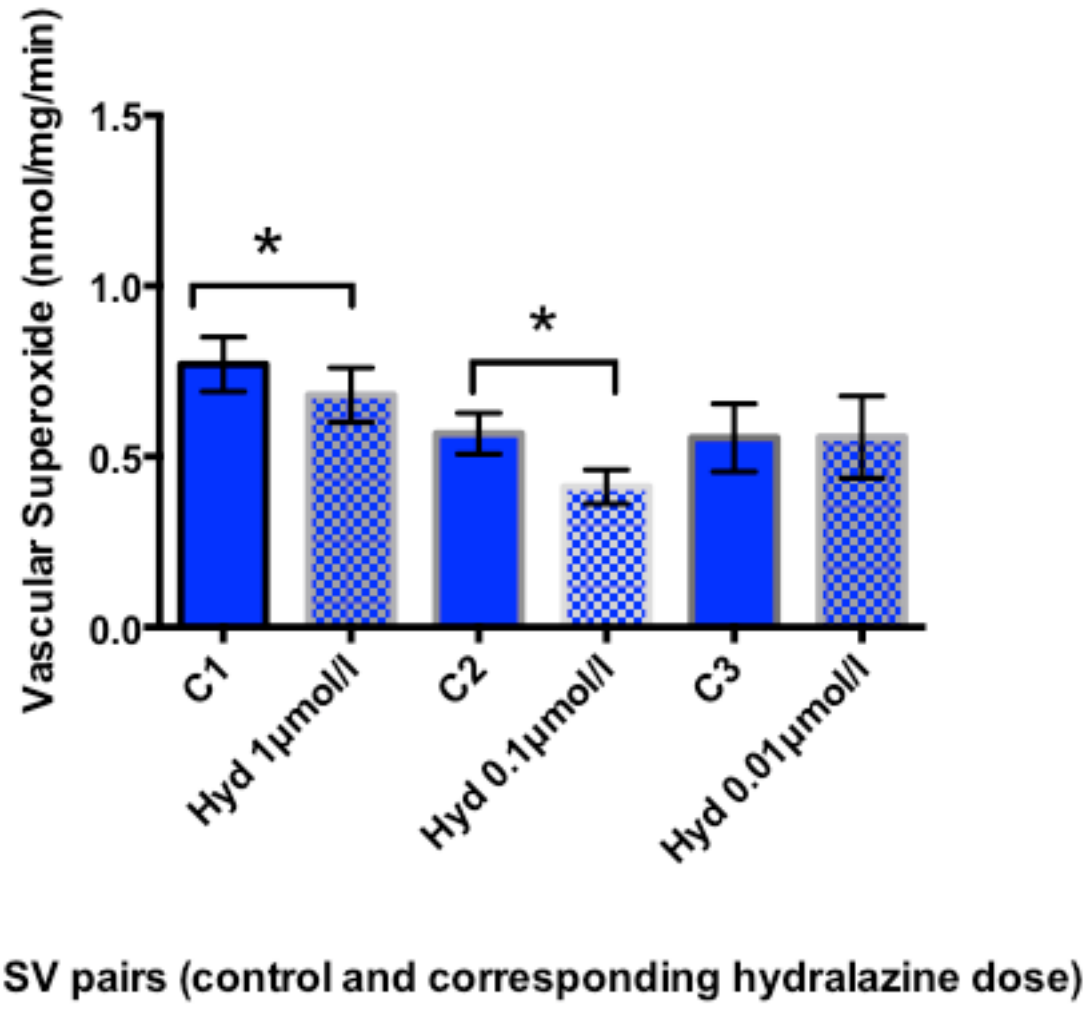
Figure 6.3 Effect of hydralazine on O_2^- production in human SVs. Blood vessels were incubated in the presence or absence of hydralazine (1 μmol/L) for 30 minutes prior to quantification of O_2^-. Results are expressed as nmol/mg/min and are shown as mean ± SEM. Blue column represents control SVs (n=12) and shaded column hydralazine-treated (n=12).



6.4.4. Dose-response relationship to hydralazine

In a series of experiments we sought to determine if there was a dose-response relationship to hydralazine. Paired rings of SV were incubated with either diluent control or a range of concentrations of hydralazine (0.01, 0.1, 1.0 μmol/L). Hydralazine at 1.0 μmol/L reduced O_2^- significantly [0.77 ± 0.08 nmol/mg/min vs. 0.68 ± 0.08 nmol/mg/min (P = 0.018)] and at 0.1 μmol/L [0.567 ± 0.06 nmol/mg/min vs. 0.411 ± 0.05 nmol/mg/min (P = 0.025)]. There was no apparent effect with the lowest dose increment of 0.01 μmol/L. These data suggest an apparent dose-response effect with hydralazine treatment **(Figure 6.4)**.

Figure 6.4 Effect of differing concentrations of hydralazine on O_2^- production in human SVs. Paired vessels were incubated in the presence or absence of hydralazine 1.0 µmol/L (n=12), 0.1 µmol/L (n=7) and 0.01 µmol/L (n=6) for 30 minutes prior to quantification of O_2^-. Results are expressed as nmol/mg/min and are shown as mean ± SEM.



6.5 Discussion

Increased levels of O_2^- in heart failure have been shown to be proportionate to the clinical severity (7). In animal models of heart failure, levels of O_2^- production are reduced with anti-oxidant treatment, which is associated with cardiac protection(72, 73). In patients with established CAD, oxidative stress may persist despite the use of agents that have been shown to reduce O_2^- production such as ACE inhibitors, Angiotensin-type1-receptor antagonists (ARBs) and HMG CoA reductase inhibitors (statins)(58, 200). There therefore exists a further potential therapeutic target for intervention –so-called nitric-oxide enhancing therapies.

