ANGIOTENSIN CONVERTING ENZYME AND MITOCHONDRIA – MOLECULAR AND GENETIC MECHANISMS INVOLVING BRADYKININ RECEPTORS AND UNCOUPLING PROTEINS

SUKHBIR SINGH DHAMRAIT

UNIVERSITY COLLEGE LONDON

DEPARTMENT OF CARDIOVASCULAR MEDICINE

UCL DIVISION OF MEDICINE

A RESEARCH THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

2009

Personal Declaration

I, Sukhbir Singh Dhamrait, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, or where work has been contributed to by others, I confirm that this has been indicated in the thesis.

“If I have seen further than others, it is by standing upon the shoulders of giants.”

Sir Isaac Newton

Abstract

Low angiotensin converting enzyme (ACE) activity is associated with various cardiovascular phenotypes including reduced left ventricular (LV) hypertrophy, reduced cardiovascular events and enhanced metabolic efficiency, but precise mechanisms are unclear and direct genetic associations remain controversial. ACE degrades kinins and promotes formation of angiotensin II. Combined genetic and *in vitro* studies were used to test the hypothesis that the previously observed effects may be through alterations in kinins or mitochondrial function via novel uncoupling proteins (UCPs).

The -9 allele of the bradykinin β2 receptor *BDKRB2+9/-9* gene variant is correlated with low kinin activity and was associated with lower prospective LV growth during strenuous physical exercise and lower prospective hypertensive cardiovascular risk, as well as increased efficiency of skeletal muscle contraction (delta efficiency) in healthy volunteers ($P = 0.003$, accounting for 11% of the inter-individual variability).

Addition of angiotensin II to skeletal myocytes resulted in a 3.5 fold increase in oxygen consumption ($P = 0.03$). Incubation of isolated myocytes with an ACE inhibitor lead to mitochondrial membrane hyperpolarisation, suggesting mitochondrial coupling may be an important mediator of the cellular actions of ACE.

A common promoter variant in the *UCP2* gene was associated with a two-fold increase in prospective cardiovascular risk ($P < 0.0001$). Variation in the *UCP3/2* gene cluster accounted for 15% of the inter-individual endurance training related changes in delta efficiency and there was a surprising, but consistent, association with serum ACE activity. Finally, *in vitro* assays confirmed physiological downregulation of UCP2 in

endothelial cells was associated with increased oxidative stress and reduced ACE mRNA.

In conclusion, BDKRB2 may mediate some of the beneficial metabolic and cardiovascular effects associated with low ACE activity, possibly through changes in mitochondrial function. Mitochondrial coupling appears pivotal in cardiovascular (patho)physiology, possibly via oxidative stress or a novel ACE metabolic regulatory pathway. UCPs may be a target for future cardiovascular interventions.

Acknowledgements

I am indebted to my supervisors, Professor Hugh E Montgomery, the inspiration and drive behind this work and Professor Steve E Humphries for his unwavering guidance and support.

I would like to thank Professor John F Martin for his words of wisdom and his support in securing a BHF Chair Student Scholarship as well as funding a flow cytometry refreshers course.

I extend my gratitude to the late Professor George J. Miller, principle investigator for the Northwick Park Study, who was a great mind and keen listener and who helped in the manuscript preparation for two of the publications arising from this thesis.

I thank my compatriots, the other research fellows Dr David J Brull for his friendship and support during the first year of my study and for the collaboration on the 'Bassingbourn 3' study cohort, Dr John Payne and Mr Laurence 'Sid' James ('the boys') for all those reflective moments that are so common in research degrees and Dr Jeffrey Stephens for the fantastic collaborative last year of our PhDs working on the UCP/oxidative stress project when 'everything worked'.

I would like to thank the other team members at the Centre for Cardiovascular Genetics, BHF Laboratories, UCL: Dr Jay Acharya, lab manager, Dr Peter Wootton & Ms. Ka Wah Li for their help in genotyping some of the studies and Ms. Emma Hawe and Ms. Jackie Cooper (departmental statisticians) for statistical support.

This thesis includes several human genetic studies and I wish to thank the external collaborators: Dr Alun Williams, University of Staffordshire, for collaborative work on the genetics of performance in this thesis, Dr Ulrik Pedersen-Bjergaard and Dr Birger Thorsteinsson (Hillerød Hospital, Denmark) for an excellent collaboration on the Danish Diabetic Study, Dr Peter Gohlke (University of Kiel, Germany) for providing the serum ACE assay and Dr B. Rayner (Groote Schuur Hospital, University of Cape Town, Republic of South Africa) for the RSA study recruitment.

I would like to thank Professor Mike Duchen (UCL) and Dr Vidya Mohammed-Ali (UCL) who obtained the first confocal image of the effects of ACE inhibitors on mitochondrial membrane potential from my cell culture experiments, and in particular Dr Mohammed-Ali and her team at the Archway Campus, UCL for honing my cell culture skills and teaching me RNA isolation.

I thank Dr Dave Kelly (UCL) for his technical support and humour during long hours of cell staring in the BHF Labs microscopy suite, Dr Geff Bellingham (UCL) for lending the OxySpot system and Professor Derek Yellon and Dr Derek Hausenloy for the use of the Hatter facility at UCL and teaching me how to isolate primary ventricular myocytes.

I would like to thank my father, Ajit Singh Dhamrait, who fell ill during this thesis and who instilled in me the virtues of perseverance, hard work and honour.

A final word of gratitude to The British Heart Foundation for funding this period of research (Chair Scholarship and PhD Studentship Grant FS/2001044).

Dedicated to my wife, Nitasha and daughters, Meghana and Ashana.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

$\Delta\psi_m$	Mitochondrial membrane potential
$\Delta\mu_{H^+}$	Electrochemical gradient
Δ_P, PMF	Proton motive force
ΔpH	Proton gradient
ΔG	Gibbs energy change
ACE	Angiotensin-I converting enzyme
ACE2	ACE-related carboxypeptidase
ACEi	Angiotensin-I converting enzyme inhibitor/inhibition
ADMA	Asymmetric dimethylarginine
ADP	Adenosine diphosphate
AGE	Advanced glycation end products
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate kinase
Ang I	Angiotensin I
Ang II	Angiotensin II
AP-1	Activator protein-1
AT_1R	Angiotensin II type 1 receptor
AT_2R	Angiotensin II type 2 receptor
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BDKRB1	Bradykinin β_1 receptor
BDKRB2	Bradykinin β_2 receptor
BH2 Study	Big Heart (Bassingbourn) 2 Study
BH3 Study	Big Heart (Bassingbourn) 3 Study
BH4	Tetrahydrobiopterin
BK	Bradykinin
BMI	Body mass index
cAMP	Cyclic adenosine monophopshate
cGMP	Cyclic guanosine-3',5-monophosphate

CHD	Coronary heart disease
CHF	Chronic heart failure
CMR	Cardiac magnetic resonance imaging
CRP	C-reactive protein
CuZn-SOD	Copper-Zinc superoxide dismutase
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DDAH	Dimethylarginine dimethylaminohydrolase
DE	Delta efficiency
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
EDRF	Endothelium derived relaxing factor
EDV	End diastolic volume
EGFR	Endothelial growth factor receptor
eNOS	Endothelial isoform of nitric oxide synthase
ETC	Electron transport chain
FAD^+	Flavin adenine dinucleotide
FADH	Reduced form of flavin adenine dinucleotide
FME	Final military exercise
FMN	Flavin mononucleotide
FSC	Forward scatter
GPCR	G-protein coupled receptor
GTN	Glyceryl trinitrate
GTP	Guanosine triphosphate
H^+	Hydrogen ion (proton)
HCM	Hypertrophic cardiomyopathy
hsCRP	high sensitivity CRP
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule-1

IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
IQR	Interquartile range
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
JC-1 A:M	JC-1 aggregate to monomer fluorescence
JGA	Juxtaglomerular apparatus
KKS	Kallikrein-kinin system
LD	Linkage disequilibrium
LDL	Low density lipoprotein
LV	Left ventricle/ventricular
LVH	Left ventricular hypertrophy
MI	Myocardial infarction
mi-CK	Mitochondrial isoform of creatine kinase
MMP	Matrix metalloproteinase
Mn-SOD	Manganese-dependent isoform of superoxide dismutase
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced form of nicotinamide adenine dinucleotide
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
nNOS	Neuronal isoform of nitric oxide synthase
NO•	Nitric oxide
NOS	Nitric oxide synthase
NPHSII	Second Northwick Park Heart Study
NSTEMI	Non-ST elevation myocardial infarction

O_2	Oxygen
$O_2\bullet^-$	Superoxide
$ONOO^-$	Peroxynitrite
OS	Oxidative stress
Ox-LDL	Oxidised low density lipoprotein
PMF, Δ_P	Proton motive force
PCR	Polymerase chain reaction
PCr	Phosphocreatine
PDGF	Platelet derived growth factor
PKC	Protein kinase C
PMT	Photomultiplier tube
PPRE	PPAR response element
QTL	Quantitative trait locus
RAAS	Renin angiotensin aldosterone system
RER	Respiratory exchange ratio
RNA	Ribonucleic acid
ROI	Region of interest
ROS	Reactive oxygen species
RSA Study	Republic of South Africa Study
RT-PCR	Reverse transcriptase-polymerase chain reaction
RV	Right ventricle/ventricular
SBP	Systolic blood pressure
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SNS	Sympathetic nervous system
SOD	Superoxide dismutase
SSC	Side scatter
SV	Stroke volume
TAOS	Total antioxidant status

TMRE	Tetramethylrhodamine ethyl ester
TMRM	Tetramethylrhodamine methyl ester
TRE	Thyroid response element
tRNA	transfer RNA
UCP	Uncoupling protein
UDACS	University College Diabetes and Cardiovascular disease Study
VO_2	Rate of oxygen uptake
VSMC	Vascular smooth muscle cell
WAT	White adipose tissue

※ CHAPTER ONE ※

INTRODUCTION

The endocrine renin-angiotensin-aldosterone system (RAAS) is involved in circulatory homeostasis and has been implicated in the pathogenesis of both coronary heart disease (CHD) and one of its common sequelae - the syndrome of heart failure (SOLVD Investigators 1991; AIRE Study Investigators 1993; Yusuf *et al.* 2000; Fox *et al.* 2003). RAAS-modifying drugs improve morbidity and mortality in both these disease processes through mechanisms beyond simple blood pressure reduction (Sleight *et al.* 2001). It is now apparent that tissue RAAS exist, whereby the components (for example Angiotensin Converting Enzyme - ACE) are either generated locally within cells, tissues or organs or are actively sequestered from the circulation, and it has been hypothesised that antagonism of tissue RAAS is responsible for the observed benefits of RAAS-modifying drugs (Dzau *et al.* 2001).

It has been further postulated that lower ACE activity, either as a consequence of pharmacological manipulation or due to genetic variation, increases cellular metabolic efficiency (Montgomery *et al.* 1998), and that this may in fact may be responsible, at least in part, for the observed range of effects; from mortality and functional benefits in heart failure to increased efficiency of skeletal muscle contraction and enhanced endurance performance in athletes (Montgomery *et al.* 1998). Could some of these metabolic effects be through the alteration of mitochondrial function? Recent results support the existence of a ***local mitochondrial RAAS***: components of the RAAS, including ACE, have been localised to mitochondria (Yayama *et al.* 1995; Peters *et al.* 1996; Clausmeyer *et al.* 1999) and ACE inhibition is of experimental benefit in other

situations where aberrations of mitochondrial function have been implicated, such as oxidative stress and ischaemia-reperfusion injury (Linz *et al.* 1986; Kingma *et al.* 1994; Berry *et al.* 2001). However, the precise underlying mechanisms of such putative actions are unclear, and may be a result of alterations in tissue levels of downstream effectors of the RAAS, such as kinins, or through novel mitochondrial pathways, such as through mitochondrial uncoupling proteins (UCPs).

This thesis will examine whether common variation in the constitutive bradykinin β_2 receptor (*BDKRB2*) gene is also associated with similar cardiovascular and performance phenotypes to that previously reported for the *ACE* gene. Secondly, *in vitro* assays will be used to test whether pharmacological manipulation of cellular ACE activity can alter mitochondrial coupling and the expression of mitochondrial UCPs. If the RAAS can alter cardiovascular pathophysiology through changes in mitochondrial activity, can genetic differences in mitochondrial UCPs be directly associated with cardiovascular and performance phenotypes? This hypothesis will also be tested, as will any detected associations between ACE and uncoupling proteins *in vitro*.

1.1 CORONARY HEART DISEASE (CHD)

By the beginning of the 21st century, cardiovascular disease (CVD) accounted for almost one-half of all deaths in the developed world and one quarter of all deaths in the developing world (World Health Organisation 2002). This represents a dramatic shift from the *status quo* in the 19th century, when infectious diseases and malnutrition represented the most common causes of death worldwide. CVD includes CHD, cerebrovascular disease and peripheral vascular disease, and it is predicted to become the major cause of morbidity and mortality worldwide, with an estimated 25 million deaths per year worldwide by 2020 (World Health Organisation 2002). There are racial

variations in CVD rates, with higher rates in some ethnic minorities, particularly amongst South Asians living in Western countries (Cappuccio 1997; Cappuccio *et al.* 2002). However, there is now an explosion in CVD rates in developing countries, which is thought secondary to the adoption of 'westernised' lifestyles (Zipes *et al.* 2005). Currently, in the UK, CVD is the leading cause of death overall and the leading cause of premature death in adults (British Heart Foundation 2006). CHD itself accounted for over 105000 deaths in 2004. Recent advances in our understanding of this disease, coupled with advances in treatment, have led to a 44% reduction in deaths in adults under 65 years of age in the past 10 years (British Heart Foundation 2006). However, there is increasing morbidity from CHD, particularly in the elderly, with an estimated 2 million sufferers of angina and almost one million people with either definite or probable heart failure in the UK (British Heart Foundation 2006).

1.1.1 Atherosclerosis (from Zipes *et al.* 2005 and reviewed by Libby 2002)

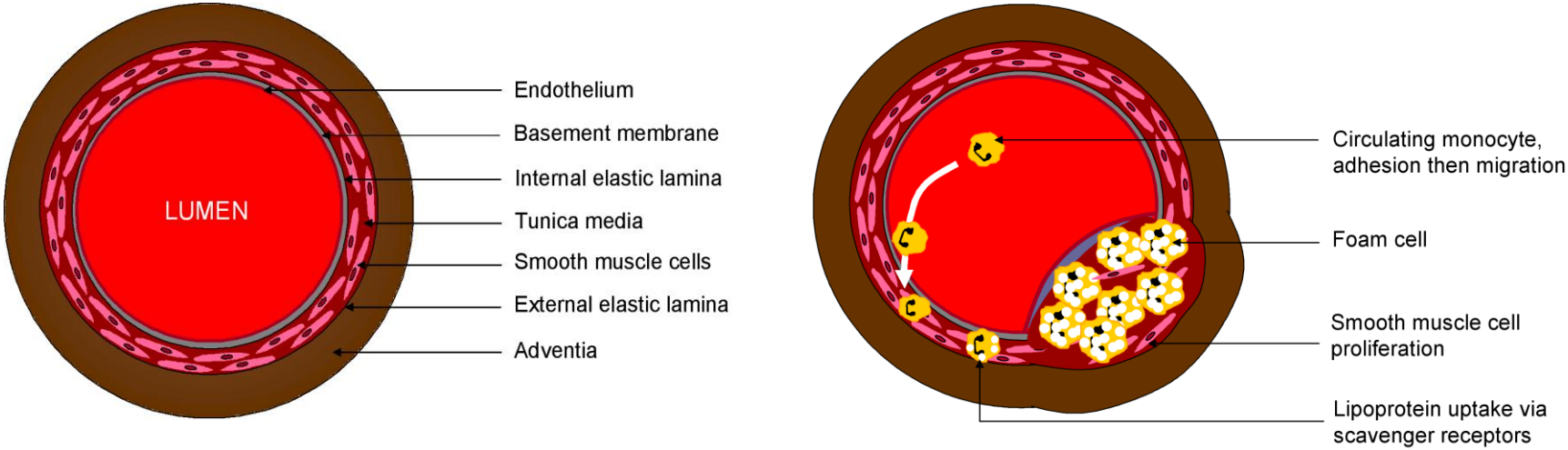
Atherosclerosis is an inflammatory disease of the artery (Ross 1999) and is caused by a complex interaction between environmental and genetic factors. The word stems from the Greek "*athera*" meaning gruel and, although its presence has been detected in Egyptian mummies, it was nevertheless uncommon until modern times. The increased prevalence of atherosclerotic disease has been thought to be a consequence of people surviving early mortality from infectious diseases together with lifestyle changes, such as the so-called atherogenic diet, and the inhalation of cigarette smoke.

The normal artery consists of three concentric layers: the tunica intima, tunica media and the adventitia (Figure 1.1). Innermost is the tunica intima, consisting of a single endothelial layer resting on a basement membrane of non-fibrillar collagens such as type IV collagen, laminin and fibronectin, and, in older individuals, there is an

underlying layer of connective tissue and smooth muscle cells. The endothelium is a highly specialised monolayer of cells which is in contact with the blood and maintains its fluidity through the expression of antithrombotic cell surface molecules such as heparin-sulphate, thrombomodulin and prostacyclin (PGI_2). The intima is separated from the underlying tunica media by the internal elastic lamina. The tunica media consists of layers of smooth muscle cells separated by elastic laminae. These layers are more pronounced in elastic arteries such as the aorta, which have a high compliance, allowing storage of the kinetic energy of systole as elastic energy, which it transmits during diastole. The external elastic lamina separates the media from the adventitia, which is a supportive loose array of collagen fibrils where the vasa vasorum and nerve endings are located.

The hallmark lesion of atherosclerosis is the fibrofatty plaque (Figure 1.1). In early atherogenesis, lipoprotein particles, particularly low-density lipoprotein (LDL), accumulate in the intima. This can be seen as the accumulation of fatty streaks in the great arteries and coronary arteries in as early as the second decade of life (Strong *et al.* 1999). Leucocytes, in the form of monocytes and T lymphocytes, are recruited into the intima, first by circulating leukocyte adhesion to the endothelium via adhesion molecules (such as VCAM-1 and selectins) then by migration towards chemokines such as monocyte chemoattractant protein (MCP-1). Monocytes within the intima have the capacity to accumulate lipid via scavenger receptors and become lipid-laden macrophages or "foam cells". Macrophages are a rich source of pro-inflammatory mediators and also recruit T lymphocytes and smooth muscle cells to the plaque. Endothelial migration into plaque has been more recently recognised and results in the formation of neovessels. The more mature plaque is formed by a complex interaction between pro- and anti-inflammatory signals between these resident cells.

Figure 1.1 The structure of the normal artery (left) and the genesis of the atherosclerotic plaque (right)



Extracellular matrix (interstitial collagens and proteoglycans) constitutes most of the advanced plaque, and its accumulation is a balance between formation by smooth muscle cells and degradation by a family of proteins called matrix metalloproteinases (MMPs).

1.1.2 The spectrum of disease in CHD

The present consensus is that coronary atherosclerosis does not develop in a smooth, progressive manner, but rather during bursts of disease activity during an individual's life. As a plaque increases in size, the artery first exhibits negative remodelling, with radial growth of the arterial wall away from the lumen and therefore relative protection of luminal diameter. Once this ability of the artery to remodel is exceeded, further plaque growth encroaches on the lumen itself. This period of plaque growth is a **chronic asymptomatic phase**. Once the effective luminal diameter is reduced by approximately 60-70%, often after several decades of plaque growth, the stenosis is likely to cause flow limitation during increased demand, such as during exercise. This leads to the syndrome of **chronic stable angina**, where the onset of ischaemic chest pain is predictable and provokable by similar degrees of exertional or emotional stress.

Acute myocardial infarction (MI) is the necrotic death of cardiac myocytes resulting from an abrupt cessation of coronary blood flow, usually due to an occlusion by thrombosis of an epicardial coronary artery (Falk 1983; Davies *et al.* 1989). This usually presents as a prolonged episode of ischaemic chest pain, resulting in transmural infarction with clinical sequelae, ranging from ventricular dysrhythmias and sudden cardiac death, to acute and chronic ventricular failure from pump or valvular failure, to myocardial rupture and aneurysm formation. Incomplete, but critical occlusion of a

coronary artery can lead to the syndromes of non-ST elevation MI (NSTEMI, previously non-Q wave infarction or subendocardial infarction) without transmural infarction and unstable (crescendo) angina, with increased frequency or severity of angina occurring at lower thresholds of stress or at rest.

It is now recognised that most cases of MI are caused by atherosclerotic lesions that were non-flow limiting at onset (Stary *et al.* 1995). Physical disruption of a plaque (plaque rupture) or, less often, plaque erosion, results in its thrombogenic core coming into contact with the circulation, leading to thrombus generation which can occlude the entire lumen. This finding has lead to the concept of the 'vulnerable plaque'. A 'stable plaque' has a large fibrous component with a thick fibrous cap and small lipid/macrophage core. The mechanical strength of the fibrous cap is regulated by plaque smooth muscle cell number and function (themselves regulated by T cells) and by matrix degradation by MMPs and cathepsins secreted by macrophages. A large lipid/macrophage core will make the plaque more vulnerable, not only due to the increased biomechanical stress this causes in the shoulder regions of the plaque, but also due to the activated macrophages contained within, which generate pro-inflammatory cytokines (causing smooth muscle cell apoptosis) and matrix-degrading enzymes.

It is likely that plaque rupture leads more often to subclinical events, whereby the ensuing thrombosis does not lead to complete luminal occlusion or an ischaemic syndrome, but rather the thrombus propagation is controlled, leading to plaque remodelling and progression (Burke *et al.* 2001). It is probably these sequential explosions in plaque growth that are responsible for progressive luminal loss.

Plaque erosion, on the other hand, has only been recently recognised and is another process which can lead to thrombus formation (Farb *et al.* 1996). It is more common in younger adults, particularly women and smokers (Burke *et al.* 1997; Arbustini *et al.* 1999).

1.1.3 Risk factors for CHD

A risk factor is a characteristic or feature of an individual or population that is associated with an increased chance of developing future disease. Conventional and novel risk factors in the development of CHD are described in brief below.

1.1.3.1 Conventional risk factors

Long recognised independent risk factors in the development of CHD are cigarette consumption (smoking), hypertension, hyperlipidaemia, the presence of diabetes or insulin resistance and obesity.

Smoking is the singlemost modifiable risk factor for CHD and its effect on risk is dose-dependent (Doyle *et al.* 1962; Zipes *et al.* 2005). Smoking more than 20 cigarettes per day increases the risk of CHD by two to three-fold. There are many adverse, atherogenic associations with smoking, including endothelial dysfunction (for example decreased endothelial nitric oxide (NO•) and increased monocyte-endothelial adhesion), oxidation of LDL cholesterol, an increase in biomarkers associated with CHD such as highly-sensitive C-reactive protein (hsCRP), ICAM-1, fibrinogen and homocysteine, increased platelet aggregation and increased oxidative stress (Adams *et al.* 1997; Tracy *et al.* 1997; Fusegawa *et al.* 1999; Barua *et al.* 2003).

The prevalence of hypertension in the adult UK population is approximately 13% and increases with age. The relative risk of CHD increases with the degree of hypertension when compared to normotensives (Kannel *et al.* 1969; Kannel *et al.* 1986; Hansson *et al.* 1993; Hansson 1996).

The universal finding of cholesterol within atherosclerotic plaque outlined the importance of circulating lipid in the pathogenesis of CHD. However, it was not until prospective cohort studies in the 1950s that the relationship between serum cholesterol and CHD risk was confirmed and fully accepted (Kannel *et al.* 1964). The lipid transport system has evolved to carry hydrophobic fats in the aqueous plasma. Lipoproteins are spherical particles consisting of a central core of cholesterol-ester and triglyceride within a cholesterol-phospholipid coating. Highly evolutionary-conserved amphipathic apolipoproteins are also embedded in the lipoprotein coating and mediate the lipid transport process. These lipoproteins vary in their size, density, lipid and apolipoprotein content. Low density lipoprotein (LDL) is the main carrier of cholesterol, containing predominantly cholesterol ester packaged in apolipoprotein B100. LDL, particularly small dense LDL (triglyceride rich) and oxidised LDL (oxLDL), is highly atherogenic, and levels correlate positively with CHD (Castelli *et al.* 1986; 1986). High density lipoprotein (HDL), whose protein content consists mainly of apolipoprotein A1, is involved with cholesterol efflux from tissue targets (*e.g.* endothelial cells). HDL promotes reverse cholesterol transport and is antiatherogenic. Epidemiological studies have shown an inverse relationship between HDL and CHD risk (Yaari *et al.* 1981; Castelli *et al.* 1986).

Early trials with drugs (bile-acid sequestrants, fibrates) with only modest reductions in LDL cholesterol reported only modest reductions in CHD risk. However, several major

trials in patients using the 'statin' class of drugs (HMG-CoA reductase inhibitors) have made possible aggressive reductions in serum LDL cholesterol, and have shown clear and reproducible beneficial reductions in CHD risk in both secondary (Scandinavian Simvastatin Survival Study Group 1994; Sacks *et al.* 1996; WoSCoPS Group 1997) and primary prevention trials (Shepherd *et al.* 1995; Downs *et al.* 1998; 2002).

The presence of obesity promotes insulin resistance and dyslipidaemia but also predicts CHD risk (Garrison *et al.* 1980; MRFIT Research Group 1986). There is a continuum between the onset of obesity and the presence of the metabolic syndrome (central obesity, hypertension, dyslipidaemia and insulin resistance). Both insulin resistance and metabolic syndrome appear to confer an independent risk of CHD (Lakka *et al.* 2002; Sattar *et al.* 2003).

The prevalence of diabetes is increasing worldwide at a dramatic rate. It is estimated that diabetes affects 5% of the world's populace and its prevalence is doubling every generation, with an estimated 300 million people likely to be suffering from diabetes by 2025 (King *et al.* 1998). At present there are 1.8 million people with diabetes in the UK (3% of the population) and this is set to increase to 3 million by 2010. There are a further 1 million people with undiagnosed type 2 diabetes (Diabetes UK 2004). Patients with diabetes have a two- to four-fold higher risk of developing CHD than matched non-diabetic individuals (Garcia *et al.* 1974; Stamler *et al.* 1993). Indeed, by diagnosis, more than half of patients will already have CVD, and CVD accounts for 80% of all deaths in diabetic patients (Beckman *et al.* 2002). Patients with diabetes but no prior history of CHD have the same level of future CHD risk as those non-diabetic patients with prior MI (Haffner *et al.* 1998).

The metabolic abnormalities in diabetes and in the pre-diabetic state, including hyperglycaemia, insulin resistance and dyslipidaemia, render arteries susceptible to atherosclerosis. The mechanisms involved are complex and include reduced bioavailability of nitric oxide (NO•) and endothelial dysfunction (Steinberg *et al.* 1996; Steinberg *et al.* 1997; Williams *et al.* 1998), increased reactive oxygen species (ROS) generation through enzymatic and non-enzymatic processes (Nishikawa *et al.* 2000; Brownlee 2001), and activation of protein kinase C (PKC) which has a vast array of actions including inactivation of the endothelial isoform NO• synthase (eNOS), augmentation of endothelial tissue factor gene expression and the increased production of proinflammatory cytokines (Terry *et al.* 1996; Koya *et al.* 1998). Diabetes also impairs vascular smooth muscle function by augmenting the production of vasoconstrictors such as endothelin and angiotensin II (Ang II) (Park *et al.* 2000; Beckman *et al.* 2002). The accumulation of advanced glycation end products (AGEs), which are formed via the non-enzymatic glycation of macromolecules, may also disturb vascular function and accelerate atherosclerosis (Stitt *et al.* 1997; Brownlee 2000).

1.1.3.2 Genetic factors

There are strong genetic influences on the development of many of the classical risk factors for CHD, shown early on in twin studies (Feinleib *et al.* 1977; Austin *et al.* 1987). However, family history is a strong independent risk factor for CHD itself (Snowden *et al.* 1982; Assmann *et al.* 1997);(1997; Hawe *et al.* 2003). Further support for a genetic contribution to CHD risk comes from twin studies, with a high concordance for age of onset of CHD and a greater risk in monozygotic twins in developing CHD at a young age compared to dizygotic twins (relative risk 8.1 compared with 3.8, respectively, (Marenberg *et al.* 1994). Rarely, CHD arises from a single gene mutation, exemplified by familial hypercholesterolaemia (FH) which results

in elevated LDL cholesterol above the 95th percentile (due predominantly to one of a number of mutations in the LDL receptor gene) and early-onset CHD in the 3rd or 4th decades. The more common form of CHD is also thought to be partly heritable, arising from the interaction between common environmental exposure (risk factors) and inheritance of many disease modifying common gene variants (polymorphisms), all with small to moderate effect (Humphries *et al.* 2004).

1.1.3.3 Novel risk factors

Almost half of all MIs occur in patients without significant hyperlipidaemia (Ridker *et al.* 2002). Even prediction models (for example based on the Framingham dataset) cannot explain approximately 1 in 5 CHD events using 'conventional' risk factors. More recently, novel risk factors have been associated with CHD, such as those involved in inflammation and thrombosis. One of the best studied examples is CRP - an acute phase reactant which plays a major role in the innate immune response and is used as a (laboratory) marker of inflammation. It has now been shown in prospective studies using highly sensitive assays for CRP (hsCRP) that CRP is an independent risk factor for CHD (Kuller *et al.* 1996; Ridker *et al.* 1997; Tracy *et al.* 1997; Ridker *et al.* 1998; Koenig *et al.* 1999). Whether CRP is directly atherogenic or is an epiphenomenon of the inflammatory process remains to be elucidated. CRP is present in atherosclerotic plaque (Torzewski *et al.* 1998) where it may have several pro-atherosclerotic actions including binding oxidised low density lipoprotein (oxLDL) (de Beer *et al.* 1982), inducing adhesion molecule expression on endothelium (Pasceri *et al.* 2000) and reducing NO• bioavailability (Venugopal *et al.* 2002)

Fibrinogen is the precursor of fibrin and, in combination with thrombin, mediates the final step in thrombus formation and also influences platelet aggregation, plasma

viscosity and plasminogen binding. Fibrinogen levels are correlated positively with smoking, obesity, LDL-cholesterol, age and insulin resistance/diabetes. Elevated plasma fibrinogen levels are associated with a moderate increase in future CHD risk (Meade *et al.* 1986; Wilhelmsen *et al.* 2001).

Other novel risk factors include inflammatory biomarkers, such as interleukin-6 (IL-6) which is the main cytokine stimulus for CRP (Ridker *et al.* 2000; Lindmark *et al.* 2001), CD40 ligand (Schonbeck *et al.* 2001), soluble ICAM-1 (Malik *et al.* 2001) and lipoprotein-associated phospholipase A_2 (Blake *et al.* 2001) amongst others. Homocysteine is a sulphydryl-containing amino acid derived from the demethylation of methionine. Patients with inherited defects in methionine metabolism develop homocystinuria and have increased risk of both venous and arterial thrombosis, which may be as a result of endothelial dysfunction, increased oxidative stress and platelet activation (Bellamy *et al.* 1998; Welch *et al.* 1998). Mildly elevated levels of homocysteine are seen in the general population (usually related to dietary deficiency of folic acid), and have been associated with increased prospective CHD risk (Wald *et al.* 2002). Lipoprotein(a) consists of an LDL particle with its apolipoprotein B100 cross-linked to apolipoprotein(a) which is a highly complex molecule with more than 25 heritable forms. Not only is it more atherogenic than LDL, but it also shares sequence homology with plasminogen and may inhibit endogenous fibrinolysis (Miles *et al.* 1989). Subjects with the top tertile of lipoprotein(a) levels are associated with a 1.6 fold increase in relative risk of CHD (Danesh *et al.* 2000).

1.2 HEART FAILURE

1.2.1 Definition

There are several definitions of heart failure. In essence, heart failure is a pathophysiological state said to be present when the heart is unable to pump blood at a rate commensurate with the requirements of the metabolising tissues, or can only do so in the face of elevated filling pressures.

Heart failure is clinically defined by the European Society of Cardiology as:

I. Symptoms of heart failure (at rest or exertion) *and*

II. Objective evidence (preferably by echocardiography) of cardiac dysfunction (systolic/diastolic) (at rest) *and*

III. Response to treatment directed towards heart failure (in cases where the diagnosis is in doubt)

Criteria I and II must be fulfilled in all cases

Heart failure has been defined by the American College of Cardiology/American Heart Association Task Force on practice guidelines as:

> "a complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill with or eject blood."

1.2.2 Epidemiology and causes

Heart failure is a complex, multisystem disorder. It is the final common manifestation of a number of disease processes that damage the heart, the most common being CHD

(Table 1.1). Heart failure carries an overall 5 year mortality of 50% (Levy *et al.* 2002) and even with current treatments available, the one-year survival of patients with a new diagnosis of heart failure in London, UK is only 62% (Cowie *et al.* 2000).

Table 1.1 The common causes of heart failure

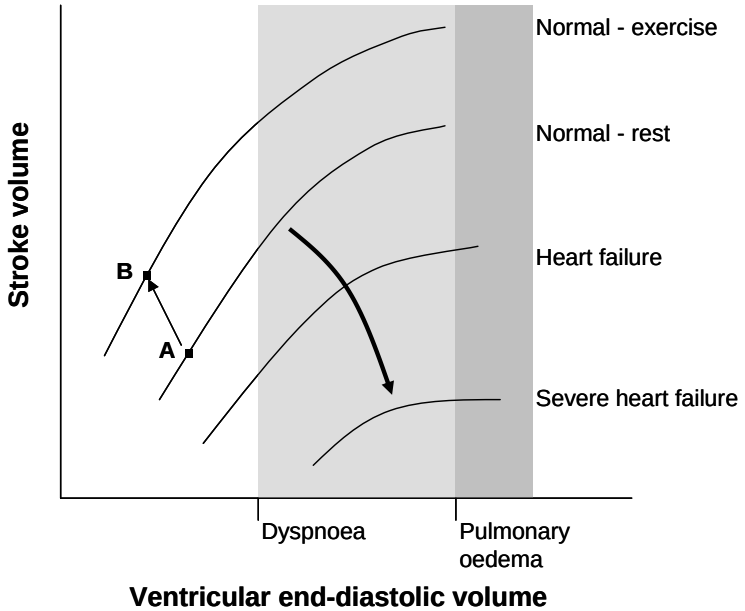
Coronary heart disease
Hypertension
Valvular heart disease
Myocardial diseases (Idiopathic) dilated cardiomyopathy Viral Metabolic Infiltrative Inherited, e.g. Hypertrophic cardiomyopathy, Fabry's, muscular dystrophy, ARVC, mitochondrial myopathies
Congenital heart disease
Drug or toxin-induced Alcohol Cardiotoxic chemotherapy
Tachymyopathy
Pericardial disease
High output failure (AV fistulae, thyrotoxicosis, Beriberi)

Heart failure is a common condition and increases in prevalence with age. It has a huge social and economic impact, with an estimated cost to the NHS of over £600 million in 2000. In the UK in 2001, heart failure was directly responsible for over 86000 hospital admissions and over 11500 deaths (data from www.heartstats.org).

1.2.3 Pathophysiology

The cardinal manifestations of heart failure are dyspnoea, fatigue and fluid retention. These clinical symptoms and signs were initially explained by either *backward* (reverse) or *forward* pump failure, first proposed by Hope (1832) and Mackenzie (1910), respectively. In *backward* heart failure, it is suggested that ventricular end-diastolic volume (EDV) and pressure increase as a function of the inability of the cardiac muscle to shorten. This then results in an increase in atrial volumes and pressures and these then result in an increase in upstream venous and capillary pressures. This increase in pressure results in increased transudation of fluid from the capillary bed into the interstitium causing either pulmonary oedema (in the case of left ventricular dysfunction) or peripheral interstitial tissue/organ oedema (in right ventricular dysfunction). In left ventricular failure, *forward* pump failure results in reduced cardiac

Figure 1.2. Diagram showing the interrelationship between ventricular end-diastolic volume (EDV) and stroke volume (SV). The points at which symptomatic dyspnoea and pulmonary oedema develop are represented on the x-axis. At rest, an increase in EDV results in increasing SV due to increased myocardial contractility. This relationship is shifted to the left during exercise (for example walking shown as moving from A to B) when adrenergic stimuli and tachycardia augment cardiac output. With the development of heart failure, the curve is shifted down and to the right with higher filling pressures and lower SV developed, and with symptoms at rest and lower myocardial reserve (adapted from Zipes *et al* 2005).



output and relative hypoperfusion of vital organs such as the heart, gut, kidneys and brain.

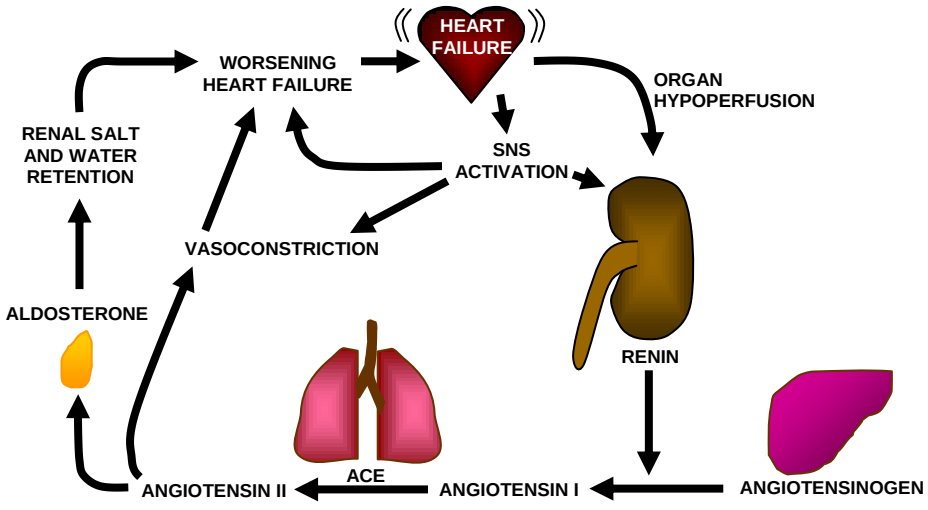
These explanations were, however, overly simplistic. Early on in heart failure, the heart depends on several adaptive mechanisms to maintain its pumping action. The most important are the Frank-Starling mechanism (in which an increase in preload results in an increase in contractility, Figure 1.2), neuroendocrine activation (including an increase in sympathetic drive, in endothelin and natriuretic peptide release and in RAAS activation) and myocardial remodelling. When cardiac output is depressed, arterial pressure is maintained by systemic vasoconstriction and salt and water retention by a number of neurohormones. These mechanisms appear to be important for the short term gain in acute circulatory failure but are deleterious in chronic heart failure (CHF), contributing to oedema formation but also having direct adverse effects on the heart (Figure 1.3). Generalised adrenergic stimulation and parasympathetic withdrawal results in vasoconstriction, stimulates myocardial contractility and tachycardia and promotes sodium retention and renin secretion from the juxtaglomerulus apparatus (JGA) in the kidney.

Activation of the sympathetic nervous system (SNS) is the primary mechanism for increasing cardiac output in the normal heart via an increase in contractility and heart rate (Bristow 1984). The mechanism for this increased contractility has been well characterised, involving activation of β-adrenergic receptors and subsequent activation of adenylate cyclase, increase in cAMP and protein kinase A and phosphorylation of calcium regulatory proteins (Li *et al.* 2000). This results in increased calcium influx into cardiomyocytes, increased calcium flux through the sarcoplasmic reticulum, decreasing inhibition of the cardiac isoform of the sarcoplasmic-endoplasmic reticulum

calcium uptake pump (SERCA2a) and decreased binding to troponin. The increase in cardiac contractility and relaxation occurs at the expense of an increase in myocardial energy demand (Houser *et al.* 2003).

A characteristic feature of heart failure is the activation of the SNS coupled with blunting of the normal adrenergic effects on myocyte contractility. Epinephrine is exceptionally cardiotoxic at levels found in the failing human heart, levels of which are elevated three-fold at rest, but also rise rapidly during exercise (Chidsey *et al.* 1962; Grassi *et al.* 1995). These elevated levels are thought to result in receptor desensitisation, possibly as a cardioprotective mechanism, with reduced β1 receptor density and uncoupling of β2 receptors from downstream effector molecules with a resultant reduction in inotropic responsiveness (Bristow 1993). However, the enhanced

Figure 1.3. **Maladaptive mechanisms in heart failure** include sympathetic (SNS) activation and RAAS activation. In the traditional endocrine model of RAAS activation, renal-derived renin cleaves hepatic-derived angiotensinogen to generate circulating angiotensin I. This is cleaved by ACE in the pulmonary circulation to generate the vasoconstrictor angiotensin II which stimulates adrenal aldosterone release. However, tissue RAAS systems also exist (see section 1.4).



epinephrine levels promote apoptosis via β receptors and hypertrophy via upregulated α receptors contributing to cell death and negative remodelling (Milano *et al.* 1994; Communal *et al.* 1998). The augmented adrenergic drive may also precipitate myocardial ischaemia, ventricular dysrhythmia and sudden cardiac death.

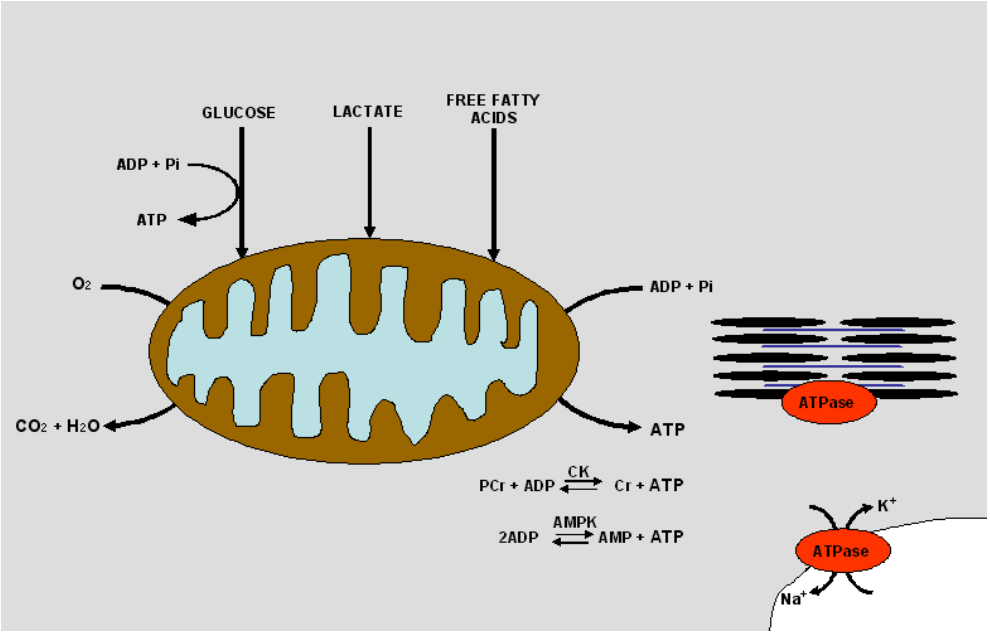
Relative renal hypoperfusion in heart failure results in renin secretion from the juxtaglomerular apparatus, with subsequent generation of Ang II and aldosterone, which promote systemic vasoconstriction and further salt and water retention as a protective mechanism to increase systemic blood pressure and hence renal perfusion pressure (Figure 1.3). These effects may be deleterious both acutely and in the long-term, leading to oedema formation. Furthermore, Ang II and aldosterone have direct effects on the heart, including cardiomyocyte necrosis (Tan *et al.* 1991), apoptosis (Leri *et al.* 1998; Mano *et al.* 2004), hypertrophy (Sadoshima *et al.* 1993; Karmazyn *et al.* 2003) and myocardial fibrosis (Weber *et al.* 1991).