To my knowledge this is the first demonstration that hydralazine reduces basal O_2^-production in human blood vessels. This effect was seen to a similar degree in both IMA and SVs. The experiments conducted were not paired with SV and IMA from the same subjects. As such, no conclusion can be made about the relative potency of hydralazine on IMA versus SV. Berry *et al* previously demonstrated that arteries are the dominant source of vascular O_2^- production in humans, owing presumably to the greater density of VSMC in the arterial media(170). Nevertheless, we have demonstrated that hydralazine significantly reduces O_2^- production in both vessel types. In SVs (which were much more readily available than IMA) there was an apparent dose-response relationship.

It should be borne in mind that this study has a number of limitations: it is an observational study so although its results have demonstrated O_2^- levels to be elevated no information on the clinical consequences of this effect can be proved. Furthermore, results are only available for a small cohort of patients limiting further subgroup analysis. Age has been associated with increased levels of oxidative stress(201, 202). Gender may also have effects on levels of oxidative stress. This study focused only on one ROS and although O_2^- is felt to be the key ROS others may also be important. It was impractical to perform paired experiments with SVs and IMAs taken from the same patients in order to explore the relative potency of hydralazine on these vessels. This was largely because of the availability of human tissue (both vessels were not consistently available from individual study participants).

This study *functionally* demonstrates a reduction in vascular O_2^- production with hydralazine but does not explore this *mechanistically*. The main enzymatic sources of O_2^- production within the vascular wall are NAD(P)H oxidase, xanthine oxidase, and endothelial NO synthase (eNOS)(203-205). Compared to historical data from this study group, the magnitude of effect of hydralazine on vascular O_2^- production appears comparable to that of the xanthine oxidase inhibitor allopurinol (0.1mmol/L) and NADH/NAD(P)H oxidase inhibitor apocynin (0.1mmol/L) in blood vessels taken from patients with CAD(198).

To further develop understanding of hydralazine-reduced vascular production of O_2^- experiments could be designed to directly compare the effects of hydralazine with a similar range of enzyme-inhibitors including those of (nitric oxide synthase (N^G-nitro-L-arginine-methyl ester). Hydralazine has also been purported to have ROS scavenging properties(206) which is also worthy of investigation. A number of confirmatory studies could be undertaken to support my findings using lucigenin chemiluminescence. Oxidative fluorescent microptography using hydroethidine allows localisation and semi-quantification of O_2^- production and has good specificity for SO(172). Briefly, frozen section of vessel are prepared and incubated with the nuclear marker 4', 6-diamidino-2-phenylindole (DAPI; 0.5 μg/ml for 2 min) followed by hydroethidine (2 μmol/L for 20 min). Fluorescence is then detected, and quantified, using a laser scanning confocal microscope. In parallel Electron Paramagnetic Resonance (EPR) spectroscopy would confirm data on O_2^- generation. EPR spectroscopy is a highly specific method to unambiguously detect free radicals such as the O_2^- anion(207). Vessel rings are placed in buffer containing the spin probe 1-hydroxy-3-carboxy-2,2,5,5 tetramethylpyrrolidine (CPH 500 μM) in 24-well plates and incubated at 37°C(207). Aliquots of this buffer are then analysed in an EPR spectrometer fitted with a temperature controller 0, 3, 6, 10 and 15 minutes to examine the time-course of O_2^- release. Experiments would be performed at basal conditions and after pre-incubation with superoxide-dismutases to guarantee specificity for O_2^- (208).

To my knowledge this is the first study to demonstrate an apparent ability for hydralazine to reduce basal O_2^- production in human blood vessels (in both arteries and veins). This outcome is important and may explain a component of the therapeutic benefits of hydralazine in combination with isosorbide dinitrate in patients with chronic heart failure, and, in particular the observed ability of hydralazine to reduce nitrate tolerance.

Chapter 7 – Effects of hydralazine on *ex vivo* angiotensin-II stimulated superoxide production in human internal mammary arteries

7.1 Summary

As described in chapter 1.1.7 and 6.1, oxidative stress plays an important role in the pathophysiology of heart failure, and may be a future therapeutic target. Hydralazine may interact with a number of vascular enzyme systems including key regulators of superoxide (O_2^-) production such as NADH/NAD(P)H-dependent oxidases. These systems may be regulated *in vivo* and *ex vivo* by the neurohormones angiotensin-II (Ang II) and aldosterone, and are believed to be pivotal in the development of endothelial dysfunction; one of the cardinal pathophysiological processes in heart failure(144, 196). Ang II-mediated O_2^- production appears to be driven by NAD(P)H oxidase, further endorsing the critical role of this enzyme system in cardiovascular disease. Ang II potently stimulates NAD(P)H oxidase activity in a variety of models. Infusions of Ang II up-regulate production of the subunits of NAD(P)H oxidase and increase O_2^- production in animal studies(209, 210). Ang II may also be an important stimulant of NAD(P)H oxidase activity in humans and additionally has been shown to induce LOX-1 expression, the human endothelial receptor for oxidised LDL(195, 211). Therefore the pathophysiological effects of Ang II may be pleotropic.

Our group has previously demonstrated that O_2- production in blood vessels from patients with established coronary artery disease (CAD) is greater in internal mammary artery (IMA) than in saphenous (veins). This may be due to larger vascular smooth muscle cell (VSMC) content (170). NAD(P)H oxidase and xanthine oxidase contributed to the production of O_2^- in these vessels. This was the first study demonstrating that Ang II could increase O_2^- production in human blood vessels, although this effect was only apparent in arteries. It is known that Ang II exerts its pathophysiological effects differently, in different vascular beds(212). Berry *et al* also demonstrated that Ang II-mediated O_2^- production could be attenuated by drug therapy (the angiotensin type 1 receptor (ATR1) antagonist, losartan). It is, however, recognised that Ang II can increase O_2^- production via non-ATR1 or ATR2- mediated receptor mechanisms in some animal models(213, 214). Whether this effect is species dependent, or whether yet unexplained intracellular mechanisms exist, remains to be fully investigated. As discussed in chapters 1.3.2 and 1.4.3, hydralazine may interact with NAD(P)H oxidase to improve nitroso-redox balance and potentially improve nitrate tolerance(106, 128). Assuming that Ang II largely stimulates this enzyme system, it would be interesting to demonstrate the effect of hydralazine on Ang II-stimulated O_2- production in human blood vessels

7.2 Aims

I sought to determine if co-incubation of human IMA vessels with hydralazine could attenuate the Ang II-stimulated increase in O_2^- production and thus partly explain its favourable effects in heart failure (a clinical syndrome characterised by Ang II excess).

7.3 Patients

Vascular O_2^- measurements were performed in internal mammary arteries (IMAs) taken from patients undergoing elective CABG. All patients were recruited as part of the VASCAB study as described in **Chapter 2.2.1.2**.

Participant characteristics and demographics for the entire cohort are presented in **table 2.1**.

7.4 Angiotensin-II stimulated superoxide production

7.4.1 Vessel preparation

Superoxide production was measured in 3-4 mm rings of IMA by chemiluminescence using lucigenin. Samples were analysed in a liquid scintillation counter (Hewlett Packard Tricarb 2100TR) in the out of coincidence mode. Readings were taken every 10 seconds for 3 minutes and absolute counts quantified with a xanthine / xanthine oxidase calibration curve for O_2^- generation and standardised to wet weight of the tissue. Detailed methods are described in Chapter 2.5.4. Paired rings of IMA were incubated at 37°C in the absence (control) and presence of hydralazine (1 μmol/L) *and/or* Ang-II (1 μmol/L) for 4 hours prior to quantification of O_2^- production as described above.

7.4.2 Angiotensin-II stimulated superoxide production in IMAs

Vascular O_2^- production was significantly greater in vessels stimulated with Ang-II (1 μmol/L) (1.84 ± 0.618 nmol/mg/min; n=6) compared with paired un-stimulated controls (0.98 ± 0.163 nmol/mg/min) (*P*=0.045). **(Figure 7.1)**

7.4.3 Angiotensin-II stimulated superoxide production in hydralazine treated IMAs

Incubation with hydralazine (1 μmol/L) significantly attenuated Ang-II-stimulated increase of O_2^-. In control vessels (n=9), O_2^- production was 1.637 ± 0.434 nmol/mg/min vs. 0.843 ± 0.144 nmol/mg/min in hydralazine-treated vessels (n=9) (*P*=0.032). **(Figure 7.2)**

Figure 7.1 Effects of Ang II on O_2^- production in IMAs. Blood vessels were incubated in presence or absence of Ang II (1 μmol/L) for 4 hours prior to quantification of O_2^-. Results are expressed as nmol/mg/min and are expressed as mean ± SEM. Blue bars indicate control vessels (n=6); red bars Ang II exposed (n=6).

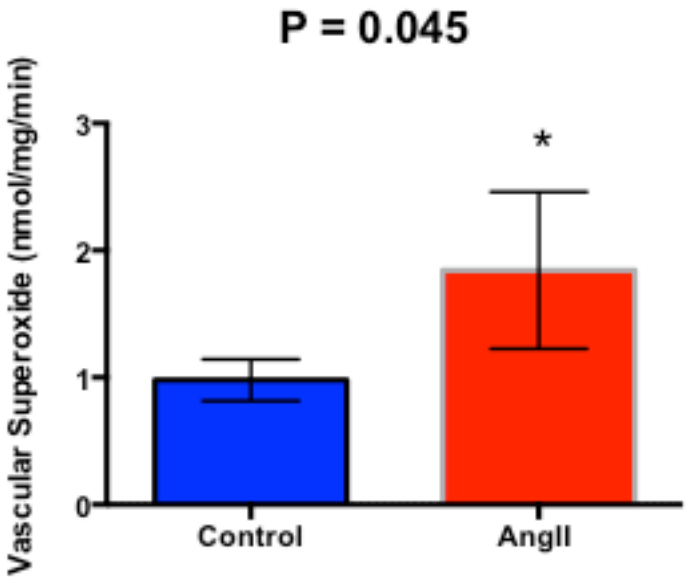
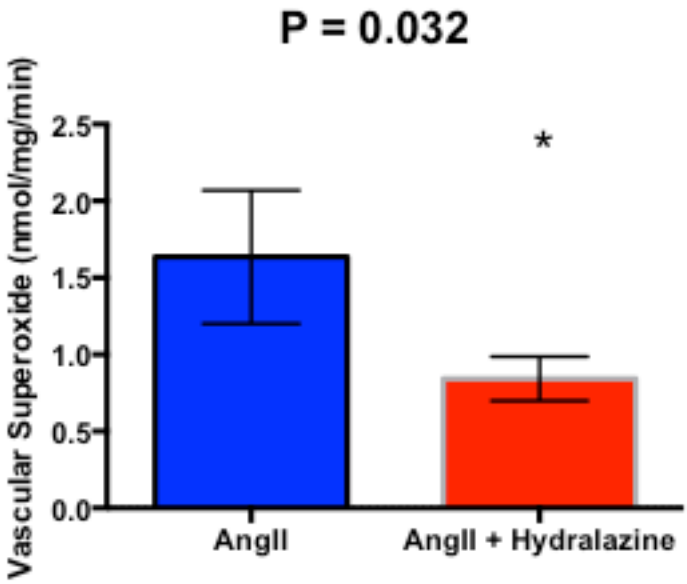


Figure 7.2 Effects of co-incubation with hydralazine (1 µmol/L) on Ang II-stimulated O_2^- production in IMAs. Blood vessels were incubated in presence or absence of hydralazine (1 µmol/L) or diluent control and Ang II (1 µmol/L) for 4 hours prior to quantification of O_2^-. Results are expressed as nmol/mg/min and are expressed as mean ± SEM. Blue bars indicate control vessels (n=9); red bars hydralazine-treated (n=9).