In the compensated phase of CHF, ventricular remodelling - comprising changes in mass, volume, shape and composition - results in hypertrophy (pressure overloaded ventricle) and dilatation (volume overloaded) (Jacob *et al.* 1998; Francis 2001; Zipes *et al.* 2005). When the haemodynamic stress on the failing heart is prolonged, however, myocardial contractility becomes further depressed, with the redevelopment of overt heart failure (Houser *et al.* 2000). Remodelling at the cellular level is characterised by myocyte hypertrophy and elongation, changes in myocyte phenotype with re-expression of foetal genes, abnormalities in calcium handling, myocyte necrosis and apoptosis and myocardial fibrosis (Anversa *et al.* 1997; Olivetti *et al.* 1997; Houser *et al.* 2000).

1.2.4 Central *vs.* peripheral abnormalities in chronic heart failure

The mammalian heart is an obligate aerobic organ, consuming approximately 8-15 ml O_2.min^{-1}.100 g tissue at rest but rising to more than 70 ml O_2.min^{-1}.100 g tissue during vigorous exercise. Molecular O_2 itself is a double-edged sword: not only is it essential to maintain normal energy requirements, but is also central to the generation of ROS. For over 20 years, an 'oxygen-wasting' contractile phenotype has been identified in the failing heart, suggestive of metabolic inefficiency (Horak *et al.* 1983; Buser *et al.* 1989; Sawyer *et al.* 2000), but the idea that the failing heart is energy-starved is decades old (Olson *et al.* 1951). More recently, the use of positron emission tomography has confirmed such an increased inefficiency of energy utilisation in the failing human heart (Bengel *et al.* 2000; Taylor *et al.* 2001), to which impaired mitochondrial function seems a fundamental contributor in animals (Sabbah *et al.* 1992; Lesnefsky *et al.* 2001; Liu *et al.* 2001; Marin-Garcia *et al.* 2001; Casademont *et al.* 2002) and humans (Sharov *et al.* 2000).

Figure 1.4. ATP synthesising and utilising reactions in the cardiomyocyte.



1.2.4.1 Normal cardiac metabolism.

Energy derived from adenosine triphosphate (ATP) hydrolysis provides the majority of the energetic requirement in the healthy adult human heart (Figure 1.4), with approximately two-thirds used for contractile work (hydrolysed by the actomyosin-ATPase) and one-third used by ion pumps such as the sarcoplasmic reticulum Ca^{2+}-ATPase and the sarcolemmal Na^{+}/K^{+}-ATPase (Suga 1990). In the healthy heart, ATP hydrolysis is matched to ATP re-synthesis, with the concentration of ATP, [ATP], kept relatively constant at approximately 10 $mmol.L^{-1}$ even during periods of increased ATP turnover. ATP-requiring processes are inhibited by the products of ATP hydrolysis: namely adenosine diphosphate (ADP) and inorganic phosphate (P_i).

To understand this process further one must consider basic thermodynamics. An increase in disorder of a system, *entropy*, is the driving force for a reaction in an isolated system such as the Universe. In a closed system such as a cell, a reaction will occur spontaneously if the entropy of that system plus its surroundings increases. An assessment of the entropy change in the surroundings (or Universe) caused by energy flow across the boundary of a closed system can be made under constant temperature and pressure, as it is equal to the flow of heat or *enthalpy* out of the closed system. The Gibbs energy change, ΔG, is the quantitative measure of the driving force for this reaction and is defined by the Gibbs-Helmholtz equation:

$$\mathbf{\Delta G = \Delta H - T\Delta S}$$

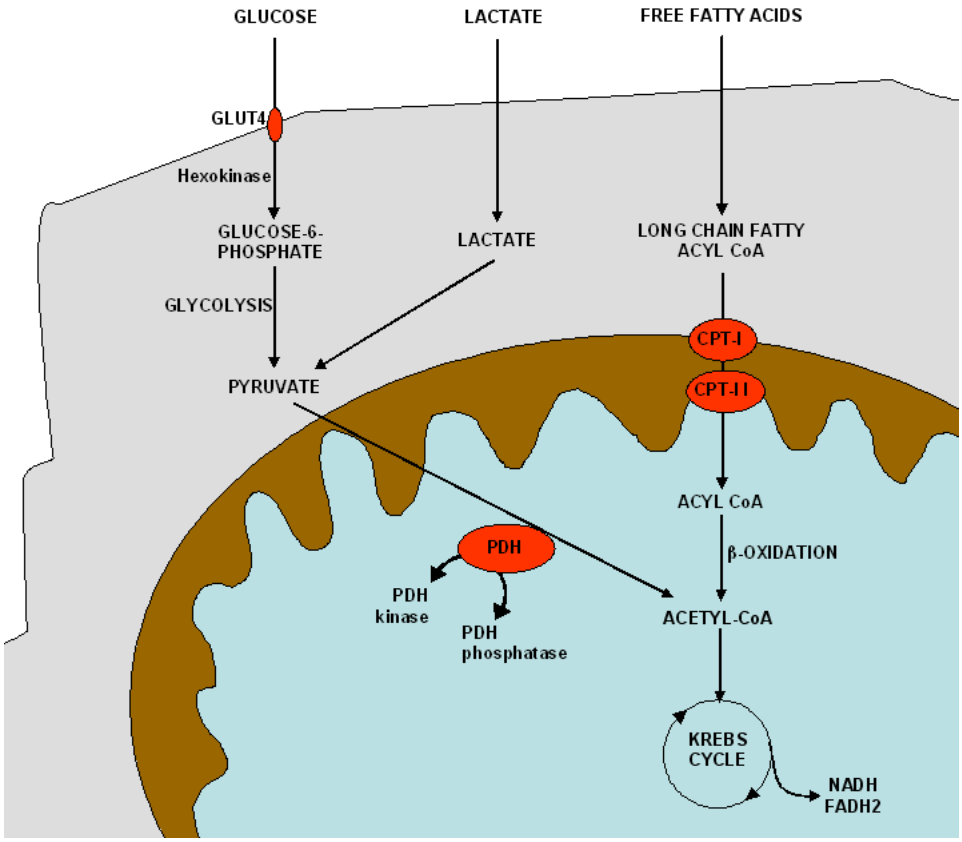
ΔG = Gibbs energy change, ΔH = enthalpy change, T = temperature
ΔS = entropy change of the system

ΔG is at a minimum for a particular reaction when the mixture of reactants and products are at equilibrium, whereas increasing displacement of the reactants and products from equilibrium results in an increase in ΔG. The ΔG required for ATP synthesis (also known as the phosphorylation potential) is obtained from the ΔG for ATP hydrolysis by

changing the sign. Intracellular [ATP], [ADP] and [P_i] in normal ventricular tissue are approximately 10 mmol.L^{-1}, <50 μmol.L^{-1} and <1 mmol.L^{-1}, respectively.

The heart uses energy reserve systems to maintain a high ΔG for ATP hydrolysis to drive ATPase reactions during variations in work output. The primary energy reserve compound in the heart is phosphocreatine (PCr), which is present in concentrations twice that of ATP. The enzyme creatine kinase (CK) transfers the phosphoryl group between PCr and ADP at a rate 10-times faster than the rate of ATP synthesis by oxidative phosphorylation, maintaining [ATP] during conditions of acute stress, such as ischaemia, but also maintaining a high ΔG for ATP hydrolysis by maintaining low levels of ADP and P_i. The enzyme adenylate kinase also functions to maintain high

Figure 1.5. **Cartoon depiction of myocardial metabolism.** The major fuel substrates in the healthy adult heart are free fatty acids (60-90%) and pyruvate (10-40%). Pyruvate dehydrogenase (PDH) - an irreversible step in pyruvate metabolism - is positively regulated by PDH phosphatase and negatively by PDH kinase. Carnitine palmitoyltransferase I and II catalyse transfer of long chain fatty acyl CoA into the mitochondrial matrix and are key regulators of fatty acid metabolism.



levels of ATP by transferring phosphoryl groups amongst the adenine nucleotides: $2ADP \rightarrow ATP + AMP$.

Almost all (98%) of the ATP resynthesis requirements of the human heart under aerobic conditions originates from oxidative phosphorylation (Figures 1.4-1.5 and section 1.5.3), with the remaining fraction (2%) arising from glycolysis (Stanley *et al.* 2002). The substrates for oxidative phosphorylation are the reducing equivalents, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). These in turn can be generated by either the β-oxidation of fatty acids, glycolysis, the oxidation of pyruvate or the Krebs cycle. At birth, the human heart switches from glucose as a substrate to fatty acid as the predominant substrate. In the healthy human heart in the fed state, about 60–90% of mitochondrial ATP generation comes from beta-oxidation of free fatty acids, and 10–40% originates from pyruvate. Free fatty acids are esterified by long-chain fatty acyl-CoA synthetase with coenzyme A to form long-chain fatty acyl-CoA, which is transferred across the mitochondrial membranes by three carnitine dependent enzymes (CPT-I, CPT-II and carnitine acylcarnitine translocase) yielding long-chain acyl-CoA in the mitochondrial matrix for β-oxidation. Successive spirals through the β-oxidation pathway then generates one acetyl-CoA and one NADH/$FADH_2$ pair.

Glucose and lactate are converted to pyruvate in the cytosol and subsequently oxidised to CO_2 in the mitochondria. The GLUT1 and GLUT4 isoforms of the glucose transporter family are responsible for glucose uptake into cardiomyocytes. Glucose uptake depends on the cell transmembrane glucose gradient and density of glucose transporters. Thereafter, glucose is locked within the cell by phosphorylation by hexokinase to form glucose 6-phosphate, and then either stored as glycogen or enters

the glycolytic pathway, which itself produces 2 NADH + 2ATP + pyruvate for each glucose molecule. Lactate supplies approximately 50% of the pyruvate oxidised by the healthy heart at rest (Gertz *et al.* 1988), being taken up and rapidly oxidised by lactate dehydrogenase. During exercise, lactate can become the predominant fuel for the heart (Gertz *et al.* 1988; Stanley *et al.* 2002).

Pyruvate decarboxylation is the key irreversible step in carbohydrate metabolism and is under metabolic control. This step is catalysed by pyruvate dehydrogenase (PDH) which is itself activated by phosphorylation. PDH phosphorylation status is under complex control by a specific PDH kinase and by PDH phosphatase (Figure 1.5). This enzyme complex, and thus pyruvate oxidation, is negatively regulated by free fatty acids and by the presence of acetyl CoA and NADH, whereas promotion of carbohydrate metabolism at the level of PDH results in less fatty acid oxidation via inhibition of CPT-I through elevated malonyl-CoA levels.

1.2.4.2 Cardiac muscle metabolic abnormalities in heart failure

There is increasing evidence that cardiac substrate utilisation is critical for cardiac function (Stanley *et al.* 2002). Indeed, defects in enzymes involved in fatty acid oxidation cause childhood cardiomyopathies (Kelly *et al.* 1994), and pharmacological inhibition of cardiac fatty acid import induces cardiac lipid accumulation and causes rapid death in peroxisome proliferator activator receptor α (PPARa)-knockout mice (Djouadi *et al.* 1998). Transgenic mice that overexpress long-chain acyl-CoA synthetase and take up excess long chain fatty acids initially exhibit cardiac hypertrophy, followed by LV dysfunction and death (Chiu *et al.* 2001). The hyperadrenergic state of CCF causes an increase in circulating free fatty acid

concentration paralleled by a 50% increase in myocardial lipid oxidation and a 60% reduction in carbohydrate oxidation (Paolisso *et al.* 1994). Fatty acids inhibit pyruvate dehydrogenase (*vide supra*) resulting in reduced pyruvate oxidation and intracellular lactate accumulation. Fatty acids generate more ATP per mol of substrate, but at the expense of a greater oxygen requirement per mol of ATP than either glucose or lactate (Nicholls *et al.* 2002), and also induce a proton leak across the inner mitochondrial membrane (Borst *et al.* 1962), increasing cardiac oxygen consumption for the same amount of cardiac external work in both the isolated (Challoner *et al.* 1966) and whole animal model (Mjos 1971), resulting in an even lower actual ratio of ATP:oxygen consumed than that predicted (Brand *et al.* 1994).

Mitochondria form 40% of the dry weight of the heart, and primary mitochondrial defects result in diverse forms of cardiac dysfunction (Casademont *et al.* 2002). A secondary decrease in PCr, ATP and mitochondrial function has long been observed in both the hypertrophied and failing heart (Schwartz *et al.* 1962; Wollenberger *et al.* 1965; Chandler *et al.* 1967; Lindenmayer *et al.* 1968; Sanbe *et al.* 1995). PCr decreases in the failing heart because of a mismatch in ATP supply and demand, followed by up to 60% loss of the total creatine pool and decreases in both muscle and alterations in the amounts of the different CK isoforms. Myocardial creatine depletion and the decrease in the [PCr]/[ATP] ratio measured by NMR predicts CHF severity and prognosis (Conway *et al.* 1991; Neubauer *et al.* 1997; Nakae *et al.* 2003). From human biopsy and ^{31}P NMR studies in patients, [ATP] is 25-30% lower in CHF (Starling *et al.* 1998; Beer *et al.* 2002). Animal models of CHF suggest the decline in [ATP] is approximately 0.35% per day and is caused by a loss of the total adenine nucleotide pool (Shen *et al.* 1999) with a resultant increase in [ADP], thereby lowering $\Delta G_{\approx ATP}$. A consequence of the rise in [ADP] is an increase in cytosolic [AMP] from the adenylate

kinase reaction (Shen *et al.* 1999) which itself is a 'double-edged sword'. High [AMP] activates AMP kinase (AMPK) leading to activation of the "low fuel warning system", switching off ATP consuming pathways such as fatty acid and sterol synthesis and activating ATP generating pathways such as fatty acid oxidation and enhanced glucose uptake (Hardie *et al.* 1997) and promoting mitochondrial biogenesis via increased $PPAR_{\gamma}$- co-activator (PGC-1α) expression (Zong *et al.* 2002). High [AMP] activates a specific cytosolic nucleotidase, which converts AMP to adenosine, and may lead to gradual reduction in the total adenine pool (Bak *et al.* 1994).

In CHF, both morphological and metabolic mitochondrial abnormalities are present, with a loss of overall capacity for oxidative phosphorylation. Such abnormalities in mitochondrial function are not confined to the myocardium. Indeed, they may occur in diffuse cell types, where they may also lead to symptom progression (Minotti *et al.* 1991; Harridge *et al.* 1996; Kong *et al.* 2001; Marin-Garcia *et al.* 2001). This may be especially true for skeletal muscle.

1.2.4.3 Skeletal muscle metabolic abnormalities in heart failure

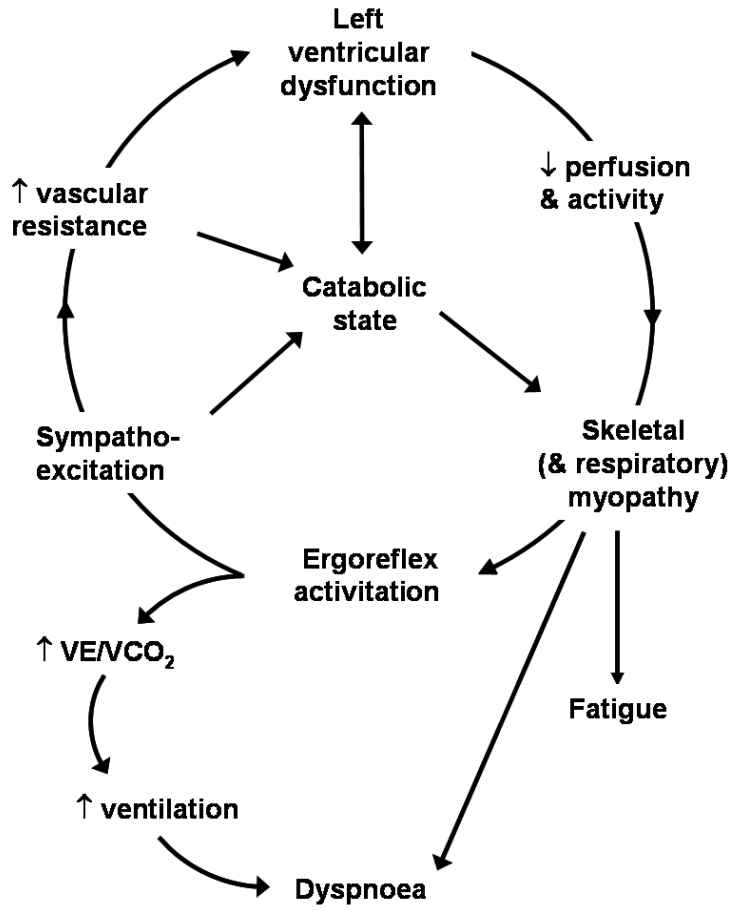
The severe exertional handicap in CHF represents one of the best predictors of mortality (Clark *et al.* 1996), but is not explained by the limitations in central haemodynamic performance (Brown *et al.* 1954; Katsuki *et al.* 1995; McKelvie *et al.* 1995; Clark *et al.* 1996). In CHF, exercise limitation occurs in the absence of pulmonary congestion (Clark *et al.* 1996), correlates poorly with the degree of left ventricular dysfunction (Sullivan *et al.* 1995) and occurs well before the limits of cardiopulmonary reserve have been reached (Jondeau *et al.* 1992). There is a lack of correlation between the peak rate of oxygen uptake (VO_2) and LV filling pressures (Fink *et al.* 1986). Conversely, it has

been long observed that successful improvement of the central haemodynamic correlates of CHF with vasodilators, inotropes or cardiac transplantation results only in a gradual improvement in patient exercise capacity over weeks or months (Maskin *et al.* 1983; Drexler *et al.* 1989).

In healthy individuals, the addition of arm exercise to *maximal* leg exercise to increase the exercising muscle bulk, does not result in an increase in VO_2, suggesting that cardiac output and oxygen delivery are maximal and that exercise capacity is therefore limited by cardiac output. Conversely, in patients with CHF, the addition of arm exercise to maximal leg exercise *does* result in an increase in VO_2, suggesting that the major determinant of exercise capacity in CHF is the ability of exercising muscle to extract oxygen rather than a limitation of oxygen supply (Jondeau *et al.* 1992). This suggests that peripheral rather than central factors may be more important in determining exercise performance in CHF.

An increase in the slope of the relation between ventilation and carbon dioxide production (V_E/V_{CO_2} slope) is also seen in CHF (Higginbotham *et al.* 1983; Franciosa *et al.* 1984). However, several lines of evidence suggest that the abnormal ventilatory drive in CHF is not as a consequence of increased CO_2-drive (Rubin *et al.* 1982; Franciosa *et al.* 1984; Clark *et al.* 1992) with a tendency to hyperventilation (Rajfer *et al.* 1987) with little or no change on arterial blood gases (Clark *et al.* 1994).

Figure 1.6. Muscle hypothesis of chronic heart failure (adapted from Coats & Clark 1994).



Non-pulmonary, peripheral causes are the likely explanation for the abnormal ventilation seen in CHF. Increased peripheral vasoconstriction is observed in CHF, with increased activation of the sympathetic nervous system (*vide supra*) and RAAS, and decreased muscle blood flow during exercise (Lindsay *et al.* 1996), coupled with endothelial dysfunction (sec 1.4.3). However, it is thought that abnormalities of skeletal muscle are the major determinant of both the symptoms and the abnormal ventilatory drive seen (Clark *et al.* 1996) – the muscle hypothesis of CHF (Figure 1.6).

Several different abnormalities in skeletal muscle have been described in heart failure. There is generalised muscle atrophy coupled with reduced capillarisation (Lipkin *et al.* 1988; Sullivan *et al.* 1990; Drexler *et al.* 1992). Although there may be an overall change in capillary density (Drexler *et al.* 1992; De Sousa *et al.* 2000), reductions in resting muscle blood flow, and in exercise-induced increases in muscle blood flow have also been observed (Sullivan *et al.* 1991; Lindsay *et al.* 1996), which may be as a result of decreased microvascular distensibility (Sorensen *et al.* 1999), reduced NO bioavailability and an increase in vasoconstrictor tone from RAAS and sympathetic overactivity (Drexler *et al.* 1988). A shift from fatigue resistant muscle to fatiguable type II fibre types occurs (Drexler *et al.* 1992), with the proportion of oxidative to glycolytic fibres being significantly correlated with VO_2 max in CHF patients (Mancini *et al.* 1989).

Intrinsic defects in metabolic function of skeletal muscle unrelated to oxygen supply may be responsible for the observed limitation in exercise capacity (Minotti *et al.* 1991; Harridge *et al.* 1996), making muscles less resistant to fatigue (Harridge *et al.* 1996) and resulting in decreased metabolic or mitochondrial efficiency (Massie *et al.* 1987; Massie *et al.* 1988; Mancini *et al.* 1994; Kemp *et al.* 1996). The resultant impairment of muscle endurance performance correlates closely with both reduced functional capacity (Minotti *et al.* 1991) and maximal oxygen uptake (Harridge *et al.* 1996). Compared to controls, oxidative capacity is reduced by 30% in CHF patients but "effective functional muscle mass" by up to 65% (Kemp *et al.* 1996). Resting lower limb oxygen consumption is raised despite reduced muscle mass (Opasich *et al.* 1997), suggestive of a wasteful phenotype.

There is a reduction in the activity of oxidative enzymes (Sullivan *et al.* 1990; Opasich *et al.* 1996; Mettauer *et al.* 2001) and early reliance on anaerobic metabolism during exercise (Sullivan *et al.* 1991). Abnormalities at the mitochondrial level may explain the observed increased lactate production (Sullivan *et al.* 1989; Opasich *et al.* 1997), the increased phosphocreatine (PCr) depletion and intracellular acidosis (Massie *et al.* 1987; Mancini *et al.* 1994), and the decreased rate of ATP resynthesis (Mancini *et al.* 1994; Clark *et al.* 1996). The number, volume and effective surface area of mitochondria are reduced in CHF, irrespective of aetiology (Drexler *et al.* 1992). A 46% reduction in mitochondrial creatine kinase (mi-CK) content has been observed in patients with CHF (Hambrecht *et al.* 1999). A similar reduction in mi-CK protein content was observed in animals with CHF, together with a marked reduction in mitochondrial oxidative capacity in both type I and type II muscle fibres, as well as a decrease in the control of mitochondrial respiration by mitochondrial kinases (adenylate kinase and mi-CK) in oxidative fibres (De Sousa *et al.* 2000).

Two reflex mechanisms may be responsible for non-central ventilatory drive in CHF: the muscle ergo- or metaboreflexes and the arterial chemoreflexes. The ergoreflex system consists of intramuscular unmyelinated or small myelinated nerve endings sensitive to the metabolic state of exercising skeletal muscle which reflexly stimulate ventilation (Clark *et al.* 1996). These ergoreflex afferents appear sensitive to local prostaglandin (Scott *et al.* 2002) and bradykinin (Scott *et al.* 2004) generation. An overactivation of these neural afferents may reflexly increase sympathetic activity (Notarius *et al.* 2001), vasoconstriction (Hammond *et al.* 2000) and ventilatory drive (Piepoli *et al.* 1996) and is therefore implicated in the dyspnoea associated with CHF, and is responsive to exercise training (Piepoli *et al.* 1996). Similarly, augmented

peripheral hypoxic and central CO_2-sensitivity has been reported in CHF (Chua *et al.* 1996).

1.2.5 Treatments

Treatments for CHF are aimed at symptomatic relief and improving prognosis, as well as reducing the risk of further exacerbation of disease by treating risk factors. Drugs which target the neurohormonal maladaptations have shown clear long term benefits, with ACE inhibitors (ACEi) and selective β_1-adrenergic receptor antagonists (beta-blockers) forming the cornerstones of therapy. Treatment of patients with heart failure (mostly post MI) with ACEi significantly reduce mortality (CONSENSUS Trial Study Group 1987; SOLVD Investigators 1991; Pfeffer *et al.* 1992; AIRE Study Investigators 1993; Kober *et al.* 1995). Antagonism of the downstream effectors of the RAAS has also been effective in the treatment of heart failure, with Ang II type 1 receptor antagonists (ARBs) shown to be as effective as ACEi in the treatment of heart failure, reducing mortality and morbidity as well as hospitalisations (Pitt *et al.* 1997; Pitt *et al.* 2000; Cohn *et al.* 2001; Granger *et al.* 2003), and with aldosterone antagonism also providing additional mortality benefit (Pitt *et al.* 1999; Pitt *et al.* 2003). Selective beta-blockade has been shown in randomised placebo-controlled trials to reduce mortality (CIBIS-II 1999; MERIT-HF 1999; Packer *et al.* 2002) and hospitalisation from heart failure (Packer *et al.* 2002).

Other drug therapies such as loop diuretics and nitrovasodilators may provide symptomatic relief in heart failure. Nitrovasodilators may be of specific mortality benefit in African-American patients with advanced heart failure (Taylor *et al.* 2004) and have been much publicised as specific pharmacogenomic or ethno-

pharmacotherapeutic agents (Rahemtulla *et al.* 2005). However, this trial was not randomised according to race, so it is far too early to draw any such conclusions.

Mechanical therapies to improve cardiac output, which are reserved for severe heart failure that is refractory to maximal medical therapy, include cardiac resynchronisation therapy with bi- or tri-ventricular pacing to assist inter- and intra-ventricular systolic dyssynchrony (Abraham *et al.* 2002), and implantable ventricular assist devices in end-stage heart failure as bridging therapy to cardiac transplantation (Rose *et al.* 2001).

Whilst traditional targets for therapeutic intervention (such as pre- and afterload, or salt/water balance) may be exhausted, future therapies might target the metabolic inefficiency of cardiac and skeletal muscles. Indeed, some therapies may already be acting through this mechanism. For instance, regular moderate exercise training improves exercise tolerance in CHF patients (Afzal *et al.* 1998; Tyni-Lenne *et al.* 1998) not through changes in limb blood flow (Hambrecht *et al.* 1997), but through improvements in muscle metabolism (Stratton *et al.* 1994; Brunotte *et al.* 1995) and metabolic efficiency (Kemp *et al.* 1996), including ultrastructural changes in mitochondria resulting in increased mitochondrial oxidative capacity (Hambrecht *et al.* 1997). ACE inhibition may work in a similar way (*section 1.6).*

1.3 GENETIC ASSOCIATION STUDIES

Genetic diseases are the extreme manifestation of genetic variation. Common diseases, such as CHD, have a complex pathogenesis, arising from interaction between environment factors (risk) and common variation in multiple genes or gene-products – the common disease-common variant theory (Lander 1996; Risch *et al.* 1996; Collins *et al.* 1997). It has long been appreciated that there is common sequence variation in the

human genome and the extent of this variation has become appreciable recently with sequencing of the entire genome (Lander *et al.* 2001; Venter *et al.* 2001). Common genetic variability allows for subtle variation in the expression or activity of the encoded protein which is compatible with health and explains subtle interindividual differences in phenotype or physiological responses (Hingorani 2001). Without this genetic variability, a single environmental factor (e.g. smoking) would expect to have an identical effect in any two, otherwise matched, individuals, and response (or disease risk) would be directly proportional to the extent of the environmental stimulus (Stephens *et al.* 2003).

Most stable variation in the human genome occurs in the form of single nucleotide polymorphisms (SNP). Recent mapping of the human genome uncovered 1.42 million SNPs with an average frequency of one SNP every 1.9kb (Lander *et al.* 2001; Sachidanandam *et al.* 2001; Venter *et al.* 2001). Individual genes vary markedly in their nucleotide diversity (Cargill *et al.* 1999), with 39% of genes containing 10 or more SNPs (Sachidanandam *et al.* 2001). SNPs represent about 90% of the common variation in the genome, with sequence insertion/ deletion polymorphisms and variable repeat elements providing the rest (Altshuler *et al.* 2000; Sachidanandam *et al.* 2001). SNPs are stable, as the sequence variation arises through a single mutation event in the history of the population making the likelihood of recurrent mutation at the same site low. Common SNPs, by definition, have a minor "variant" allele frequency greater than 1% and may occur in coding and non-coding sequences.

A major difficulty arises when trying to assess functionality of a common variant. Functional genetic polymorphism may result in phenotypic changes in gene transcript levels or gene product. Initial estimates suggest that there are 22287 genes in the human

genome (www.sanger.ac.uk), with approximately 60000 SNPs occurring within exonic sequences (Sachidanandam *et al.* 2001). Non-conservative coding SNPs (resulting in an amino acid change in the gene product and therefore change in structure or function of a protein), appear to have been selected against during human history, being found at a lower allele frequency than to be expected compared to conservative or synonymous SNPs (Cargill *et al.* 1999). The majority of SNPs occur outside the coding regions of genes, and some of these will be within sequences with important regulatory function, such as the gene promoter, and such variation may be the key primary effect contributing to phenotypic variation in humans (Stranger *et al.* 2007). Indeed phenotypic variability in mRNA transcript levels may represent an intermediate stage between common genetic variation and complex traits. It was recently confirmed that there is a genetic contribution to this variability (Cheung *et al.* 2003).

These functional gene polymorphisms account for much of the biological diversity in homeostatic systems. One hypothesis is that the disease-susceptibility alleles included in this variation have persisted at moderate frequency because they have been selectively neutral, at least until the recent emergence of technological advancements creating the environment required for disease manifestation (Doris 2002).

In order to determine the *genetic* risk profile of a subject we must therefore use a polygenic strategy, and it is only by the simultaneous analysis of functional gene polymorphisms at several loci, or by stratifying by the presence of an environmental factor, that such an understanding can be obtained (gene-environment approach).

Different tools are available in the study of common disease causing candidate genes. Linkage analysis can be a powerful tool in mapping disease genes (Lander *et al.* 1994).

In such an approach, the recombination fraction between two genetic loci is estimated using genotype data from collections of related individuals with family members who manifest a complex trait or disease (e.g. hypertension, CHD). The recombination fraction is the probability that recombination (during meiosis) will take place between 2 loci, ranging between 0 (no recombination – loci are very close) and 0.5 (essentially random assortment because loci are far apart or on different chromosomes). In linkage analysis, one locus is known (e.g. genotyped SNP) and the other is unobserved (the disease locus). The test therefore examines the co-inheritance with the complex trait of several widely-distributed genetic markers, in order to infer the genomic position of the allele(s) contributing to that trait.

Linkage disequilibrium (LD) refers to a non-random relationship between two alleles, typically because they are close together on a chromosome and less likely to be separated by gene recombination. The degree of LD also reflects human population history as it is influenced by recombination, by the historical size and pattern of expansion and contraction of the population, by migration followed by admixture, by selection and by random drift. A common standardised measure of LD is *D'* (Lewontin 1964). *D'* ranges between 0 (two loci are completely unlinked, *e.g.* different chromosomes) and 1 (the two loci are never seen separately).

Gene association studies look for statistical association between common genetic variants and a complex trait. Typically, case-control studies have been used to assess the impact of genetic variation in candidate genes on the presence of CHD (Hingorani 2001; Stephens *et al.* 2003). In such studies, the frequency of a genetic variant in unaffected controls is compared with that in a sample of unrelated cases. This approach has potentially greater statistical power than linkage-based designs (Risch *et al.* 1996).

However, statistical power can be reduced by several factors including a lower frequency of the variant allele, genotyping error, phenotyping error (including locus heterogeneity) and strength of association between variant genotype and phenotype (genotype relative risk or effect size). False positives may arise in this approach for several reasons: as a function of sample size, because the gene variant under study may not be directly functional but may be in LD with other SNPs or neighbouring variants which are functional, or because of population stratification or undetected ethnic admixture, both of which may alter allele frequencies (Montgomery *et al.* 2002; Montgomery *et al.* 2003).

1.4 RENIN ANGIOTENSIN ALDOSTERONE SYSTEM (RAAS)

The first chapter in the story of the RAAS was penned by Tigerstedt and Bergman at the end of the 19th Century in their description of a pressor agent produced by the renal cortex (Tigerstedt *et al.* 1898) termed renin, and later identified as an aspartyl protease which cleaves the α_2 globulin angiotensinogen to yield the decapeptide, angiotensin I (Ang I). Subsequently elucidated, the endocrine RAAS was ascribed the sole function of maintaining circulatory homeostasis. However, it has now become clear that local tissue RAAS also exist, where they have paracrine, autocrine and intracrine ('between cells' and 'intracellular') roles.

1.4.1. Tissue ACE

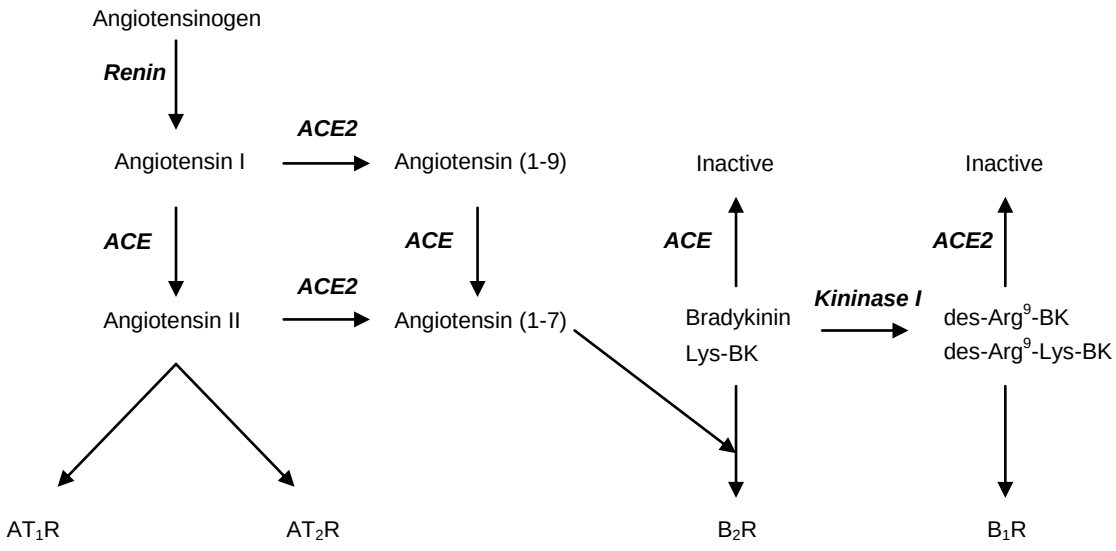
The RAAS and Kallikrein-Kinin System (KKS) are intimately enmeshed at the tissue level (Figure 1.7). First isolated in 1956, the 158kDa zinc metalloproteinase Angiotensin I Converting Enzyme (ACE; kininase II) plays a pivotal role in both the RAAS and KKS: it acts as a dipeptidyl carboxypeptidase, removing the C-terminal dipeptide from Ang I to form Ang II, from the vasodilator nonapeptide bradykinin (BK) to form BK(1-7), and from BK(1-7) to form BK(1-5). Kinetic studies have shown that the preferred substrate for ACE is BK (Jaspard *et al.* 1993).

ACE can exist in two forms: a somatic form containing two homologous zinc-binding catalytic domains arising from tandem gene duplication, and a truncated testicular form containing only the C-terminal catalytic domain which is critical to fertility. ACE is anchored to the plasma membrane of cells by its C-terminus, which is subsequently cleaved by ACE secretase to yield circulating ACE (Wei *et al.* 1991). The somatic *ACE* promoter has a typical TATA box, 3 Sp1 binding sites, 4 glucocorticoid response elements and an early growth response-1 (egr-1) factor response element. Endothelial ACE mRNA is induced by steroids within 4 hours of treatment (Dasarathy *et al.* 1992). *ACE* gene expression is also upregulated by a variety of factors such as vascular endothelial growth factor (Saijonmaa *et al.* 2001) and atrial natriuretic peptide (Saijonmaa *et al.* 1998) via secondary messengers such as protein kinases, cyclic GMPs and MAP kinases. Protein kinase C (PKC) activation upregulates ACE gene expression, possibly via egr-1, and may be a common pathway through which a variety of mechanisms (shear stress and hypoxia) can activate endothelial cells (Iwai *et al.* 1987; Villard *et al.* 1998).

The ACE-related carboxymonopeptidase ACE2 is a homologue of ACE, discovered only recently (Donoghue *et al.* 2000; Tipnis *et al.* 2000). It is an 89.6kDa endothelial-bound enzyme whose expression appears limited to the heart and renal vasculature (Donoghue *et al.* 2000; Tipnis *et al.* 2000). It has a single zinc-binding catalytic domain which shares 40% sequence homology with the ACE catalytic domains (Donoghue *et al.* 2000). ACE2 can cleave Ang I to inactive Ang(1-9) (Donoghue *et al.* 2000; Vickers *et al.* 2002), can generate vasodilator Ang(1-7) from Ang II (Crackower *et al.* 2002; Vickers *et al.* 2002) and can also cleave kinin and opioid peptides (Vickers *et al.* 2002). The ACE2 knockout mouse has cardiac dysfunction, but the ACE/ACE2 double knockout has a normal cardiac phenotype (Crackower *et al.* 2002). Therefore the ACE and ACE2 enzymes may counterregulate one another.

Circulating ACE contributes less than 10% of total body ACE, and it is now recognised that the largest proportion of ACE is to be found in tissue compartments (Cushman *et al.* 1971). Other RAAS components have also been identified in diverse tissues

Figure 1.7. The Renin-Angiotensin and Kallikrein-Kinin Systems



including those of the heart (Danser 1996; Neri Serneri *et al.* 1996), vasculature (Dzau 1993), brain (Saavedra 1992; Hilbers *et al.* 1999), lung (Pieruzzi *et al.* 1995), kidney (Alhenc-Gelas *et al.* 1989; Harris *et al.* 1996; Zhuo *et al.* 1998) and pancreas (Sernia 2001; Lam *et al.* 2002), as well as in adipose tissue (Jonsson *et al.* 1994; Schling *et al.* 1999), and skeletal muscle (Reneland *et al.* 1994).

In some situations, complete RAAS seem to exist: in others, local generation of some RAAS components is supplemented by the uptake of others from the circulation (Danser *et al.* 1999). Local RAAS may influence the inflammatory process (Brull *et al.* 2002). Indeed macrophages contain an active RAAS (Eklund *et al.* 1987; Potter *et al.* 1998; Sun *et al.* 2001) which here, as elsewhere, may drive synthesis of proinflammatory cytokines such as IL-6 (Schieffer *et al.* 2000; Keidar *et al.* 2001). Fibrotic responses in tissues such as heart (Weber *et al.* 2000; Sun *et al.* 2001), lung (Marshall *et al.* 2000) and kidney (Mezzano *et al.* 2001) are similarly RAAS-dependent.

Several hundred thousand patient years of experience underscore the safety and efficacy of pharmacological ACE inhibition in CHF (CONSENSUS Trial Study Group 1987; Flather *et al.* 2000) and after myocardial infarction (Pfeffer *et al.* 1992). ACEi reduce the generation of Ang II, reduce degradation of BK (Swartz *et al.* 1980) and mediate cross-talk between membrane-bound ACE and the bradykinin β_2 receptor (BDKRB2), leading to a reduction in BDKRB2 desensitisation, reduced receptor endocytosis and an increase in BK-receptor affinity (Minshall *et al.* 1997). More recently, fundamental roles of ACEi in vascular disease modification, beyond circulatory homeostasis (Fonarow *et al.* 1992), have been confirmed by the Heart Outcome Prevention Evaluation (HOPE) study (Yusuf *et al.* 2000). In this randomised controlled trial of 9297 high risk patients with established atherosclerotic disease, the ACEi ramipril

significantly decreased the incidence of MI, stroke and death from cardiovascular causes, as well as the incidence of new-onset diabetes and diabetic complications. It also induced regression of, and inhibited development of, left ventricular hypertrophy (LVH). Minor associated reductions in blood pressure (3/2 mmHg) explain, at most, only one-third of these benefits (Mancini *et al.* 2001; Sleight *et al.* 2001). Such effects may be mediated through inhibition of tissue, rather than circulating, ACE activity. Similar benefits were later confirmed in a large multicentre, placebo-controlled trial of patients with CHD but without clinical heart failure (Fox *et al.* 2003).

1.4.2. Receptors for angiotensin II and kinins

Ang II acts via two different types of G-protein-coupled receptors (GPCRs) – the Ang II type 1 (AT_1R) and type 2 (AT_2R) receptors. Ang II is the effector peptide of the RAAS cascade, acting via the AT_1R to cause potent vasoconstriction, renal salt and water retention via stimulated secretion of aldosterone, fibrosis, cell proliferation, inflammation and reactive oxygen species (ROS) generation. The AT_2R is thought to oppose the actions of the AT_1R (Matsubara 1998).

Kinins are a family of peptides, whose original functions in pain, inflammation and thrombosis have been extended to vasodilatation and metabolism (Scholkens 1996). Kinins are released from the precursor glycoproteins 'low molecular weight' or 'high molecular weight' kininogen (LMWK and HMWK, respectively – both coded by a single kininogen gene by alternative splicing) by serine proteases termed kallikreins. Plasma kallikrein catalyses the conversion of kininogens to BK (Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9) and tissue kallikreins catalyse the conversion of kininogens

to Lys-BK (also termed kallidin) which differs only by the addition of an N-terminal lysine (Lys^{1}-Arg^{2}-Pro^{3}-Pro^{4}-Gly^{5}-Phe^{6}-Ser^{7}-Pro^{8}-Phe^{9}-Arg^{10}). The subsequent cleavage of the C-terminal arginine residue by carboxy-peptidase yields the active fragments des-Arg^{9}-BK and Lys-des-Arg^{9}-BK.

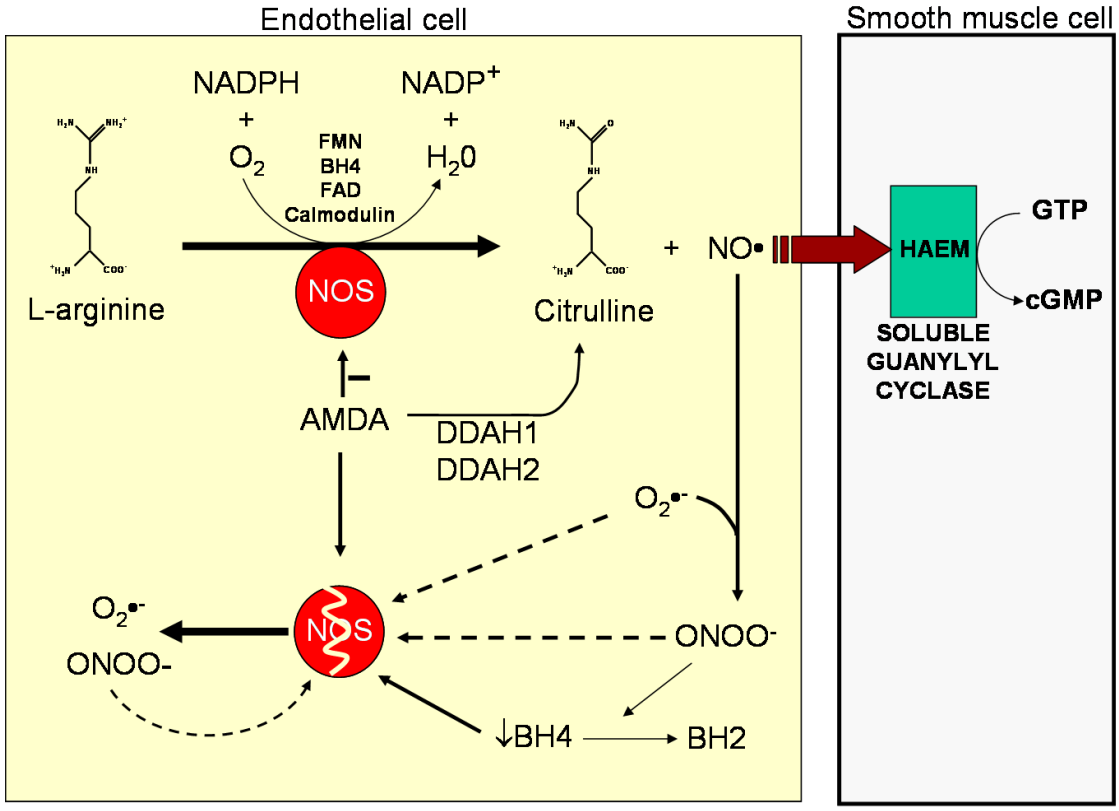
Intact kinins and their fragments act upon β_1 and β_2 G-protein coupled cell-surface receptors (termed BDKRB1 and BDKRB2, respectively), which share only 36% sequence homology and differ greatly in their expression and pharmacology (Regoli *et al.* 1997). Thus, the *constitutive* BDKRB2 is expressed in diverse cell types including the endothelium, myocardium and skeletal muscle, and is responsive to intact kinin peptides (Faussner *et al.* 1999). Conversely, the *inducible* BDKRB1 is activated by C-terminal arginine-deficient kinin fragments (Faussner *et al.* 1999). Kinin receptor activation leads to calcium-dependent nitric oxide (NO•) and prostaglandin release.

Animal studies have suggested that kinins have cardioprotective effects, including immediate protection of the myocardium from ischemia-reperfusion injuries (Yang *et al.* 1997; Zhang *et al.* 1997; Kitakaze *et al.* 1998; Su *et al.* 2000) and, in the longer-term, a reduction in left ventricular hypertrophy (LVH) and prevention of heart failure (Linz *et al.* 1992; Linz *et al.* 1993; Ishigai *et al.* 1997; Liu *et al.* 1997). Kinin receptor activation may also play a role in the modulation of atherosclerotic risk through promotion of microangiogenesis (Parenti *et al.* 2001), inhibition of vascular smooth muscle cell growth (Murakami *et al.* 1999), coronary vasodilatation (Su *et al.* 2000), increased local NO• synthesis (Kichuk *et al.* 1996), and anti-thrombotic actions (Schmaier 2000). Reduced kinin degradation may therefore contribute to the beneficial effects of ACE inhibition on vascular risk, LVH and heart failure (Linz *et al.* 1995), but this is unproven in humans.

1.4.3. Nitric oxide (NO•)

It was as early as 1867 that Brunton first reported the use of amyl nitrite for the relief of angina (Brunton 1908). He also noted that glyceryl trinitrate (GTN, nitroglycerin) had a similar effect, but felt unable to work with this compound because of the terrible headaches it caused. Over a century passed before there was more interest in nitrous compounds and their effects on the circulation. In 1980, it was shown that endothelial cells were necessary for the acetyl choline-induced relaxation of rabbit aorta (Furchgott *et al.* 1980), but that the artery denuded of endothelium still responded to exogenous GTN. The endogenous mediator of this effect was termed endothelium derived relaxing factor (EDRF) (Furchgott *et al.* 1980) and later proven to be NO• (Ignarro *et al.* 1987; Palmer *et al.* 1987).

Figure 1.8. Cartoon depiction of classical nitric oxide (NO•) generation within endothelial cells by nitric oxide synthase (NOS). NO• can diffuse across the plasma membrane to act on soluble guanylyl cyclase to form cGMP within adjacent smooth muscle cells to promote vasodilatation. NOS can become uncoupled by reactive oxygen species (ROS), low levels of BH4 or high levels of AMDA to generate further ROS. See text for discussion.



NO• is a diffusible, lipophilic, highly reactive gaseous molecule with a short half life (<4 seconds in biological solutions). It is rapidly oxidised to nitrite and then nitrate by oxygenated haemoglobin before being excreted into the urine (Moncada *et al.* 1993). NO• is synthesized from the amino acid L-arginine by different isoforms of nitric oxide synthase (NOS) (including constitutive neuronal type 1 isoform (nNOS, or NOS1), inducible type 2 isoform (iNOS or NOS2) and constitutive endothelial type 3 isoform (eNOS or NOS3)), yielding L-citrulline as a byproduct (Figure 1.8). Several co-factors are required for NO• biosynthesis, including nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), tetrahydrobiopterin (BH4), and calmodulin. NOS has two catalytic domains consisting of a C-terminal reductase where NADPH, FMN, and FAD bind, and an N-terminal oxygenase domain where heme, BH4, oxygen and L-arginine bind. The catalytic mechanisms of NOS involve flavin-mediated electron transport from C-terminal–bound NADPH to the N-terminal heme centre, where oxygen is reduced and incorporated into the guanidine group of L-arginine, yielding NO• and L-citrulline.

An important physiological target of NO• is the heme protein soluble guanylyl cyclase. NO• diffuses across the endothelial cell membrane and activates guanylyl cyclase by interacting with its heme, generating cyclic guanosine-3',5-monophosphate (cGMP) from guanosine triphosphate (GTP). As a second messenger, cGMP mediates many of the biological effects of NO• including the control of vascular tone and platelet function. Subcellular NO• signal transduction is complex, involving cGMP-dependent protein kinases (PKGs) and cGMP-stimulated and cGMP-inhibited cyclic nucleotide phosphodiesterases (cGsPDE and cGiPDE, respectively). PKG-dependent signal transduction involves the phosphorylation of various targets, whereas cGsPDE- and cGiPDE-dependent pathways involve decreases or increases (respectively) in cAMP

levels. In addition, NO• has other molecular targets which include metal and thiol-centred proteins and DNA, supporting additional reactions with key enzymes or ion channels. NO• also interacts with enzymes of the mitochondrial respiratory chain (Brown *et al.* 1994; Cleeter *et al.* 1994).

Impaired synthesis or bioavailability of NO• may underlie the endothelial dysfunction seen in individuals with cardiovascular risk factors but without clinical CHD (smokers, hypertensives, hypercholesterolaemics), and may predispose to the development of atherosclerosis (Calver *et al.* 1992; Chowienczyk *et al.* 1992; Celermajer *et al.* 1993; Cooke *et al.* 1997). NO• bioavailability can be reduced by several factors, including a reduction in cofactors for NOS, for example reduced BH4 as seen early in endothelial dysfunction, diabetes and hypercholesterolaemia.

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of NOS which has been found to be increased in atherosclerotic disease, hypertension, diabetes and chronic renal failure. ADMA is formed from L-arginine by the protein methylase-I and subsequently metabolised by dimethylarginine dimethylaminohydrolase (DDAH) to produce citrulline (Vallance *et al.* 1992).

There are also important interactions between NO• and ROS. Superoxide (O_2•$^-$) can react with equimolar concentrations of NO• to form peroxynitrite ($ONOO^-$), thus reducing the bioavailability of NO•. Peroxynitrite at high concentrations is highly toxic producing protein nitration and oxidation and irreversible inhibition of mitochondrial electron transport (Beckman *et al.* 1990; Radi *et al.* 1994). NOS can also become a potent generator of ROS (Figure 1.8): in relative substrate (L-arginine) or cofactor (BH4) deficiency, NOS can become 'uncoupled', generating O_2•$^-$ and peroxynitrite

(Mayer *et al.* 1997; Xia *et al.* 1997). Peroxynitrite itself can directly or indirectly (by rapidly metabolising BH4 to BH2) uncouple NOS, thereby perpetuating ROS generation (Zou *et al.* 2002).

It has become increasingly apparent that NO• has divergent roles. The effects of NO• depends on several factors including the cellular source and targets of NO• as well as target cell redox/antioxidant status, the amount of NO• released or studied, stimuli such as coronary flow rate and heart rate or co-existing neurohumoral stimuli, or the presence of immune activation or disease (Shah *et al.* 2000). For example, NO• has positive and negative inotropic responses at low and high concentrations, respectively (Mohan *et al.* 1996) and a similar biphasic response is seen with respect to apoptosis (Kim *et al.* 1999). Exposure to bacterial lipopolysaccharide (LPS) or cytokines such as TNFα results in increased vascular iNOS expression and the resultant NO• has bactericidal, antiviral and leukostatic effects. Endotoxaemia rapidly results in hypotension, with dysregulation of vasomotor tone and myocardial depression, and excess NO• generation is directly implicated in the pathogenesis of septic shock in both animals and humans (Petros *et al.* 1991).

NO• has also been implicated in the development of LVH and heart failure. Endogeneous NO• has a small baseline inotropic effect in the human heart *in vivo* which is lost in patients with heart failure (Cotton *et al.* 2001). eNOS and nNOS are constitutively expressed in the human heart, but the expression pattern may change in heart failure, with an increase in cytokine-induced calcium-insensitive iNOS expression (de Belder *et al.* 1993; Drexler *et al.* 1998). Cardiac eNOS and iNOS gene expression correlates positively with indices of cardiac performance in heart failure and negatively with the severity of LV dysfunction (Heymes *et al.* 1999). However, cardiac iNOS

expression in the failing heart has also been implicated in blunting β-adrenoceptor responsiveness *in vitro* (Hare *et al.* 1998) and *in vivo* (Drexler *et al.* 1998) . Paracrine NO• release from the coronary microvasculature enhances myocardial relaxation and diastolic LV function and reduces myocardial oxygen consumption (Shah *et al.* 2000). In animal models of LVH, NO•-dependent LV relaxation is markedly impaired independent of coronary flow (MacCarthy *et al.* 2000). This may be due to a significant increase in superoxide generated by NADPH oxidase which can be acutely reversed by administration of antioxidants (MacCarthy *et al.* 2001). The precise spatial and temporal expression patterns of the NOS isoforms and relationship to cardiac failure remains controversial at present (Drexler 1999; Shah *et al.* 2000).

1.5 MITOCHONDRIA

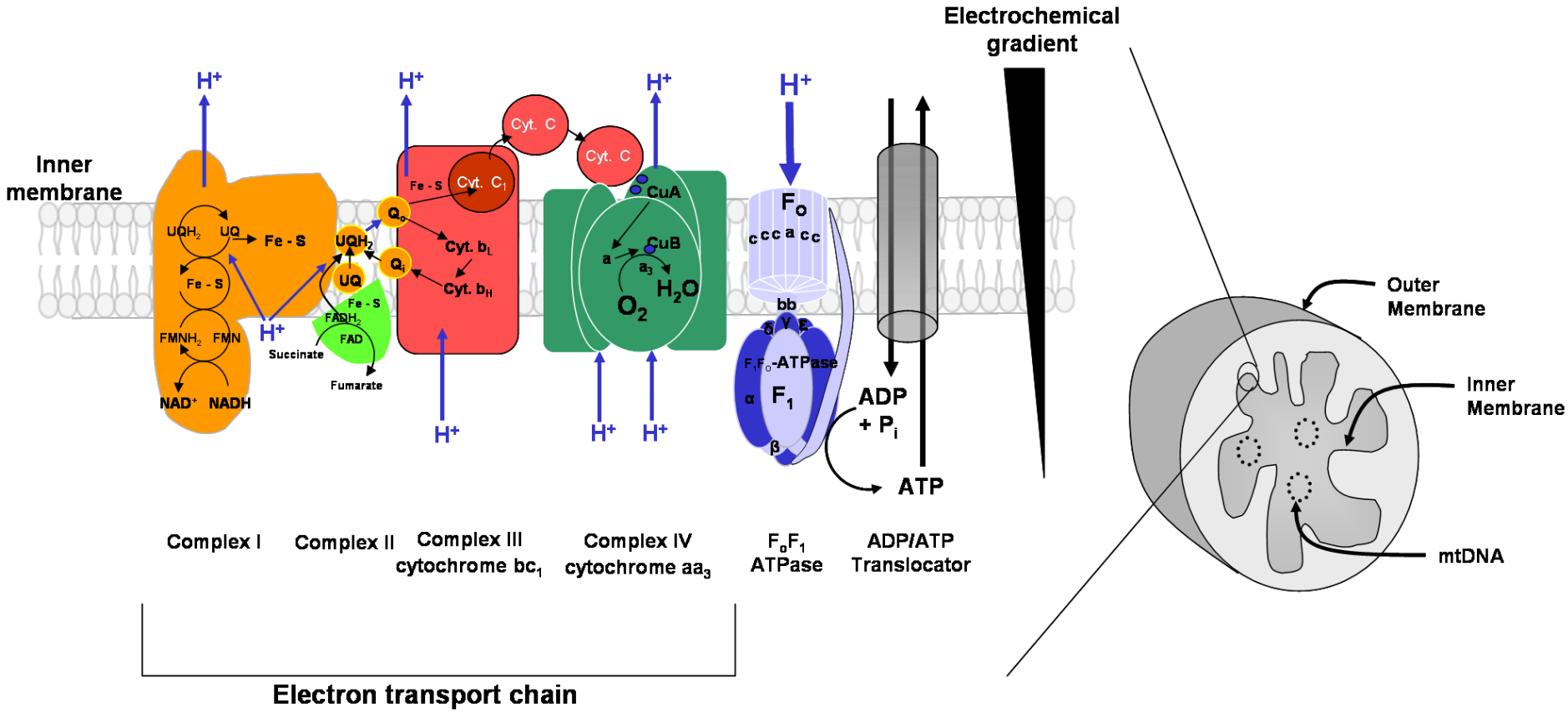
1.5.1 Overview

The endosymbiosis hypothesis suggests that mitochondria are organelles derived from ancient protobacteria about 1500 million years ago (Lang *et al.* 1997). Mitochondria play a critical role in survival of the 'host' cell; they house energy-yielding oxidative reactions, metabolise amino acids, fatty acids and ketone bodies, and are central in calcium homeostasis, oxidative stress and the process of programmed cell death or apoptosis.

The mitochondrion (Figure 1.9) consists of an outer membrane and an inner membrane which define the intermembranous space and the inner matrix (Graff *et al.* 1999). The inner membrane is a sophisticated energy-transducing membrane and is folded into cristae, increasing its surface area. All energy-transducing membranes contain two types of proton pump. In the case of the mitochondrion, it contains 'primary pumps' known as the electron transport chain (ETC) which consist of four complexes (numbered I to IV) that catalyse the transfer of electrons from substrates to the final acceptor, molecular oxygen (Figure 1.9). These are coupled to highly evolutionary-conserved 'secondary' pumps termed ATP synthase ($F_1.F_o$-ATPase or complex V) which are seen as 'knob-like' structures on the matrix side of the inner membrane on electron microscopy, consisting of a proton channel (F_o subunit) and an ATP synthase (F_1 subunit). If the secondary pump were in isolation, it would hydrolyse ATP to ADP and P_i.

The mitochondrial matrix contains 2-10 copies of a 16,569 base pair circular molecule of double stranded DNA (mitochondrial or mtDNA) which is distinct from nuclear DNA. Human mtDNA encodes 37 genes: 22 encoding transfer RNAs, 2 ribosomal RNAs and 13 polypeptides which are components of the ETC or ATP synthase. The ETC complexes contain at least 70 nuclear-encoded peptides, which are synthesised within the cell cytoplasm and targeted to the mitochondrion using specific import pathways.

Figure 1.9. Cross section through a mitochondrion, showing the inner and outer membranes. A section of inner membrane has been magnified to show the complexes of the electron transport chain which generate an electrochemical gradient by transporting protons (H^+) out of the matrix during a series of redox reactions depicted. H^+ re-enters the matrix through F_OF_1-ATPase down this gradient, providing energy for generation of ATP. ATP exits the matrix through the ADP/ATP translocator.



1.5.2 Mitochondrial metabolism (reviewed in (Nicholls *et al.* 2002)

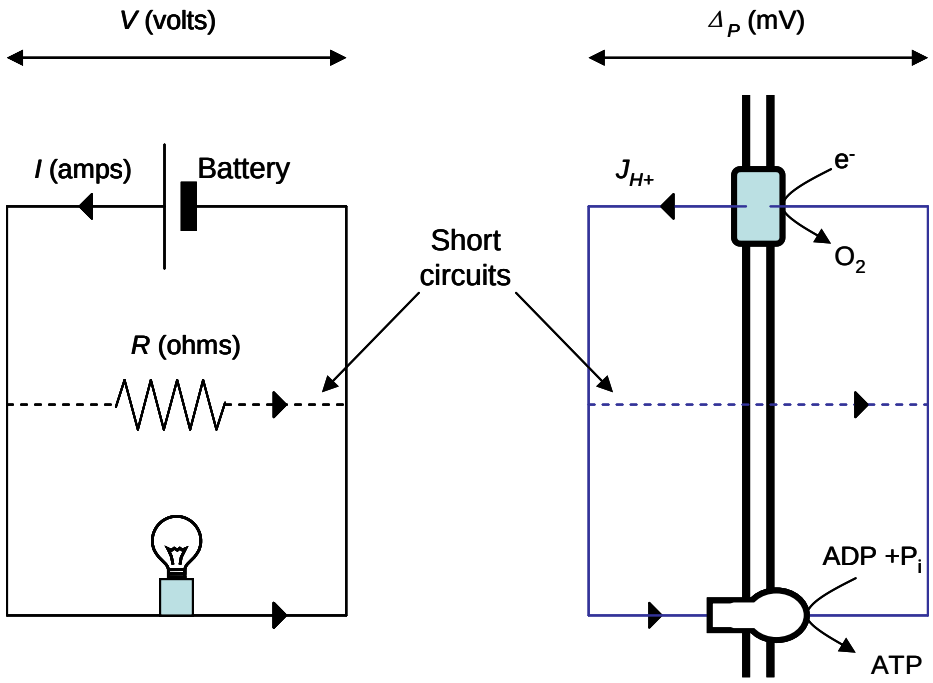
Oxidative phosphorylation is the process by which oxidation of the reducing equivalents NADH, FADH and succinate is coupled to the generation of ATP in the mitochondrion (Figure 1.9). NADH, FADH and succinate (derived from the oxidation of fuel substrate) transfer electrons into the respiratory chain (ETC) and ultimately to O_2. The ensuing series of redox reactions releases free-energy which is used to translocate protons (H^+) at complexes I, III and IV, out of the mitochondrial matrix and into the intermembranous space. The outer membrane contains proteins, termed porins, which render it freely permeable to molecules up to 10kDa in weight. The inner membrane is permeable only to gases such as O_2 and carbon dioxide (CO_2) and certain lipophilic compounds (discussed later). Hence the translocation of H^+ across the inner membrane results in an electrochemical gradient ($\Delta\mu_{H^+}$) or proton motive force (PMF, Δ_P) which drives the ATP synthase to synthesise ATP from ADP and P_i. This process has been termed the chemiosmotic theory of energy transduction (Mitchell *et al.* 1967). Δ_P consists of two components: a H^+ gradient (pH gradient or ΔpH) and a gradient due to the electrical potential generated between the matrix and intermembranous space ($\Delta\psi_m$). In the mitochondrion, the Δ_{pH} component is small, only 0.5pH units, and $\Delta\psi_m$ approximates Δ_P.

The ETC can be thought of as a 'proton circuit' analogous to an electrical circuit (Nicholls *et al.* 2002) (Figure 1.10). Both circuits have generators of a potential difference (expressed in volts) used to perform useful work. The current flowing (I) is defined by Ohm's Law, **V = I x R,** where R is the resistance of the circuit (inverse of conductance). Both circuits can be shorted and the potential falls if the current drawn increases. A typical $\Delta\psi_m$ achieved is 200mV, equivalent to a charge of 300000V.cm^{-1} across the inner membrane. Ionophores are lipophilic compounds which possess a

hydrophilic core, able to carry or shield charge within. They can therefore enter a charged lipid bilayer, such as the inner membrane, and act as mobile charge carriers or channel formers and are used to study membrane properties.

Protonphores, or uncouplers, are molecules which can insert into lipid bilayers. They contain extensive π-orbital rings, which allow them to dissociate their proton charge, facilitating proton cycling across the inner mitochondrial membrane, increasing proton conductance and collapsing $\Delta\psi_m$. The rate of proton flow can be estimated from the respiration rate of mitochondria measured, for example, with an oxygen electrode.

Figure 1.10. <u>The mitochondrial proton circuit is analogous to an electrical circuit (after Nicholls and Ferguson 2002).</u> Voltage (V) is equivalent to proton motive force (Δ_p); current (I) is equivalent to proton flux (J_{H+}); conductance, resistance and short circuits can be derived.

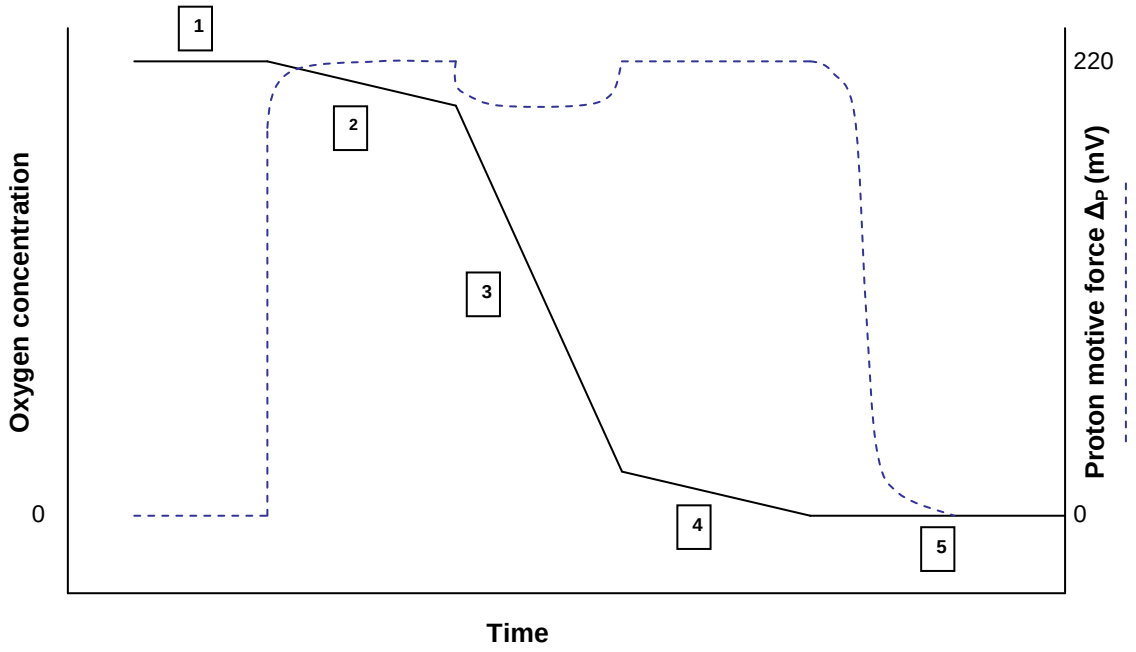


Classically, five states of respiration are recognised in isolated mitochondria according to the availability of substrate, ADP and O_2, presented in Figure 1.11. Maximal respiration occurs (state 3) under optimal conditions when substrate, ADP and O_2 are all available. That any respiration occurs during state 4 is due to the inherent 'proton leak' of the inner membrane, whereby any leak of protons is counterbalanced by proton extrusion by the respiratory chain (see chapter 1.7.1). Protonphore uncouplers can initialise rapid respiration, similar to state 3, by collapsing $\Delta\psi_m$, driving futile respiration. In this situation, glycolytically formed ATP is further depleted by reversal of the ATP synthase. The respiratory control ratio is defined as state 3 (or uncoupled) divided by state 4 respiration (absence of uncoupler) and is related to the integrity or efficiency of the mitochondria.

1.5.3 Measuring mitochondrial membrane potential in living intact cells

Traditionally, respiration was measured in isolated mitochondria using an oxygen (Clark) electrode (Nicholls *et al.* 2002), but studying mitochondrial function in intact cells remained problematic until recently (Griffiths 2000; Duchen *et al.* 2003). The explosion of knowledge in this area has been aided by the development of fluorescent dyes and fluorescence technologies, which have been used to measure $\Delta\psi_m$, intramitochondrial calcium, redox state, ROS generation, apoptosis and mitochondrial distribution and movement within cells.

Figure 1.11. States of respiration in isolated mitochondria. Changes are related to changes in membrane potential (Nicholls 2002).



State 1: mitochondria alone

State 2: substrate added; low respiration due to lack of ADP

State 3: limited quantity of ADP added allowing rapid respiration

State 4: all ADP converted to ATP, therefore respiration slows again.

State 5: anoxia

Lipophilic cationic compounds will concentrate within mitochondria, which are more negatively charged than the surrounding cytoplasm, according to Nernstian principles (Emaus *et al.* 1986; Duchen *et al.* 2003):

$$[C]_i/[C]_o = e^{-F\Delta\psi m/RT}$$

where $[C]_i$ is the concentration of cations inside and $[C]_o$ is the concentration of cations outside the mitochondria, R is the gas constant, T is the temperature in degrees Kelvin

and *F* is the Faraday constant. This characteristic has been exploited to develop fluorescent lipophilic cationic dyes which will concentrate within mitochondria according to the magnitude of $\Delta\psi_m$. At very low probe concentrations, fluorescence intensity is proportional to dye concentration. However, some important problems exist. Firstly, as dye concentration within a compartment increases, the fluorescence fails to increase because of the phenomenon of "quenching": the dye is thought to form multimers, with energy transferred between molecules rather than expressed as fluorescence (Duchen *et al.* 2003). Secondly, the dye will concentrate in the cell cytoplasm according to the plasma membrane potential ($\Delta\psi_p$) and then within mitochondria according to $\Delta\psi_m$. Thirdly, an ideal fluorescent dye would not bind to any mitochondrial component, but be free to move according to changes in $\Delta\psi_m$. This binding would cause apparent deviation from Nernstian behaviour and would lead to enhanced mitochondrial accumulation (Rottenberg 1984; LaNoue *et al.* 1986). Lastly, almost all of these fluorescent probes are photosensitising agents and prolonged laser illumination can cause phototoxicity leading to mitochondrial oxidative damage and depolarisation (Duchen *et al.* 2003).

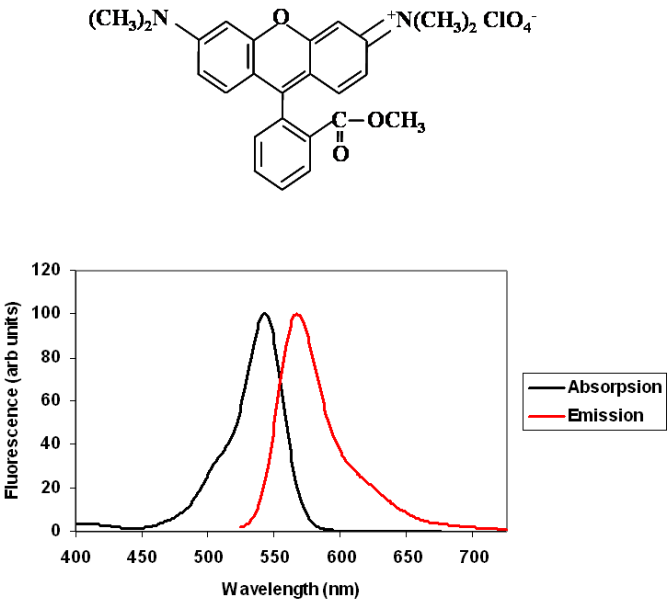
Many of the dyes first used were developed during a systematic search for dyes which would enable measurement of $\Delta\psi$ in neurones (Cohen *et al.* 1978). Several types of dye are now available for study including rhodamines (Farkas *et al.* 1989; Duchen *et al.* 1998; Floryk *et al.* 1999; Diaz *et al.* 2000; Ward *et al.* 2000), rosamines, and

carbocyanine derivatives. Two of these fluorescent probes are discussed in greater detail below.

1.5.3.1. TMRM

The rhodamine derivatives TMRE (tetramethylrhodamine ethyl ester) and TMRM (tetramethylrhodamine methyl ester) have been used extensively to measure $\Delta\psi_m$ in living cells (Duchen *et al.* 1998; Antonicka *et al.* 1999; Floryk *et al.* 1999; Scaduto *et al.* 1999; Collins *et al.* 2000; Diaz *et al.* 2000; Ward *et al.* 2000; Krieger *et al.* 2002; Michelakis *et al.* 2002; Michelakis *et al.* 2002). Both dyes equilibriate rapidly according to the magnitude of $\Delta\psi_m$. However, TMRM demonstrates much less mitochondrial toxicity and less mitochondrial binding than TMRE at the same dose (Scaduto *et al.* 1999). TMRM fluorescence demonstrates red spectral shift on accumulation in mitochondria, with maximal mitochondrial red fluorescence at 573nm (Scaduto *et al.* 1999). The chemical structure and excitation/emission spectra of TMRM is shown in Figure 1.12.

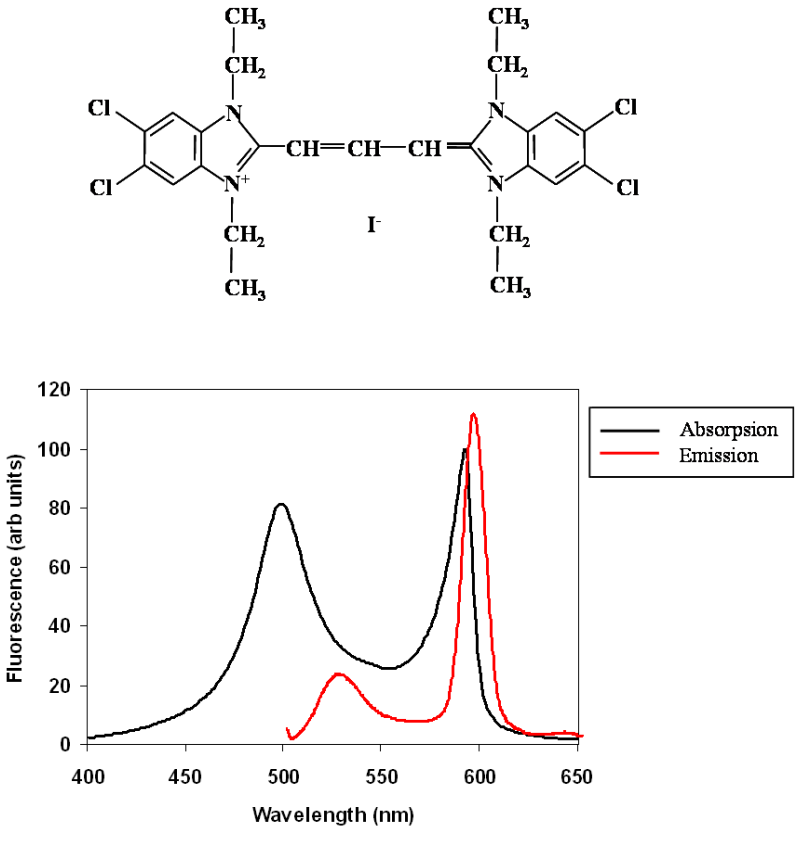
Figure 1.12. <u>Chemical structure and excitation/emission spectra for TMRM</u>



1.5.3.2. JC-1

JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide, Figure 1.13) is a carbocyanine derivative, which has been used widely to measure $\Delta\psi_m$ (Reers *et al.* 1991; Smiley *et al.* 1991; Cossarizza *et al.* 1993; Di Lisa *et al.* 1995; Cossarizza *et al.* 1996; Nuydens *et al.* 1999; Mathur *et al.* 2000; Minners *et al.* 2001; Michelakis *et al.* 2002; Michelakis *et al.* 2002). It exhibits dual fluorescence: at low concentrations, JC-1 exists as a monomer which fluoresces green (peak fluorescence 527nm); at higher concentration (>0.1M in solution or upon mitochondrial concentration), it forms so-called "J-aggregates" which exhibit red "resonance fluorescence" at a peak of 590nm. The ratio of red/green fluorescence has been used for potentiometric assessment of $\Delta\psi_m$ (Reers *et al.* 1991; Michelakis *et al.* 2002) and has been found to be more reliable than several other compounds, such as $DiOC_6$, Rhodamine 123 and CMXRos, being less

Figure 1.13. Chemical structure and excitation/emission spectra for JC-1



sensitive to changes in $\Delta\psi_p$ (Salvioli *et al.* 1997; Mathur *et al.* 2000). J-aggregates are slow to disperse and therefore JC-1 does not give a dynamic representation of $\Delta\psi_m$ (Smiley *et al.* 1991)

1.5.4 Defects in mitochondrial metabolism

Mutations in mtDNA result in neurological, myopathic and cardiomyopathic abnormalities (Graff *et al.* 1999). Not only is mtDNA highly susceptible to damage because it lacks protective histones and effective DNA repair mechanisms, but it also resides within an environment prone to ROS generation (Shigenaga *et al.* 1994; Graff *et al.* 1999; Ide *et al.* 2001). mtDNA is almost exclusively inherited from the mother, but typically several allelic forms are inherited. Each mitochondrion contains 2-10 copies of mtDNA, and each cell can contain up to 10^4 mitochondria. Heteroplasmy (the coexistence of more than one type of mtDNA within a cell, tissue, or organism) explains why there is variation in which cells within a tissue, or which tissues within an individual, are affected by a mtDNA mutation, and also the variable penetrance. The propensity for the CNS or myocardium to be affected probably reflects the high metabolic demand within these tissues and possibly also higher oxidative stress within these tissues (two-hit phenomenon).

Oxidative damage to mtDNA, mitochondrial protein and lipid accumulates as a function of age. A decrease in respiratory rate of mitochondria isolated from ageing tissues is also seen, allied with a generalised decline in ETC complex and cytochrome c oxidase activity, perpetuating further oxidative damage. These processes are thought to be a major determinant in the decline of organ function attributable to ageing (Shigenaga *et al.* 1994).

1.5.5 ACE and mitochondria

A handful of studies have localised components of the RAAS in mitochondria, mostly in relation to aldosterone biosynthesis which is highly energy dependent. Ang II and potassium regulate the synthesis and secretion of aldosterone from adrenal mitochondria via calcium. Physiological concentrations of Ang II induce oscillating cytoplasmic calcium signals (Quinn *et al.* 1988; Kramer 1990), associated with parallel mitochondrial calcium and NAD(P)H oscillations (Pralong *et al.* 1994; Rohacs *et al.* 1997). This activates mitochondrial respiration and the generated ATP is used to drive aldosterone biosynthesis (Xu *et al.* 1991; Kowluru *et al.* 1995). Extra-adrenal production of aldosterone has been found in the heart (Young *et al.* 2000), vasculature (Hatakeyama *et al.* 1994) and brain (Gomez-Sanchez *et al.* 1997), and the mechanism of production and secretion are presumed at present to be the same as in adrenal glomerulosa cells. Most of the components of the RAAS have been located in adrenal cells (Hilbers *et al.* 1999; Mazzocchi *et al.* 2000). Interestingly, biochemically active renin has been found within adrenal mitochondria, located in inclusion bodies of high electron density (Peters *et al.* 1996) and targeted to mitochondria rather than the cytoplasm by alternative transcription and splicing of the renin gene (Clausmeyer *et al.* 1999). Ang II droplets have also been immunolocalised within rat brain, liver, and adrenal mitochondria (Erdmann *et al.* 1996).

In a study of the effects of ACE inhibition on ageing, enalapril was added to the drinking water of CF1 mice shortly after weaning until death (approximately 24 months(Ferder *et al.* 1993). Enalapril treatment was associated with longevity, lower cardiac weight, lower myocardial and glomerular sclerosis and, interestingly, an increase in the number of mitochondria in cardiomyocytes and hepatocytes. In a further study, Ferder *et al* again found a protective effect of enalapril on cardiac mitochondrial

number with ageing in the CF1 mouse, an effect which was significant by 18 months. This effect was related to higher mitochondrial superoxide dismutase, higher cardiomyocyte replicative capacity (marked by higher cyclin expression) and reduced apoptosis (Ferder *et al.* 1998).

1.6 OXIDATIVE STRESS

ROS, including superoxide ($O_2\bullet^-$), hydrogen peroxide (H_2O_2), hydroxyl radicals (HO•), NO• and peroxynitrite ($ONOO^-$), are important mediators of both physiological and pathological processes. ROS can damage cellular macromolecules including DNA, protein and lipid and have been implicated in ageing, atherosclerosis, diabetes, tumourogenesis and neurodegeneration (Shigenaga *et al.* 1994; Ide *et al.* 2001). Oxidative stress refers to an imbalance between the production of ROS and the endogenous antioxidant defence mechanisms, and this may result in toxicity to lipids, DNA or protein.

As discussed above, ROS include $O_2\bullet^-$, H_2O_2, HO•, NO• and $ONOO^-$, as well as lipid radicals. Any one ROS has the potential to generate more ROS via chain reactions. For instance ROS may react with cell membrane fatty acids to produce a fatty acid peroxyl radical (R-COO•) that can attack adjacent fatty acid side chains and produce other lipid radicals, which may accumulate to have deleterious effects on cell and organelle integrity such as membrane leak, dysfunction of membrane-bound receptors, as well as cytotoxicity and mutagenesis (Herbst *et al.* 1999). ROS may modify proteins to cause enzyme inactivation or protein denaturation (Stadtman *et al.* 2003). ROS may also contribute to mutagenesis of DNA by inducing strand breaks, purine oxidation, protein-

DNA cross-linking and chromatin remodelling to affect gene expression (Konat *et al.* 2003). Oxidative stress may thus contribute to age-related changes in the cardiovascular system, but this remains the subject of debate (Lakatta 2003).

ROS are not solely deleterious, having important physiological roles. They have important modulatory roles through redox-sensitive proteins and enzymes to effect cytokine, growth factor, and hormone action and secretion, ion transport, transcription, neuromodulation and apoptosis (Finkel 1999). These effects may be imparted by action on tyrosine-phosphatases which have a redox-sensitive cysteine residue at the active site (Hecht *et al.* 1992), dimerisation of receptors or enzymes at redox-sensitive cysteine cross-bridges (Finkel 1999), altering protein-protein interaction (Wang *et al.* 1996) and direct effects on transcription factors such as nuclear factor Kβ (NF-Kβ) (Schreck *et al.* 1991).

As mentioned above, atherosclerosis is now thought to be an inflammatory disease and abnormalities of redox signalling may be a contributing factor (Ross 1999; Libby 2002; Harrison *et al.* 2003). ROS appear to promote all stages of atherogenesis, through reduced endothelial-dependent vasodilatation (Rubanyi *et al.* 1986), lipid peroxidation (Steinberg *et al.* 1996), increased platelet aggregation and monocyte adhesion (Marui *et al.* 1993), endothelial cell apoptosis (Herbst *et al.* 1999), vascular smooth muscle cell hypertrophy (Zafari *et al.* 1998; Harrison *et al.* 2003) and plaque instability caused by MMP activation (Rajagopalan *et al.* 1996). There is also a growing body of evidence that ROS production in response to traditional risk factors such as hypertension, hypercholesterolaemia and diabetes results in alteration in signal transduction pathways in target cells, thereby altering a plethora of genes implicated in atherogenesis such as inflammatory gene transcription (Sen *et al.* 1996; Kunsch *et al.* 1999). In this regard,

both the transcription factors NF-Kβ and activator protein-1 (AP-1) are redox sensitive (Ares *et al.* 1995). Indeed, AP-1 is activated by a variety of ROS, including superoxide, H_2O_2, oxLDL and the lipid peroxidation product 4-hydroxy-2-nonenal. NF-kB was the first eukaryotic transcription factor shown to respond directly to oxidative stress and its activation mechanisms, which directly lead to NF-Kβ nuclear translocation, involve ROS as a common step (Schreck *et al.* 1991). However, the ROS that activate NF-Kβ are more restricted to hydroperoxidases, such as H_2O_2. NF-Kβ has been directly implicated in atherogenesis, and activated NF-Kβ is specifically localised in atherosclerotic but not healthy vessels (Brand *et al.* 1996).

Increased ROS production or decreased antioxidant activity may favour LVH. Ang II, TNFα and adrenergic stimulation all induce ROS-dependent cardiomyocyte hypertrophy (Nakamura *et al.* 1998; Amin *et al.* 2001) and partial inhibition of cytoplasmic CuZn-SOD (SOD 1) also induces cardiomyocyte hypertrophy (Siwik *et al.* 1999). Ang II induces cardiomyocyte hypertrophy in a non-pressor dependent manner via NAD(P)H oxidase ROS production (Bendall *et al.* 2002). Graded increases in ROS induce a graded phenotype shift in cardiac myocytes, from hypertrophy and induction of foetal gene expression at low levels to apoptosis at high levels of oxidative stress (Siwik *et al.* 1999; Pimentel *et al.* 2001). Mechanical strain may also induce ROS-dependent cardiomyocyte hypertrophy via MAP kinase (Pimentel *et al.* 2001).

ROS have been implicated in the pathophysiology of CCF. Elevated levels of ROS have been found in both animal models of heart failure (Hill *et al.* 1996) and in clinical trials of heart failure treatment (McMurray *et al.* 1993; Keith *et al.* 1998; Yucel *et al.* 1998), and may contribute to the endothelial dysfunction identified in CCF (Kubo *et al.* 1991) and to post infarct myocardial remodelling due to effects on hypertrophy, fibrosis

(via MMP activation) and apoptosis (Spinale 2002; Sabri *et al.* 2003). $ONOO^-$ may inhibit mitochondrial substrate supply via inactivation of the Kreb's cycle enzyme aconitase (Castro *et al.* 1994) and can inhibit mitochondrial electron transport, with the potential to generate further ROS from the ETC (Radi *et al.* 1994), with marked depression of myocardial contractile function and reduced cardiac efficiency in the isolated perfused rat heart (Schulz *et al.* 1997). $O_2\bullet^-$ can rapidly and irreversibly inhibit calcium-induced force generation in isolated rat cardiomyocytes (Miller *et al.* 1995).