7.5 Discussion

This is the first study to demonstrate that hydralazine can attenuate Ang II-stimulated increased vascular O_2^- production in human blood vessels. This is of clinical importance as Ang II is known to be one of the key component in the cascade of neurohormonal activation that is characteristic of heart failure, and is prognostically important (4, 7). Ang II-stimulated increase in vascular O_2^- production is thought to be pathophysiologically important: leading to hypertrophic effects on VSMCs contributing to increased vascular tone in animal models of hypertension(144, 215). Our group and others have consistently demonstrated that Ang II increases O_2^- production through stimulation of NAD(P)H oxidase in a variety of animal models and in the human vasculature (144, 195-197). Moreover, Berry *et al* confirmed that the principal source of O_2^- production in human blood vessels was mediated through stimulation of NAD(P)H oxidase (which is regulated *in vivo* and *ex vivo* by Ang II)(170). They also demonstrated that the AT_1-specific receptor antagonist losartan had no effect on *basal* O_2^- production (unlike hydralazine in the present study), only Ang II-stimulated production.

Hydralazine is thought to be an inhibitor of NAD(P)H oxidase. This may be relevant to the apparent ability of hydralazine to reduce nitrate tolerance. NAD(P)H oxidase is strongly implicated in the development of nitrate tolerance(106, 140, 141). Whilst I have not mechanistically demonstrated that hydralazine inhibits NAD(P)H oxidase activity *per se*, its apparent ability to significantly block Ang II stimulated O_2^- production is compelling. That said, other potential anti-oxidant effects of hydralazine have been proposed, including its direct scavenging effect(206). This could - potentially - help restore the nitroso-redox balance even in the presence of a potent stimulator of vascular O_2^- production such as Ang II.

As already noted, this study has a number of limitations. As discussed in **Chapter 6**, as this is principally an observational study no conclusions can be drawn with regard to the direct clinical consequences of these findings. Whilst I have demonstrated that hydralazine can block Ang II-stimulated O_2^- production, the mechanisms underlying this remain unclear. As discussed above, confirmatory studies could be undertaken oxidative fluorescent microptography using hydroethidine (to allow localisation and semi-quantification of O_2^- production) and EPR spectroscopy to unambiguously detect and quantify production of the O_2^- anion (172, 207).

In order to further elaborate whether hydralazine attenuates enzymatically generated O_2^- production (specifically NADH/NAD(P)H oxidase and xanthine oxidase) a series of experiments comparing hydralazine with that of the known inhibitors of these enzyme systems may deepen the findings of this study. Protocols deployed in such further studies could then be repeated in vessels pre-treated with hydralazine in order to establish if hydralazine has any additional effect.

Whatever the underlying mechanism, I have, for the first time, demonstrated that hydralazine functionally inhibits Ang II mediated O_2^- production in human vascular tissue. This is clinically relevant and may partly explain the favourable effects of hydralazine in heart failure and its interaction with organic nitrates to reduce nitrate tolerance.

Chapter 8 – General discussion

8.1 General discussion

In the era prior to the emergence of evidence-based medicine, heart failure treatment was largely limited to symptomatic relief with diuretics and digoxin. Emphasis was then placed on modulating the haemodynamic abnormalities of heart failure, with small observational studies highlighting a favourable effect of vasodilator drugs on left ventricular compliance and function(216-218). A number of orally acting agents were then investigated in patients with refractory symptoms(142, 219, 220).

The concept that "balanced vasodilatation" with the combination of hydralazine and ISDN (simultaneous reduction in preload with ISDN and afterload with hydralazine) would produce net clinical benefits then emerged with encouraging results(221). Later randomised-controlled studies demonstrated a survival advantage over placebo(83). This drug combination was subsequently shown to be inferior to the ACE inhibitor enalapril despite observations that H-ISDN produced greater improvements in ejection fraction and exercise tolerance(84).

The A-HeFT trial was biologically and ethically contentious, both in its concept and subsequent race-specific licensing of the fixed dose combination of H-ISDN. The US Federal Drug Administration's approval of a prospective randomised-controlled trial of H-ISDN *exclusively* in self-identified African Americans was profoundly controversial(222). On scientific grounds there appears to be excess in morbidity and mortality in African American patients with heart failure as well as lesser responses to neurohormonal antagonists (85, 223). Biological plausibility has been derived from observations of reduced NO activity and worsened endothelial dysfunction in patients(182, 224). With specific reference to the apparent association between race and drug response in V-HeFT I/II, these data are based on retrospective analyses, which were not powered to determine non-inferiority.

Race is a poor surrogate for genetic background and consequently drug responsiveness. The Genetic Risk Assessment and Heart Failure (GRAHF) sub-study of A-HeFT explored the influence of genetic heterogeneity of*NOS3* (the gene encoding e-NOS) on clinical response to H-ISDN. When analysed by genotype, H-ISDN improved outcome in patients with the Glu298 polymorphism of *NOS3* but not in those with the Asp298 variant(225). Conversely, in the Genetic Risk Assessment of Cardiac Events (GRACE) registry of nearly 500 patients with low ejection fraction heart failure, 77.8% of African Americans carried the Asp298 polymorphism compared to 40% of Caucasians with the Glu298 variant(226). Whilst the former was associated with a worse event-free survival clearly a large proportion of Caucasian patients may carry a therapeutically relevant polymorphism; suggesting that many Caucasian patients may benefit from H-ISDN. Despite the apparent lack of treatment response in Caucasian patients in the V-HeFT studies, there was in fact a significant improvement in LVEF and exercise tolerance with H-ISDN compared to enalapril in V-HeFT II, and no difference between hospital admissions between African American or Caucasian patients in either study(85). Many therefore believe that the incremental clinical benefits achieved in A-HeFT are transferrable to a wider heart failure population. In this thesis I have investigated the effects of hydralazine on blood vessels from a European Caucasian population of patients with heart failure.

Nitroso-redox balance is central to the pathophysiology of heart failure and is a potential therapeutic target. Imbalance results in an excess of reactive oxygen species with consequent reduced S-nitrosylation of physiologically important signalling molecules. This includes the cardiac ryanodine receptor (RyP2), which regulates intracellular calcium concentration and excitation-contraction coupling(227). Impaired S-nitrosylation directly leads to reduced contractility. Impaired NO bioavailability is also associated with reduced guanylyl cyclase activation and endothelial dysfunction. The degree of endothelial dysfunction appears to be proportionate to the severity of the clinical syndrome. Whilst the studies are relatively small, they consistently demonstrate an independent association between measurable endothelial dysfunction and poor functional class and outcome in heart failure, independent of aetiology (228-230).