NO• has a plethora of roles in the myocardium (see section 1.4.3) including effects on myocardial diastolic and systolic function and oxygen consumption (Shah *et al.* 2000) and cardiac NOS isoform expression correlates positively with indices of cardiac performance in heart failure and negatively with the severity of LV dysfunction (Heymes *et al.* 1999).

Despite this wealth of data, clinical trials of antioxidants in patients at high risk of cardiovascular disease have yielded disappointing results (Hennekens *et al.* 1996; Yusuf *et al.* 2000) and it remains speculative whether oxidative stress is causal or an epiphenomenon of cardiovascular diseases.

1.6.1 Mitochondrial ROS generation

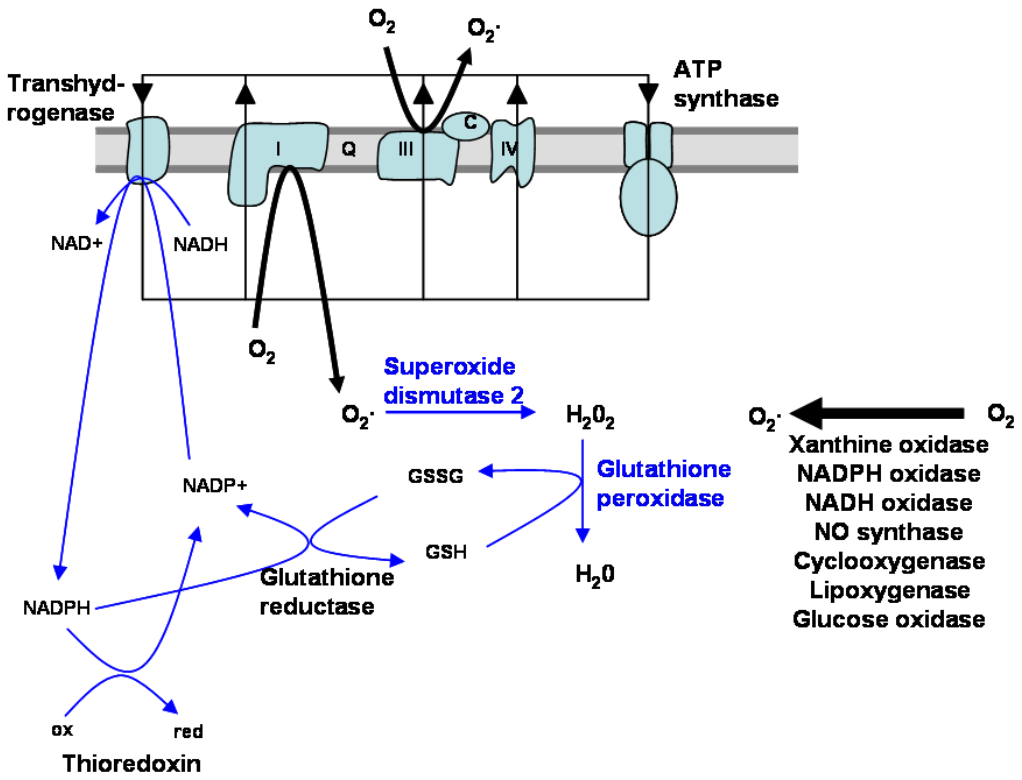
Mitochondria are the major cellular source of ROS (Chance *et al.* 1979) (Figure 1.14). Quantitative data on isolated mitochondria suggest that 2-6% of total O_2 consumption is due to $O_2\bullet^-$ production**.** O_2 appears to have access to the electron donating sites in complexes I and III, to allow formation of $O_2\bullet^-$. At complex III, this tendency increases as $\Delta\psi_m$ increases, for instance as mitochondria move from state 3 to state 4 respiration.

$O_2\bullet^-$ generated at the quinine-binding site of complex III is released into the intermembranous space, as it cannot cross the inner membrane because of the negative $\Delta\psi_m$. $O_2\bullet^-$ generated in this way is probably detoxified by cytoplasmic Cu-Zn-superoxide dismutase (CuZn-SOD or SOD1).

Mitochondria have also developed elaborate enzyme systems to protect against ETC-generated ROS. Complex I is thought responsible for most of the ROS within the mitochondrial matrix. This $O_2\bullet^-$ is first reduced to diffusible H_2O_2 by the mitochondrial manganese-dependent isoform of superoxide dismutase (Mn-SOD or SOD2). The mouse homozygous SOD2 knockout show neonatal lethality due to selective mitochondrial injury resulting in dilated cardiomyopathy, neuronal degeneration, accumulation of lipid in liver and skeletal muscle and metabolic acidosis (Li *et al.* 1995; Lebovitz *et al.* 1996) emphasising the toxicity of mitochondrial ROS.

The glutathione couple (GSSG/GSH representing the oxidised and reduced forms) is responsible for detoxifying H_2O_2 to water via the enzyme glutathione peroxidase. Glutathione is maintained in a reduced state at the expense of nicotinamide adenine dinucleotide phosphate (NADPH) via glutathione reductase. Furthermore, a highly reduced $NADP^+$ pool is maintained at the expense of NADH by the Δ_P-driven mitochondrial transhydrogenase. Thioredoxin and thioredoxin reductase form another redox regulatory couple which can catalyze the regeneration of many antioxidant molecules, including ubiquinone (Q10) and ascorbic acid, and as such constitute an important antioxidant defence against ROS. Thioredoxin reductase gene deletion results in congenital cardiac abnormalities and a severe dilated cardiomyopathy (Conrad *et al.* 2004). Non-enzymatic antioxidants include the vitamins E, C and β-carotene, ubiquinone, lipoic acid, and urate (Nordberg *et al.* 2001).

Figure 1.14. Reactive oxygen species (ROS) are generated at complexes I and III of the electron transport chain. Superoxide dismutase and the glutathione reductase systems form part of the mitochondrial defence against ROS. Other non-mitochondrial sources of ROS, such as xanthine oxidase are also shown (adapted from Nicholls and Ferguson 2002).



1.6.2 Non-mitochondrial sources of oxidative stress

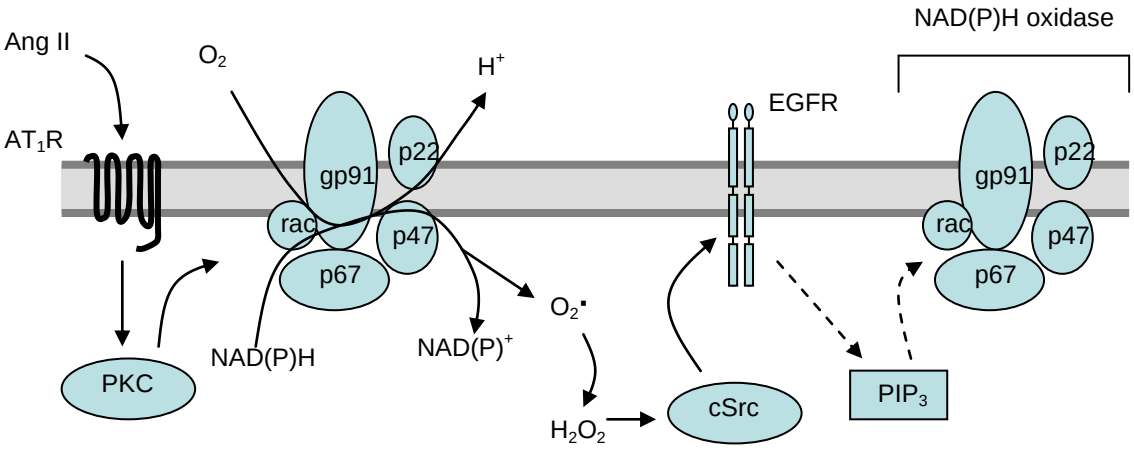
Important non-mitochondrial sources of cellular ROS in the myocardium and endothelial cells include NAD(P)H oxidase, xanthine oxidase and NOS (Figure 1.15). The membrane-bound, non-phagocytic NAD(P)H oxidase is a flavocytochrome b heterodimer which is a major source of intracellular ROS generation from endothelial cells, vascular smooth muscle cells and cardiomyocytes. This process is regulated by Ang II via the AT_1R, shear stress and by cytokines such as TNFα and platelet derived

growth factor (PDGF) (Rajagopalan *et al.* 1996; Zhang *et al.* 1999; Harrison *et al.* 2003). Receptor binding activates protein kinase C (PKC) which both directly stimulates ROS from NAD(P)H oxidase, and indirectly via transactivation of the endothelial growth factor receptor (EGFR), which causes further assembly of NAD(P)H oxidase subunits over a period of hours (Figure 1.15).

Several studies have shown that NAD(P)H oxidase mediates Ang II–induced hypertension and ROS generation (Rajagopalan *et al.* 1996; Ushio-Fukai *et al.* 1998; Zhang *et al.* 1999). This cascade is important in transducing Ang II intracellular signalling to mediate cellular hypertrophy/proliferation/migration/apoptosis (Griendling *et al.* 2000), as well as in endothelial dysfunction and atherogenesis (Harrison *et al.* 2003) and in part explains endothelial ROS generation in both diabetes and hypercholesterolaemia (Guzik *et al.* 2000; Brownlee 2001). Ang II – NAD(P)H oxidase mediated ROS production is reduced by ACE inhibition and AT_1R antagonism, and is enhanced by AT_2R antagonism (Zhang *et al.* 1999).

Xanthine oxidoreductase catalyses the oxidation of xanthine and hypoxanthine during purine metabolism and can exist in two forms: xanthine dehydrogenase which reduces NAD^+ and xanthine oxidase which reduces molecular oxygen to generate $O_2\bullet^-$ and H_2O_2. There is some evidence that ROS generated by xanthine oxidase on endothelial cells may reduce NO• bioavailability and its activity is increased in patients with atherosclerosis (Harrison *et al.* 2003). NOS uncoupling to generate ROS was discussed in section 1.43.

Figure 1.15. <u>Angiotensin II (Ang II) activation of membrane bound NAD(P)H oxidase system.</u> Ang II type 1 receptor binding activates protein kinase C (PKC) which can either directly stimulate ROS generation from NAD(P)H or via transactivation of other growth promoting receptors, such as endothelial growth factor receptor (EGFR) and insulin-like growth factor receptor (IGFR), via the tyrosine kinase cSrc, causing further assembly of NAD(P)H oxidase subunits (rac, gp91phox, p22phox, p47phox, p67phox) over a period of hours, protein synthesis and cell growth and proliferation.



1.7 <u>RAAS AND METABOLISM</u>

There is now a wealth of evidence that ACEi improve morbidity and mortality in cardiovascular disease. Early, large clinical trials showed a reduction in mortality and mortality associated with ACEi treatment of patients with severe (NYHA class IV) to moderate (class II-III) heart failure (CONSENSUS Trial Study Group 1987; SOLVD Investigators 1991). ACEi therapy appeared to prevent further left ventricular dilatation and dysfunction compared to conventional therapy (SOLVD Investigators 1991). These benefits were extended to patients treated with ACEi post-MI who had a left ventricular ejection fraction below 40% but without overt heart failure (Pfeffer *et al.* 1992) and then

to any patient post-MI who had any clinical evidence of heart failure (either transient or ongoing) (AIRE Study Investigators 1993). However, no reduction in mortality was seen when enalapril was started within 24hours of MI in the CONSENSUS II trial (Swedberg *et al.* 1992).

The HyC (Hydralazine *vs.* Captopril) trial in 1992 pointed to a role of ACE inhibition in CCF beyond blood pressure reduction (Fonarow *et al.* 1992). 117 patients with severe CCF were randomised to either captopril or vasodilator therapy (hydralazine plus nitrate). The haemodynamic status was then closely matched using invasive monitoring in the two groups. Captopril treatment conferred a significant survival benefit, with projected one year survival rates of 81% and 51% in captopril and hydralazine groups, respectively. The HOPE Study (Flather *et al.* 2000) further focussed the idea that inhibition of ACE has biological effects above and beyond those associated with simple systemic blood pressure reduction (Dzau *et al.* 2001; Mathew *et al.* 2001).

Quality of life, in particular dyspnoea, has also been reported to be improved by ACEi treatment in heart failure (Rector *et al.* 1993; Rogers *et al.* 1994). Exertional dyspnoea, which is characterised by narrow and rapid respiration during exercise, is the main symptom of patients with CHF. In a small study of patients with CHF, ACEi therapy for one week was associated with a decrease in the ratio of minute ventilation to expired CO_2, a marker of excessive ventilation during exercise. In those patients with the least exercise capacity, ACEi improved excess ventilation without an increase in peak oxygen consumption (VO_2) (Kitaoka *et al.* 2000).

ACEi also reduce the decline in physical function and exercise capacity in patients with CHF (Gambassi *et al.* 2000). ACEi were shown to have a dramatic effect in a study of

755 women from the Women's Health and Ageing Study who had hypertension but not CHF: subjects on ACEi showed a lower decline in muscle strength and in mean walking speed over 3 years compared with women who received other or no antihypertensives (Onder *et al.* 2002). This effect in elderly women suggested a dose-dependent relationship of ACEi with physical function.

Forearm oxygen consumption during maximal exercise is lower in patients with severe CCF, and is increased by the administration of the ACEi captopril (Imaizumi *et al.* 1990). In rats with CCF, muscle ATP and PCr levels decline more quickly with exercise than in controls, and lactate levels rise faster. Six weeks of an ACEi reverses these effects, suggesting that ACE inhibition may restore skeletal muscle metabolic efficiency (Yamaguchi *et al.* 1999).

These studies suggest that the benefit of ACE inhibition may not be simply due to central (cardiac), but also due to peripheral factors, such as alterations in or preservation of skeletal muscle function due to alterations in tissue RAAS. Genetic studies have provided further evidence to support a role for peripheral tissue RAAS in metabolic efficiency (*vide infra*).

1.7.1 The ACE gene and physical performance

Global indices of human athletic performance (Rankinen *et al.* 2002), as well as more precise measures of human skeletal muscle function (Thomis *et al.* 1998), are strongly influenced by genetic as well as environmental factors. To date, few genetic loci of influence have been identified (Yang *et al.* 2003). One such is the gene for angiotensin-I converting enzyme (*ACE*) (Montgomery *et al.* 1998; Myerson *et al.* 1999; Folland *et al.* 2000; Williams *et al.* 2000; Woods *et al.* 2001).

A common variant in the human gene for *ACE* exists in which the presence (Insertion or I), rather than the absence (Deletion or D), of a 287bp *Alu* repeat sequence in intron 16 is associated with lower circulating (Rigat *et al.* 1990) and tissue ACE activity (Costerousse *et al.* 1993; Danser *et al.* 1995). The *ACE* I/D gene variant is therefore a useful *marker* for ACE activity, accounting for up to 50% of the inter-individual variation in activity (Rigat *et al.* 1990).

Montgomery *et al* have performed a series of prospective gene-environment studies to test the hypothesis that low muscle ACE activity, as marked by the *ACE* I allele, is associated with endurance performance. A homogeneous human population was selected (i.e. young healthy adult, male, white Caucasians), thus limiting genetic heterogeneity and differences in previous environmental exposures, both of which would serve to limit the power of any subsequent test. A uniform or near-uniform stimulus was then applied to all study participants and any phenotypic changes related to differences in a candidate gene. This approach has also been termed "stressing the genotype". Using such an approach, the maximum duration of repetitive elbow flexions whilst holding a 15kg bar bell (**the phenotype**) was assessed before and after 10 weeks of military training (**the uniform stressor**) in 78 young healthy male British army recruits (**homogeneous population**) (Montgomery *et al.* 1998). There was no difference in performance by *ACE* I/D genotype pre-training, but the change in performance following training was highly genotype dependent, with II individuals improving on average by 66.2% and DD individuals by 5.9% (P=0.001). In a follow-up study, the efficiency of muscular contraction was better quantified by measuring delta efficiency (DE; the percentage ratio of the change in work performed per minute to the change in energy expended per minute) during bicycle ergometry using the same

stimulus of 11 weeks military training in 58 training-naïve male recruits (Williams *et al.* 2000). Again, the baseline efficiency was identical by *ACE* genotype pre-training (II 24.5% and DD 24.9% delta efficient). However, training related benefits were highly *ACE* I/D genotype dependent, with II gaining muscular efficiency (percentage increase in efficiency 8.62% in II and -0.39% in DD; $P<0.025$).

Further association studies have shown a predilection for *ACE* I allele carriers for endurance sports or extreme performance, including high altitude mountaineering without oxygen (Montgomery *et al.* 1998), elite rowers (Gayagay *et al.* 1998) and distance-running events amongst British Olympic athletes (Myerson *et al.* 1999). Professional athletes from mixed sporting disciplines have also been shown to have an excess of the I allele (Alvarez *et al.* 2000).

The I allele has also been associated with a greater anabolic response to exercise training (Montgomery *et al.* 1999). Conversely, the D allele has been associated with greater skeletal muscle strength gain following isometric strength-training (Folland *et al.* 2000) and with predominantly explosive / anaerobic sports (Myerson *et al.* 1999). Clinical studies have shown an association of the *ACE* D allele with a disturbance in peripheral oxygen utilisation and hyperlactataemia during exercise in patients with COPD (Kanazawa *et al.* 2002), and the D allele was a strong, independent predictor of severe hypoglycaemia in patients with type 1 diabetes (Pedersen-Bjergaard *et al.* 2001).

However, there is some contradictory evidence regarding the influence of the *ACE* gene I/D polymorphism on endurance performance, but these studies used heterogeneous, rather than homogeneous, study groups which may have diluted any genotypic effect (Karjalainen *et al.* 1999; Rieder *et al.* 1999; Taylor *et al.* 1999; Rankinen *et al.* 2000).

1.7.2 RAAS and metabolic efficiency in cardiac and skeletal muscle

How might some of these metabolic differences be explained? The downstream effector peptides angiotensin II and bradykinin may both be responsible for the metabolic effects of ACE. Angiotensin II has recognized effects on metabolism (Brink *et al.* 1996) and is a recognized growth factor necessary for the hypertrophy of skeletal muscle in response to mechanical load (Gordon *et al.* 2001). Levels of bradykinin are dependent on *ACE* genotype (Murphey *et al.* 2000) and may influence skeletal muscle glucose uptake and muscle blood flow (Wicklmayr *et al.* 1983) as well as mitochondrial oxygen utilisation (Moncada *et al.* 2002). Some of these effects of bradykinin have been shown to be dependent on the constitutive B_2 receptor (BDKRB2) (Taguchi *et al.* 2000).

There are no human data relating these downstream effectors to physical performance. Much of the data arise from animal models of heart disease, which suggest that ACE inhibition not only preserves cardiac function, but also levels of high energy phosphates and indices of mitochondrial function in acute ischaemia (Watanabe *et al.* 1997; Divisova *et al.* 2001), chronic CHF (Nascimben *et al.* 1995; Sanbe *et al.* 1995; Hugel *et al.* 1999) and left ventricular hypertrophy (Gohlke *et al.* 1994). The beneficial effects of ACEi on myocardial metabolism may be kinin mediated (Linz *et al.* 1996). Kinins are released from ischemic hearts (Hashimoto *et al.* 1977), whilst BK perfusion during ischaemia improves cardiac function and myocardial energy metabolism (Linz *et al.* 1987) and metabolic benefits of ACEi in hypertrophy are BDKRB2 dependent (Gohlke *et al.* 1994). Ang II, acting via the AT_1R, mediates cardiac fibrosis and hypertrophy (Kato *et al.* 1991). However, the protective effect of AT_1R blockade on the myocardium may be due to unopposed stimulation of AT_2R, which is kinin and NO• dependent (Gohlke *et al.* 1998; Jalowy *et al.* 1998). Perfusion with a combination of an

ACEi and an AT_1R antagonist may have an additive benefit on ATP levels and intracellular pH during ischaemia (Kawabata *et al.* 2000). It was originally suggested some ACEi such as captopril had free radical scavenging activity, explained by the sulphydryl group in their structure, and that this helped attenuate myocardial dysfunction during reperfusion injury (Westin *et al.* 1988). However, this seems unlikely since this cardioprotection has been extended to other non-sulphydryl-containing ACEi, suggesting a direct effect on tissue RAAS (Rabkin 1992), which may also be dependent on NO• synthesis via the BDKRB2 (Jin *et al.* 1998).

Skeletal muscle also contains a complete kallikrein-kinin system (Mayfield *et al.* 1996), can liberate kinins locally (Langberg *et al.* 2002), and expresses functional BDKRB2 (Figueroa *et al.* 1996; Rabito *et al.* 1996). BK is thus generated within exercising skeletal muscle (Langberg *et al.* 2002), can increase skeletal muscle glucose uptake (Wicklmayr *et al.* 1979) and produces an endothelial-dependent increase in muscle blood flow (Wicklmayr *et al.* 1983). In fact, through the BDKRB2 (Taguchi *et al.* 2000), BK enhances insulin-stimulated tyrosine kinase activity of the insulin receptor, which stimulates phosphorylation of insulin receptor substrate-1 with subsequent GLUT-4 translocation in adipose (Isami *et al.* 1996), cardiac (Rett *et al.* 1996) and skeletal muscle tissue during exercise (Motoshima *et al.* 2000). Thus ACE inhibition can increase skeletal muscle glucose uptake, insulin sensitivity and GLUT-4 translocation (Henriksen *et al.* 1995; Jacob *et al.* 1996) via BDKRB2 agonism (Henriksen *et al.* 1999). Indeed, ACEi have been associated with hypoglycaemia in diabetic patients (Herings *et al.* 1995) and the ACE I/D polymorphism has also been associated with decreased insulin resistance and baseline insulin levels in diabetes (Katsuya *et al.* 1995; Panahloo *et al.* 1995; Kennon *et al.* 1999; Takezako *et al.* 1999).

BDKRB2 activation can lead to transient rises in inositol 1,4,5-trisphosphate (Rabito *et al.* 1996), which is involved in excitation-coupling of skeletal muscle via increases in cytoplasmic calcium (Hidalgo *et al.* 1989; Lopez *et al.* 1991; Foster 1994). This process is enhanced by both insulin (Kudoh *et al.* 2000) and by inhibition of ACE (Kudoh *et al.* 2000). Conversely, chronic Ang II infusion in rats has been shown to induce cachexia with profound skeletal muscle wasting (Brink *et al.* 1996), due to enhanced protein catabolism, with concomitant downregulation of skeletal muscle insulin-like growth factor 1 (IGF-1) and IGF-1 binding proteins (Brink *et al.* 2001), and increased energy expenditure and changes in oxygen consumption associated with a metabolic cachexia (Cassis *et al.* 2002). Skeletal muscle does express Ang II receptors (Stoll *et al.* 1995; Rabito *et al.* 1996), and paracrine adipose RAAS may play a role in the mobilization of triglycerides as a metabolic fuel (Goldman *et al.* 1987; Campbell *et al.* 1993; Hennes *et al.* 1996; Jones *et al.* 1997). Moreover, ACEi (Flather *et al.* 2000) and ARBs (Dahlof *et al.* 2002) have both been associated with a reduction in the risk of developing type II diabetes in patients at high risk of CHD events.

Could the mitochondrial effects of ACE inhibition be mediated by NO•? NO• is both a vasodilator and a second messenger which, at physiological (nanomolar) concentrations, reversibly inhibits cytochrome c oxidase (complex IV) in competition with O_2 (Brown *et al.* 1994; Cleeter *et al.* 1994). It thus reduces O_2 uptake in skeletal muscle and heart mitochondria (Cleeter *et al.* 1994; Poderoso *et al.* 1996) and in heart submitochondrial particles isolated from the rat heart (Poderoso *et al.* 1996). It has been suggested that the interplay between NO• and O_2 allows cytochrome c oxidase to act as an O_2 sensor within cells, with the affinity of NO• for cytochrome c oxidase dependent on the O_2 tension (Clementi *et al.* 1999). The large amount of NO• induced by cytokines such as TNFα and IF-γ via iNOS, is able to inhibit mitochondrial respiration in vascular smooth

muscle cells (Geng *et al.* 1992). NO• donors also have the potential to reversibly inhibit oxygen utilisation in rat skeletal mitochondria (Cleeter *et al.* 1994). Tissue and whole animal studies have shown that kinins can suppress oxygen consumption via endogenous NO• in skeletal (Shen *et al.* 1995) and cardiac muscle (Zhang *et al.* 1997), an effect mimicked by ACEi and prevented by blockade of BDKRB2 (Zhang *et al.* 1997). Poderoso observed a dose-dependent reduction in myocardial oxygen consumption and release of H_2O_2 when isolated rat heart was perfused with bradykinin (Poderoso *et al.* 1998). Co-administration with enalapril resulted in further reductions in oxygen uptake. Moreover, bradykinin, the ACEi ramiprilat, calcium channel blocker amlodipine, and the neutral endopeptidase inhibitor thiorphan, all stimulate endogenous NO• release while significantly decreasing oxygen consumption in failing human heart tissue explanted at transplantation (Loke *et al.* 1999). This effect is attenuated in the presence of a NOS inhibitor. Vavrinkova *et al* demonstrated that 4 weeks pre treatment with the ACEi captopril was associated with a significant reduction in the content of free radical forms of the mitochondrial respiratory chain during ischaemia in isolated rat hearts perfused with L-arginine and that this was associated with enhanced NO• formation (Divisova *et al.* 2001). NO• modulation of mitochondrial respiration and membrane potential can have protective effects in apoptosis (Beltran *et al.* 2000) and ischaemia-reperfusion injury (Rakhit *et al.* 2001).

1.8 UNCOUPLING PROTEINS (UCPs)

1.8.1 Overview

Not all of the energy released by oxidation of fuel substrate by the ETC generates ATP, implying that the coupling of respiration to ATP synthesis is imperfect. A basal leak of protons is found in all mitochondria and is responsible for state 4 respiration (section 1.2.2), when all the available ADP has already been converted to ATP (Nicholls *et al.* 2002). In resting hepatocytes and skeletal muscle, this proton leak is responsible for up to 20-30% and 50% of oxygen consumption, respectively, and together may contribute up to 20-30% of basal metabolic rate (Rolfe *et al.* 1996). Moreover, differences in skeletal muscle energy expenditure accounts for the majority of the observed interindividual variation in both basal and exercising metabolic rate (Zurlo *et al.* 1990; Ravussin *et al.* 1992).

Endothermic organisms can produce facultative heat in a cold environment from skeletal muscle and brown adipose tissue (BAT); termed shivering and non-shivering thermogenesis, respectively. Uncoupling of respiration resulting in 'wasteful energy' or theromogenesis would therefore be important for energy balance and body weight control, and may be particularly important in metabolic diseases such as obesity and diabetes. Indeed, the mitochondrial uncoupler dinitrophenol was shown to greatly increase metabolic rate in patients (Cutting *et al.* 1932) and was widely prescribed in the USA in the 1930s as a treatment for obesity, until it was discredited due to its narrow 'therapeutic window' and its significant hepatic, cardiac, nerve, muscle (and other) toxicities, as well as blindness and fatal malignant hyperpyrexia (Colman 2007).

BAT makes an important contribution to non-shivering thermogenesis in mammals and is regulated principally by the SNS (Himms-Hagen 1990). In humans, this process is important at birth and in infancy, but reduces in importance as BAT atrophies to 2% of total body adipose tissue mass in adults. In BAT, energy derived from fuel substrate is not used to generate ATP, but is dissipated via proton leak to generate useful heat. Interest in the high thermogenic capacity of BAT began in the 1960s, when BAT was shown to have a high density of mitochondria which respired rapidly, but displayed no respiratory control (Nicholls 2001). The proton leak or conductance was subsequently shown to be nucleotide-sensitive and activated by fatty acids, but it was not until 1977 that the protein responsible, thermogenin or uncoupling protein (UCP, later termed UCP1), was identified and shown to constitute up to 10% of inner mitochondrial membrane protein content (Heaton *et al.* 1978). BAT from UCP1 knockout mice have low spontaneous respiration (Enerback *et al.* 1997). It is now accepted that UCP1 is exclusively responsible for non-shivering thermogenesis in BAT (Krauss *et al.* 2005).

UCP1 expression is, however, limited to BAT, so the search for UCP1 homologues which might be responsible for the protein leak observed in tissues other than BAT was spurred. UCP2 and UCP3 were subsequently cloned in 1997 (Boss *et al.* 1997; Fleury *et al.* 1997; Gimeno *et al.* 1997; Gong *et al.* 1997; Solanes *et al.* 1997; Vidal-Puig *et al.* 1997) and found to have 59% and 57% amino acid sequence homology with UCP1, respectively. The *UCP2/UCP3* gene locus has been mapped to human chromosomal region 11q13, with the *UCP2* gene 7kB downstream from the *UCP3* gene, a region that has been linked to hyperinsulinaemia and obesity in humans (Fleury *et al.* 1997). *UCP2* and *UCP3* themselves share 72% sequence homology. It is now thought that *UCP2* is in fact the ancestral *UCP*, and that *UCP3* was caused by a gene duplication event (Borecky *et al.* 2001).

UCPs belong to the mitochondrial anion-carrier superfamily, which has been described across various animal and plant species and includes the adenine nuclear translocator (ATP/ADP translocator; ANT), dicarboxylate, citrate, carnitine and oxoglutarate/maleate carriers, as well as the mammalian homologues UCP1-5 (Borecky *et al.* 2001). The UCPs are nuclear-encoded, mitochondrial-targeted proteins. Phylogenetic analysis (Figure 1.16) suggests that there is a subfamily of closely related UCPs, consisting of UCP1, UCP2, UCP3 and bird UCPs (Borecky *et al.* 2001). UCP4 and UCP5 (brain mitochondrial carrier protein 1, BMCP1) share lower sequence homology with UCP1 (30% and 33%, respectively) and do not appear to be true members of the UCP sub-family (Mao *et al.* 1999; Yu *et al.* 2000).

Figure 1.16. Phylogenetic tree of the mitochondrial anion-carrier superfamily (adapted from Borecky 2001).

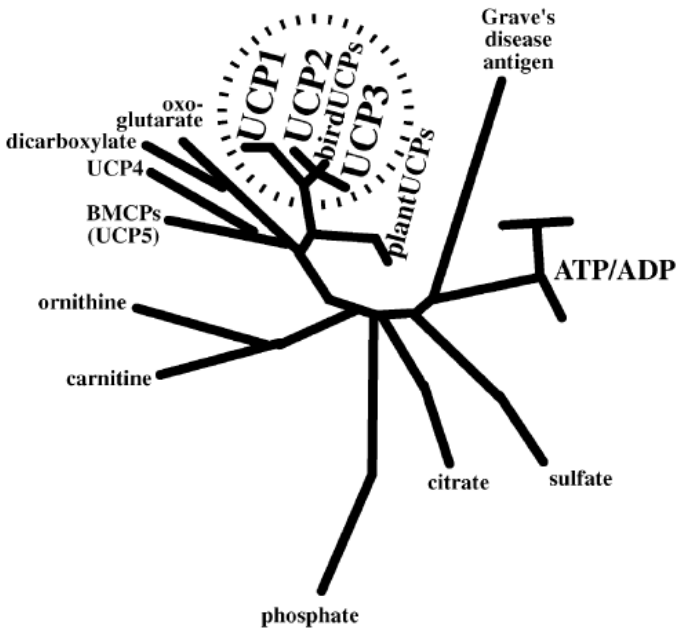
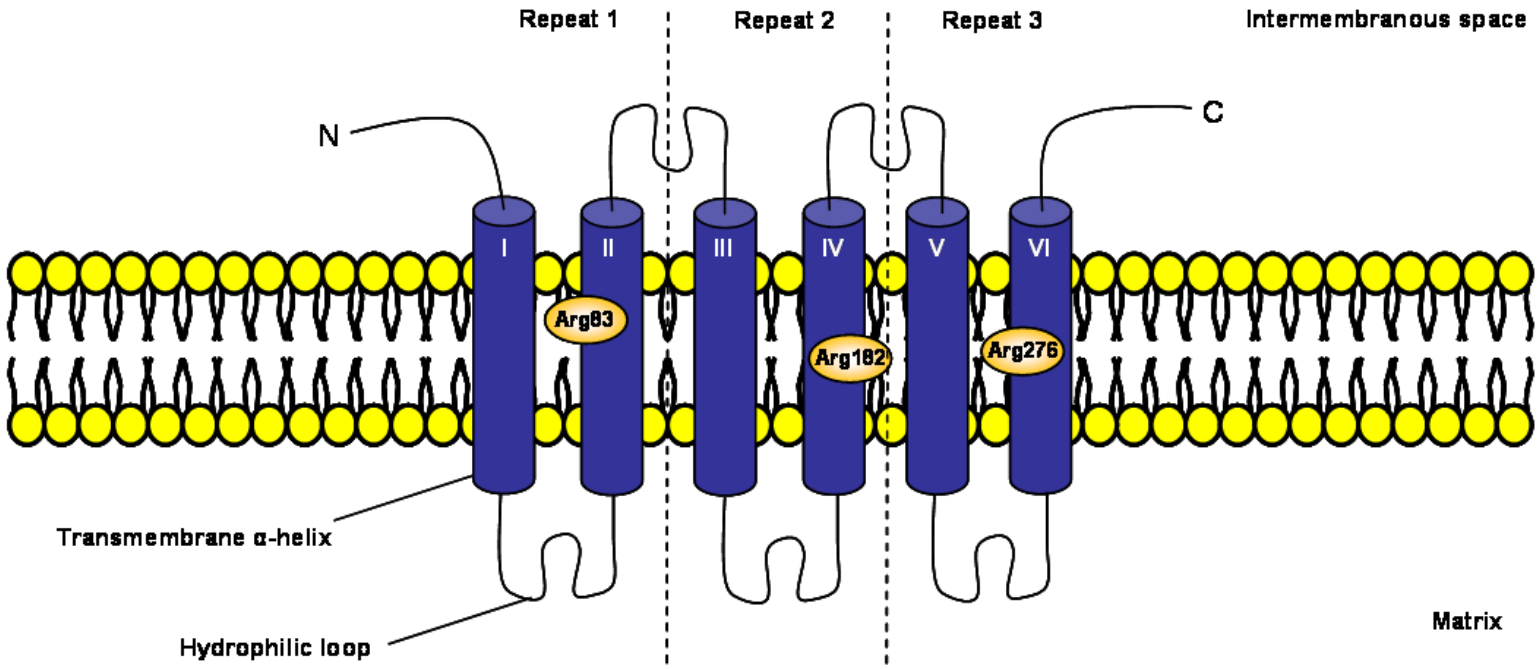


Figure 1.17. <u>Postulated tripartite structure of uncoupling proteins.</u> The conserved arginine residues shown are necessary for nucleotide binding.



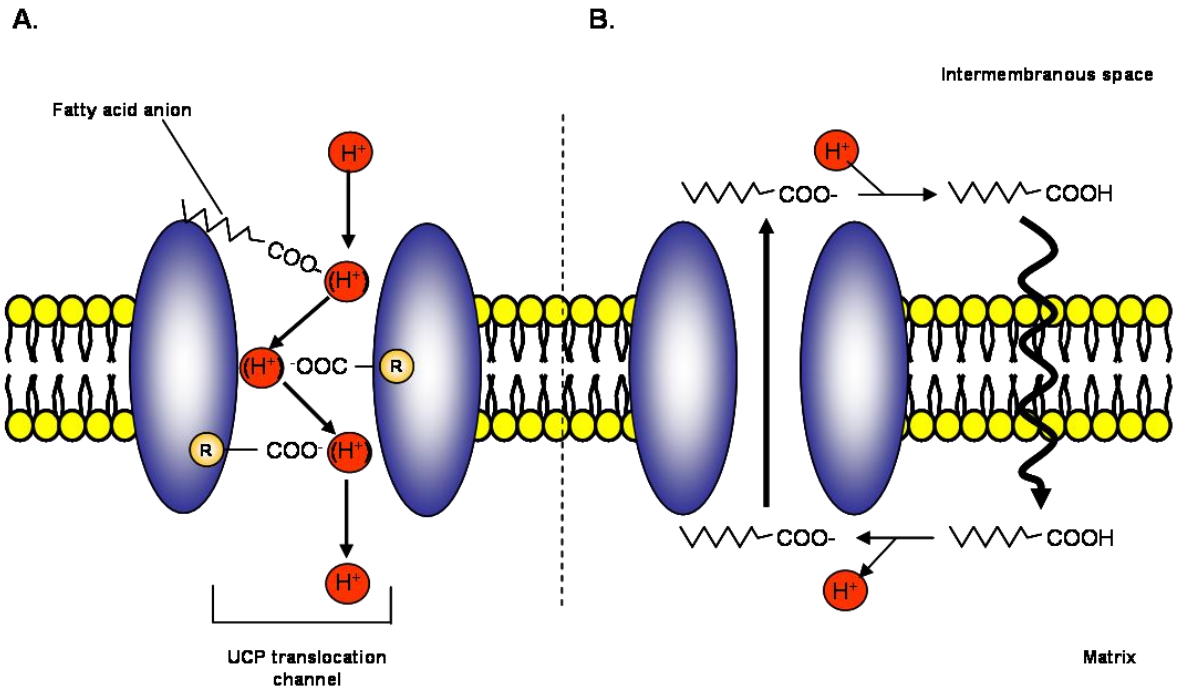
1.8.1.1 Structure

UCP 1, 2 and 3 contain 306, 308 and 311 amino acid residues, bearing tripartite structures containing signature motifs for mitochondrial carrier proteins (Figure 1.17). The crystal structure has not been determined. However, each of the three repeat domains consists of two transmembrane, hydrophobic α-helices linked by hydrophilic loops. The central matrix-facing loop is important for membrane insertion, as there is no typical N-terminal mitochondrial targeting sequence (Schleiff *et al.* 2000) and, once inserted in the inner mitochondrial membrane, UCPs are thought to dimerise (Lin *et al.* 1980).

Figure 1.18. <u>Possible mechanisms of uncoupling protein proton translocation</u>

A. Proton buffering model. UCPs can translocate protons directly, but fatty acid anion can facilitate this process.

B. Fatty acid cycling model. Fatty acid anions can pass from the matrix through the UCP channel, becoming protonated in the intermembrane space. The protonated fatty acid is then able to flip-flop back through the inner membrane, releasing protons on the matrix side.



Most is known about the function of UCP1. Mutational analysis suggests that the UCP helices together form a hydrophilic channel with the loops controlling access (gating domains) (Arechaga *et al.* 2001). Site-directed mutagenesis has suggested nucleotide binding domains within the transmembrane α-helices at three conserved arginine residues (Arg 83, Arg 182 and Arg 276), binding to which results in a conformational change in the pore and inhibition of function (Modriansky *et al.* 1997).

UCP1 can transport protons and several anions, such as chloride and nitrate (Nicholls *et al.* 1973; Nicholls 1974). The exact mechanism of proton transport is unknown but is fatty acid sensitive. Two main hypotheses exist. In the first model, proposed by Klingenberg and known as the "proton buffering model", it is suggested that UCP1 actually transports protons, and fatty acids provide an essential free carboxy group which augment proton movement through the channel (Figure 1.18A). The second model, proposed by Garlid and termed the "fatty-acid cycling model" suggests that UCP1 transports charged fatty acid anions to the intermembrane space, which would otherwise be confined to the matrix. On the positively charged outer face of the inner mitochondrial membrane, the fatty acid anions can accept protons. In their protonated, hydrophobic form, the fatty acids can flip-flop back directly through the inner membrane to release protons on the matrix side to complete the circuit (Figure 1.18B). It has been suggested that both of these mechanisms may exist (Ricquier *et al.* 2000); with a proton pathway accounting for spontaneous respiration in state 4 which could be further enhanced by small concentrations of fatty acids. At higher concentrations, fatty acid cycling may occur as described above.

1.8.2 Uncoupling protein 2

In contrast to UCP1, UCP2 is expressed ubiquitously at the mRNA level (Fleury *et al.* 1997; Gimeno *et al.* 1997), in particular in cells of the immune system, white adipose tissue (WAT), skeletal and cardiac muscle, brain, lung and liver. Studies with specific antibodies have been difficult, and it has been suggested that UCP2 protein expression may be somewhat restricted in mice (to the spleen, stomach, lung and gonadal WAT) (Pecqueur *et al.* 2001) but has also been found in the heart (Murray *et al.* 2004) and liver (Taniguchi *et al.* 2002) in humans. To date, no study has shown the presence of any UCP within endothelial cells. However, UCP2 mRNA content of any adipose tissue examined was 3-4x higher in the stromal-vascular fraction (rich in endothelial cells, pre-adipocytes, fibroblasts and monocyte/macrophages) than in the adipocyte fraction (Prunet-Marcassus *et al.* 1999). The amount of UCP2 mRNA increases during adipocyte differentiation (Do *et al.* 1999), so the excess mRNA in the stromal-vascular fraction must be due to the expression of UCP2 in non-adipocyte cell types. In the same study, UCP3 mRNA was found only in adipocyte fractions.

1.8.2.1 Gene structure

The human *UCP2* (*hUCP2*) gene consists of 8 exons, of which exons 1 and 2 are non-coding (Pecqueur *et al.* 1999). The 3.3kb promoter differs significantly from those of the *hUCP1* and *hUCP3* genes, lacking a typical TATA or CAAT box (Tu *et al.* 1999). The transcription start site is preceded by a GC rich region with multiple Sp-1 binding sites and AP-1 and AP-2 motifs. This region contains a strong, *cis*-acting positive regulatory element (-141 to -65) which may underlie the ubiquitous expression of UCP2 (Tu *et al.* 1999), analogous to the situation in the mouse (Yoshitomi *et al.* 1999).

Several consensus sequences exist for transcription control elements, such as C/EBP-beta (important for IL6 gene expression), CREB-1 (cAMP response element binding protein 1), 2 PPREs (PPARγ response elements), two TREs (thyroid hormone response elements), MyoD (muscle regulatory protein) and NF-κB (Tu *et al.* 1999). Promoter construct work has delineated regions containing further positive (-1398 to -884) and negative (-3271 to -1398, -1398 to -884, -884 to -141) control elements (Tu *et al.* 1999).

1.8.2.2 Regulation

Due to a lack of specific antibodies for UCP2, much work has focussed on changes in UCP2 mRNA expression in response to metabolic challenges. UCP2 mRNA expression is upregulated by an increase in circulating free fatty acids in rodent oxidative skeletal muscle (Samec *et al.* 1998; Samec *et al.* 1999; Vettor *et al.* 2002) and heart (Vettor *et al.* 2002). A similar response at the mRNA level has been shown in humans after lipid infusion and a high fat diet in WAT (Nisoli *et al.* 2000) and skeletal muscle (Schrauwen *et al.* 2001), respectively, and plasma free fatty acid levels have been positively correlated with UCP2 protein levels in human heart (Murray *et al.* 2004). Hence, metabolic perturbations which result in an increase in circulating free fatty acids increase UCP2 mRNA expression. As such, cold exposure, fasting and fat feeding increase UCP2 mRNA to varying degrees in WAT and skeletal muscle of rodents (Boss *et al.* 1997; Matsuda *et al.* 1997; Samec *et al.* 1998; Weigle *et al.* 1998; Gong *et al.* 1999).

Similarly, in humans, UCP2 expression is upregulated by fasting in WAT (Millet *et al.* 1997). PPARs may mediate the transcriptional effects of fatty acids on UCP2 mRNA expression. PPARγ agonists increase UCP2 mRNA levels in adipocytes and skeletal myocytes *in vitro* in some (Aubert *et al.* 1997; Camirand *et al.* 1998) but not all studies

(Kelly *et al.* 1998). PPARα agonists appear to have limited effects *in vitro*, increasing liver UCP2 mRNA expression only (Kelly *et al.* 1998).

Norepinephrine or β_3-agonism does not appear to alter UCP2 mRNA expression in murine WAT, but 3 weeks of β_3-agonism in mice decreased skeletal and cardiac muscle UCP2 mRNA expression together with a decrease in circulating free fatty acids and insulin (Aubert *et al.* 1997; Yoshitomi *et al.* 1998). As to be expected, tri-iodothyronine (T_3) increased UCP2 mRNA expression in WAT, skeletal and cardiac muscle in rodents (Lanni *et al.* 1997; Masaki *et al.* 1997; Lanni *et al.* 1999) and in WAT and skeletal muscle in humans (Barbe *et al.* 2001).

In rats, an 8 week endurance training programme was associated with 54% and 41% decreases in UCP2 mRNA expression in heart and tibialis anterior (type IIa and IIb fast twitch fibres) muscle, respectively, with no associated changes in WAT and soleus (slow twitch) muscle (Boss *et al.* 1998). Denervation increased UCP2 mRNA expression in mixed muscle (gastrocnemius) in both the rat and mouse (Cortright *et al.* 1999). In the same study, only acute exercise in the mouse increased UCP2 mRNA expression, whereas chronic exercise had no effect in rats (Cortright *et al.* 1999). The differences may have been produced by differences in concomitant feeding in the experimental protocols.

1.8.2.3 Function

Unlike UCP1, the precise function of UCP2 remains to be elucidated and is the matter of much debate. UCP2 has been implicated in the development of diabetes and obesity (O'Rahilly 2001). UCP2 is expressed in pancreatic islet β cells and overexpression can lead to a reduction in glucose-stimulated insulin secretion (GSIS) – an ATP-dependent

process (Chan *et al.* 1999). UCP2 knockout mice exhibit markedly increased circulating insulin levels, and islets isolated from these animals have enhanced GSIS (Zhang *et al.* 2001). Overexpression in mice of the human *UCP2* gene was associated with reduced fat mass, with a tendency to increased basal metabolic rate, but with no effects on thermoregulatory feeding behaviour (Horvath *et al.* 2003). However, the *UCP2* knockout mouse is not obese and has a normal response to cold exposure (Arsenijevic *et al.* 2000), suggesting an alternative function.

Many of the early cell overexpression studies suggested that UCP2 was an uncoupler of mitochondrial respiration (Fleury *et al.* 1997; Gimeno *et al.* 1997; Chan *et al.* 1999; Jaburek *et al.* 1999; Rial *et al.* 1999; Teshima *et al.* 2003). However, it has been demonstrated that UCP overexpression results in non-physiological uncoupling of mitochondria, either because the high levels of expression causes perturbation of inner mitochondrial membrane integrity or because of incorrect membrane insertion (Heidkaemper *et al.* 2000; Stuart *et al.* 2001; Stuart *et al.* 2001). In contrast, isolated thymocytes from UCP2 knockout mice were shown to have higher $\Delta\psi_m$ and ATP levels than thymocytes isolated from wild-type animals (Krauss *et al.* 2002). It was calculated that up to 50% of the basal proton leak was dependent on the expression of UCP2.

Gene deletional studies and the ubiquitous expression of UCP2 suggest the primary role may not be thermogenesis (Arsenijevic *et al.* 2000; Couplan *et al.* 2002). An alternative conclusion from the widespread expression and electrochemical actions of UCP2 is that it is a plausible and powerful regulator of mitochondrial ROS production (Negre-Salvayre *et al.* 1997; Casteilla *et al.* 2001). In keeping with this proposal, UCP2 expression is induced by oxidative stress (Lee *et al.* 1999; Pecqueur *et al.* 2001) and UCP-induced proton conductance is activated by $O_2^{\bullet -}$ (Echtay *et al.* 2002; Echtay *et al.*

2002). The $O_2\bullet^-$ activated uncoupling is, predictably, dependent on the presence of fatty acids and inhibited by purine nucleotides (Echtay *et al.* 2002) and occurs from the matrix side of the mitochondria (Echtay *et al.* 2002) directly activating proton conductance, rather than by futile cycling of reactive species through UCPs. UCP2 may therefore be involved in inflammatory processes through ROS generation. Mitochondria isolated from UCP2 knockout mice are resistant to infection from toxoplasmosis and their macrophages generate more ROS than wild-type mice (Arsenijevic *et al.* 2000). UCP2 protein is increased (at the transcriptional level) by inflammatory cytokines and endotoxin in hepatocytes and pulmonary tissue (Pecqueur *et al.* 2001).

1.8.3 Uncoupling protein 3

UCP3 has 73% sequence homology with UCP2 but its mRNA expression in humans and rodents is limited predominantly to skeletal muscle and BAT, with smaller amounts in WAT and in cardiac tissue (Boss *et al.* 1997; Larkin *et al.* 1997; Vidal-Puig *et al.* 1997). The human *UCP3* gene consists of 7 exons, the first of which is not translated (Solanes *et al.* 1997). Human UCP3 mRNA exists in two forms, UCP3S (short) and UCP3L (long) due to the presence of translation termination (TGA) codons in exon 6 and 7, respectively, which appear to be transcribed in equal amounts (Solanes *et al.* 1997). UCP3L codes the 312 amino acid protein, whereas UCP3S codes a putative 275 amino acid protein lacking the last transmembrane domain which is not transcribed (Solanes *et al.* 1997).

1.8.3.1 Gene structure

The human UCP3 promoter contains several potential binding sites for muscle-specific factors (E Box, MyoD, MEF2), CAAT boxes, PPREs and a TRE containing a CRE-like element (Acin *et al.* 1999; Tu *et al.* 1999). A consensus sequence for a TATA box is present at position -45 from initiation of transcription. There are no GC rich SP-1 regions. Strong negatively and enhancing cis-acting regions were found between -2983 to -1585 and -982 to -622 in the promoter, respectively (Tu *et al.* 1999).

1.8.3.2 Regulation

As is the case for UCP2, the study of UCP3 protein expression has also been hampered by a lack of specific antibodies, and again, most work has focussed on UCP3 mRNA expression. Its expression in skeletal muscle is upregulated in a similar way to UCP2 by fatty acids, in both rodents (Samec *et al.* 1998; Weigle *et al.* 1998; Hwang *et al.* 1999; Samec *et al.* 1999; Vettor *et al.* 2002; Costello *et al.* 2003) and humans (Schrauwen *et al.* 2001). A feedback loop involving fatty acid supply, UCP3 and mitochondrial fatty oxidation has been suggested, and may vary according to muscle fibre type (Samec *et al.* 1999). Hence, high fat feeding and fasting/calorie restriction have been associated with elevations of UCP3 mRNA in skeletal muscle in rodents (Boss *et al.* 1998; Gong *et al.* 1999), and with elevations of both UCP3 mRNA and protein in humans in some (Millet *et al.* 1997; Hesselink *et al.* 2003), but not all studies (Pedersen *et al.* 2000; Cameron-Smith *et al.* 2003).

In humans and rodents, UCP3 mRNA in skeletal muscle is upregulated during treatment with thyroxine, and downregulated following endurance training (Larkin *et al.* 1997; Boss *et al.* 1998; Cortright *et al.* 1999; Schrauwen *et al.* 1999; Masaki *et al.* 2000). In humans, UCP3 mRNA expression correlates negatively with maximal aerobic power

(Schrauwen *et al.* 1999). UCP3 protein content differs according to muscle fibre type (most abundant in type 2b fast glycolytic > type 2a fast oxidative-glycolytic >type 1 slow oxidative fibres) (Hesselink *et al.* 2001) and is 46% lower in the skeletal muscle of endurance trained cyclists compared to healthy untrained men, although the same hierarchy of content exists (Russell *et al.* 2003). Skeletal muscle mitochondrial density is known to increase during endurance training secondary to neo-mitochondrial biogenesis (Freyssenet *et al.* 1996). Fernström *et al* demonstrated that *vastus lateralis* muscle mitochondrial volume increased by 47% during a 6 week endurance training programme in healthy men, but relative UCP3 protein content and uncoupled mitochondrial respiration decreased by 53% and 18%, respectively (Fernstrom *et al.* 2004).

Adrenergic stimulation may also increase muscle UCP3 mRNA expression, but there has been conflicting data in both rodents (Gomez-Ambrosi *et al.* 1999; Nagase *et al.* 2001; Nakamura *et al.* 2001) and in humans (Boivin *et al.* 2000; Hoeks *et al.* 2003). Leptin administration increases skeletal muscle UCP3 mRNA expression (Gomez-Ambrosi *et al.* 1999) and this effect may be thyroid hormone-dependent (Cusin *et al.* 2000). PPARγ agonists decrease UCP3 mRNA expression skeletal myocytes in culture (Cabrero *et al.* 2000), but their administration *in vivo* increased UCP3 expression in BAT but not in skeletal muscle or WAT (Kelly *et al.* 1998). In contrast, PPARα activation (with bezafibrate or Wy-14 643) increased UCP3 mRNA threefold in adipocytes in culture (Cabrero *et al.* 2000), but had no effect on skeletal myocytes in culture without the presence of exogenous fatty acids (Cabrero *et al.* 2000).

1.8.3.3 Function

As is the case with UCP2, the precise function of UCP3 is unclear. The *hUCP3* overexpressing (*UCP3tg*) mouse produces approximately 66 times more UCP3 and is hyperphagic but lean, with less adipose tissue (Clapham *et al.* 2000). Isolated skeletal muscle mitochondria from these *UCP3tg* mice have an increase in mitochondrial uncoupling (Clapham *et al.* 2000). Indeed isometric stress-testing in isolated soleus muscle from the *UCP3tg* mouse has the same contractile performance as muscle from wild-type animals, but produces greater heat energy (*i.e.* is less efficient) (Curtin *et al.* 2002). However, it has been shown that *UCP3* overexpression studies suffer from the same controversy as those of *UCP1* and *UCP2* overexpression (*vide supra*), as uncontrolled uncoupling ensues (Cadenas *et al.* 2002; Harper *et al.* 2002).

The study of *UCP3* gene deletion (*UCP3ko*) mice, produced independently by several laboratories, has shed some light on function. Isolated skeletal muscle mitochondria from these animals were shown to have increased mitochondrial coupling due to reduced proton leak (state 4 respiration) and increased ATP/ADP ratios by two independent groups (Gong *et al.* 2000; Vidal-Puig *et al.* 2000) but not by a third (Cadenas *et al.* 2002). However, these animals exhibit no obvious phenotype, with no difference in body weight, resting energy expenditure (fed or fasting), or response to exhaustive exercise and cold temperature compared to wild-type mice (Vidal-Puig *et al.* 1997; Gong *et al.* 2000).

In line with observations with UCP2, isolated skeletal muscle mitochondria from *UCP3ko* mice produced significantly more ROS as assessed by a lucigenin assay and by mitochondrial aconitase activity (Vidal-Puig *et al.* 1997) and superoxide induced

proton conductance in isolated skeletal muscle mitochondria through a UCP3 dependent mechanism (Echtay *et al.* 2002).

Few studies have examined the role of UCP3 protein in human skeletal muscle. In one such small study, dietary manipulation was used to alter skeletal muscle UCP3 content: ingestion of a high fat diet resulted in an elevation of UCP3 protein compared to a low fat diet (Hesselink *et al.* 2003). High-intensity exercise with limb blood flow occlusion was used to deplete the primary muscle energy reserve compound, phosphocreatine (PCr), and the PCr resynthesis rate was measured biochemically as a marker of efficiency of ATP generation. There was no difference in PCr resynthesis rate between the high and low fat dietary groups, leading the authors to conclude that an increase in UCP3 content within the physiological range did not affect mitochondrial coupling *in vivo* in human skeletal muscle (Hesselink *et al.* 2003). However, there was a positive linear association between UCP3 protein content and PCr resynthesis rate overall, suggesting that the authors were measuring a surrogate marker of total mitochondrial protein or volume rather than the relative amount of UCP3. Indeed, the relationship between UCP3 protein content and PCr resynthesis rate appeared to change during high fat feeding compared to normal diet, but the study was not powered to detect such a difference (N=6 subjects). In support of this counter-argument, it has been shown that during 6 weeks of endurance training, total UCP3 protein remains unchanged in human skeletal muscle, but when indexed to a marker of mitochondrial volume (citrate synthase), the relative amount of UCP3 protein decreased significantly by 53% (Fernstrom *et al.* 2004). The data overall remain inconclusive.

A further alternative hypothesis is that UCP3 protein functions to remove fatty acid anions from the mitochondrial matrix as part of a detoxifying mechanism. This may be

either to remove excess fatty acid anion oversupply which would accumulate passively within the matrix according to $\Delta\psi_m$ (Schrauwen 2003), or alternatively as part of a fatty acid cycle, whereby intramitochondrial fatty acyl-CoAs release the CoA moiety (by the action of thioesterase) during periods of increased bursts of mitochondrial Kreb's cycle and β-oxidation activity with resultant surplus matrix fatty acid anion which must be removed (Himms-Hagen 2001).

1.8.4 Cardiac UCPs

Some controversy exists as to whether UCPs are expressed within the heart and, if so, what function they may serve. Certainly, both UCP2 and UCP3 have been consistently shown to be expressed at the mRNA level in the heart, although UCP2 mRNA is expressed at a much higher level than UCP3 mRNA in both the rodent and human heart (Boss *et al.* 1997; Fleury *et al.* 1997; Gimeno *et al.* 1997; Vidal-Puig *et al.* 1997). As stated above, research into protein expression in the heart has been hampered by the lack of specific antibodies. Western blot analysis using sensitive antibodies failed to detect UCP2 protein in the mouse (Pecqueur *et al.* 2001). However, both UCP2 and UCP3 protein has been detected in the rat (Boehm *et al.* 2001) and, more recently, UCP2 protein was detected in human right atrial tissue samples (Murray *et al.* 2004).

Changes in UCP2 or UCP3 expression may be associated with an alteration in the balance between cardiac glucose and fatty acid utilisation. In rodent studies, cardiac UCP2 mRNA levels are present in low level in the foetus and increase shortly after birth, mirroring the rise in fatty acid utilisation in the heart (Van Der Lee *et al.* 2000). Cardiac UCP2 mRNA levels are five fold higher in the adult than before birth. In contrast, UCP3 mRNA levels are undetectable in the foetus and rise in concert with

suckling in the newborn in a fatty acid-dependent manner, declining once chow is introduced (Skarka *et al.* 2003). *In vitro* administration of fatty acids induce UCP2 mRNA expression in isolated neonatal cardiomyocytes and both tri-iodothyronine and phenylephrine induced UCP2 mRNA expression in a fatty acid dependent manner (Van Der Lee *et al.* 2000). However, elevation of serum free fatty acids (by high fat feeding, fasting or induction of diabetes) increases cardiac UCP3 mRNA in the rat in a PPARα-dependent manner but does not alter cardiac UCP2 mRNA (Depre *et al.* 2000; Van Der Lee *et al.* 2000; Van der Lee *et al.* 2001; Young *et al.* 2001).

Both UCP2 and UCP3 mRNA and protein expression in the rat heart are increased in models of pressure overload in a fatty acid-dependent manner (stroke-prone hypertensive rat, aortic constriction, chronic aortic regurgitation), and decreased by pressure unloading and by acute aortic regurgitation (Fukunaga *et al.* 2000; Noma *et al.* 2001; Young *et al.* 2001). During acute volume overload in acute aortic regurgitation, UCP2 mRNA was reduced by 53% by 3 days and was inversely proportional to LV diastolic dimension (Noma *et al.* 2001). In contrast, UCP2 mRNA was increased 3.8 fold by 100 days in the left ventricle in the chronic phase of heart failure (Noma *et al.* 2001). The degree of LV dysfunction, together with the increase in UCP2 mRNA expression was mitigated by treatment with an ACEi (Murakami *et al.* 2000; Murakami *et al.* 2002). Similarly, cardiac UCP2 mRNA expression was increased in different mouse models of dilated cardiomyopathy caused by genetic enhancement of β-adrenoceptor signalling (Gaussin *et al.* 2003).

Cardiac UCP mRNA levels have been regulated by hormones, inflammatory mediators and hypoxia. Induction of hyperthyroidism in rats to induce a physiological alteration in mitochondrial function, resulted in a 32% and 48% increase in UCP2 and UCP3

protein respectively, combined with a 71-100% increase in palmitate-induced proton leak and a 38% overall decrease in cardiac efficiency (work performed by unit oxygen consumed) (Boehm *et al.* 2001). TNFα administration in rats reduced UCP2 and UCP3 mRNA expression in the heart (Young *et al.* 2001). Isolated cardiac mitochondria from male Wistar rats which had spent one week in a hypobaric hypoxic (11% O_2) environment showed similar reductions in rates of oxygen consumption and ATP synthesis without a change in mitochondrial coupling (respiratory control ratio). There was no change in UCP2 mRNA levels, but a 63% decrease in cardiac UCP3 mRNA levels, together with decreases in mRNA level of enzymes involved in fatty acid metabolism (Essop *et al.* 2004).

In vitro UCP2 gene overexpression using an adenoviral vector resulted in an approximately two-fold increase in UCP2 protein level in isolated rat cardiomyocytes, a partial depolarisation of $\Delta\psi_m$ and decreased cell death, calcium overload and loss of $\Delta\psi_m$ in response to oxidative stress (Teshima *et al.* 2003). No cardiac phenotypic alterations have been reported in mouse models of either UCP2 or UCP3 gene overexpression

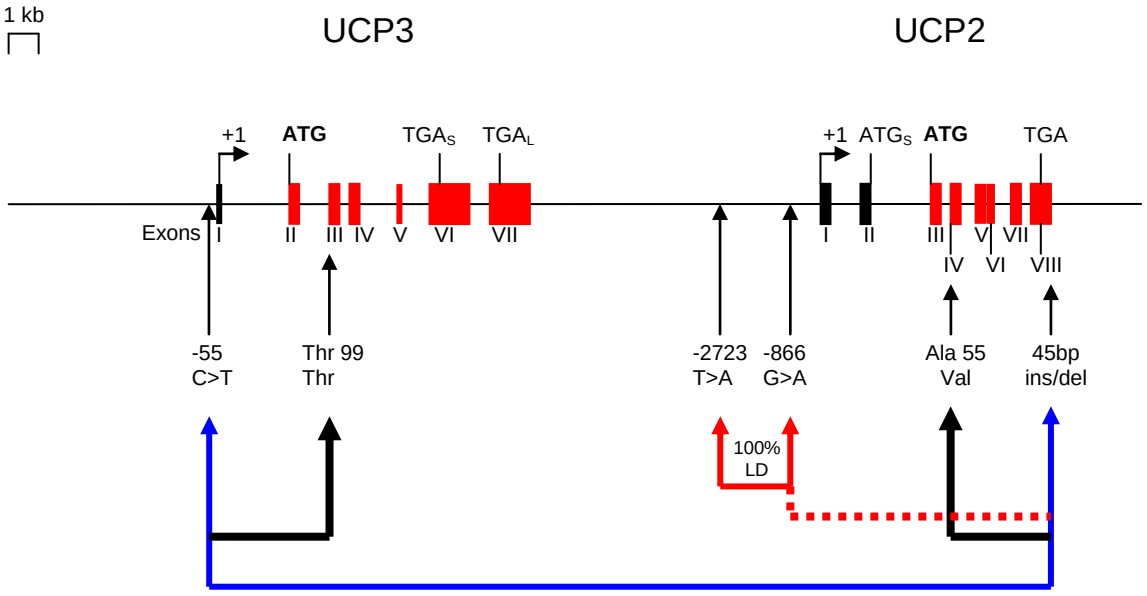
There have been few human studies to date. UCP2, but not UCP3, mRNA was significantly reduced in the left ventricle of patients with severe left ventricular failure (ejection fraction <20%) due to dilated cardiomyopathy (idiopathic, ischaemic or peripartum). This was in concert with a change to a foetal metabolic gene profile with down-regulation of enzymes involved in both lipid and glucose metabolism (Razeghi *et al.* 2001). Murray *et al* demonstrated the presence of both UCP2 and UCP3 protein in the hearts of patients (n=39) undergoing elective coronary artery bypass grafting with impaired LV systolic function (mean 46±10%). Both UCP2 and UCP3 protein

concentrations were positively correlated with fasting plasma free fatty acid concentrations but not with the presence of clinical heart failure, diabetes or drug treatment with ACEi, β blockers or statins (Murray *et al.* 2004).

1.8.5 Genetic variants of UCP2 and UCP3

The UCP3/UCP2 locus has shown positive association with resting metabolic rate (Bouchard *et al.* 1997) and is in a region that has been associated with diabetes and obesity (Fleury *et al.* 1997). Several common variants have been described in both genes (Figure 1.19). In UCP2, a common exon 4 variant (+164C>T) exists that encodes an amino acid change (Ala55Val), in which the heterozygote state (Ala/Val) was associated with higher sleeping metabolic rate in a sample of Pima Indians (Walder *et al.* 1998), but no association with metabolic indices or body mass was found in other ethnic groups (Urhammer *et al.* 1997; Kubota *et al.* 1998; Otabe *et al.* 1998; Yanovski *et al.* 2000). The positive data in the large sample of Pima Indians may have been because of significant linkage disequilibrium (LD) with an exon 8, 3'UTR 45 base pair insertion deletion (ins/del) variant. The heterozygote state was associated with increased sleeping metabolic rate in the Pima Indian sample (Walder *et al.* 1998), higher body mass index (BMI) in South Indian women and higher leptin in obese European women (Cassell *et al.* 1999) and childhood obesity (Yanovski *et al.* 2000). However no associations have been reported with obesity in men (Dalgaard *et al.* 1999) or with the presence of diabetes (Walder *et al.* 1998; Cassell *et al.* 1999).

Figure 1.19. Human UCP2/UCP3 gene locus (chromosome 11q13) and common variants



UCP3 mRNA has two forms: a short (S) form due to translation termination at a premature stop codon in exon 6 or a long (L) form due to termination in exon 7 which codes for UCP3 protein. A premature translation start codon exists in UCP2 exon 2 (ATG_s) which is not thought to give rise to an mRNA species. Common gene variants known to be in linkage disequilibrium (LD) are joined by arrows. Interrupted red line signifies UCP2-866G>A variant explains 70% variation in mRNA transcript ratio seen in exon 8 45bp ins/del heterozygotes.

Recently, a common, functional promoter variant has been described (Esterbauer *et al.* 2001), UCP2-866G>A, which is in complete LD with a second promoter variant (-2723T>A), and explains 71% of the variation in mRNA transcript ratio of the exon 8 ins/del variant. The -866G>A variant is at the junction between negative and positive cis-acting DNA regions, and within a region containing binding sites for hypoxia, inflammation and pancreatic β-cell-specific binding factors. The rare (A) allele has been associated with lower gene transcription (repression) in somatic non-β cells (Krempler *et al.* 2002), but more effective gene transcription in pancreatic β cells with

reduced markers of β cell function (Krempler *et al.* 2002) as well as measures of reduced GSIS (Sesti *et al.* 2003). The A allele has been associated with protection from obesity (Esterbauer *et al.* 2001), but is associated with the presence of diabetes in obese subjects (Krempler *et al.* 2002).

A common promoter variant has also been described in the UCP3 gene (-55C>T) which is in LD with the UCP2 exon 8 ins/del polymorphism (Cassell *et al.* 2000). The variant allele has been associated with obesity in a recessive manner in several studies (Cassell *et al.* 2000; Otabe *et al.* 2000; Halsall *et al.* 2001) and higher LDL-cholesterol in a large sample from the MONICA study (Meirhaeghe *et al.* 2000). Silent sequence changes (C99T, C200T) and rare variants (+5G>A, -155C>T) have been described (Cassell *et al.* 1999; Otabe *et al.* 2000). Rare dysfunctional mutations have been described in African-American patients with morbid obesity and diabetes (Argyropoulos *et al.* 1998).

Table 1.2. Published studies of common variants in the human *UCP2* gene and association with various phenotypes

Author (Year)	*UCP2* variant	Study group (n)	Phenotype	Association
Klannermark (1998)	Ala55Val	Caucasian (55 with metabolic syndrome, 51 controls)	BMI, resting energy expenditure	No association
Urhammer (1998)	Ala55Val	Caucasian (144 juvenile onset obesity, up to 369 healthy controls)	Obesity, insulin resistance	No association
Otabe (1998)	Ala55Val 3' UTR 45bp D/I *Rare:* 5'UTR Exon 1 C^{19}>T 5'UTR Exon 1 C^{27}>G 5'UTR Exon 2 C^{97}>T Exon 4 Gly85Ser	Caucasians: Controls (210 – 226) Obese (44 – 966)	Obesity	No associations
Kubota (1998)	Ala55Val Ala232Thr	Japanese (470)	Obesity, type 2 Diabetes	No associations
Walder (1998)	Ala55Val 3' UTR 45bp D/I	Pima Indians (82) Pima Indians (790)	Sleeping and daily metabolic rate (SMR, DMR) Diabetes, obesity	Ala/Val higher SMR (P=0.007) D/I higher SMR (P=0.02), DMR (P= 0.04) No associations
Cassell (1999)	3' UTR 45bp D/I	South Indian (708) UK Caucasian (247)	Obesity, type 2 diabetes, leptin	II raised BMI in South Indian Women (P=0.018) DI lowest leptin in obese Caucasian women (P=0.006)
Dalgaard (1999)	3' UTR 45bp D/I	Danish Caucasian men (791 obese; 915 lean)	Body mass index	No association

Table 1.2.(contd). Published studies of common variants in the human *UCP2* gene and association with various phenotypes (contd.)

Nordfors (2000)	3' UTR 45bp D/I	Swedish Caucasian (41)	Body fat accumulation during peritoneal dialysis	DD increase in body fat and body weight (P<0.05)
Evans (2000)	3' UTR 45bp D/I	German Caucasians (579)	Morbid obesity, BMI	I allele increased in obesity and higher BMI (P=0.002, 0.005)
Yanovski (2000)	3' UTR 45bp D/I	Children age 6-10 years (105) African American, Caucasian, Asian	BMI, body fat, resting energy expenditure	DD lower BMI, lower body fat.
Esterbauer (2001)	-866G>A	Danish Caucasian (340 obese, 256 lean) German Caucasian (791)	Obesity / BMI	AA reduced risk of obesity (RR 0.61 P = 0.007) GG increases population risk of obesity by ~ 15%
Buemann (2001)	Ala55Val	Caucasian (8 Ala/Ala, 8 Val/Val)	Incremental exercise efficiency (energy expenditure at increasing workload)	Efficiency higher in Val/Val (P<0.05)
Kimm (2002)	3' UTR 45bp D/I Ala55Val	African American (77) Caucasian (75)	Resting energy expenditure	No association

Table 1.3. Published studies of common variants in the human UCP3 gene and association with various phenotypes

Author (Year)	***UCP3* variant**	**Study group (n)**	**Phenotype**	**Association**
Meirhaeghe (2000)	-55C>T	French Caucasian (116 obese, up to 718 controls, 171 type 2 diabetes)	Type 2 diabetes, obesity, lipids	CC increased risk of diabetes (P<0.05), TT higher LDL (P=0.001)
Urhammer (1998)	Tyr99Tyr Tyr200Tyr *Rare*: Gly84Ser 4 intron 3 variants	Caucasian (156 juvenile onset obesity, up to 380 healthy controls)	Obesity, insulin resistance	No association
Otabe (2000)	-55C>T Tyr99Tyr *Rare:* +5G>A -155C>T -439A Ins Exon 4 Gly85Ser	Caucasians: Controls (231) Obese (401)	Obesity	-55TT higher BMI in lean (P=0.03) and obese (P=0.003) No other associations
Walder (1998)	Tyr99Tyr	Pima Indians (82) Pima Indians (790)	Sleeping and daily metabolic rate (SMR, DMR) Diabetes, obesity	No associations
Cassell (1999)	-55C>T	South Indian (540) UK Caucasian (150)	BMI, waist-to-hip ratio, type 2 diabetes	CC lower waist-to-hip ratio in women. No association with BMI, type 2 diabetes and no associations in men.

Table 1.3 (contd) Published studies of common variants in the human *UCP3* gene and association with various phenotypes.

Argyropoulos 1998	Val102Ile Rare: R143X Exon 6 splice-donor mutation	Several ethnic populations Mende tribe of Sierra Leone African-Americans Caucasians	Obesity, type 2 diabetes	Rare mutations associated with morbid obesity and diabetes
Kimm (2002)	-55C>T Tyr99Tyr Val102Ile Tyr210Tyr	African American (77) Caucasian (75)	RER	Tyr210Tyr associated with RER in African-American women

1.9 SUMMARY

The RAAS is important in normal cardiovascular physiology and is implicated in the genesis of CHD and in the perpetuation of heart failure. Heart failure is associated with ultrastructural, metabolic and mitochondrial abnormalities of both cardiac (central) and skeletal (peripheral) muscle, leading to an intrinsic deficiency of fatigue resistance and endurance performance. ACE inhibitors, and other therapies which modulate the RAAS, are of proven clinical benefit in CHD and heart failure, through downstream mechanisms which have not been clearly defined, but may include improvements in mitochondrial function. Genetic studies suggest ACE is involved in the metabolic efficiency of skeletal muscle. Novel inner mitochondrial proteins, UCPs, have been described which uncouple mitochondrial respiration from the generation of ATP and may be involved in muscle contractile efficiency and also may protect against oxidative stress.

1.10 HYPOTHESES & AIMS OF THIS THESIS

1.10.1 Hypotheses

i. The association of low ACE activity with improved cardiovascular mortality and morbidity is mediated, in part, by elevated kinin activity.

ii. The association of low ACE activity with increased skeletal muscle efficiency is mediated, in part, by elevated kinin activity.

iii. Reduction of ACE activity increases the coupling of substrate oxidation to ATP generation within mitochondria of both cardiac and skeletal muscle.

iv. ACE modifies mitochondrial function, either directly or indirectly via downstream effectors such as kinins or angiotensins or through novel pathways such as uncoupling proteins.

v. Mitochondria are an important source of cellular ROS generation and, as such, may be fundamental in the genesis of atherosclerotic disease and cardiac growth.

vi. Uncoupling proteins 2 and 3 have a role in cardiac and skeletal physiology and pathophysiology.

1.10.2 Aims

i. To use the prospective gene-environment approach to test the hypothesis that bradykinin, acting via the constitutive β2 receptor (BDKRB2), is an important regulator of human cardiac physiology and pathophysiology. A functional promoter variant in the *BDKRB2* gene (*BDKRB2 +9/-9*) has been described, in which the -9 allele is associated with higher mRNA and receptor activity. We would expect that the -9 allele would be associated with prospective LV growth in response to physical exercise and lower prospective CHD risk, particularly in hypertensive individuals.

ii. Similarly, common functional promoter variants exist in both the human *UCP2* and *UCP3* genes. The *UCP2*-866A (as opposed to G) allele is associated with lower gene transcription in somatic non-β cells and would be expected to be associated with elevated ROS and increased prospective CHD risk. The *UCP3*-55T allele (rather than C) has been associated with obesity phenotypes and may also be associated with CHD risk. If UCP2 and UCP3 influence cardiac metabolism and ROS generation, then *UCP2/UCP3* genotypes may also be associated with prospective LV growth. We aim to test this hypothesis.

iii. To use prospective gene-environment studies to test the hypothesis that the BDKRB2 and uncoupling proteins 2 and 3 are important regulator of muscle efficiency.

iv. To examine intermediate phenotypes in these studies, including inflammatory markers, in relation to variation in the UCP2/UCP3 gene locus.

v. To examine the effect of the RAAS on mitochondrial coupling by assessing mitochondrial membrane potential and oxygen consumption in whole live cells.

vi. To examine the *in vitro* relationship between ACE and uncoupling proteins.

※ CHAPTER TWO ※

MATERIALS & METHODS

2.1. HUMAN STUDIES

Local ethics committees gave approval for each study. All study subjects gave written, informed consent. I carried out genotyping and data analysis for all studies, unless otherwise stated.

2.1.1. Bassingbourn (Big Heart) 2 Study (BH2)

This was a prospective gene-environment study of the effects of exercise as a stimulus to heart growth. It has been previously shown that intense physical exercise amongst military recruits is an effective model for studying gene-environment interaction (Montgomery *et al.* 1996). Subjects comprised 141 Caucasian male British Army recruits from the Bassingbourn (Big Heart) 2 Study (Myerson *et al.* 2001) who completed 11 weeks of basic army training. Genomic DNA was isolated from peripheral blood leukocytes. Subjects were selected by homozygosity for the ACE I/D gene variant (section 2.3) and randomised to receive either placebo or a low dose (25mg) of the AT_1R antagonist, losartan. Baseline age, height and body mass were recorded. Left ventricular mass and cardiac dimensions were assessed by cardiac magnetic resonance imaging (CMR) before and after training. Body composition was assessed in 117 recruits by whole body MRI and lean body mass calculated. Dr Saul Myerson was responsible for subject recruitment and CMR scanning. For this thesis, subjects were further genotyped for the *BDKRB2* (+9/-9), *UCP2*-866G>A and *UCP3*-55C>T promoter variants (section 2.3).

2.1.2. Second Northwick Park Heart Study (NPHSII)

This is a prospective study of subjects comprising 3012 unrelated healthy Caucasian middle-aged men (mean age 56.1 ± 3.5 years) recruited from nine UK general practices who, at the time of my studies, had been followed for a median of 10.2 years (range 9 days to 13.3 years). At recruitment, subjects were devoid of a history of prior myocardial infarction (MI), cerebrovascular disease, life threatening malignancy or regular medication with aspirin or anticoagulants. A 5ml EDTA blood sample was drawn, from which genomic leukocyte DNA was extracted by salting out (section 2.2). Genotypes were subsequently determined using polymerase chain reaction amplification (PCR) and confirmed by two independent technicians blind to subject outcome, with discrepancies resolved by repeat genotyping (section 2.3). Subjects were subsequently divided according to the principles of Mendelian randomisation at study onset. Entry systolic and diastolic (Korotkoff V) blood pressures (SBP and DBP respectively) were recorded twice with a random zero mercury sphygmomanometer after the subject had been seated for 5 minutes, and mean values used in the statistical analysis. Systolic and diastolic hypertension was defined as SBP≥160mmHg and DBP≥95mmHg respectively, according to the accepted criteria at trial conception (World Health Organisation 1962; Miller *et al.* 1995), and was reported to the subject's general practitioner for action. Baseline demographics and conventional risk factors for CHD were documented. Patients were monitored with annual examinations and regular review of medical records. Those who moved from their recruitment location were similarly followed up. Deaths were recorded through the UK National Health Service Central Register. Information for events presenting clinically / symptomatically were assembled by systematic enquiries through the participating practices, hospitals attended and for fatal events through coroners' offices. CHD events were defined as sudden cardiac death,

symptomatic MI (based on history, ECG, cardiac enzymes and pathology that were assessed by an independent reviewer who classified events by criteria of the World Health Organization (1976), silent MI (the appearance of a new major Q wave on the follow up ECG, using Minnesota codes 1_1,$1_{2.1\ to}$ $1_{2.7}$, $1_{2.8}$ plus 5_1 or 5_2 (Prineas *et al.* 1982)), or coronary revascularisation (either surgical or percutaneous). In addition, any (rare) subclinical events were identified though routine electrocardiography at baseline and the sixth annual examination. Time to first coronary event was recorded, yielding only one event per subject. The likelihood of detecting any component endpoint event is thus considered equal across all sites. At the time of my analysis, there had been 204 events comprising 148 (72.5%) acute MI, 38 (18.6%) coronary revascularisation and 18 (8.8%) silent MI.

2.1.3. University College diabetes and cardiovascular disease study (UDACS)

Consecutive subjects attending the outpatient diabetes clinic were recruited by Dr JW Stephens to the University College Diabetes and Cardiovascular Disease Study (UDACS). Analyses were confined to Caucasian men only (n=465; mean age 61.1±13.3 years). Baseline characteristics are presented in the results chapter 6 (Table 6.17). Presence of CHD was defined as either a history of angina, documented myocardial infarction (MI) or coronary revascularisation (percutaneous or surgical). Genomic DNA was isolated from peripheral blood leukocytes (section 2.2). Venous blood was also drawn from a left forearm vein for measurement of serum ACE activity (section 2.1.9) and assays for assessment of plasma markers of oxidative stress (Appendix 4).

2.1.4. Human physical performance

Healthy subjects were drawn from two sources and were free from significant cardiorespiratory or musculoskeletal disease and were taking no medication. Recruitment and the exercise training programme were supervised by Dr Alun Williams (University of Staffordshire).

Males were drawn from consecutive Caucasian male British army recruits, selected for homozygosity for the *ACE* I/D variant, and studied at the start and end of 11 weeks of basic army training (Williams *et al.* 2000). This training is target-orientated and comprises a mixture of upper body strength, and lower limb strength/strength–endurance exercise of graded workload. Recruits are tested to ensure that they have reached a set of minimum standards at the end of the training period.

Fifty healthy female Caucasian volunteers (aged range 18-39) who had not been involved in any structured training programme during the previous 6 months, were recruited from the student and staff populations of the University of Staffordshire. They underwent an 8 week endurance training program consisting of 3 non-supervised sessions per week at 70-80% of maximum heart rate (as derived from the test of maximal oxygen uptake), with 20 minute sessions for weeks 1-4 increased to 30 minute sessions for weeks 5-8. Subjects were trained to regulate their exercise intensity using a Polar™ heart rate monitor (Polar Electro, Kempele, Finland) and regular contact was maintained throughout training to ensure compliance.

Measures of height and body mass were taken at baseline and after endurance training. Resting blood samples were drawn from a superficial forearm vein before and after

training. Genomic DNA was extracted from 5ml whole blood (section 2.2). Serum was separated from 10ml whole blood by centrifugation at 1500g for 10 minutes and was stored at -20°C until analysis for serum ACE activity (section 2.1.9).

Delta efficiency (DE) is a measure of efficiency of skeletal muscle contraction and is the ratio of the external work performed to the internal energy expended. DE was assessed during cycle ergometry before and after training (Cooke, 1996). Briefly, subjects cycled on an electrically braked cycle ergometer (Lode RehcorTM, Lode, Netherlands) at 60 r.p.m. at external power outputs of 40, 60 and 80 W for 4 min per stage. Expired air was analysed breath-by-breath using an Oxycon analyser (Mijnhardt, Netherlands) and heart rate was monitored telemetrically (Polar Electro™, Polar, Kempele, Finland). A conversion factor dependent on respiratory exchange ratio was applied to the oxygen uptake measured, to give rate of energy expenditure (Brouwer, 1957). DE was calculated as:

$$\frac{\Delta \text{ work performed per min}}{\Delta \text{ energy expended per min}} \text{ x100 \%}$$

2.1.5. British Olympic athletes (Williams, Dhamrait *et al* 2004)

All 1,086 athletes selected for competition by the British Olympic Association were contacted in 1998 (Myerson *et al.* 1999). Of the 495 respondents, 91 were elite runners (48 men, 43 women; 79 Caucasian) who competed over 12 distances ranging from 100m to 100km (sprinters to ultramarathon runners). Genomic DNA was isolated from buccal cells (section 2.2). The twelve distance running events were grouped according to the type of muscle metabolism involved (Newsholme *et al.* 1992): ≤200 m

(predominantly anaerobic), 400–3,000 m (mixed aerobic and anaerobic), and ≥5,000m (predominantly aerobic or endurance trained). This segregation was confirmed by two independent sources (D. Jones, Professor of Sport and Exercise Physiology, Birmingham University, and R. Godfrey, Chief Physiologist, British Olympic Medical Centre). Individuals were genotyped for the *ACE* I/D, *BDKRB2* (+9/-9), *UCP2-866*G>A and *UCP3-55*C>T gene variants. Genotype and haplotype effects were examined by grouping according to distance run.

2.1.6. Systemic inflammatory response to intense physical exercise. The Bassingbourn (Big Heart) 3 Study

This was a prospective gene-environment study of the effects of strenuous physical activity on serum markers of acute inflammation and on serum ACE activity. Every fortnight, new recruits start an 11-week period of basic army training designed to emphasise physical fitness. At the end of this period, recruited soldiers embark on an intensive 48 hour final military exercise (FME), with free access to water to prevent dehydration. Constraints on recruit availability meant that only a maximum of 6 individuals per fortnightly recruitment could be accurately and prospectively studied at the latter time points during basic training in order to take serial blood samples following FME, without disrupting the troop's training program.

The study was performed at the Army Training Regiment, Bassingbourn, UK, with recruitment, venesection and sample handling carried out by Dr David Brull and myself. Between January 2000 and January 2001, consecutive Caucasian male recruits were asked to participate. The study sample consisted of 250 healthy male Caucasian British

Army recruits (mean age 19.4 ± 2.2 years) (Brull *et al.* 2002; Brull *et al.* 2003). Venous blood was drawn as part of the recruits' initial routine medical examination. Blood was also taken for DNA extraction (10ml EDTA) and for serum analysis (4.5ml citrated sample) at baseline, 6 weeks (during a rest week) and immediately (2 hours) after returning from FME. Citrated samples were centrifuged (3000g, 10 min) and the serum aspirated, aliquoted and transported (2hr, 4°C), then immediately frozen at –20°C for subsequent analysis for serum IL6, fibrinogen, CRP and ACE activity.

Serum ACE activity was assayed by Dr Peter Gohlke (U. Kiel, Germany, section 2.1.9). CRP, IL-6 and fibrinogen were measured by staff blind to all subject data in the laboratory of Professor GDO Lowe, Department of Medicine, University of Glasgow, UK. hsCRP was measured on a BN Prospec® System (Dade Behring, Germany), which is an automated analyser using nephelometry. Interassay and intraassay coefficients of variation were <4% and <2%, respectively, with a detection limit of 0.20mg.L^{-1}. IL-6 concentration was measured using a commercial assay (R&D Systems, UK). Interassay and intraassay coefficients of variation were 5% and 3%, respectively, with a detection limit of 0.70pg.mL^{-1}. Fibrinogen concentration was determined using a semi-automated Clauss assay (MDA-180 coagulometer, Organon Teknika, Cambridge), using the manufacturer's reagents and calibrated with the 7th British Standard (NIBSC). Individuals were genotyped for the *ACE* I/D, *BDKRB2* (+9/-9), *UCP2*-866G>A and *UCP3*-55C>T gene variants and data analysed according to genotype.