Other adverse effects of impaired nitroso-redox balance include excess formation of peroxynitrite, driving a myriad of deleterious actions including lipid peroxidation, direct DNA damage and induction of apoptosis(231, 232). Any intervention that restores nitroso-redox balance in heart failure could conceivably translate to improved clinical outcome. Improvement in endothelial function has however, not always translated into clinical outcomes. The beneficial effects of many strategies have been demonstrated only in short-term trials inadequately powered to establish outcome benefit(58, 233). These agents, for example anti-oxidants, have a heterogeneous mechanism of action and are not endothelium-specific. Oxidative stress may also persists despite the use of guideline-directed optimal medical therapies, which have proven clinical benefits and have been shown to impact measurably on levels of reactive oxygen species(200). Finally, even measureable improvement in endothelial function may not necessarily translate to clinical outcome in the complex syndrome of heart failure.

In this thesis I have investigated for the first time the *direct* vasodilator effects of hydralazine on blood vessels taken from patients with heart failure secondary to coronary artery disease and interaction with a range of organic nitrates. The body of evidence in the literature suggested hydralazine reduced contractile responses to a range of vasoconstrictor agonists but had not explored direct vasodilator activity. This was also largely restricted to animal models. The scarce human data available (from *post mortem* studies) suggested a greater effect on arteries than veins (thought to be proportionate to the mass of vascular smooth muscle)(102, 234).

I have demonstrated that hydralazine (at therapeutically relevant concentrations) had no significant *ex vivo* vasodilator effect on blood vessels from patients with heart failure secondary to CAD. At supra-therapeutic concentrations there was very modest vasodilatation, largely restricted to capacitance arteries and veins. I confirmed previous data demonstrating a greater maximal effect on arteries, although at therapeutically relevant concentrations the effect was similarly absent.

The apparent lack of effect on SRAs was surprising. Vessels were appropriately pre-constricted using standard protocols and intrinsic prostanoid pathways were inhibited by the addition of indomethacin to the PSS buffer. We understand that these vessels contribute most to resting vascular tone and blood pressure(235). Structural alterations in the microcirculation are one of the most powerful predictors of cardiovascular events in at risk patients(236). There was, however, no apparent effect of hydralazine even at supra-therapeutic doses. This was of course an *ex vivo* model using blood vessels taken from patients receiving guideline-directed optimal medical therapy including in all cases an ACE inhibitor or ARB. Impaired vasodilator responses are well documented in SRAs from patients with heart failure(162). A measurable additional response in vessels from patients receiving optimal drug therapy could perhaps be difficult to demonstrate. There is a body of evidence documenting disparate vascular responses of neurohormonal therapies in large and small calibre blood vessels(235). There is also some heterogeneity of endothelial function within the circulation and between large and small calibre blood vessels and also in their inherent responses to different vasoconstrictor agents (237-239). My data could suggest that the therapeutic effects of hydralazine may not simply be dependent on arterial vasodilatation and direct vasodilator activity and that the observed clinical benefits of combination therapy with isosorbide dinitrate may be partly explained by favourable effects elsewhere e.g. through restoration of the nitroso-redox balance.

We understand that the clinical benefits of hydralazine in combination with ISDN were *independent* of blood pressure lowering effect and also recognise that hydralazine may have effects beyond simple vasodilatation(175). Recently hydralazine has been shown to improve Ca^{2+} cycling and contractility in isolated cardiomyocytes in an animal model of oxidative stress induced cardiac injury(240). This is perhaps through antagonism of post-translational modifications of the RyP2 receptor associated with excess ROS (241). As described earlier, hydralazine has been proposed to inhibit endoplasmic reticulum Ca^{2+} release in vascular smooth muscle through regulation of the IP_3 receptor (a member of the same receptor family as RyR2)(99, 112). There is considerable evidence that oxidative stress induces cardiac injury by oxidizing cellular constituents including proteins critical for excitation-contraction coupling(241). It is therefore conceivable that the positive effects of H-ISDN on LVEF and outcome in patients with heart failure could relate to enhanced contractility and not simply balanced haemodynamic effect.

The combination of H-ISDN has favourable effects beyond the systemic vasculature. Pulmonary hypertension is common in heart failure and influences prognosis(242). Elevated pulmonary vascular resistance is a product of vascular remodelling of the pulmonary vasculature; partially attributed to endothelial dysfunction resulting from impaired NO availability and increased endothelin expression(243). In low ejection fraction heart failure H-ISDN has marked short-term effects on pulmonary vascular resistance. This has been shown to correspond to improvements in right ventricular function(244, 245). Right ventricular dysfunction is a marker of poor prognosis in low ejection fraction heart failure(246). The measurable positive effects of H-ISDN on right ventricular function (which may be independent of systemic vascular activity) could also contribute to the clinical effects.

In this thesis, hydralazine treatment produced a trend towards augmented *ex vivo* endothelium-dependent vasodilatation in large and small calibre vessels although this effect was not significant when corrected for multiple comparisons. These protocols were limited by small numbers of vessels and likely underpowered. Nevertheless, the results suggest a trend of potential biological significance. Endothelial dysfunction may be a feature of heart failure of any aetiology, but is best characterised in heart failure secondary to coronary artery disease, where co-morbidities such as atherosclerosis, diabetes mellitus and hypertension contribute (178). Although there is heterogeneity of endothelial function within the circulation there are also data supporting correlation in large and small calibre vessels in cardiovascular disease(247). Whilst atherosclerotic lesions do not affect veins to the same extent as arteries, endothelial dysfunction has been demonstrated in both veins and arteries taken from patients with coronary artery disease and heart failure(147, 179). My data are consistent with the hypothesis that the mechanism of action of hydralazine could be partially mediated through improved endothelium-dependent vasodilatation. This effect could conceivably contribute to the clinical benefits of H-ISDN, particularly in patients with excessive endothelial dysfunction. As previously discussed, the presence of endothelial dysfunction influences outcome in heart failure. Strategies that may positively affect endothelial function could be therapeutically important. This thesis was undertaken exclusively in with heart failure secondary to coronary artery disease. Larger studies are needed to determine if this effect is significant and using other vascular preparations and *in vivo* techniques to assess endothelial function in patients with heart failure of varying aetiology.