2.1.7. Republic of South Africa (RSA) study

Subjects were 200 unrelated male black South African volunteers recruited from the Xhosa ethnic group during 2002-3 from a local community centre in Cape Town. Recruitment was carried out by Dr B. Rayner (Division of Hypertension, Groote Schuur Hospital, University of Cape Town, Republic of South Africa). Subjects were healthy and not on regular medication (mean age 39.8±10.0 years). Mean resting blood pressure was SBP 120.9±14.9mmHg and DBP 78.6±11.0mmHg. Venous blood was obtained at rest from the antecubital fossa from all subjects for genomic DNA extraction (section 2.2) and measurement of serum ACE activity (section 2.1.9). I carried out DNA extraction, genotyping and data analysis.

2.1.8. Danish type 1 diabetics hypoglycaemia study

The study consisted of 262 consecutive adult (≥ 18 years old; mean age 45.5±13.7 years; 117 female) type 1 diabetic patients drawn from the outpatients department Hillerød Hospital, Denmark by Dr Ulrik Pedersen-Bjergaard and colleagues (Pedersen-Bjergaard *et al.* 2001). These patients had had diabetes for more than 2 years, with type 1 diabetes defined as need for insulin treatment from the time of diagnosis and unstimulated C-peptide concentrations below 300 $pmol.L^{-1}$ or stimulated (venous blood glucose concentration >12$mmol.L^{-1}$) C-peptide concentrations below 600 $pmol.L^{-1}$. These patients were part of a wider study examining the association of ACE activity and hypoglycaemic events. Blood was drawn for extraction of DNA and for measurement of serum ACE activity using a kinetic assay (Sigma Diagnostics, St Louis, MO, USA). The within-assay and between-assay variabilities were 13% and 11%. I extracted genomic DNA, and performed genotyping and data analysis.

2.1.9. Serum ACE activity

Serum ACE activity was assayed by Dr Peter Gohlke (Institute of Pharmacology, University of Kiel, Germany) by a modified fluorometric method using carbobenzoxy-phenyl-alanyl-histidyl-leucine (Z-phe-his-leu) as a substrate (Depierre *et al.* 1975) for all studies apart from the Danish Hypoglycaemia study. Interassay and intraassay coefficients of variation were 13% and 11%, respectively.

2.2. DNA EXTRACTION

2.2.1. Materials for DNA extraction

All reagents were supplied by Sigma (Poole, UK) and were as follows:

- **1M $MgCl_2$**: 20.33 $MgCl_2$ dissolved in 100ml dH_2O;
- **1M Tris pH 7.5**: 12.11g Tris made up to 100ml in dH_2O, pH corrected to 7.5 and autoclaved;
- **Sucrose lysis mix**: 109.54g sucrose. 5ml 1M MgCl2, 10ml Tris pH 7.5, 10ml Triton-X-100, made up to 1000ml in dH_2O and stored at 4ºC;
- **0.5M Na_2EDTA**: 37.22g EDTA, up to 200ml in dH_2O, adjust with NaOH to pH 8.0;
- **10% SDS**: 10g Sodium dodecyl sulphate , made up to 100ml dH_2O;
- **Nuclear lysis mix**: 1ml Tris-HCl pH 8.2, 2.34g NaCl, 0.4ml 0.5M Na_2EDTA pH 8.0, 10ml 10% SDS, made up to 90ml in dH_2O;
- **5M sodium perchlorate**: 70.24g sodium perchlorate, up to 100ml in dH_2O;
- **TE buffer pH 7.6**: 1.21g Tris, 0.37g EDTA, made up to 100ml dH_2O.

2.2.2. DNA Extraction Protocol from peripheral blood leucocytes

Genomic DNA was extracted from 5ml potassium-EDTA or citrated anticoagulated blood using a salting out method (Miller *et al.* 1988). Samples were meticulously logged and entered into a double-password protected computer database.

2.2.2.1. Cell and Nuclear Lysis

1. 3-5ml blood was added to a 30ml polypropylene tube, to which 20ml of ice-cold sucrose lysis buffer was added.
2. The mixture was inverted several times to ensure thorough mixing.
3. The resulting suspension was centrifuged at 1300*g* (10 000rpm in Sorvall RC5 centrifuge using SA-600 rotor) at 4°C for 10 minutes. The supernatant was carefully decanted and discarded without disturbing the pellet into waste tubes.
4. The pellet was resuspended in 20ml sucrose lysis buffer using a Pasteur pipette, mixed as before and centrifuged at 1300*g* for 10 minutes. The supernatant was carefully discarded once more.
5. The pellet was resuspended in 2ml nuclear lysis buffer using a Pasteur pipette.

2.2.2.2. Deproteinisation & extraction

1. 1ml 5M sodium perchlorate was added and the suspension mixed by inverting at least 10 times by hand. The suspension was transferred to shaker for 10 minutes.
2. 2ml of ice-cold chloroform (-20°C) was added using a glass pipette and the suspension mixed by inversion 10 times by hand.
3. The suspension was then centrifuged at 1300*g* for 3 minutes.

2.2.2.3. DNA precipitation

1. The upper aqueous phase (containing the DNA) was transferred by pipetting to a fresh 15ml polypropylene tube without disturbing the organic phase.
2. 10ml cold (-20°C) 100% ethanol was added slowly *down the side* of the tube to the aqueous phase, and the tube inverted gently to precipitate the DNA.
3. A sterile pipette was used to carefully spool and remove the white "woolly" DNA, washed quickly in fresh 70% ethanol and plunged into a sterile microtube containing 1ml TE buffer
4. The DNA was allowed to dissolve by incubating overnight at 37°C.
5. If no DNA was seen to precipitate, the DNA mixture was cooled to -20ºC overnight. The mixture was subsequently centrifuged at 1300*g* and the supernatant decanted off quickly without disturbing the pellet. The pelleted DNA was allowed to air dry prior to the addition of 0.5ml TE buffer and the DNA allowed to dissolve overnight at 37°C. The resulting DNA suspension was transferred to a sterile microtube.
6. The microtubes were arranged in 96 well boxes and stored at -20ºC.

2.2.3. <u>DNA extraction protocol from saline mouthwash (buccal cells)</u>

Lysis Buffer consisted of 1000ml dH_2O containing 400mM Tris, 50mM EDTA, 150mM NaCl and 1% SDS. DNA extraction from buccal cells was a similar process to that from blood leucocytes, although the vessels used were scaled down to 1.5ml eppendorrf containers:

1. A universal container containing 10ml saline mouthwash was centrifuged at 3000rpm for 3 minutes to pellet the cheek cells.

2. The saline supernatant was discarded and the cell pellet resuspended in 500µl lysis buffer. The resulting solution was transferred to a 1.5ml eppendorrf.
3. 150µl 5M sodium perchlorate was added followed by 500µl chloroform. The mixture was shaken vigorously.
4. The mixture was centrifuged at 1400rpm for 3 minutes, resulting in the formation of an aqueous (DNA) layer above a chloroform (organic) layer.
5. The 500µl aqueous layer was carefully drawn off into a second 1.5ml eppendorrf.
6. 1000µl of 100% ethanol was added and the eppendorrf slowly inverted 10 times to precipitate the DNA.
7. The DNA was pelleted by centrifuging the mixture for 2 minutes at 14000rpm and the supernatant discarded.
8. The DNA was allowed to air dry for 10 minutes.
9. 500µl sterile distilled water was added and the sample left at 37ºC for 24 hours and stored at 4ºC (for 2 weeks) before analysis.

2.3. HIGH THROUGHPUT GENOTYPING FOR COMMON VARIATION IN HUMAN STUDIES

2.3.1. PCR amplification of a target DNA sequence

Genotyping involves amplifying a target DNA sequence by PCR. This requires a unique set of oligonucleotide sequences (termed 'primers') designed to be complementary to either end (5' and 3') of the target DNA sequence and are termed forward and reverse. These are approximately 20 bases long. The temperature (T_m) at which these primers anneal to the target sequence is unique and is a function of the sequence. Genomic DNA is initially heated to 95ºC to 'melt' the DNA into single

strands. The DNA is then allowed to cool and, as it does so, the primers will anneal to the target sequence. A DNA polymerase will then synthesise a single strand from the 3' end of each primer by the addition of nucleotides complementary to the target sequence, taking approximately 3 to 5 minutes depending on the size of the target sequence. To separate the two strands of DNA (target and new complementary sequence) the strands must be again heated to a higher temperature (typically 95ºC). As the reactant mix is further cooled, excess primer anneals to the target sequence. This cycle of annealing, extension and melting is repeated, typically 30 times, thereby exponentially amplifying the target DNA sequence. This process is mechanised by virtue of a computerised thermal cycler block which can rapidly cycle through preset temperatures by means of heater elements and cooling fans.

If the genetic variation under investigation involves the absence or presence of a short sequence of DNA, a so-called insertion/deletion polymorphism, then the amplified alleles will accordingly vary in length. Potentially, these alleles can be directly discerned by gel electrophoresis. Genetic variations involving a difference in a nucleotide ('single nucleotide polymorphism' or SNP) can be discerned utilising restriction endonucleases. These are bacterial enzymes which cut dsDNA at a specific short sequence (usually 4-6bp long) that it recognises. A restriction enzyme can therefore be chosen which will splice at the SNP site of just one of the variant alleles. The resulting differences in DNA fragment sizes can be discerned by gel electrophoresis.

2.3.2. PCR experimental protocol

Dissolved genomic DNA samples were prepared in a 96 well format for subsequent Microtitre Array Diagonal Gel Electrophoresis (MADGE). The order was recorded on

paper and electronic array sheets and the identical format was replicated on DNA storage arrays, DNA working arrays and PCR plates through to the gels used for electrophoresis. DNA was diluted to 45ng.µl^{-1} in 96 well working arrays, leaving at least 4 out of the 96 wells blank. These arrays were centrifuged at 200*g* for 1 minute to ensure the DNA was at the bottom of the wells to prevent cross-contamination. A Finnipippete multichannel dispenser (Life Sciences, Basingstoke, UK) was used to transfer 2.5µl of each sample DNA into a 96 well PCR plate (Omniplate, Hybaid). Positive control DNA samples of known genotype were added to 2 out of the 4 blank wells, leaving two blank wells (negative controls). The plates were centrifuged at 200*g* for 1 minute to ensure the DNA was at the bottom of the wells and the DNA then dried on a 96 well thermal cycler block (MJ Tetrad DNA Engine Thermocycler) at 80ºC for 10 minutes.

Table 2.1 PCR mix for 100 reactions

Reagent (µl)	**Genotype**				
	ACE I/D	*BDKRB2 (+9/-9)*	*UCP2-866G>A*	*UCP3-*55C>T	*UCP2 D/I*
Polmix	200	200	200	200	200
Magnesium 50mmol.l^{-1}	60	100	100	100	100
Forward primer	2.75	8	8	8	8
Reverse primer	1.5	8	8	8	8
Third primer	7.5				
dH_2O	1620	1617	1617	1617	1617
Taq polymerase 5u/µl	4	7	7	7	7
BSA	-	60	60	60	60
W1 (1%)	100				

20µl of PCR mix (unique for each genetic variant; Table 2.1) was added to each well of the PCR plate using an automatic Biohit repeating dispenser (Alpha Laboratories, UK). The PCR mix contains forward and reverse primers, 'Polmix' (containing 50mM KCl, 10mM Tris-HCl (pH 8.3) and 0.2mM of each of the nucleotides dATP, dGTP, dTTP, dCTP), *Thermus aquaticus* (*Taq*) DNA polymerase and magnesium (a cofactor required for DNA polymerase). *Taq* polymerase is a thermostable DNA polymerase (isolated from thermophiles which exist in thermal vents on the oceanic floor) which remains active at the high temperatures required for the denaturation-naturation cycles.

Each sample was overlaid with 20µl mineral oil to prevent evaporation. Plates were then sealed with sterile clear plastic adhesive lids and again centrifuged at 200g for 1 minute prior to PCR amplification on an MJ Tetrad.

2.3.3. MADGE

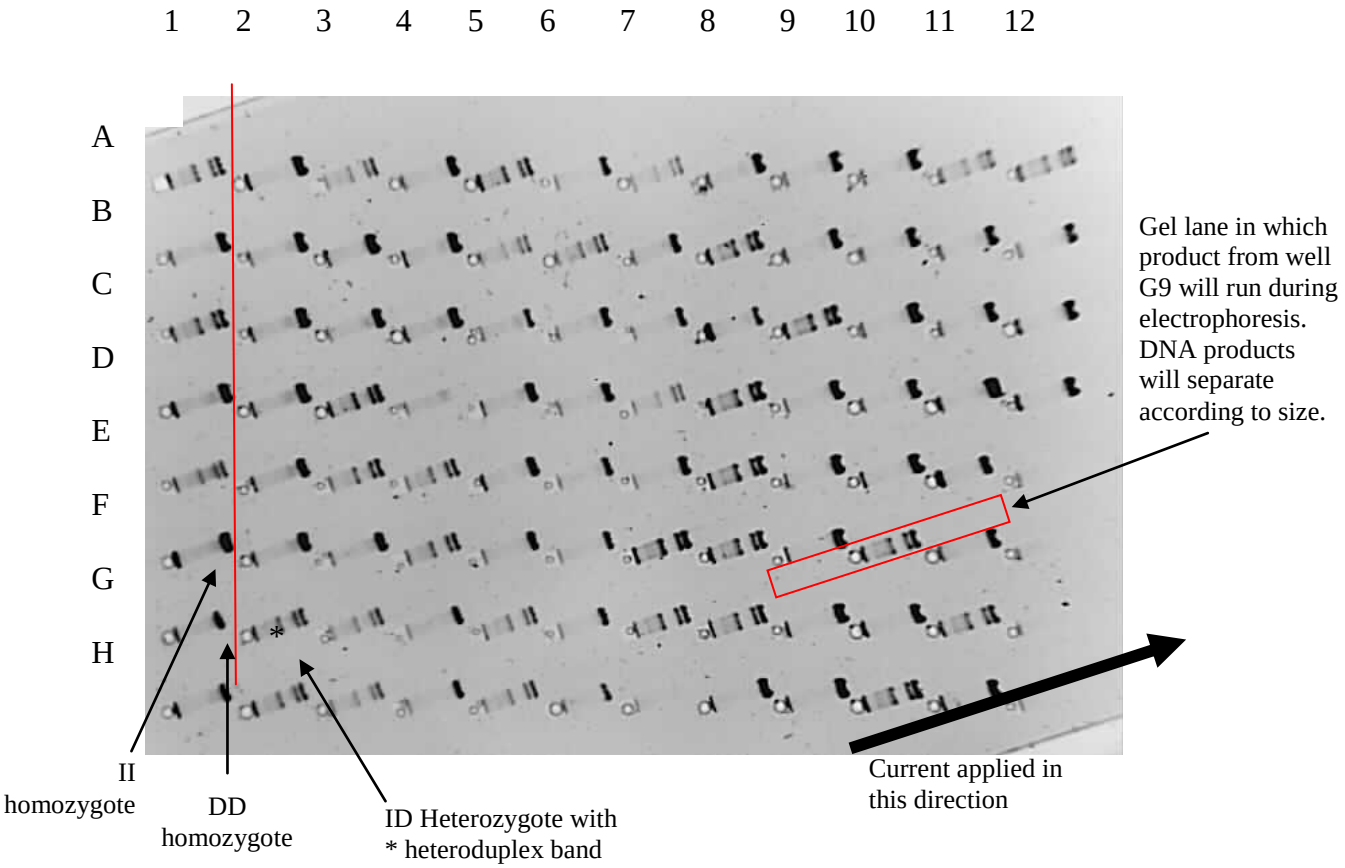
DNA fragments in the PCR product were separated using electrophoresis on a non-denaturing polyacrylamide gel using MADGE (Day *et al.* 1995). MADGE consists of an open arrangement of 8x12 wells each 2mm deep and arranged at an angle of 71.2º to the short axis of the array, but perpendicular to the long axis of the Perspex formers. Thus, the maximum track length is 26.5mm allowing sufficient travel for genotype resolution. All 96 wells can therefore be seen on a single gel (Figure 2.1).

MADGE gels were made up in batches as follows. Glass plates (160x100x2mm) were rigorously cleaned and hand-dried using 70% ethanol. Once dry, 2 ml of γ-methacryloypropyltrimethoxysilane ('sticky silane') was spread across the plates and left to air dry. Silane was used to ensure the gel adhered to the glass plate.

The gel was made using 5ml 10xTBE, 12.2mls 30% acrylamide-bisacrylamide (ratio 19:1, Protogel National Diagnostics, Hull, UK), 32.5mls dH_2O and 150µl N'-tetramethylethylenediamine (TEMED, BDH, Leicestershire, UK). Polymerisation of the gel was initiated by the addition of 150µl 25% ammonium persulphate (APS, BDH, Leicestershire, UK). TBE (10x Tris-Boric acid-ethylenediaminetetraacetic acid) contained: 0.9M Tris, 0.9M orthoboric acid and 0.2M ethylendiaminetetraacetic acid (all from BDH, Leicestershire, UK).

Immediately after the addition of APS, the solution was poured into the perspex MADGE former and a glass plate (silanised face down) was then carefully placed

Figure 2.1. <u>Example of a MADGE gel.</u> 96 genomic DNA samples (including 4 blanks) have been amplified by PCR for the *UCP2*D/I variant. The gel was then loaded with each individual PCR product. The wells are arranged in a 12x9 grid with lanes arranged at 71.2º to the short axis which enables more samples to run on a single gel.



across the liquid surface without trapping any air bubbles. A 0.5kg weight was placed on top of the plate to aid adherence of the gel and the gel allowed to set for 15 minutes. Excess gel was trimmed off and the gel-plate was lifted off the former. MADGE gels were stored in 1xTBE.

The MADGE gels were stained with ethidium bromide prior to loading (1000ml 1xTBE containing 100µl ethidium bromide for 20 minutes). 5µl PCR product was mixed with 2µl formamide dye (98% formamide, 10mmol.l^{-1} EDTA, 0.025% xylene cyanol, 0.025% bromophenol blue) in a fresh round-bottomed 96 well plate. 5µl of this mixture was transferred into the wells of the MADGE gel which had been placed in an electrophoresis tank containing 1L of 1xTBE. 120V was applied across the gel for approximately 50 minutes or until genotypes were distinguishable. The gel was viewed using the Gene Snap gel documentation system under UV light.

2.3.4. Genotyping for variation in the *ACE*, *BDKRB2*, *UCP2* and *UCP3* genes

2.3.4.1. *ACE I/D* Variant

Reagents were optimised (Table 2.1) and published primers (Table 2.2) were used (Evans *et al.* 1994). The PCR conditions were:

95C 5 min

95C 45 sec
54C 45 sec
72C 30 sec
35 cycles

72C 5 min

and yielded two products that could be resolved on MADGE (7.5% gel): an 84bp product (D allele) and a 65bp product (I allele).

2.3.4.2. *BDKRB2* (+9/-9) Ins/Del variant

The Bassingbourn 2 study volunteers were genotyped for the *BDKRB2* (+9/-9) polymorphism by Dr J Erdmann, with products resolved on a heteroduplex gel (Lung *et al.* 1997). For all subsequent studies, genotyping was carried out using a MADGE system with primers (Table 2.2) and reagents (Table 2.1) designed and optimised by Dr John Payne. This yielded a 100bp (deletion) and 109bp (insertion) fragments that could be discerned by MADGE using a 7.5% gel, with a third heteroduplex band in heterozygotes (209bp).

2.3.4.3. *UCP3-55*C>T variant

Published primers were used (Cassell *et al.* 2000). Reagents and conditions were optimised for MADGE (Table 2.1). PCR conditions were as follows:

95ºC for 4mins

95ºC for 40s
55ºC for 30s
72ºC for 1m
30 cycles

72º for 5 mins

The 194bp product was digested overnight at 37ºC with the restriction enzyme *BsuR*1 to yield 110, 64, 20bp fragments for the C allele and 110 and 84bp fragments for the T allele. For 100 reactions, 60µl *BsuR1* was mixed with 300µl buffer R^+ and 640µl dH_2O.

2.3.4.4. *UCP2-866*G>A variant (Esterbauer *et al.* 2001)

Primer sequences were a kind gift from Harald Esterbauer (Department of Laboratory Medicine, Landeskliniken Salzburg, Austria; Table 2.2). The PCR reagents were optimised for a 7.5% MADGE (Table 2.1) and conditions were as follows:

95ºC for 4mins

95ºC for 40s
65ºC for 30s
72ºC for 1min
30 cycles

72º for 5 mins

Mlu1 breaks dsDNA at the following recognition sequence, found only in the G allele:

Mlu1

5'-A↓CGCGT
3'-TGCGC↑A

On digestion, the 360bp PCR product would therefore yield 290+70bp fragments in G allele carriers only. For 100 digestion reactions, 20µl Mlu1 was mixed with 150µl buffer R^+ and this diluted with 320µl dH_2O. 5µl of this mixture was added to 10µl PCR product and incubated overnight at 30C. 5µl of the resulting digestion mixture was mixed with 2µl formamide dye and loaded on an ethidium stained MADGE gel as above.

2.3.4.5. *UCP2Del/Ins* variant

Primers were as previously published (Cassell *et al.* 1999); Table 2.2). Reagents (Table 2.1) and conditions were optimised for 7.5% MADGE:

95ºC for 3 mins

95ºC for 30s
65ºC for 30s
72ºC for 30s
35 cycles

72º for 5 mins

The PCR yielded a 412bp (deletion) and 457bp (insertion) fragments that could be discerned by MADGE using a 7.5% gel, with a third heteroduplex band in heterozygotes.

2.4. CELL CULTURE

Cell culture was performed in a dedicated tissue culture suite (Rayne Institute, UCL). Gloves, cell culture dedicated laboratory coats and overshoes were worn at all times. Sterility was maintained by handling all open cell culture media, cultureware and liquid materials within a class II microbiological safety cabinet with unidirectional laminar downflow (Envair (UK) Ltd). Surfaces were cleaned with 1% Virkon and 70% ethanol solutions. Materials for use within the laminar flow hoods were pre-sprayed with 70% ethanol. Contaminating fluids such as used culture media were removed with suction into 1% Virkon containing vessels and stored therein for at least 24 hours before disposal. Cells were cultured in Galaxy R CO_2 incubators (Wolf Laboratories, UK), humidified and set to 5% CO_2, 37°C unless otherwise stated.

Table 2.2. Forward and reverse primers for genotyping common gene variants.

Gene variant	Forward primer	Reverse primer	Restriction enzyme or 3rd primer
***BDKRB2* (+9,-9)**	5'-TCTGGCTTCTGGGCTCCGAG-3'	5'-AGCGGCATGGGCACTTCAGT-3'	-
UCP3-55C>T	5'-GGATAAGGTTTCAGGTCAGGC-3'	5'-AAGGGATGAGGGAGGAGAAA-3'	*BsuR1*
UCP2-866G>A	5'-CACGCTGCTTCTGCCAGGAC -3'	5'-AGGCGTCAGGAGATGGACCG -3'	*Mlu1*
UCP2 Del/Ins	5'-CAGTGAGGGAAGTGGGAGG-3'	5'-GGGGCAGGACGAAGATTC-3'	-
ACE I/D	5'–CATCCTTTCTCCCATTTCTC-3'	5'-TCGGATTACAGCCCTGATACAG-3'	5'-ATTTCAGAGCTGGAATAAAATT- 3'

2.4.1. General cell culture methods for immortalised cell lines

2.4.1.1. Thawing and seeding cells for culture

A single cryotube (Nunc™, Fisher Scientific, UK) containing 0.5ml frozen cell suspension, stored in the vapour phase of liquid Nitrogen (-170°C), was thawed for 1 minute in a 37°C water bath (Grant Instruments,Cambridge, UK). The cell suspension was diluted into 5ml of warm (37°C) growth medium contained in a 50ml centrifuge tube and then immediately pelleted by centrifuging at 1000rpm for 5 minutes (Eppendorrf centrifuge 5804R). The supernatant was removed and the pellet gently resuspended in 5ml warm growth media (37°C). The resultant suspension was then added to a 75ml culture flask containing 15ml warm growth medium which had been allowed to equilibriate with a humidified 5% CO_2, 37°C atmosphere for 10 minutes in the incubator. Once the cells were adequately dispersed, the flask was placed in an incubator at 37°C, in a 5% CO_2 humidified atmosphere. Every 2-3 days, growth media was suctioned off with a sterile glass pipette and replaced with fresh media.

Adherent cells were split at subconfluency either at a ratio of 1:3 or from 75cm^2 into 175cm^2 flasks. First, growth media was removed by suction, cells washed with PBS, and 1-2ml trypsin-EDTA (GIBCO BRL) added. Once the cells had detached (usually within 1-2 minutes), the cell suspension was removed to a 50ml centrifuge tube and centrifuged at 1000rpm for 5 minutes. The supernatant was then discarded and the cell pellet resuspended in 5ml warmed (37°C) growth medium. The cell suspension was then divided as above or added to a 175cm^2 flask with a 40ml final volume of growth media.

2.4.1.2. Cryopreservation of cells

Following trypsinisation, cells were resuspended in cell freezing media (GIBCO BRL) at a concentration of 10^6cells.ml^{-1} and 0.5ml of this cell suspension was then aliquoted into cryotubes. The cryotubes were immediately stored at -80°C for 24 hours and thereafter moved to -170°C in liquid nitrogen for long-term storage.

2.4.2. Growing characteristics of C_2C_{12} cell line

The C_2 cell line was originated by selective serial passage of myoblasts obtained from the thigh muscle of 2 month old normal mice, prepared 70 hours after crush injury (Yaffe *et al.* 1977). The C_2C_{12} subclone was derived by further passaging (Blau *et al.* 1985) and was shown to differentiate rapidly and to produce extensive myotubes expressing characteristic muscle proteins. The C_2C_{12} cell line was obtained from ATCC (product CRL-1772, ATCC-LGC, Teddington, UK).

Myoblast growth medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) containing 4500mg.L^{-1} D-glucose (Sigma D6429) supplemented with penicillin and streptomycin at a final concentration of 100u.ml^{-1} (GIBCOBRL No 15140-122) and 10% heat-inactivated foetal bovine serum (GIBCOBRL 10106-169). Myoblasts were differentiated into myotubes at confluency by changing to serum deprived (differentiation) media consisting of DMEM containing 4500mg.L^{-1} D-glucose (Sigma D6429) supplemented with penicillin and streptomycin at a final concentration of 100u.ml^{-1} (GIBCOBRL No 15140-122) and 2% horse serum (Sigma Cat No H1138).

2.4.3. Growing characteristics of H9C2

The H9C2 cardiomyoblast cell line was originally derived from embryonic heart tissue using selective serial passage (Kimes *et al.* 1976). The cell line was a kind gift from

Professor D. Yellon (Hatter Institute, UCL). Growth media was identical to that used for C_2C_{12} myoblast culture. Differentiated H9C2 cardiomyoblasts are of a similar phenotype to skeletal myotubes (Kimes *et al.* 1976) and were not used in subsequent experiments.

2.4.4. Culture of primary cells

2.4.4.1. Adult ventricular cardiomyocytes isolated from the Sprague Dawley rat

All experiments were conducted in accordance with the animal care guidelines of the Ethics Committee of University College London, adhering to the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986. Adult ventricular cardiomyocytes were isolated using retrograde perfusion in a modified Langendorff apparatus (Stemmer *et al.* 1991) in the laboratory, and under the supervision, of Professor Yellon (Hatter Institute, UCL). Isolation buffers were prepared as follows:

Buffer 1 (1000ml)

Reagents	mM	$g.L^{-1}$	MWt
Sterile distilled water 1000ml			
Sodium chloride (BDH)	116.3	6.8	58.44
Potassium chloride (BDH)	5.4	0.4	74.55
Magnesium sulphate (BDH)	0.4	0.1	246.48
HEPES (BDH)	20.0	4.76	238.3
Sodium dihydrogen orthophosphate (BDH)	0.9	0.12	137.99
D-Glucose (BDH)*	5.6	1	180.16

Buffer 1 was adjusted to pH 7.35-7.4 and sterilised by autoclaving.

* Sterile filtered D-glucose was added after autoclaving.

Buffer 2 (200ml/heart)

Buffer 1 + 3.3µM EGTA (132µl 5mM EGTA pH 7.4)

1mg.ml^{-1} (0.2g) bovine serum albumin (fraction V fatty acid free)

Buffer 3 (200ml)

Buffer 1 + 1mg/ml (0.2g) collagenase

25µM (50µl of 100mM) $CaCl_2$

Buffer 4 (200ml)

Buffer 1 + 50µM (100µl of 100mM) $CaCl_2$

Buffer 5 (200ml) – Restoration Buffer

Reagents	
Buffer 1 +	
BSA	2g
Na pyruvate (kept at 4C)	10mg
5.0mM Taurine	125mg
2.0mM Carnitine	64mg
1.0mM Creatine	131mg
75µM $CaCl_2$	150µl of 100mM

Buffers were oxygenated with 100% oxygen. Adult male Sprague Dawley rats (SDR) were fed *ad libitum* and kept at a 12hour day/night cycle. SDR (300-350g) underwent terminal anaesthesia with an intraperitoneal injection containing 0.7ml phenobarbitone (60mg.ml^{-1}) and 0.3ml sodium heparin (1000u.ml^{-1}). The heart was rapidly excised, arrested in chilled (4°C) Buffer 1 and mounted on an aortic cannula. The heart was

perfused (in a non-circulating manner) with calcium-free Buffer 2 (warmed to 37°C) for 5 minutes, followed by 1mg.ml^{-1} Type 2 collagenase (Buffer 3) for 10 minutes (37°C). During this time, the heart assumes a globular shape, yellows and softens. Finally, the heart was perfused with 50μM calcium Buffer 4 for 5 minutes (37°C), before the ventricles were excised and minced in oxygenated restoration Buffer 5 (37°C). Dispersed cardiomyocytes were recovered and any remaining intact ventricular tissue underwent further cycles of digestion with collagenase (Buffer 3) at 37°C with subsequent recovery of dispersed cardiomyocytes by aspiration and centrifugation (1000rpm 5 minutes). Isolated cardiomyocytes were resuspended in 25ml oxygenated restoration Buffer 5 and the calcium concentration increased stepwise with aliquots of 50μl 100mM calcium chloride solution at 5 minute intervals to a final concentration of 1.25mM. Cardiomyocytes were then ready for use in subsequent experiments.

2.4.4.2. Isolated human ventricular cardiomyocytes

Isolated human ventricular myocytes were obtained from the laboratory of Dr Sian Harding (National Heart and Lung Institute, Imperial College School of Medicine, London, United Kingdom). Ventricular myocardium was obtained from explanted human hearts comprising recipient hearts with chronic end-stage heart failure (ischaemic or dilated cardiomyopathy). Myocytes had been isolated by enzymatic digestion of left or right ventricular myocardium (Harding *et al.* 1992; Davies *et al.* 1995; Peeters *et al.* 1995) and only rod shaped cells were used in the confocal studies. Cells were incubated in M199 medium (GIBCOBRL) supplemented with human insulin 1μM, creatine 5mmol, taurine 5mmol, carnitine 5mmol, 0.2% BSA (no ascorbate).

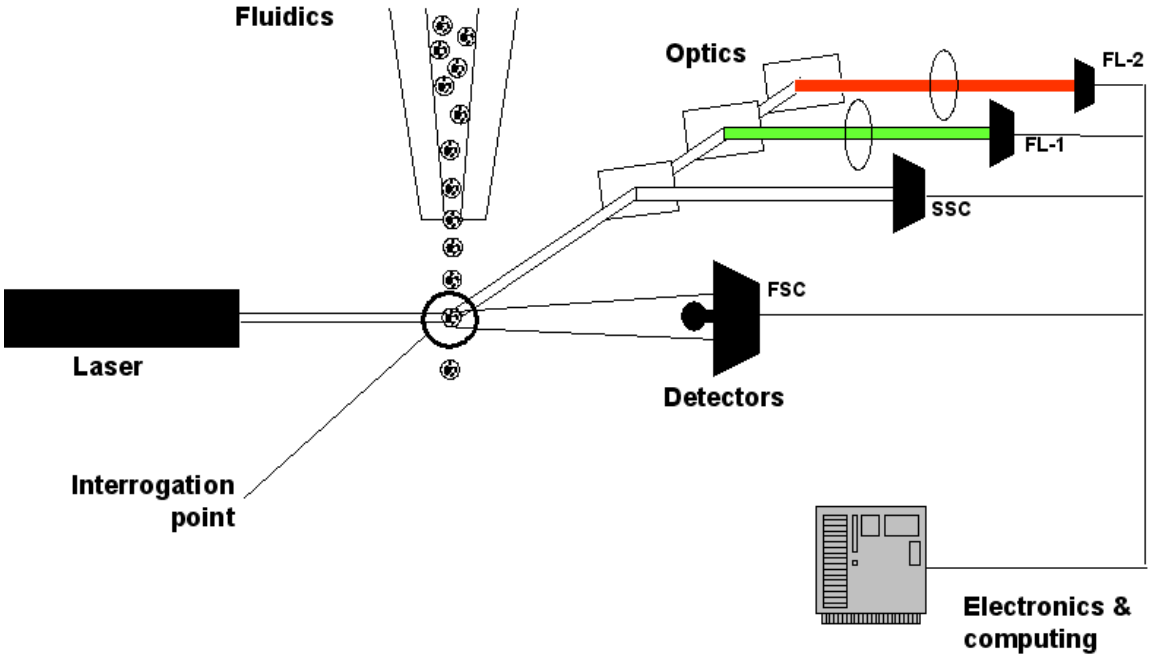
2.4.4.3. Culture of human umbilical vein endothelial cells (HUVECs)

Human umbilical vein endothelial cells (HUVECs) were obtained from PromoCell (Catalogue number C-12200, Heidelberg, Germany) at passage 2. They were cultured in tissue culture vessels coated with 1% gelatine (GIBCOBRL) at 37°C with an atmosphere of 20% O_2 and 5% CO_2. The culture medium was endothelial growth medium (EGM™ BulletKits® Cambrex Clonetics, Wokingham, Cat No CC-3124) containing bovine brain extract (with heparin), human endothelial growth factor, 1000 units of gentamicin and amphotericin B and supplemented with 10% heat-inactivated bovine calf serum (BCS, GIBCOBRL). HUVECs at passage 3-5 at confluence were used in experiments, having been seeded to Nunclon™Δ 6 well culture dishes at a density of 5000 cells.cm^{-2}. Cells achieved 80% subconfluency with standard growth medium and then incubated in serum-free EGM™ for 48 hours to confluency prior to all experiments. For experiments in prolonged hypoxia, cells were incubated in an atmosphere of 5% O_2 and 5%CO_2.

2.5. MEASURING MITOCHONDRIAL FUNCTION IN LIVE CELLS

Novel approaches using fluorescent indicators have been recently applied to the study of mitochondrial function *in vitro*. These approaches coupled to digital imaging fluorescence microscopy or flow cytometry are allowing new insights into mitochondrial physiology and pathophysiology such as apoptosis, free radical generation, intramitochondrial calcium and cell death. In this thesis, both flow cytometry and confocal microscopy have been used to study alterations in mitochondrial membrane potential ($\Delta\psi_m$) and ROS generation. The fluorescent probes TMRM and JC-1 were obtained from Molecular Probes (Eugene, Oregon).

Figure 2.2. Overview of a flow cytometer. Cells are focussed through a laser light at the interrogation point where cell size, structural complexity and fluorescence characteristics are measured and recorded for subsequent analysis (see text).



2.5.1. Flow cytometric measurement of $\Delta\psi_m$

2.5.1.1. Principles of flow cytometry

Flow cytometry is a powerful tool for measuring the physical and fluorescent characteristics of individual cells from a homogeneous population, sampled at high speed (up to 10000 cells.s^{-1}). Fluorescently labelled (e.g. mitochondrial or antibody-conjugated) cells in suspension are passed through an argon laser light within a pressurised fluidic stream. Hydrodynamic focussing ensures that the incident argon laser light (488nm) hits the cells, one cell at a time, The incident laser light is scattered in all directions and is registered on various detectors set at different angles to the incident light. The forward scatter (FSC) detector is set directly in front of the laser and detects cell size. Cells passing through the beam will scatter light in a forward direction

which is proportional to cell size. A detector converts this light into an electrical signal which is further amplified and registered by the attached computer. The side scatter (SSC) detector is set at 90° to both the incident laser light and FSC. SSC detects scattered light as a cell passes through the beam, and therefore gives an indication of granularity or structural complexity of the cell, being particularly important in differentiating leucocytes.

A further series of filters and dichroiic mirrors is also set at 90° to the incident laser light. The filters allow fluorescent light of particular wavelengths to pass through. This range of wavelengths is termed the bandpass width. The first dichroiic mirror allows all light above 560nm to pass directly through, but light below this wavelength is reflected at 90° through a 535-560nm bandpass filter, which only allows green light to pass through. This light will then be detected on the FL-1–green or 'fluorescein' channel. Similar arrangements allow detection of orange-red (FL-2) and far-red (FL-3) light. Again each signal can be amplified to allow detection. The signal is transmitted to a computer for data collection. For a sample of 10000 cells labelled with 2 fluorochromes, 40000 data points will be collected. In these experiments, FACScan and FACSCalibur (Becton Dickinson, Oxford, UK) flow cytometers were used, attached to a G4 computer (Apple Macintosh) using Cell Quest and Cell Quest Pro Software for acquisition and analysis of data. WinMDI (for flow cytometry; version 2.8) freeware was used for further data analyses and data presentation.

2.5.1.2. Flow cytometric analysis of $\Delta\psi_m$

Adherent cell lines were seeded at $0.5x10^5$ cells per well in 6-well plates, grown to confluency and treated with appropriate intervention or vehicle. Thereafter, the cells were washed twice with 2ml warmed Krebs' solution and then incubated with

potentiometric fluorochrome (in Krebs' solution) in the dark at 37°C (5% CO_2). After this period, the cells were washed once in Kreb's solution and detached with 0.5ml trypsin/EDTA. Once detached, trypsinisation was halted by the addition of 0.5ml cooled (4°C) trypsin-neutralising solution and the cell suspension pipetted into a 5ml round-bottomed falcon tube (Becton Dickinson, Oxford, UK) and then placed in ice and kept in the dark for transfer to the flow cytometer and immediately analysed.

Table 2.3. Typical flow cytometer detector settings for analysis of $\Delta\psi_m$ in live cells using JC-1 fluorescent dye

Parameter	Detector	Voltage	Amp Gain	Mode
P1	FSC	E-1	4.87	Lin
P2	SSC	280	5.41	Lin
P3	FL-1	420	1	Log
P4	FL-2	385	1	Log
P5	FL-3	150	1	Log

FSC threshold 400; Compensation: FL-1 minus 1.5% FL2 and FL-2 minus 35.3% FL1

2.5.2. Confocal scanning laser microscopic measurement of $\Delta\psi_m$

Confocal laser scanning microscopy (CLSM) uses similar fluorescent technology to flow cytometry. However, CLSM allows more detailed examination of a magnified field of view, for example a histological section or live single cells grown on coverslips. CLSM uses a conventional, high powered microscope attached to a computer. The sample is illuminated by laser light, from a selection of lasers, which is focussed at a specific point and focal plane within the specimen and scans across the specimen in a series of lines to build up the image. The laser wavelengths can also be selected to match the excitation / emission properties of the fluorescent probes used. The light

emitted by the specimen is captured through an objective lens, and is transmitted through a series of dichroiic mirrors and filters to detectors in a similar manner to the flow cytometer discussed above. Again, the fluorescence signal can be amplified by photomultiplier tubes (PMT) and thereafter converted to a digital signal at a detector which is then sent to the computer. The CLSM is software driven. The software is used to acquire the image. Indeed, the image can be captured at any focal plane, rotated and digitally zoomed before capture. The strength of the laser light can be reduced by reducing the power and also the iris size of the shutter above the specimen. This would also have the effect of improving focus and reduce laser photobleaching of the specimen as well as oxidant and thermal stress. The emitted light signal can be post processed by amplification by the PMTs and digital amplification to aid image capture and also reduce the strength of laser light needed.

The CLSM therefore allows a field of cells to be captured at low magnitude (x20), or single cells at higher magnitude (x40) and intracellular examination (x60 and above). This therefore allows separation of mitochondrial from cytoplasmic signal and single cell-based physiological responses to be tested. However, it is slower and materials more expensive than flow cytometry. CLSM and flow are therefore complementary.

In these experiments, a Nikon Eclipse TS100 inverted microscope attached to a Bio-Rad Radiance2000 confocal system was used. This has 3 lasers: an argon laser capable of emitting laser light at 4 different wavelengths (457, 476, 488, 514nm), a helium-neon laser (543nm) and a krypton laser (637nm). Images were acquired using LaserSharp 2000 software, and analysed using LaserPix and LaserVox software (Bio-Rad, UK) as appropriate.

For live cell experiments, adherent cells were seeded to 2 well quartz-glass chamber slides (Nunc™, Fisher Scientific, UK) at 10^3 cells per well. For cardiomyocyte experiment, chambers were pre-coated with 150µl laminin (30µg.ml^{-1}; Roche, Mannheim, Germany).

2.5.3. Measurement of cellular ROS generation

Cell monolayers were washed with Krebs medium and then incubated with either DCF-DA at a final concentration of 1 µM or DHE at 10 µM. The cells were then washed in Krebs medium and suspended in solution in order to be analysed by flow cytometry. Cells were excited by an argon laser at 488nm and DCF-DA fluorescence measured in the green (FL1) channel and DHE fluorescence in the red (FL2) channel.

To manipulate $\Delta\psi_m$, cells were pre-treated with mClCCP (Mathur *et al.* 2000) or nigericin (Keij *et al.* 2000; Zhang *et al.* 2001). To increase cellular OS, cells were treated with mClCCP (Nishikawa *et al.* 2000), high glucose (Nishikawa *et al.* 2000), rotenone (Duranteau *et al.* 1998; Pearlstein *et al.* 2002), menadione (Roychowdhury *et al.* 2002) or incubated in a hypoxic environment (Pearlstein *et al.* 2002).

2.5.4. Measurement of *in vitro* ACE activity

Cell culture media was aspirated and immediately frozen at -20ºC. ACE activity was subsequently measured by Dr P Göhlke as above (section 2.1.9).

Figure 2.3. 'Optics' folder within the Laser Sharp confocal microscopy software. This gives a graphic representation of the settings used to obtain a confocal image. To the right are the percentage power outputs of the lasers in use as well as the wavelength of the laser light. This light is reflected onto the specimen. Emitted light then passes back through a series of mirrors and then to dichroiic mirrors with subsequent filtering of light to allow detection of certain emitted fluorescence wavelengths (see text). This confocal system is set to detect green fluorescence which is reflected at the first dichroiic mirror (reflects light below 560nm) through a green light filter (515±30nm) to PMT1. Red-orange fluorescence is reflected at the second dichroiic mirror and through the red filter (590±70nm) to PMT2. Far red signal is detected at PMT3.

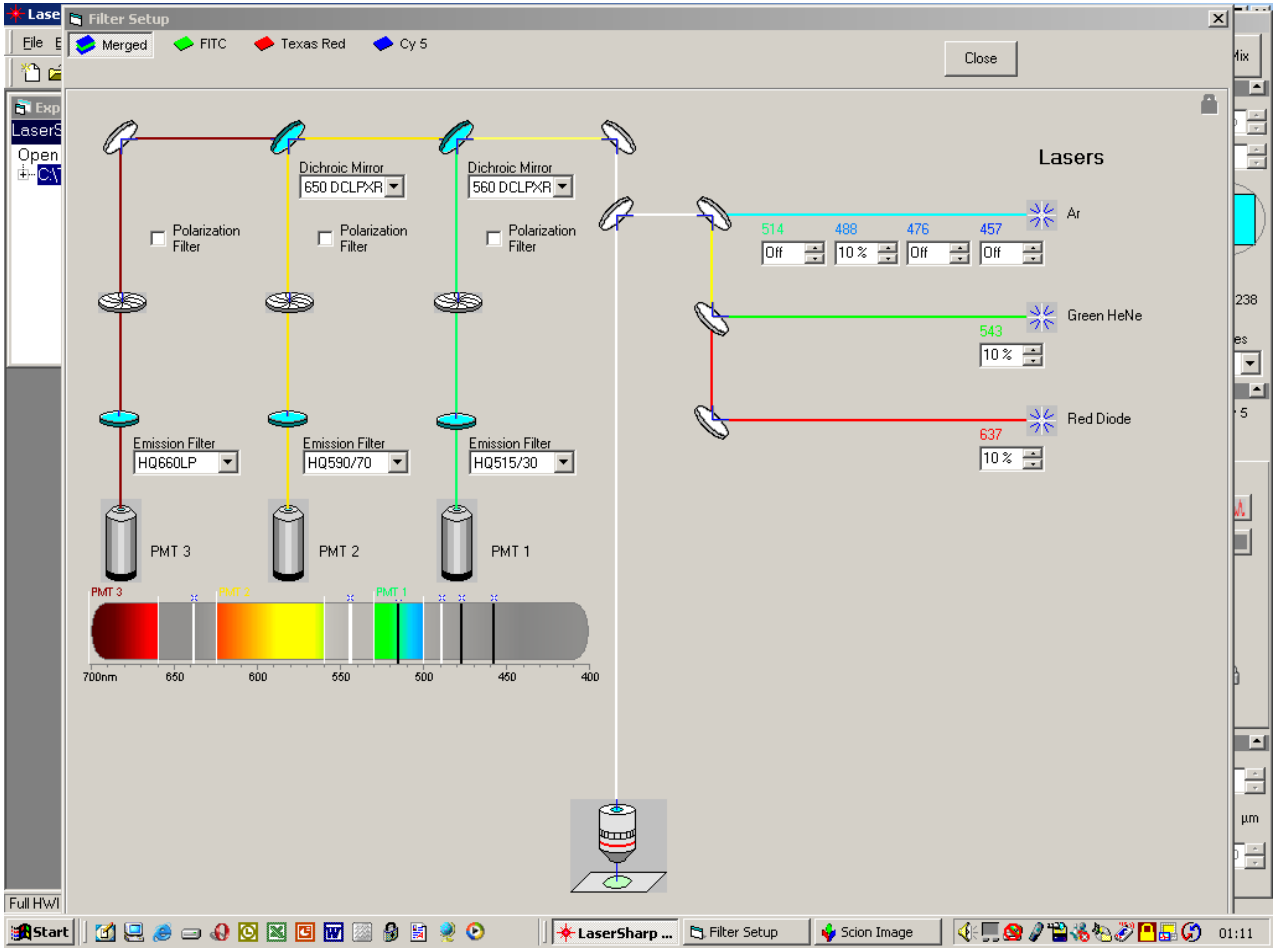
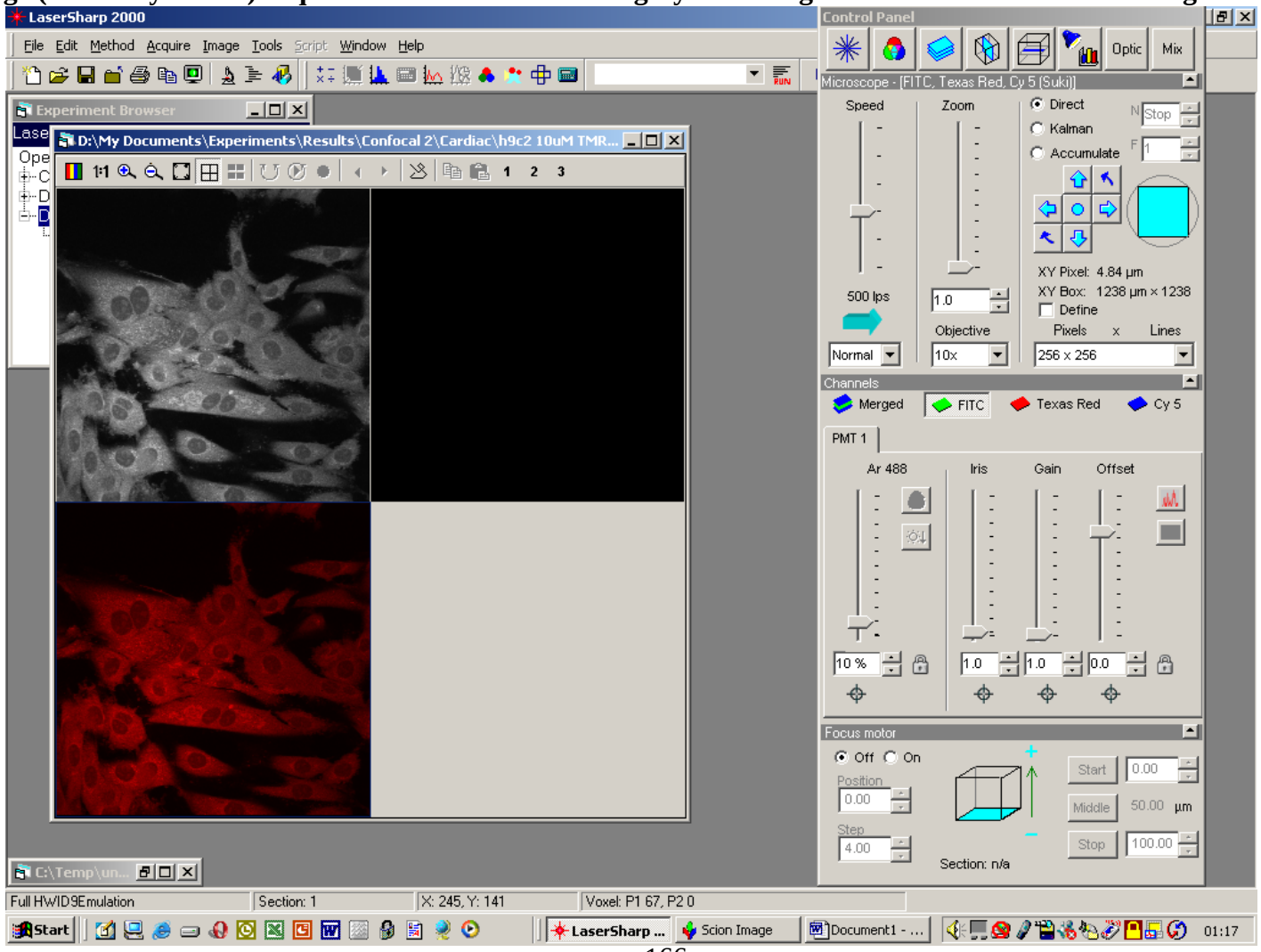


Figure 2.4. Laser Sharp imaging software during live confocal image acquisition. The scanning parameters are in the folder to the right. The laser scanning line speed, laser power, iris size as well as post processing digital zoom and image orientation are seen. The image (H9C2 myoblasts) acquired is to the left with the greyscale image above the red colourised image.

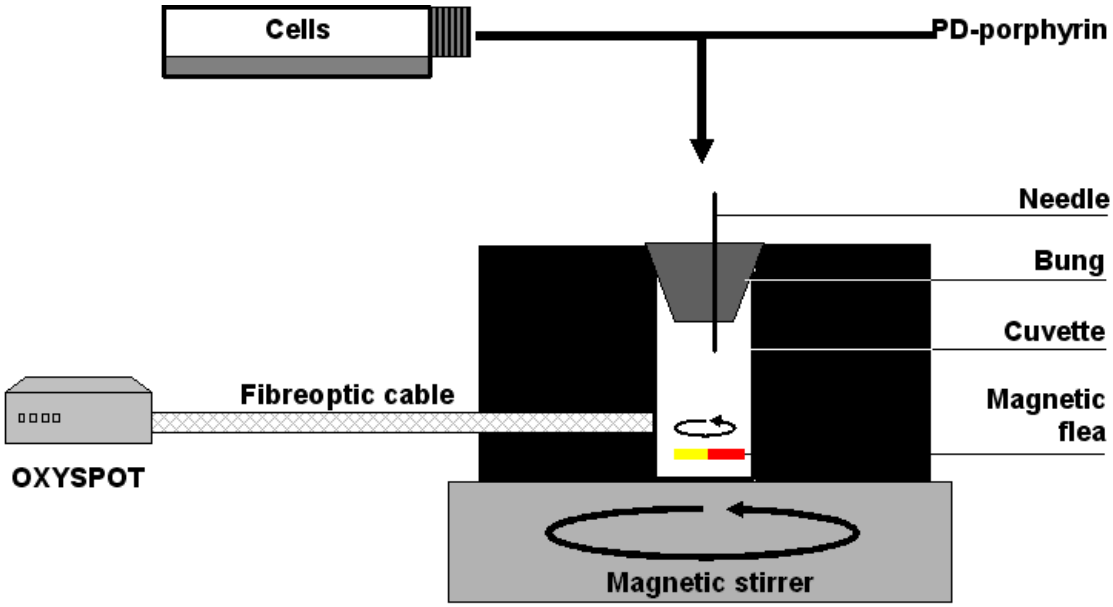


2.5.5. Measurement of cellular oxygen consumption

Cellular O_2 consumption was measured using the OxySpot system (Rosser *et al.* 1998; Knopp *et al.* 1999). Cells were grown to confluency as described. Cells were trypsinised and concentration of cells calculated using a haemocytometer. Cells were resuspended in aerated Kreb's solution containing 10% albumin at $4x10^6$ cells per ml. The cell suspension was placed in a 3 cm^3 disposable glass cuvette, containing 37.5 mg of palladium mesotetra porphyrin (Medical systems, via Digitimer, Welwyn Garden City, UK) and a magnetic stirrer (micro magnetic flea, Fisher Scientific).

The cuvette was then sealed with a rubber stopper to prevent the entry of atmospheric oxygen and placed within a dark chamber with the tip of the fibre optic cable from the OxySpot system (Medical Systems Corp.) abutting against the side. A 22 gauge needle (Sherwood Medical) was pushed through the stopper and filled with medium to form an effective diffusion barrier. Drug additions were through a second needle. Continuous uniform mixing was achieved with the magnetic stirrer. Oxygen tension was measured

Figure 2.5. Cartoon depiction of oxyspot system to measure cellular oxygen consumption (described fully in the text).



by the oxygen dependent quenching of porphyrin phosphorescence (Vanderkooi *et al.* 1986; Lo *et al.* 1996) using the OxySpot. In these experiments the phosphorescence half-life of five flashes delivered by the fibreoptic cable were averaged. Measurements of oxygen tension were recorded every 10 s once oxygen tension had fallen to 60 torr, the upper limit of accuracy using this porphyrin, and were continued until oxygen consumption had ceased, or for 30 minutes. Oxygen consumption at room temperature was calculated from the rate of fall of oxygen tension in the sealed cuvette ($torr.s^{-1}$). Respiratory control ratio was calculated as the uncoupled rate / baseline oxygen consumption rate.

2.6. MEASUREMENT OF mRNA

2.6.1. RNA isolation

All materials were handled with gloves, pipettes and Eppendorrfs autoclaved prior to use for RNA work only, and benchspace cleaned meticulously with 70% ethanol. RNA was isolated from cells grown in 6 well plates using the RNeasy® Mini Kit (Qiagen, Crawley, UK) spin protocol. This entails the use of a high-salt buffer system which allows up to 100 µg of RNA longer than 200 bases to bind a silica-gel-based membrane within an RNeasy® mini column. Briefly 350 µl lysis buffer ('Buffer RLT') was added directly to cells in culture, and resultant cellular suspension homogenised for 2 minutes by pipetting and added to a labelled eppendorrf. 350 µl of 70% ethanol was added and the suspension mixed well. The sample was then added to a spin-column, and centrifuged for 15s at ≥ 10000 rpm. The flow-through was discarded. 350 µl of Buffer RW1 was added to the spin column, and the sample centrifuged for 15s at ≥ 10000 rpm to wash. 80µl DNase I solution (Qiagen, Crawley, UK) was added directly to the silica-

gel membrane of the spin column for 15 minutes followed by a second RW1 wash. The sample was then washed twice with 500 µl Buffer RPE. Finally, the RNA was eluted with 50 µl RNase-free water added directly to the silica-gel membrane with subsequent centrifugation for 1 minute at ≥ 10000 rpm into a fresh eppendorrf. The RNA was stored at -80°C.

2.6.2. cDNA synthesis

The concentration of RNA was determined by measuring the absorption at 260 nm (A_{260}) in a spectrophorimeter. Then 1.5 µg RNA was added to 2 µl random primers (pd$[N]_6$; Pharmacia) and the reaction volume made up to 12 µl with RNase-free dH_2O. The sample was heated to 70°C for 5 minutes and then chilled on ice for 1 minute. 4 µl 1st strand buffer (Invitrogen), 2 µl 0.1M DTT (Invitrogen) and 1 µl 10mM dNTP mix (Gibco BRL) was mixed in gently and the sample centrifuged, followed by incubation at 42°C for 2 minutes. After this stage, 1 µl Superscript™ II reverse transcriptase (Invitrogen) was added, the sample mixed and incubated at 42°C for 1 hour. The incubation temperature was increased to 55°C for 5 minutes with subsequent addition of 1 µl RNaseH (Invitrogen) and further incubation for 10 minutes at 55°C. Resultant cDNA samples were stored at -20°C.

2.6.3. Design of primers

Forward and reverse primers were designed using *Oligo* (v 3.4) and *LightCycler Probe Design Software* (v.1.0, Idaho Technology Inc.). DNA/cDNA sequences were obtained from the National Centre for Biotechnology Information (NCBI) public database (www.ncbi.nlm.nih.gov./PubMed) and exon-exon boundaries identified. Primers were typically 20*mer* and either crossed exon-exon boundaries or the amplified sequence

would have at least one intervening intronic sequence to avoid amplification of genomic DNA. Primers were designed for polymerase chain reaction (PCR) conditions of ≥ 3.0 mmol.l^{-1} for use with the LightCycler®.

Table 2.4. Source of DNA sequences for primer design

cDNA sequence	Accession number	cDNA sequence	Accession number
Human *GAPDH*	J04038	Mouse *UCP3*	AF032902
Human *UCP2*	NM_003355 / AF019409	Mouse *ACE*	M55333
Human *UCP3*	U84763 / AF050113	Rat *GAPDH*	AF106860
Human *ACE*	J04144	Rat *UCP2*	AB017043
Mouse *GAPDH*	NM_008084	Rat *UCP3*	U92069
Mouse *UCP2*	AB012159	Rat *ACE*	U03734

2.6.4. Reverse-transcription (RT)-PCR

RT-PCR was performed on DNA Engine Tetrad thermal cyclers (MJ Research, now Bio-Rad, USA). 1 µl cDNA was used in 20 µl reaction volume. Primers were added at 8pmol per reaction. 'Polmix' buffer containing 0.2mM dNTPs, 50mM KCL and 10mM Tris-HCl (pH 8.3) was added. PCR was optimised using temperature gradients and magnesium titrations (2.0 – 5.0mM, ≥3.0mM for Light Cycler® work). 5 µl RT-PCR product was mixed with 2 µl of MADGE dye, loaded alongside a 1kB DNA ladder (GIBCO BRL) on 1-2% agarose gels (stained with ethidium bromide) in a gel tank containing 1% TRIS. Appropriate current was applied for 30-45 minutes and gels were

visualised with UV light (GeneSnap gel documentation system, SynGene, Cambridge, UK).

2.6.5. Quantitative real-time RT-PCR using the LightCycler®

The LightCycler® (Roche, Mannheim, Germany) was used to perform quantitative real-time PCR. cDNA was amplified using 'FastStart DNA Master SYBR Green I' (Roche). This is a ready-to-use reaction mix, containing FastStart *Taq* DNA polymerase (a modified form of thermostable recombinant *Taq* polymerase, whose activity is blocked by heat-labile blocking groups at room temperature, preventing non-specific binding of primers) and SYBR Green I dye, which binds specifically to DNA and whose fluorescence is proportional to the quantity of DNA. The LightCycler® has a motorised 32 sample carousel, with a heating coil/rapid air cooling system for rapid temperature ramping and microvolume fluorimetry system to measure sample fluorescence. The machine is connected to a computer and data acquired and analysed on LightCycler Software (v.3.0, Roche). Quantification in the log-linear phase of PCR amplification is calibrator normalised with PCR efficiency correction using the LightCycler Relative Quantification Software ('RelQuant' v.1.0, Roche). Standards were prepared from both target and reference (GAPDH) DNA sequences, by serial dilution from a starting known concentration (1:10, 1:100, 1:1000…etc.). The standards were then run in triplicate by RT-PCR, and a standard curve plotted and exported to RelQuant (Appendix 4). The samples were then run in triplicate with target and reference calibrators and negative controls, with results exported for analysis on RelQuant software using the relative standard curves.

2.7. STATISTICAL ANALYSES

I conducted all statistical analyses using Microsoft® Excel (Microsoft Corporation 2002), SPSS for Windows v.10 (SPSS Inc. Chicago, USA) and Graphpad Prism v3.00 (Graphpad Software Inc.) on double-password protected databases. Log-transformations were conducted for data which were not normally distributed. Data are represented as mean ±standard deviation unless otherwise stated. Two-sided tests were performed throughout. For genetic studies, allele frequencies were estimated by gene counting. A χ^2 test was used to compare the observed numbers of each genotype with those expected for a population in Hardy-Weinberg equilibrium. Linkage disequilibrium between sites in pairwise combination was estimated (Chakravarti *et al.* 1984). One way analysis of variance (ANOVA) and linear trend analysis was used to assess the effect of genotypes on baseline characteristics, using either the raw values or log transformed values, as appropriate. One-way analysis of covariance (ANCOVA) was performed to test for confounders. Univariate and multivariate analysis was used to measure significance of association. Pearson's correlation coefficient was used to assess the degree of correlation.

Complex analysis for the NPHSII study was performed by Jackie Cooper and Emma Hawe (departmental biostatisticians, Cardiovascular Genetics, UCL) using 'Intercooled STATA' software (version 7.0, STATA Corporation, Texas, USA) under my direction. Data are reported for those individuals amongst whom high-throughput genotyping was successful. There was no evidence of heteroscedacity between groups, considered via Bartlett's test. Survival analysis with respect to genotypes was carried out using Cox's proportional hazards model, thus allowing for varying follow-up intervals and censoring due to competing events. For this modelling, 'failure time' was taken as the time to the first CHD event. The significance of the parameters in the Cox model was assessed

using the Likelihood Ratio (LR) Test. 95% confidence intervals (CI) for the estimates were calculated from the standard errors assuming a normal distribution. Results are presented as hazard ratios (HR) with their corresponding 95% CI and CHD event rate per 1000 patient years calculated from survival analysis. To allow for differences in baseline data according to age and practice, age was included as a covariate in the model and data stratified by practice (using the strata option in STATA). To adjust for established CHD risk factors, BMI, blood pressure, smoking, diabetes, cholesterol, triglyceride, fibrinogen and CRP were also included as covariates in the model. Interactions were considered as deviations from multiplicative effects in the survival model. The assumption of proportional hazards was checked by testing for a non-zero slope in a generalized linear regression of the scaled Schoenfeld residuals on time (using the stphtest command in STATA). The relative excess risk due to interaction (RERI) was used as a measure of deviation from additive effects. A value of zero represents no deviation from additive effects, and 95% CI were calculated using bootstrapping (Assmann *et al.* 1996).

No adjustment was made for multiplicity of testing. Whilst making such an adjustment reduces the type I error, it leads to increases in the type II error, and fewer errors of interpretation occur when no adjustment is made (Rothman 1990). In all cases a *P* value of <0.05 was considered statistically significant.

※ RESULTS CHAPTER THREE ※

IS THERE AN ASSOCIATION BETWEEN GENETIC VARIATION IN THE BRADYKININ B2 RECEPTOR AND PROSPECTIVE CARDIOVASCULAR PHENOTYPES?

Coronary heart disease (CHD) is caused by a complex interaction between environment (risk) and genetic background resulting in atherosclerosis, a progressive inflammatory disease of the large arteries, associated with the deposition of lipid plaques in the arterial wall characterised by episodic erosion or rupture complicated by thrombosis and remodelling. Left ventricular hypertrophy (LVH) itself is an independent risk factor for CHD (Levy *et al.* 1990) with several determinants including age, race, sex, blood pressure and body mass. Even after correcting for such confounders, twin studies suggest that more than 50% of LV mass in the adult has a genetic component (Swan *et al.* 2003).

CHD and LVH are thus both complex disorders with multifactorial pathogenesis including a strong genetic component (Marenberg *et al.* 1994; Swan *et al.* 2003) which is rarely monogenic. More commonly, both will have a polygenic component, with each functional genetic variant contributing a variable, often minor, amount to overall risk. Without genetic variation, the change in phenotype or disease risk as a result of exposure to the same environmental stimulus (such as smoking or hypertension) would be identical in all humans or directly proportional to the extent of the environmental stimulus.

Prospective gene-environment studies test the interaction between a given environmental stimulus and common sequence genetic variation (polymorphism) with

resultant differences in phenotypic expression. Carriage of each genetic variant is subject to Mendelian randomisation.

ACE activity plays an important role in the development of both LVH and CHD, and pharmacological inhibition of ACE activity has proven benefits in preventing coronary events and in the regression of LVH (Pfeffer *et al.* 1992; Yusuf *et al.* 2000; Fox *et al.* 2003). It has been accepted that many of these observations are due to alterations in angiotensin II generation, but bradykinin is the preferred substrate of ACE (Jaspard *et al.* 1993). A common genetic variation in the human *ACE* gene exists in which the D allele (rather than I) is associated with higher circulating (Rigat *et al.* 1990) and tissue ACE activity (Costerousse *et al.* 1993; Danser *et al.* 1995). The *ACE* D allele has been associated with prospective LV growth (Montgomery *et al.* 1997; Myerson *et al.* 2001; Jamshidi *et al.* 2002) and with CHD (Cambien *et al.* 1992). I tested the hypothesis that genetic variation in the constitutive bradykinin B2 receptor (BDKRB2) may similarly be associated with LVH and CHD using a gene-environment approach in a prospective army training model of LV growth and in a large prospective study of healthy UK men.

3.1 BASSINGBOURN (BIG HEART) 2 STUDY AND *BDKRB2* +9/-9 GENE VARIANT

The baseline characteristics are shown in Table 3.1 of the 141/212 study subjects who completed army training. There was no difference in baseline characteristics between those who entered and those who completed the study. There was no effect of losartan therapy on baseline characteristics or on LV mass change during exercise (Myerson *et*

al. 2001); therefore *BDKRB2* genotype analyses combined both losartan-treated and untreated subjects.

Basic training was associated with a significant prospective increase in LV mass (8.4 ±13.9g $P<0.001$), LV stroke volume (5.0 ±16.4ml $P<0.001$) and RV stroke volume (5.0 ±14.5ml $P<0.001$).

Table 3.1. Baseline characteristics of Big Heart 2 Study subjects

Trait	Mean (SD)
Age (years)	19.6 (2.4)
Systolic blood pressure (mmHg)	117.6 (11.7)
Diastolic blood pressure (mmHg)	66.1 (10.5)
Body mass index ($kg.m^{-2}$)	23.1 (2.2)
Lean mass (kg)	58.8 (6.0)
Adipose tissue (kg)	12.2 (3.9)
LV mass (g)	183.9 (25.1)
BDKRB2 +9+9/+9-9/-9-9 (n) -9 allele frequency	33 / 60 / 16 0.422 [0.356-0.488]

Of the study subjects, 109/141 (77%) were successfully genotyped for the *BDKRB2* (+9/-9) gene variant. The genotype frequency (Table 3.2) was consistent with the Hardy-Weinberg equilibrium (χ^2=1.8; *P*=0.18) and rare (-9) allele frequency (0.422) was similar to previous reports (Houle *et al.* 2000). The (-9) allele frequency was 0.49 and similar to previous published reports for a white Caucasian population (Lung *et al.* 1997; Brull *et al.* 2001). There was no association between *BDKRB2* genotype and any baseline characteristic including LV mass (Table 3.3). In particular there was no association with DBP or SBP.

BDKRB2 genotype was associated with LV growth response (a gain of 4.6 ±11.3g *vs.* 8.3 ±13.1g *vs.* 13.7 ±13.9g for the 16, 60 and 33 individuals of -9/-9 , -9/+9 and +9/+9 genotypes respectively: ANOVA *P*=0.06, linear trend *P*=0.02; Table 3.3, Figure 3.1) and this persisted after adjustment for lean body mass (Brull *et al.* 2001).

BDKRB2 +9/+9 subjects had larger right and left end-diastolic and end-systolic volumes after training, reflected in greater increases in stroke volume related to training, significant for the right ventricle (Table 3.3).

As previously published, *ACE* genotype was significantly associated with prospective LV growth (*ACE* II 4.3 ±14.1g vs. DD 11.5 ±12.9g *P*=0.02). Tests for statistical interaction demonstrated that *ACE* and *BDKRB2* genotypes interacted additively, with growth being greatest amongst those of DD/+9+9 genotype (lowest kinin and BDKRB2 activity), and least amongst those of II/-9-9 genotype (highest kinin and BDKRB2 levels): 15.7 ±14.2g *vs.* –1.4 ±10.7g respectively: *P*=0.009 for comparison of homozygotes: *P*=0.003 across all genotypes; Figure 3.2). Although the numbers were small, subgroup analysis suggested evidence of biological interaction when the

influence of *BDKRB2* genotype was examined separately according to *ACE* homozygosity. Amongst those of II genotype, LV growth rose by –1.4 ±10.7g, 6.2 ±11.6g and 11.5 ±13.6g for those of *BDKRB2* -9/-9, -9/+9 and +9/+9 genotype respectively (*P*=0.02). However, such a gradient was far less clear (9.3 ±10.7g, 9.7 ±14.1g and 15.7 ±14.2g) amongst those of DD genotype, and failed to reach statistical significance (*P*= 0.18).

Table 3.2. Baseline characteristics of Big Heart 2 Study sample by *BDKRB2* +9/-9 gene variant

Trait	*BDKRB2* +/-9 Genotype		
	+9/+9 n = 33	**+9/-9 n = 60**	**-9/-9 n = 16**
Age (years)	19.3 (2.1)	19.6 (2.2)	19.8 (2.4)
Systolic blood pressure (mmHg)	117.0 (11.9)	118.4 (10.7)	121.6 (10.4)
Diastolic blood pressure (mmHg)	63.2 (8.9)	64.8 (10.4)	68.5 (9.2)
Body mass index ($kg.m^{-2}$)	22.9 (1.9)	23.2 (2.2)	22.7 (2.4)
Lean mass (kg)	58.4 (4.1)	59.3 (6.7)	55.8 (6.1)
Adipose tissue mass (kg)	11.7 (4.1)	12.4 (3.8)	11.3 (3.7)

Table 3.3. Training related changes in cardiac parameters by *BDKRB2* +9/-9 genotype and *ACE*/*BDKRB2* haplotypes

Cardiac traits	*BDKRB2* +9/-9 Genotype			*P* ANOVA	*P* Linear
	-9/-9	-9/+9	+9/+9		
LV mass pre-training (g)	180.0 (19.8)	184.2 (27.3)	181.9 (24.6)	0.82	0.93
$LVMI^{LM}$ pre-training ($x10^{-3}$)	3.25 (0.26)	3.13 (0.35)	3.11 (0.39)	0.43	0.27
LV mass post training (g)	184.6 (22.0)	192.5 (28.2)	195.6 (22.8)	0.38	0.19
$LVMI^{LM}$ post training ($x10^{-3}$)	3.26 (0.35)	3.16 (0.36)	3.30 (0.34)	0.24	0.47
Change in LV mass (g)	4.6 (11.3)	8.3 (13.1)	13.7 (13.9)	0.06	0.02
Change in LV mass (%)	2.7 (6.4)	4.8 (7.2)	8.1 (8.5)	0.04	0.01
Change in $LVMI^{LM}$ (%)	0.1 (5.3)	2.3 (7.7)	5.8 (9.1)	0.06	0.02
Change in LV stroke volume (%)	2.36 (27.0)	10.8 (23.5)	13.5 (19.1)	0.28	0.14
Change in RV stroke volume (%)	3.1 (23.1)	8.8 (21.2)	16.5 (18.0)	0.08	0.03
	***BDKRB2* / *ACE* haplotypes ***				
	-9-9 II N=7	**-9-9 DD N=9**	**+9+9 II N=16**	**+9+9 DD N=17**	***P* ANOVA**	***P* Linear trend**
LV mass pre-training (g)	176.0 (21.2)	183.1 (19.4)	181.4 (24.5)	182.5 (25.4)	0.8	0.7
$LVMI^{LM}$ pre-training ($x10^{-3}$)	3.20 (0.32)	3.28 (0.24)	3.10 (0.41)	3.12 (0.37)	0.4	0.1
LV mass post training (g)	174.6 (15.1)	192.4 (24.1)	192.9 (26.2)	198.2 (19.6)	0.9	0.5
$LVMI^{LM}$ post training ($x10^{-3}$)	3.14 (0.35)	3.35 (0.35)	3.21 (0.36)	3.38 (0.31)	0.3	0.3
LV mass change (g)	-1.4 (10.8)	9.3 (10.7)	11.5 (13.6)	15.7 (14.2)	0.07	0.003
LV mass change (%)	-0.4 (6.0)	5.0 (6.0)	6.6 (7.9)	9.4 (9.1)	0.05	0.001
LV MI^{LM} change (%)	-1.9 (5.0)	1.6 (5.3)	3.6 (7.5)	8.0 (10.2)	0.03	0.001

* Homozygotes only shown, statistics calculated on all genotypes

Figure 3.1. Proportional change in LV mass as assessed by CMR according to *BDKRB2* +9/-9 genotype in the BH2 study.

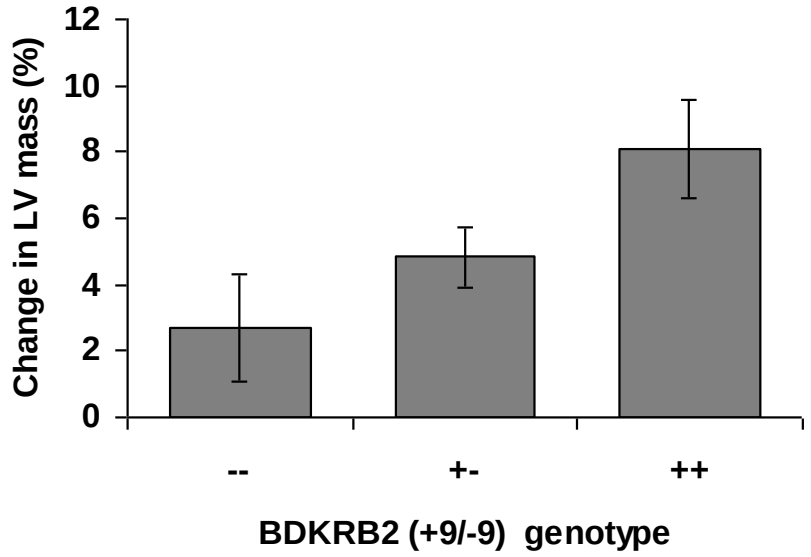
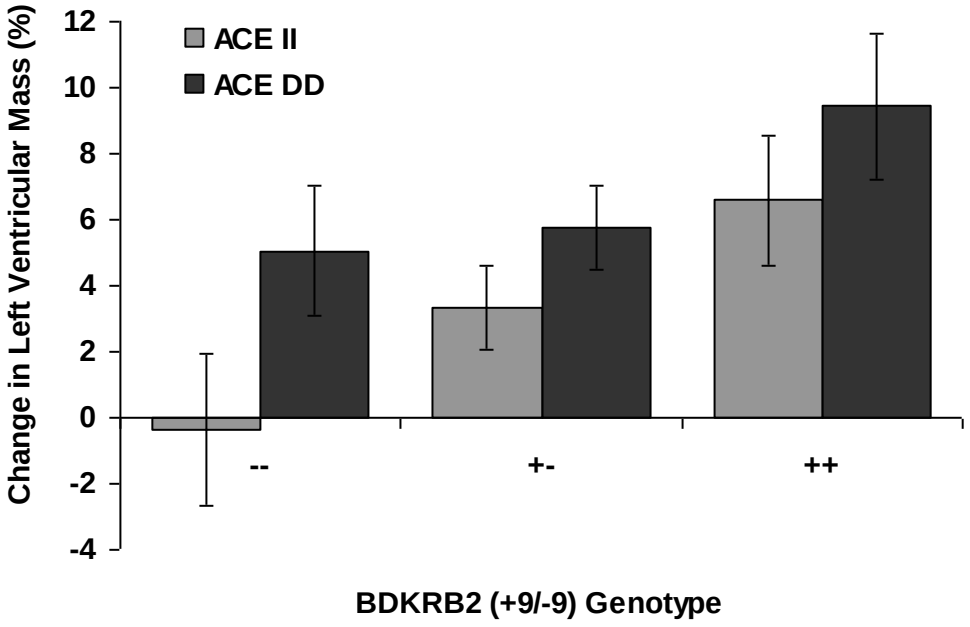


Figure 3.2. Proportional change in LV mass as assessed by CMR in the BH2 study in recruits grouped according to *BDKRB2* +9/-9 and *ACE* I/D genotypes.



3.2 NPHSII AND THE *BDKB2* GENE VARIANT (DHAMRAIT *ET AL.* 2003)

BDKRB2 genotype was obtained for 2364 (87.4%) participants and genotype frequencies were as expected for a population in Hardy-Weinberg equilibrium (χ^2=0.003; *P*=0.96; Table 3.4). There was no difference in baseline characteristics in those genotyped and those not successfully genotyped for the *BDKRB2*. The (-9) allele frequency was 0.49 and similar to previous published reports for a white Caucasian population (Lung *et al.* 1997) and to the BH2 study (Brull, Dhamrait *et al* 2001). Baseline characteristics did not vary by *BDKRB2* genotype. In particular, there was no association with DBP or SBP. The (-9) allele frequency was 0.50 and 0.48 amongst normotensives and subjects with systolic hypertension at baseline (*P*=0.59) and there was no association with the development of hypertension over the follow up period. There was no difference in *BDKRB2* genotype between cases and controls (-9 allele frequency 0.51 and 0.49 respectively, *P*=0.20).

Cardiovascular event probability in relation to increasing systolic blood pressure is shown in Figure 3.3a, demonstrating the expected strong relationship between elevated blood pressure and CHD event rate. For further analysis of the effects of genotype in relation to hypertension, subjects were also divided into normotensive or hypertensive (SBP≥160mmHg, DBP≥95mmHg) groups as defined at the onset of the trial (Miller *et al.* 1995).

Coronary event probability in relation to increasing SBP in subjects divided by homozygosity for the *BDKRB2* variant is depicted in Figure 3.3b. Risk increased significantly with increasing blood pressure in (+9,+9) and (+9,-9) groups, but there was no significant increase risk amongst (-9,-9) carriers (hazard ratio for a 1 standard

deviation increase in SBP 1.58 [1.18-2.11]; *P*=0.002, 1.33 [1.09-1.61]; *P*=0.004 and 1.12[0.82-1.52]; *P*=0.47 for (+9,+9), (+9,-9) and (-9,-9) respectively). There was no significant association between *BDKRB2* genotype and cardiovascular events in the study overall. Amongst normotensives, compared to the (+9,+9) men, the CHD event rate tended to be higher in those carrying one or more (-9) alleles (HR 1.67 [1.05–2.64]; *P*=0.03) and this effect was statistically significant in the larger (+9,-9) heterozygote group normotensive at baseline (HR (+9,+9): 1.0; (+9,-9): 1.70 [1.05–2.74];p=0.03). However, as shown in Table 3.5, hypertension significantly increased the cardiovascular risk in *BDKRB2*(+9,+9) individuals when compared to their normotensive counterparts (HR 3.51 [1.69-7.28]; *P*=0.001 and HR 2.65 [1.31 – 5.38]; *P*=0.007 for systolic and diastolic hypertension respectively), but not in *BDKRB2*(-9,-9) homozygotes (HR 1.25 [0.51-3.04]; *P*=0.63 and HR 1.23 [0.62 – 2.44]; *P*=0.55 for systolic and diastolic hypertension respectively, with *P*=0.21 for the interaction of *BDKRB2* genotype, SBP and risk). Risk estimates for homozygotes remained significant after adjustment for other CHD risk factors (Table 3.5) and when CHD events were confined to acute (fatal and non-fatal) MI events.

Table 3.4. Baseline characteristics of men from NPHS II according to *BDKRB2*(+9, -9) genotype.

Trait	*BDKRB2*(+9, -9) genotype			Probability
	(+9,+9) n = 607	(+9,-9) n = 1183	(-9,-9) n = 574	
Age (years)	55.8 (3.4)	56.1 (3.5)	56.0 (3.5)	0.23
Systolic blood pressure (mmHg)	138.3 (20.0)	137.9 (18.9)	137.9 (19.4)	0.92
Diastolic blood pressure (mmHg)	84.6 (12.0)	84.2 (10.7)	84.6 (12.2)	0.69
Body mass index ($kg.m^{-2}$)	26.2 (3.3)	26.5 (3.6)	26.5 (3.4)	0.24
Current smoking % (n)	29.2% (177)	31.0% (365)	26.1% (150)	0.13
Diabetes % (n)	2.1% (13)	2.1% (25)	2.6% (15)	0.79
Cholesterol ($mmol.l^{-1}$)	5.75 (1.01)	5.76 (1.01)	5.68 (1.00)	0.32
Triglyceride ($mmol.l^{-1}$) *	1.78 (0.95)	1.79 (0.94)	1.77 (0.94)	0.95

Data are mean (SD) unless otherwise stated.
*For triglyceride, the mean is geometric (approximate SD).

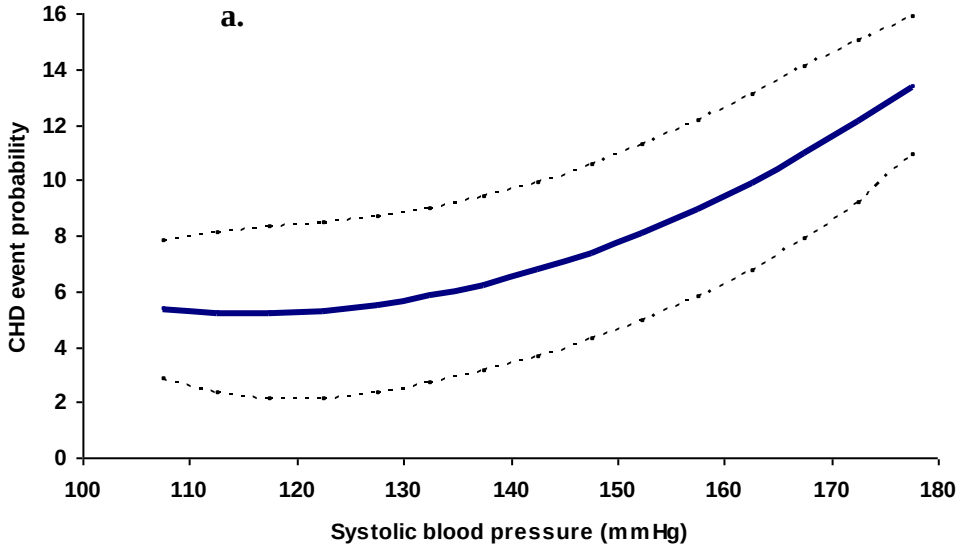
Table 3.5. Coronary heart disease (CHD) events in subjects stratified by presence of systolic hypertension (SBP≥160mmHg) by *BDKRB2*(+9,-9) genotypes. Relative hypertensive risk is CHD risk of hypertensive vs. normotensive subjects within each respective genotype group.

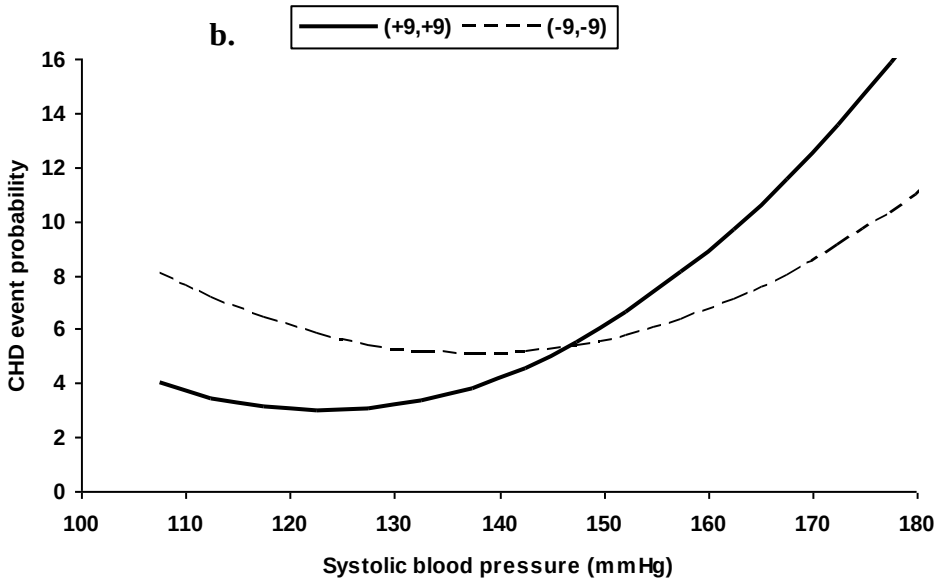
BDKRB2 Genotype	Normotensives SBP<160mmHg Events / Total No. (Events per 1000 patient years)	Hypertensives SBP>160mmHg Events / Total No. (Events per 1000 patient years)	Relative Hypertensive Risk* [95% CI]	Probability	Adjusted Relative Hypertensive Risk† (95% CI)	Probability
(+9,+9)	22 / 514 (4.6)	13 / 93 (16.0)	3.51 [1.69 – 7.28]	0.001	3.17 [1.46 – 6.87]	0.003
(+9,-9)	73 / 1016 (7.8)	22 / 165 (16.0)	1.87 [1.15 – 3.03]	0.01	1.49 [0.90 – 2.46]	0.12
(-9,-9)	33 / 498 (7.2)	6 / 76 (8.8)	1.25 [0.51 – 3.04]	0.63	1.15 [0.46 – 2.88]	0.77

***** Risk adjusted for age and smoking for *BDKRB2* genotypes.
† Risk adjusted for age, practice, smoking, diabetes, body mass index, cholesterol, triglycerides.
SBP indicates systolic blood pressure; *BDKRB2*, bradykinin B2 receptor.