I sought to investigate the direct interaction between hydralazine and organic nitrates in vascular preparations. The combination of H-ISDN produces clinical results yet neither drug used in isolation influences prognosis and indeed long term treatment with organic nitrates may be deleterious. There is evidence that chronic treatment with most of the organic nitrates causes endothelial dysfunction(131, 248). This may correlate with worse clinical outcome, particularly post-MI(249). Although nitrate tolerance is a complex phenomenon, one of the most compelling hypotheses is that nitrate therapy stimulates production of reactive oxygen species such as superoxide and peroxynitrite(250). Hydralazine has been shown to possess powerful peroxynitrite-quenching properties, which could explain in part its attenuation of experimental nitrate tolerance(145). Oxidative stress in response to chronic nitrate therapy may also activate a cross-talk phenomenon with vascular NAD(P)H oxidase resulting in further reactive oxygen species formation and peroxynitrite(251). Hydralazine has been purported to inhibit this enzyme system

I have demonstrated only modest *ex vivo* vasodilator interaction in vessels treated *acutely* with hydralazine. This is consistent with the hypothesis that the therapeutic synergy is not simply dependent on vasodilator effect. However, we should be mindful that this was an *ex vivo* study. Organic nitrates undergo biotransformation processes that may be differently active in the *in vivo* state. GTN and PETN undergo mitochondrial activation, whist ISMN and ISDN are thought to undergo cytochrome P450 dependent biotransformation in the endoplasmic reticulum. The mechanistic interaction with hydralazine *in vivo* may be driven by intracellular accumulation and membrane localisation of the drug, which may not occur during acute administration. I demonstrated modest augmentation of the direct NO donor SNP in large calibre veins and SRAs. There is conflict in the literature about this interaction; some groups suggest that hydralazine attenuates SNP-mediated vasodilatation (and subsequent cGMP production) whilst others found no effect(128, 192, 193). These disparities may be partly explained by the diverse animal models used. In human blood vessels from patients with heart failure and coronary artery disease, this interaction has not been previously characterised.

There is an increasing body of evidence suggesting a favourable effect of hydralazine on nitroso-redox balance. Mechanistically a number of enzyme systems and processes have been implicated including increased soluble guanylate cyclase expression, inhibition of semicarbazide-sensitive amine oxidase and NAD(P)H oxidase(122, 126). Our group has established that angiotensin-II increases superoxide production in human internal mammary artery through enhanced NAD(P)H oxidase activity(170). The pathological effects of angiotensin-II may not be completely antagonised by conventional neurohormonal antagonists and as such there may be a therapeutic indication for drugs that could antagonise this enzyme.

This is to our knowledge the first demonstration that hydralazine reduces basal O_2- production in human blood vessels. No conclusion can be drawn on the relative potency of hydralazine on arteries or veins, as experiments were not paired. The observed effect on veins was however similar to that previously observed with potent enzyme-inhibitors such as allopurinol. I have however, demonstrated that hydralazine significantly reduces O_2- production in both vessel types with an apparent dose-response relationship in SVs. This may explain part of the therapeutic benefits of H-ISDN in patients with chronic heart failure. As discussed previously this study has a number of limitations owing the small cohort of patients studied, the single reactive oxygen species under investigation and the semi-quantitative assay used. As an observational study, no conclusions can be firmly drawn between reduced superoxide production and clinical outcome; it is however, hypothesis generating. This study *functionally* demonstrates a reduction in vascular O_2- production with hydralazine but does not explore this *mechanistically*.

This is the first study to demonstrate that hydralazine can *functionally* attenuate Ang II-stimulated O_2^- production in human blood vessels. This is clinically and functionally important as Ang II is central to the cascade of neurohormonal activation in heart failure, and is prognostically important (4, 7). Ang II increases O_2^- production through stimulation of NAD(P)H oxidase which is the dominant source of O_2^- in the vasculature(144, 195-197). The apparent ability of hydralazine to block Ang II stimulated O_2^- production is therapeutically interesting. As discussed above, other potential anti-oxidant effects of hydralazine should be considered, including a direct scavenging effect(206). Hydralazine could potentially help restore the nitroso-redox balance even in the presence of Ang II and other potent agonists of ROS.

The *in vivo* effects of hydralazine and nitroso-redox balance remain uncertain. Superoxide production from blood can be stimulated by ADP-induced platelet aggregation and measured by semi-quantitative techniques such as lucigenin chemiluminescence(252). In a small controlled crossover trial of 14 patients with chronic heart failure (receiving at least one neurohormonal antagonist), short-term (2 week) administration of 25mg BD of hydralazine failed to impact positively on superoxide generation(253). This was in contrast to *ex vivo* studies from the same group. Interestingly this study was undertaken in European Caucasians. The dosage and duration of hydralazine therapy was significantly less than that proven in clinical trials (50mg/day vs. 140mg/day) and whilst sufficient to produce a haemodynamic response may not have impacted on platelet superoxide production.

Besides quantitative difference in ROS production between vascular tissues and blood, there is undoubtedly also different utilisation of various ROS (such as superoxide and hydrogen peroxide) both in physiological cell signalling and in pathophysiological states(254, 255). There are also several features of NAD(P)H oxidase enzymes expressed in blood vessels that distinguish them from those in blood cells. Superoxide production from the phagocyte is considerably lower than that of vascular tissue, which displays a largely constitutive activity that is further increased by agonists such as Ang-II(256). Without a doubt the major source of ROS in the cardiovascular system (and as such, potential pathological effectors) are the NAD(P)H oxidases. In the present study I have consistently demonstrated a favourable effect of hydralazine in human vascular tissue taken from patients with chronic heart failure, albeit in an *ex vivo* preparation. The potential positive effects of hydralazine on nitroso-redox balance (and nitrate tolerance) merit future investigation in appropriately designed *in vivo* studies.

In conclusion, the findings presented in this thesis provide insight into the mechanism of action of hydralazine in blood vessels from patients with heart failure. In terms of clinical perspective there appears to be direct correlation between the development of optimal medical therapy, as directed by randomised controlled clinical trials, and improved outcomes in patients with low ejection fraction heart failure. Despite these tangible benefits, a gap exists between guideline recommendations and real world prescribing of evidence-based therapies, notably H-ISDN.

8.5 Limitations of this work and future directions

The major weaknesses of this thesis are evident: the number of patients recruited for this study was relatively small as were the number of vessels utilised in individual experiments. Whilst the numbers of paired samples in each study protocol were small, these reflect similar studies, which have yielded scientifically meaningful results(163, 257, 258). All similar experimental protocols are limited by both the availability and responsiveness of vascular tissue. Healthy controls were not included in our experimental protocols. Our group have previously demonstrated impaired vasodilator responses in blood vessels taken from patients with heart failure when compared with healthy controls(259). We have also recently demonstrated that superoxide production is greater in vessels taken from subjects with CAD when compared with those from healthy controls(198). As such, protocols were designed to specifically examine the effects of hydralazine in blood vessels taken from patients with heart failure and CAD. Nevertheless, expanding this research to include healthy control subjects could provide useful insight, particularly our observed finding of no significant direct *ex vivo* vasodilator activity of hydralazine. A comparative healthy control study would be valuable to confirm and distinguish this intriguing result. The composition of the control group may be difficult to balance with that of a contemporary heart failure population.

Heart failure is a heterogeneous syndrome comprised of a spectrum of phenotypes from the acutely decompensated *de novo* patient to a chronic stable state. The haemodynamic and neurohormonal profiles of these stages may not be accurately reflected in an *ex vivo* model. In this thesis every attempt was made to include a cohort of individuals who were representative of the general heart failure population (secondary to CAD) and who were receiving guideline-directed optimal medical therapy (which at that time included an ACE inhibitor/or ARB and a beta-blocker). Experimental work comprised entirely of an *ex vivo* model and therefore may not adequately reflect natural variation in the response of blood vessels to endogenous and exogenous vasoactive substances. With respect to *ex vivo* functional investigation, we only used one endothelium-dependent agonist carbachol, selected on the basis of published work from our group and others(185, 186). As discussed in **Chapter 4** comparison of the effects of hydralazine treatment on vessels with and without endothelium would allow a more complete assessment of the role of eNOS in the observed vasodilator activity and a more confident attribution of the observed differences to endothelial mechanisms. Beyond the functional

observation of *ex vivo* interactions with the endothelium, mechanistic assessment of the effects of hydralazine could be more thoroughly explored through a series of experiments to determine the effects of hydralazine on cyclic nucleotide production – particularly cGMP – using enzyme immunoassay. *Ex vivo* techniques are clearly limited by the availability of tissue but the results also need to be interpreted carefully as the samples may behave differently compared to when *in vivo*. Further *in vivo* studies in a range of vascular beds in the typical heart failure patient would be desirable to confirm or refute these findings. This could include dorsal hand veins studies using a modified Aellig technique to document local effects of hydralazine and organic nitrate interaction on dorsal hand veins or forearm venous occlusion plethysmography to study local arterial effects on the brachial circulation.

Whilst we have consistently demonstrated a *functional* reduction in vascular O_2^- production with hydralazine this has yet to be elaborated *mechanistically*. Emphasis should be placed on the NAD(P)H oxidase family of enzymes which are the dominant source of O_2^- in the vasculature and are strongly implicated in the development of nitrate tolerance Confirmatory studies could be undertaken using oxidative fluorescent microptography using hydroethidine (to allow localisation and semi-quantification of O_2^- production) and EPR spectroscopy to unambiguously detect and quantify production of the O_2^- anion (172, 207). Finally, the impact of hydralazine on NAD(P)H oxidase stimulated superoxide production could be assessed by messenger ribonucleic acid expression of relevant NAD(P)H oxidase isoforms (particularly NOX4) transcripts, quantified by real-time polymerase chain reaction(179). This would confirm our observations with mechanistic data not only at an enzymatic, but transcriptional, level.

Chapter 9 – Supplementary Data

9.1 Appendix 1: Letter of ethical approval for VASCAB study

North Glasgow University Hospitals
Division

NHS
Greater
Glasgow

West Glasgow Ethics Committee
Western Infirma
Dumbarton Ro
Glasgow G11 6

Telephone: 0141 211 62
Facsimile: 0141 211 19

04 October 2006

Prof Anna F. Dominiczak
Director BHF Glasgow Cardiovascular Research Centre
BHF GCRC
126 University Place
University of Glasgow
Glasgow
G12 8TA

Dear Prof Dominiczak

Full title of study: **VAScular function in Coronary Artery Bypass patients (VASCAB)**

REC reference number: **06/S0703/110**

The Research Ethics Committee reviewed the above application at the meeting held on 03 October 2006.

Ethical opinion

The Committee thanked Professor Dominiczak and Dr. Jane Dymott for attending the meeting to discuss this study.

The Committee has one or two questions to the investigators which were answered to their satisfaction i.e.

a) A34 visits 0-12 - the investigators indicated that this was indeed an error.
b) The Committee wondered what the role of the fitness fanatics was and this was answered.
c) The Committee are of the opinion that the initial approach shouldbe at the pre-admission clinic visit.
d) The Committee indicated to the investigators that any further tests on the samples will require a further submission to an ethics committee.
e) A58 in respect of funding should be completed.
f) The Committee are of the view that GPs should be informed and a GP letter should be drawn up and passed to the committee for review.
g) The committee strongly feel that patients who have an intolerance of Salbutamol or GTN should be added to the Exclusion criteria.

Patient Information Sheet CABG should be amended as under:

a) The word "minor" should be deleted prior to "scratch".
b) Volume of blood taken should be stated in "tablespoonfuls".
c) A sentence should be added in respect of gifting of the samples.
d) Page 3 - 2nd top line - should read "treat patients better who" etc

Healthy Volunteer PIS:

a) Any reference to "disease" should be deleted.
b) A sentence should be added to the effect that there is a risk that something might be picked up which could have future implications for insurance/mortgage purposes.
c) A sentence should be added in respect of "gifting of the samples".
d) "current medication" should read "any medication".
e) A sentence should be added in respect of notifying your private medical insurer of your taking part in the study.
f) Page 3 - 2nd top line should read "treat patients better" etc
g) Blood vols should be expressed in tablespoonfuls.
h) Page 2 - 2nd bottom bullet point should read "allergy against glycerol trinitrate please tell us.

All Consent Forms:

a) A tick box should be added in respect of telling GPs.

The above minor amendments should come back to the Secretary for checking and filing.

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation.

Ethical review of research sites

The Committee agreed that all sites in this study should be exempt from site-specific assessment (SSA). There is no need to complete Part C of the application form or to inform Local Research Ethics Committees (LRECs) about the research. The favourable opinion for the study applies to all sites involved in the research.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Application	5.1	06 September 2006
Investigator CV	CI	06 September 2006
Protocol	1.0	01 September 2006
Covering Letter		06 September 2006
Summary/Synopsis	CABG 1.0	01 September 2006

Questionnaire: non-validated follow up 2 years	F4 1.0	01 September 2006
Questionnaire: non-validated follow up 1 year	F3 1.0	01 September 2006
Questionnaire: non-validated follow-up 30 days	F2 1.0	01 September 2006
Questionnaire: Non-validated follow up 7 days	F1 1.0	01 September 2006
Questionnaire: Non-validated	Main MQ 1.0	01 September 2006
Participant Information Sheet	HE 1.0	01 September 2006
Participant Information Sheet	VV 1.0	01 September 2006
Participant Information Sheet	CABG 1.O	01 September 2006
Participant Consent Form	HE 1.0	01 September 2006
Participant Consent Form	VV B 1.0	01 September 2006
Participant Consent Form	VV A 1.0	01 September 2006
Participant Consent Form	CABG 1.0	01 September 2006
Summary Synopsis	HE 1.0	01 September 2006
Summary Synopsis	VV 1.0	01 September 2006
CV for student		06 September 2006

Research governance approval

You should arrange for the R&D Department at all relevant NHS care organisations to be notified that the research will be taking place, and provide a copy of the REC application, the protocol and this letter.

All researchers and research collaborators who will be participating in the research at a NHS site must obtain final research governance approval before commencing any research procedures. Where a substantive contract is not held with the care organisation, it may be necessary for an honorary contract to be issued before approval for the research can be given.

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/S0703/110 **Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

PP **Andrea H Torrie**
Ethics Manager – West Glasgow LREC's

Email: andrea.torrie@northglasgow.scot.nhs.uk

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*
Standard approval conditions SL-AC2

Copy to: R & D Department WIG

9.2 Appendix 2: Letter of ethical approval for gluteal biopsy study

West Glasgow Ethics Committee 1
Western Infirmary
Dumbarton Road
Glasgow G11 6NT

Telephone: 0141 211 6238
Facsimile: 0141 211 1920

07 November 2006

Professor JJV McMurray
Professor of Medical Cardiology
BHF Glasgow Cardiovascular Research Centre
University of Glasgow
126 University Place
Glasgow G12 8TA

Dear Professor McMurray

Full title of study: **Hydralazine in heart failure: A study of the mechanism of action in human blood vessels**
REC reference number: **06/S0703/130**

The Research Ethics Committee reviewed the above application at the meeting held on 07 November 2006. The Committee thanked Drs Robb and Rocchiccioli for attending the meeting to discuss this study.

Ethical opinion

Study Design:

The committee had one or two questions for the investigators which were answered to their satisfaction:

However the undernoted required addressing:

a) Question A50 amend to the correct numbers being considered 60 or 90?
b) The Committee noted that patients on Warfarin were excluded but wondered about patients on Clopidogrel?
c) The Committee indicated to the investigators that due to the multiple comparisons they should be cautious in interpreting the findings.
d) Question A 51 is duplicated within the answer.

Patient Information Sheet:

a) A further sentence should be added to the effect that any future research on the stored samples will require ethics committee approval.
b) Delete "small" in any reference to the biopsy i.e."small area of skin", "small piece of skin" etc.

The above minor amendments should come back to the secretary for filing.

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation.

Ethical review of research sites

The Committee agreed that all sites in this study should be exempt from site-specific assessment (SSA). There is no need to complete Part C of the application form or to inform Local Research Ethics Committees (LRECs) about the research. The favourable opinion for the study applies to all sites involved in the research.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Application	1	13 October 2006
Investigator CV		12 October 2006
Protocol	1	12 October 2006
Covering Letter		12 October 2006
GP/Consultant Information Sheets	1	12 October 2006
Participant Information Sheet	1	12 October 2006
Participant Consent Form	1	12 October 2006
Summary CV for supervisor	1	12 October 2006

Research governance approval

You should arrange for the R&D Department at all relevant NHS care organisations to be notified that the research will be taking place, and provide a copy of the REC application, the protocol and this letter.

All researchers and research collaborators who will be participating in the research at a NHS site must obtain final research governance approval before commencing any research procedures. Where a substantive contract is not held with the care organisation, it may be necessary for an honorary contract to be issued before approval for the research can be given.

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

none

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/S0703/130	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

Andrea H Torrie
Ethics Manager – West Glasgow LRECs

Email: andrea.torrie@northglasgow.scot.nhs.uk

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*
Standard approval conditions SL-AC2

Copy to: *R&D Office*
Administration Building,
Ground Floor, Room 9, Western Infirmary
Glasgow

Chapter 10 – References

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