Figure 3.3. <u>Association between systolic blood pressure at recruitment in NPHS II and CHD amongst</u>

a. all subjects
b. men stratified by homozygosity for the *BDKRB2* (+9,-9) variant





3.3 DISCUSSION

The *BDKRB2* (+9/-9) gene variant was associated with both prospective LV growth and prospective CHD risk.

BDKRB2(+9/-9) and prospective LV growth

To date, few genetic loci have been associated with prospective LV growth (Montgomery *et al.* 1997; Myerson *et al.* 2001; Jamshidi *et al.* 2002). That now reported for the *BDKRB2* (+9/-9) polymorphism contributed to 4% of the observed interindividual variability. These data provide important mechanistic insight. Both LV ACE activity (Danser *et al.* 1995) and circulating kinin concentrations (Murphey *et al.* 2000) are dependent on *ACE* genotype. The resultant difference in local kinin concentration acts upon genetically (*BDKRB2* genotype) determined levels of receptor (Lung *et al.* 1997). These data demonstrate a role for *BDKRB2* genotype in determining LV growth response, and suggest that alterations in kinin concentrations (marked by the *ACE* genotype) and kinin-receptor transcription (marked by *BDKRB2* genotype) interact biologically in an additive way. These data support those from *in vivo* animal and *in vitro* cell culture studies that kinins exert antitrophic effects through the BDKRB2 present on cardiomyocytes (Linz *et al.* 1996) and vascular endothelial cells (Rosenkranz *et al.* 1999). In animal models, the antihypertrophic effects of ACE inhibition may be substantially accounted for by increases in local kinin levels (Zhu *et al.* 1996) whereas *BDKRB2* gene knockout mice develop LVH (Emanueli *et al.* 1999). These data therefore support a role for bradykinin in the regulation of human LV growth, and suggest that the effects of ACE could be partly mediated through alterations in kinin concentrations. Nonetheless, care must be taken in the extrapolation of these findings to the genesis of pathological hypertrophy, in which the magnitude of the role of kinins

might differ. These data could also have implications for the treatment of patients with pathological LVH.

The development of LVH is an independent risk factor for CHD (Levy *et al.* 1990) and its development has been shown to be *ACE* I/D genotype dependent (Montgomery *et al.* 1997; Myerson *et al.* 2001). This study has now shown that *BDKRB2*(+9/-9) genotype is also associated with prospective LV growth in healthy adult men undergoing strenuous physical exercise, with a significant interaction with *ACE* I/D genotype. Could *BDKRB2*(+9/-9) genotype also be associated with prospective CHD risk, particularly in individuals in whom a hypertrophic stimulus, such as hypertension, is present? In NPHSII, the cardiovascular risk associated with hypertension amongst middle-aged men was influenced by functional variation in the *BDKRB2* gene: CHD risk climbed steeply as blood pressure increased in the presence of the *BDKRB2*(+9) allele – an effect not identified amongst those homozygous for the *BDKRB2*(-9) allele. The impact of genotype was exemplified by the substantial elevation of risk amongst those suffering systolic hypertension (≥160mmHg, as dichotomously defined at trial inception some 14 years ago). Even when modern definitions are applied (e.g. SBP≥140mmHg) the impact of genotype on risk remained statistically significant. These findings persisted after multivariate adjustment for all potential confounders, and genotype was unrelated to the presence or development of hypertension itself, in keeping with past observations (Gainer *et al.* 2000). These are the first prospective data to demonstrate a role for the BDKRB2 in pathogenesis of human coronary vascular disease.

Genotype strongly influenced the CHD risk associated with hypertension, whilst risk was genotype-independent amongst normotensives. These observations are consistent

with the previously-reported excess frequency of another *BDKRB2* polymorphism (*BDKRB2*-58C rather than -58T) amongst hypertensive (rather than normotensive) sufferers of acute MI (Aoki *et al.* 2001). Such a phenomenon may relate to a genotype-dependent difference in (potentially protective) receptor upregulation in the hypertensive state. Plaque growth and risk of rupture correlate strongly with the levels of pro-inflammatory markers, through which 'classical' risk factors may partly mediate their effects (Farmer *et al.* 2002). Hypertension is similarly associated with a systemic (Koenig *et al.* 1999) and local vascular (Parissis *et al.* 2000) inflammatory response which, through interleukin-driven activation of NF-κβ, induces (potentially protective) vascular BDKRB1 expression (Ni *et al.* 1998). Indeed, β_1 (and, to a lesser extent β_2) receptors are highly expressed in the atheromatous plaque (Raidoo *et al.* 1997). Whether BDKRB2 expression may be similarly modulated is, however, not known.

Altered activity in the human coronary vascular KKS (Kichuk *et al.* 1996) might also underlie these findings. Kallikreins catalyse the conversion of kininogens to autacoid peptide kinins such as bradykinin and Lys-bradykinin, whose subsequent cleavage of the C-terminal arginine residue by carboxy-peptidase yields the fragments des-Arg^9-bradykinin and Lys-des-Arg^9-bradykinin. Intact kinins and their fragments act upon the β_1 and β_2 G-protein coupled cell-surface receptors, which share only 36% sequence homology and differ greatly in their expression and pharmacology (Regoli *et al.* 1997). Thus, the *constitutive* BDKRB2 is expressed in diverse cell types including those of the endothelium, and is responsive to intact kinin peptides (Faussner *et al.* 1999). Conversely, the *inducible* BDKRB1 is activated by C-terminal arginine-deficient kinin fragments (Faussner *et al.* 1999). The (-9) variant of the *BDKRB2* gene is associated with greater *BDKRB2* gene promoter activity (Braun *et al.* 1996) and mRNA expression (Lung *et al.* 1997). These functional effects may underlie the reported associations with

clinical disease states: the *BDKRB2*(-9) allele is associated with symptomatic hereditary angioedema in cases of C1 inhibitor deficiency (Lung *et al.* 1997) and now with lower cardiac physiological hypertrophic responses.

These data offer some insight into the mechanisms of atherosclerosis, with the genotype predicted to lead to *higher* BDKRB2 expression/activity seemingly associated with *reduced* cardiovascular risk in the hypertensive state. KKS protection may be mediated through a number of potential mechanisms including BDKRB2-mediated inhibition of vascular smooth muscle cell growth (Murakami *et al.* 1999), coronary vasodilatation (Su *et al.* 2000), and local nitric oxide synthesis (Kichuk *et al.* 1996). KKS activation also exerts a potent anti-thrombotic role. Factor XII activation and prekallikrein activation participate in a positive feedback loop, leading to rapid bradykinin release (Schmaier 2000). This stimulates formation of tissue plasminogen activator and nitric oxide release, whilst kininogen breakdown products act as antithrombins. KKS activity therefore exerts antiadhesive, anticoagulant, and profibrinolytic effects, and can inhibit platelet activation at low thrombin concentrations (Colman *et al.* 1999). Thus, kinins (and, via this mechanism, the use of ACEi) may prevent coronary thrombosis (Schmaier 2000).

These results also offer important insight into the mechanisms underlying the cardiovascular protective action of drugs which target the RAAS. Treatment with ACEi not only impairs kinin degradation, raising kinin levels *in vivo* (Swartz *et al.* 1980), but also mediates cross-talk between membrane-bound ACE and the BDKRB2, leading to a reduction in BDKRB2 desensitisation, reduced receptor endocytosis and an increase in bradykinin receptor affinity (Minshall *et al.* 1997). Moreover, selective AT_1R blockade also raises tissue kinin levels and may influence crosstalk (through heterodimerisation)

between AT_1 receptors and bradykinin receptors (AbdAlla *et al.* 2000). These data would suggest that these phenomena may contribute to the marked vasculoprotective effects associated with ACE inhibition (Yusuf *et al.* 2000) and, perhaps, with ARB use (Dahlof *et al.* 2002). Such data suggest potential gains in cardiovascular risk reduction from the combined use of ARBs with ACEi, or from the use of newer drugs, such as the neutral endopeptidase-ACEi class, which greatly increase kinin levels.

A drawback of the study is that no detailed information about the specific cardiovascular medication received at baseline and after enrolment is currently available. However, the use of specific RAAS antagonists was uncommon in the timeframe of study (NPHSII was started in 1989), since their putative role in primary prevention had not yet been elucidated. No treated hypertensives, nor patients with heart failure, were included at the onset of the study. The study was initiated well over 20 years ago and, at this time, these were the only two indications for therapy with ACEi, and ARBs were not yet available. Thus, there was no potential for pharmacogenomic interaction at the outset. Similarly, the onset of heart failure (leading to ACEi treatment) would have necessitated development of one of the documented clinical endpoints (such as myocardial infarction), and as such could not have operated as a confounder. Meanwhile, the use for other reasons (such as diabetic nephropathy, or primary cardiovascular prevention) was not accepted during most of this timeframe and would, in any event, have applied to only a few individuals. Thus, it is unlikely that therapy with these (or other) agents could account for any differences observed, given that prescription would have had to have been strongly predicated by genotype to act as a significant confounder- and most indications for treatment would have been documented as a study endpoint. Evidently, however, one might postulate that hypertension itself may have been an active confounder leading to pharmacogenomic

interaction. However, this is unlikely: analysis shows no association of the candidate genotypes with hypertension- whether defined as a categorical variable, or as a continuous trait. In support of the lack of confounding associations, the survival plots indicate divergence by genotype early on in the trial. Nonetheless, pharmacogenomic studies would be warranted given the mechanistic implications of the data.

In summary, I have presented novel associations between the *BDKRB2*(+9) allele (associated with reduced receptor mRNA expression) and both an enhanced *prospective* human left ventricular hypertrophic response and with *prospective* coronary artery disease only amongst hypertensives. This suggests common mechanisms through which the deleterious effects of hypertension on LVH and CHD may be mediated.

※ RESULTS CHAPTER FOUR ※

THE BRADYKININ B2 RECEPTOR GENE AND HUMAN PERFORMANCE.

In Chapter 3, novel associations between genetic variation in the *BDKRB2* and both *prospective* human left ventricular hypertrophic response and *prospective* hypertensive CHD risk were described, providing insight into the mechanism of action of ACEi in heart disease. Pharmacological inhibition of ACE has long been associated with reductions in cardiovascular mortality and morbidity, but it is now clear that ACEi have biological effects beyond simple blood pressure reduction (Flather *et al.* 2000; Dzau *et al.* 2001; Mathew *et al.* 2001), including additional metabolic effects as detailed in Section 1.7. Both animal and human studies suggest an improvement in metabolic efficiency of both skeletal and cardiac muscle following treatment with ACEi (Imaizumi *et al.* 1990; Gohlke *et al.* 1994; Nascimben *et al.* 1995; Sanbe *et al.* 1995; Watanabe *et al.* 1997; Hugel *et al.* 1999; Yamaguchi *et al.* 1999; Gambassi *et al.* 2000; Divisova *et al.* 2001; Onder *et al.* 2002). Could an increase in local kinin activity be responsible for some of these observations? Prospective gene-environment studies are a useful tool that can answer some of these questions.

Global indices of human athletic performance (Rankinen *et al.* 2002), as well as more precise measures of human skeletal muscle function (Thomis *et al.* 1998), are strongly influenced by genetic as well as environmental factors. To date, few genetic loci of influence have been identified (Yang *et al.* 2003). One such is the gene for angiotensin-I converting enzyme (*ACE*) (Montgomery *et al.* 1998; Myerson *et al.* 1999; Folland *et*

al. 2000; Williams *et al.* 2000; Woods *et al.* 2001). As detailed in Section 1.7.1, a common genetic variation in the human *ACE* gene exists in which the I allele (rather than D) is associated with lower circulating (Rigat *et al.* 1990) and tissue ACE activity (Costerousse *et al.* 1993; Danser *et al.* 1995). The *ACE* I allele has been associated with an anabolic response to exercise training (Montgomery *et al.* 1999) and with an increased training-related gain in fatigue resistance (Montgomery *et al.* 1998) and in contractile efficiency (Williams *et al.* 2000), whereas the D-allele has been associated with improvements in strength (Folland *et al.* 2000). Similarly, the I-allele is associated with elite endurance performance at both sea-level (Myerson *et al.* 1999) and at altitude (Montgomery *et al.* 1998), and the D-allele with performance over shorter distances in runners, rowers, and swimmers (Myerson *et al.* 1999; Brull *et al.* 2001).

However, there is some contradictory evidence regarding the influence of the *ACE* gene on endurance performance (Taylor *et al.* 1999; Rankinen *et al.* 2000). Serum ACE activity appears to remain relatively constant during both acute and prolonged exercise with regards to *ACE* genotype (Woods *et al.* 2004; Huang *et al.* 2007), but it is not known whether tissue ACE activity remains constant for an individual or changes during exercise training and if any changes are *ACE* I/D genotype dependent. Furthermore, the *ACE* gene is separated by approximately 370kb from the growth hormone gene cluster, and it has been postulated that some of the observed effects may be due to linkage disequilibrium with genes within this cluster. Phenotype associations with the downstream effector peptides of the RAAS, such as angiotensin II and bradykinin, may help to strengthen any such association with *ACE*. However, there are no such human data relating the BDKRB2 to physical performance.

If the effects of ACE on human skeletal muscle function and its role in influencing more global aspects of performance are mediated through bradykinin, then we might anticipate *BDKRB2* genotype to be similarly associated with muscle function and performance. A prospective gene-environment approach was therefore used to test the hypotheses that common variation in the *BDKRB2* gene is associated with skeletal muscle contractile efficiency and with competitive running distance in Olympic standard athletes.

4.1. HUMAN PHYSICAL PERFORMANCE

Subjects were drawn from two sources: young healthy male British army recruits undergoing basic army training and young healthy female volunteers from the University of Staffordshire who underwent an endurance training programme (Methods section 2.1.4). Of the 131 subjects (89 male, 42 female) who had baseline delta efficiency (DE) measured, 85 (65%) subjects completed training (either basic army or structured). There was no difference in baseline characteristics (male age 19.4 ±2.5yr, height 1.78 ±0.06m, body mass index [BMI] 22.8±2.4 $kg.m^{-2}$; female age 23.2 ±6.2yr, height 1.66 ±0.06m, BMI 24.3 ±3.1$kg.m^{-2}$) between those who did and did not complete training. Of those with complete data, mean DE was 24.6 ±2.6%, and there was no association of gender, age, height, mass or BMI with either DE or change in DE. DE and change in DE were normally distributed (Figure 4.1)

Figure 4.1. Distribution of baseline delta efficiency and training related changes in delta efficiency.

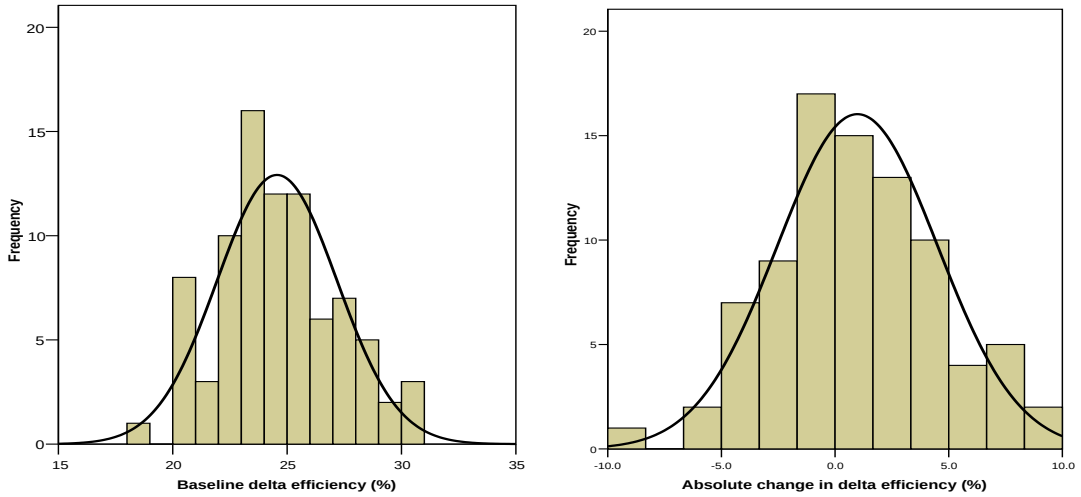


Table 4.1. Baseline delta efficiency according to *BDKRB2* (+9/-9) genotype in the study subjects overall and in those homozygous for the *ACE* I/D polymorphism.

BDKRB2	**Delta efficiency% (sd; n)**		
Genotype	***ACE* DD, n=45**	***ACE* II, n=52**	**Overall, n=115**
+9+9	23.45 (2.81; 8)	24.34 (2.51; 11)	23.84 (2.41; 25)
+9-9	24.06 (3.15; 12)	24.26 (2.41; 32)	24.25 (2.81; 61)
-9-9	25.30 (1.65; 15)	27.41 (2.61; 9)	26.05 (2.26; 29)
+9 allele	23.90 (3.03; 20)	24.28 (2.41; 43)	24.13 (2.69; 86)
ANOVA	0.233	0.005	0.003
Linear trend	0.097	0.013	0.002
+9 allele vs. -9-9	0.104	0.0008	0.001

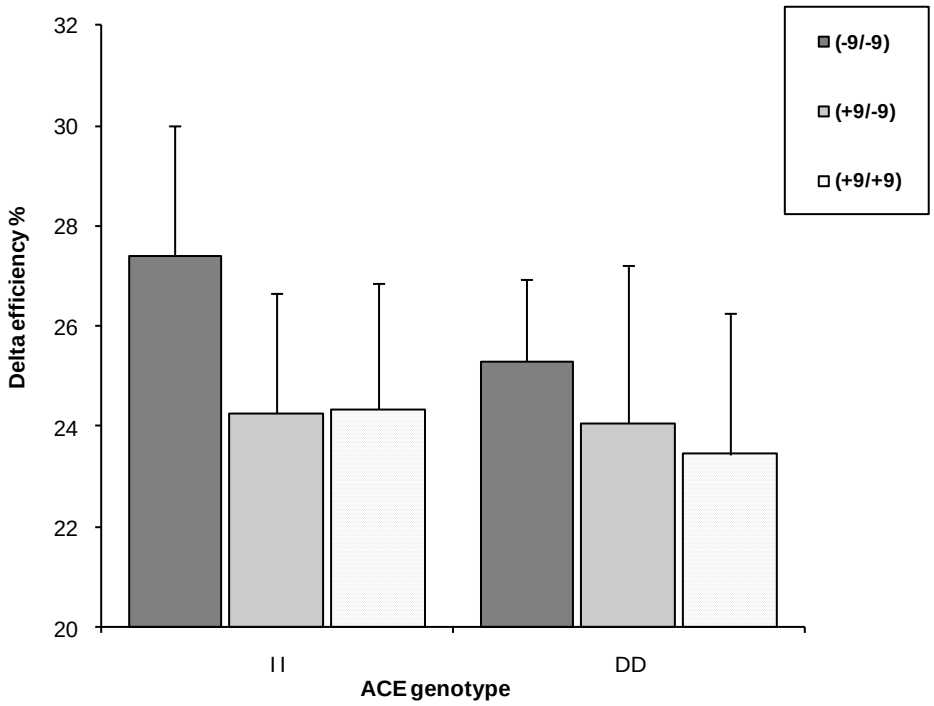
BDKRB2 (+9/-9) genotyping was successful in 115/131 (88%, with 42 female) of subjects with genotype distribution (29 *vs.* 61 *vs.* 25 for -9/-9 *vs.* -9/+9 *vs.* +9/+9) consistent with Hardy-Weinberg equilibrium (Table 4.1) and similar to that previously reported (Braun *et al.* 1996; Lung *et al.* 1997) and to the Bassingbourn 2 and NPHSII studies (Chapter 3). Age, height, mass and BMI were independent of *BDKRB2* genotype.

DE prior to training was highly significantly associated with *BDKRB2* genotype (24 ±3% *vs.* 24 ±3% *vs.* 26 ±2% for those of +9/+9 *vs.* +9/-9 *vs.* -9/-9 genotype respectively, $P = 0.003$ by ANOVA, $P = 0.001$ for +9 allele *vs.* -9/-9 carriers; Table 4.1). This significance increased after adjustment for sex ($P = 0.0008$ for ANOVA), and the data remained significant after adjustment for all demographic data ($P = 0.003$ for ANOVA). Multivariate analysis, including sex as a covariate, suggested that *BDKRB2* genotype accounted for 11% of the inter-individual variability in pre-training DE. There was no association between *BDKRB2* genotype and training related changes in DE.

As previously reported, there was no association between *ACE* genotype and DE at baseline (Williams *et al.* 2000). We sought to examine whether there was any biological interaction between *ACE* and *BDKRB2* genotypes in influencing DE prior to training (Table 4.1; Figure 4.2). Amongst the 45 of *ACE* DD genotype, there was no significant difference in DE according to B*DKRB2* -9/-9 homozygotes (24 ±3% vs. 24 ±3% vs. 25 ±2% for +9/+9 vs. +9/-9 vs. -9/-9, respectively). However, *BDKRB2* genotype significantly influenced DE for those individuals who were of *ACE* II genotype (24 ±3% vs. 24 ±2% vs. 27 ±3% for +9/+9 vs. +9/-9 vs. -9/-9 respectively, $P =$

0.005 by ANOVA, *P* = 0.0008 for +9 allele vs. -9/-9 carriers). DE was associated with *ACE/BDKRB2* ranked genotypes (*P*=0.004 for linear trend adjusted for gender, genotype groups ranked according to Figure 4.2). DE was significantly higher in individuals with the highest predicted kinin receptor activity (*ACE II*, *BDKRB2* -9/-9) compared to lowest kinin receptor activity (*ACE* DD, *BDKRB2* +9/+9; *P* = 0.0007 ANOVA adjusted for gender).

Figure 4.2. Baseline delta efficiency according to *BDKRB2* (+9/-9) genotype in study subjects homozygous for the *ACE* I/D polymorphism



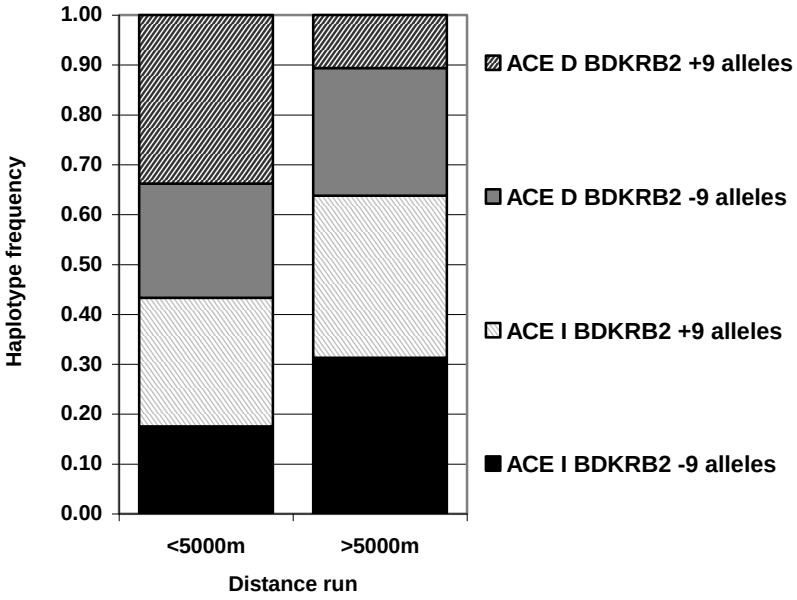
Data are mean ± SD

4.2. BRITISH OLYMPIC ATHLETES

DNA was available for 86/91 (95%) of the original sample of elite runners drawn from athletes selected for competition by the British Olympic Association in 1998 (Methods 2.1.5). Nine athletes were of Afro-Caribbean descent of whom, 7 competed at track disciplines deemed to be predominantly anaerobic (<400m distance) and two competed at middle distance (400-3000m).

The *BDKRB2* (+9/-9) genotype distribution was consistent with Hardy-Weinberg equilibrium and rare (-9) allele frequency (0.46 [0.39-0.54]) similar to previous reports (Braun *et al.* 1996; Lung *et al.* 1997; Brull *et al.* 2001). Amongst the 81 runners, analysis revealed a linear trend of increasing -9 allele frequency with distance run. The proportion of -9 alleles increased from 0.382 to 0.412 to 0.569 for those athletes running ≤200 m (n = 17), 400-3000 m (n = 35), and ≥5000 m (n = 29), respectively (P=0.04 for comparison of ≤5000 m *vs.* ≥5000 m). *ACE* and *BDKRB2* combined genotype analysis demonstrated a significant relationship with distance run (≤5000 m *vs.* ≥5000 m) both overall (P=0.001 Fisher's exact test) and for Caucasians only (P=0.003), with a greater proportion of 'low kinin receptor activity' (*ACE* D and *BDKRB2* +9 allele) activity in events shorter than 5000 m and, conversely, a greater proportion of 'high kinin receptor activity' combined genotypes (*ACE* I and *BDKRB2* -9 alleles) competing in events greater than 5000 m (Figure 4.3).

Figure 4.3. *ACE*/*BDKRB2* combined genotypes by running distance in BOA study



4.3 DISCUSSION

There were highly significant associations between the *BDKRB2* -9 (rather than +9) allele and skeletal muscle metabolic efficiency, and also with endurance athletic performance. Moreover, these associations were greatest amongst individuals with highest kinin receptor activity as marked by the *ACE* I (high kinin ligand generation) allele (Murphey *et al.* 2000) and *BDKRB2* -9 (high receptor expression) allele (Lung *et al.* 1997). Such data support recent linkage analyses which suggest an effect of a locus near to the *BDKRB2* gene on performance-related phenotypes such as cardiac output and stroke volume (Feitosa *et al.* 2002).

Such data are important for two reasons. Firstly, it has been suggested that the *ACE* I/D polymorphism is in strong allelic association with functional variants in adjacent genes

(such as that for growth hormone, GH), and that these (and not ACE phenotype) are responsible for the observed associations with *ACE* genotype (Rankinen *et al.* 2000). However, these data suggest this is not the case, given the demonstration of a similar (and biologically plausible) effect of a downstream receptor. In this regard, these data support past studies suggesting such linkage disequilibrium to be unlikely (Montgomery *et al.* 1998; Williams *et al.* 2000). Secondly, the *ACE* I-allele has been associated with increased metabolic efficiency (Williams *et al.* 2000) and with endurance performance (Gayagay *et al.* 1998; Montgomery *et al.* 1998; Myerson *et al.* 1999), and these are the first data to implicate a specific underlying mechanism. At least some of these associations between *ACE* genotype and performance seem mediated through alterations in kinin activity at the BDKRB2, given that the *ACE* I-allele is associated with increased kinin activity (Murphey *et al.* 2000), and that a genetic marker of higher kinin receptor expression (Braun *et al.* 1996; Lung *et al.* 1997) is now associated with the same performance phenotypes. Association with other genetic variants (such as the -58C>T promoter variant) or haplotypes in the *BDKRB2* gene should be sought as confirmation of these data. Further *in vivo* work is also required to relate the *ACE*/*BDKRB2* haplotypes to kinin metabolism and responses. However, these haplotypic data do support our previous observation relating these *ACE*/*BDKRB2* haplotypes to prospective exercise-induced left ventricular growth (Brull *et al.* 2001).

Skeletal muscle contains a complete KKS (Mayfield *et al.* 1996), can liberate kinins locally (Langberg *et al.* 2002), and expresses functional B_2 receptors (Figueroa *et al.* 1996; Rabito *et al.* 1996). However, it is not yet clear precisely how kinin activity affects the endurance performance phenotypes studied here. Bradykinin generated within exercising skeletal muscle (Langberg *et al.* 2002) may influence muscle blood

flow and skeletal muscle glucose uptake (Wicklmayr *et al.* 1983). In fact, through the BDKRB2 (Taguchi *et al.* 2000), bradykinin enhances insulin-stimulated tyrosine kinase activity of the insulin receptor, with subsequent GLUT-4 translocation in skeletal muscle tissue during exercise (Taguchi *et al.* 2000). BDKRB2 activation can lead to transient rises in inositol 1,4,5-trisphosphate (Rabito *et al.* 1996), which is involved in excitation-coupling of skeletal muscle (Hidalgo *et al.* 1989; Foster 1994) via increases in cytoplasmic calcium (Lopez *et al.* 1991). This process is enhanced by both insulin (Kudoh *et al.* 2000) and by inhibition of ACE (Kudoh *et al.* 2000). Bradykinin-induced nitric oxide (NO•) generation may also modulate mitochondrial respiratory control (Moncada *et al.* 2002). NO• is a vasodilator which, at physiological concentrations, reversibly inhibits cytochrome c oxidase (mitochondrial complex IV) in competition with oxygen (Cleeter *et al.* 1994) and thus reduces oxygen uptake in skeletal muscle and heart mitochondria (Cleeter *et al.* 1994; Poderoso *et al.* 1996). It has been suggested that the interplay between NO• and oxygen allows cytochrome c oxidase to act as an oxygen sensor within cells (Clementi *et al.* 1999). NO donors have also been shown to reversibly inhibit oxygen utilization in rat skeletal muscle mitochondria (Cleeter *et al.* 1994). Tissue and whole animal studies have shown that kinins can suppress oxygen consumption via endogenous NO• production in skeletal (Shen *et al.* 1995) and cardiac muscle (Zhang *et al.* 1997), an effect mimicked by ACE inhibition and prevented by blockade of BDKRB2 (Zhang *et al.* 1997). It may also be that *BDKRB2* genotype influences skeletal muscle fibre type. The relative proportion of Type I (slow-twitch, oxidative) to type IIA (fast, oxidative) and type IIB (fast glycolytic) skeletal muscle fibres has a strong influence on propensity to endurance or sprint performance (Coyle *et al.* 1992), and also influences DE (Coyle *et al.* 1992), whilst *ACE* I/D genotype has recently been associated with fibre type distribution (Zhang *et al.* 2003).

Conversely, such a role for bradykinin does not exclude a contribution for Ang II in mediating the effects of ACE. Chronic Ang II infusion results in profound metabolic cachexia in rodents (Brink *et al.* 1996) with muscle catabolism and increased energy expenditure allied with changes in oxygen consumption (Cassis *et al.* 2002). As a powerful growth factor, it is also necessary for the hypertrophy of skeletal muscle in response to mechanical load (Gordon *et al.* 2001).

Further studies are required to confirm these observations amongst other comparable groups of athletes. The association of genotype with relative ranked performance amongst endurance athletes should also be sought. Such studies should also include those of other ages and race. The small number of *ACE* ID heterozygotes (n=18) restricted the ability to assess the combined *ACE*/*BDKRB2* genotypic association with DE within this group. This inability in no way weakens the observations, but further studies should be performed if allele co-dominant influences on genotype response are to be sought. In addition, no single gene will determine (exclusively) propensity to a given sporting discipline, and any association does not demonstrate the underlying mechanism of causation. By combining association study of phenotype class with a mechanistic study, we have attempted to overcome such problems. However, these data do suggest that bradykinin, acting via the BDKRB2, has a role in regulating skeletal muscle performance. The implications of such findings go beyond sports alone, and may extend to the management of patients with cardiovascular, respiratory and metabolic diseases, in which muscle function is adversely affected.

※ RESULTS CHAPTER FIVE ※

THE EFFECT OF ACE-INHIBITION ON MYOCYTE MITOCHONDRIAL FUNCTION

Low ACE activity, such as that resulting from ACEi treatment in animal studies (Gohlke *et al.* 1994; Nascimben *et al.* 1995; Sanbe *et al.* 1995; Watanabe *et al.* 1997; Hugel *et al.* 1999; Divisova *et al.* 2001) or as marked by the *ACE* I allele in human genetic studies (Montgomery *et al.* 1999; Myerson *et al.* 1999; Williams *et al.* 2000), is associated with enhanced metabolic efficiency. However, the mechanisms responsible have yet to be elucidated. I have hypothesised that some of these associations may be through alterations in mitochondrial metabolism. Uncoupling proteins are nuclear-encoded inner mitochondrial membrane proteins which are associated closely with the electron transport chain and may serve to negatively regulate mitochondrial ATP production by dissipating $\Delta\psi_m$ (Pecqueur *et al.* 2001; Echtay *et al.* 2002). As such, UCPs may be potential mediators of any putative mitochondrial actions of ACE.

To test these hypothesis, *in vitro* assays were established for measuring $\Delta\psi_m$ in live cells by direct (confocal microscopy) and indirect (flow cytometry) visualisation using fluorescent mitochondrial potentiometric probes. The effect of ACEi treatment on $\Delta\psi_m$ was tested in both skeletal and cardiac myocytes and in both cell lines and primary cell cultures. The effect of ACEi on skeletal myocyte UCP3 expression was measured. The response of cellular oxygen consumption to ACEi was also studied.

5.1 FLOW CYTOMETRY

5.1.1. Baseline characteristics

Preliminary experiments were conducted with C_2C_{12} undifferentiated myoblasts and preadipocytes because of their ease of culture. Cytometer PMT settings (voltage, gain) were adjusted until C_2C_{12} blasts were localised on the linear FSC (size) and SSC (granularity) scales (Figures 5.1, 5.2). The FSC threshold was set at 200 to exclude the majority of cellular debris. The contour plot (Figure 5.2) demonstrates the homogeneity of spatial characteristics of this population.

Figure 5.1. Dot plot representation of murine C_2C_{12} myoblasts cells analysed by flow cytometer, with granularity (Side scatter SSC) plotted on the y-axis vs. cell size (Forward scatter FSC) on the x-axis. Each dot represents one cell event (N= 10000).

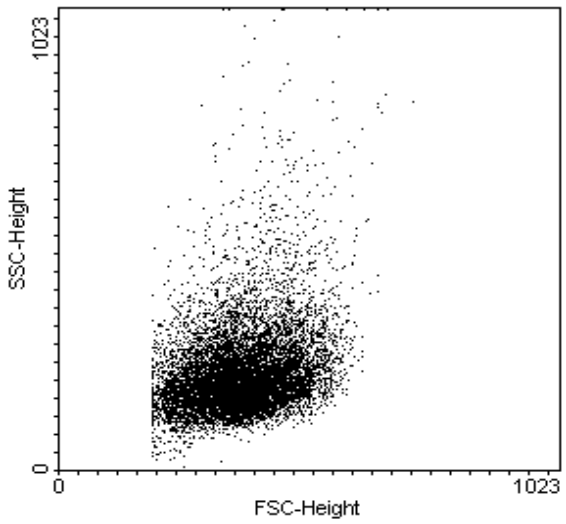
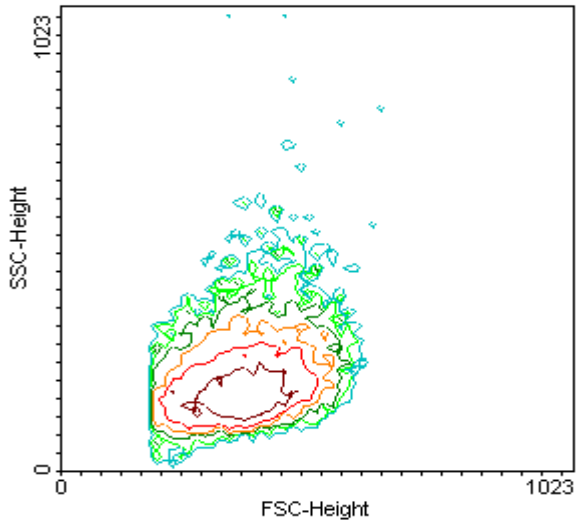


Figure 5.2. Flow cytometric contour map of the same C_2C_{12} blasts (events of similar occurrence are joined by lines of similar colour). Most frequent areas (highest density in Fig 5.1) are brown to least frequent, blue. (N=10 000)



5.1.2. TMRM mitochondrial probe

5.1.2.1. Dose titration

C_2C_{12} myoblasts were incubated with the mitochondrial probe TMRM and red (FL-2) fluorescence analysed. Figure 5.3 shows a three-dimensional representation of spatial characteristics (FSC on x-axis, SSC on y-axis) and mitochondrial TMRM fluorescence (TMRM z-axis). Figure 5.4 shows the same population of cells on a histogram plot, with TMRM fluorescence normally distributed on a logarithmic scale. Compensation was adjusted to ensure that detected FL-1 (green fluorescence) from spectral overlap was within the first log decade.

Figure 5.3. A 3D density plot of TMRM staining characteristics of C_2C_{12} blasts. Size (FSC) and granularity (SSC) are on linear x- and y-axes, respectively, and TMRM red fluorescence on the logarithmic z-axis.

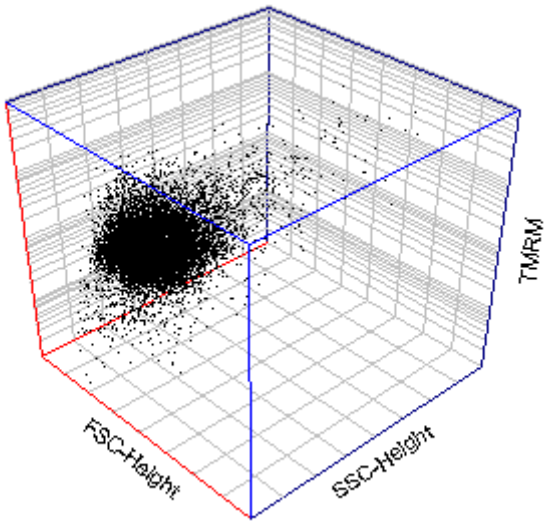
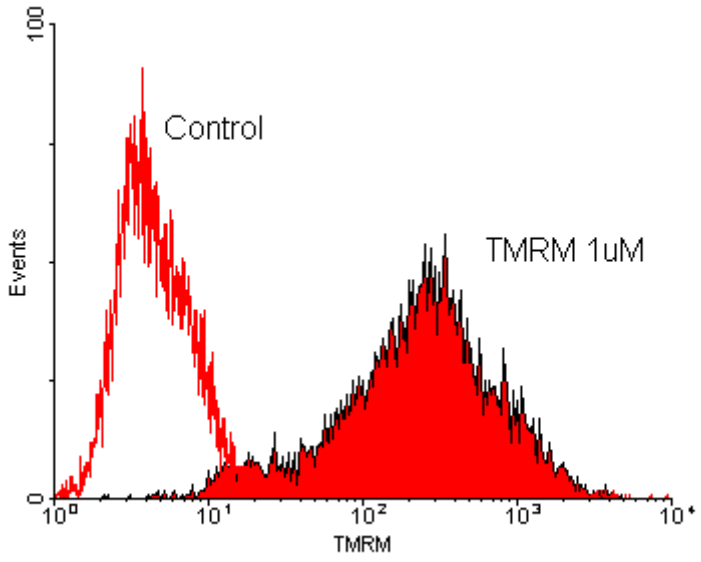
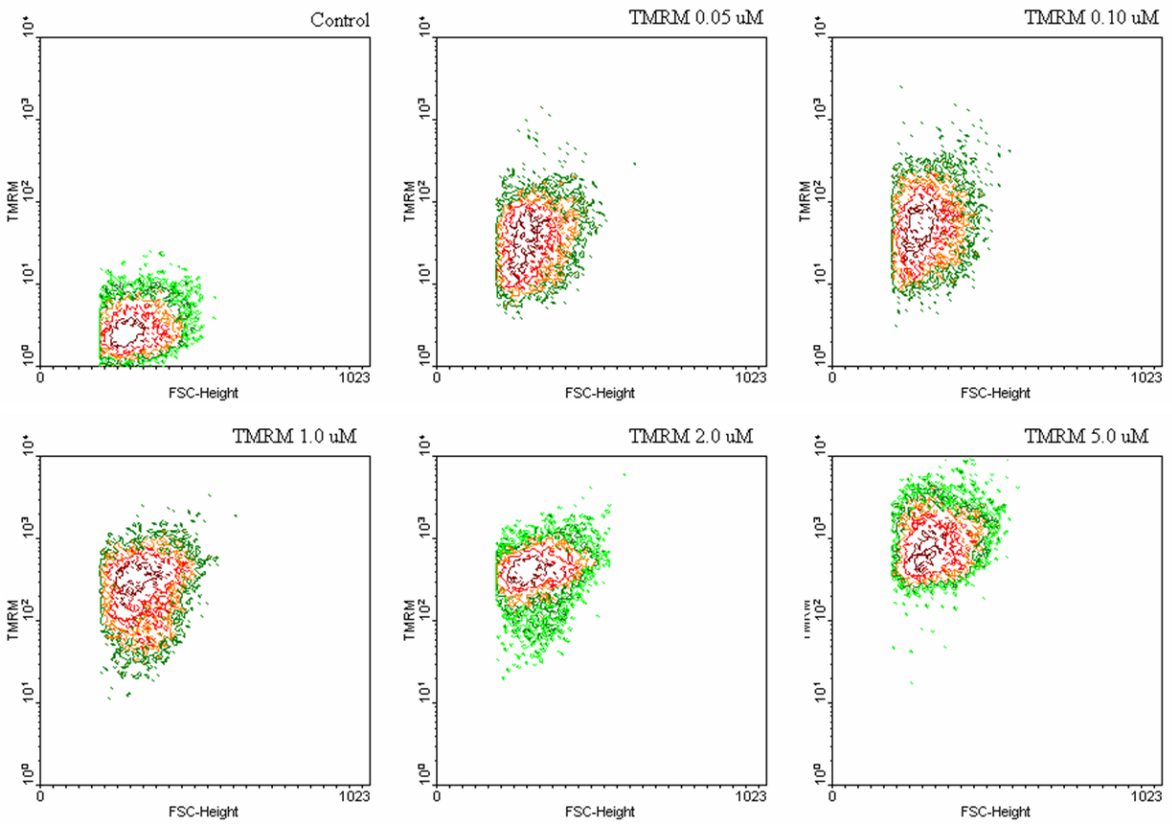


Figure 5.4. TMRM fluorescence of C_2C_{12} blasts expressed on a histogram plot, with number of events (cells; y-axis) against TMRM fluorescence (FL-2; x-axis; logarithmic scale, arbitrary units).



C_2C_{12} myoblasts were incubated with increasing concentrations of TMRM for 30 minutes (Figure 5.5). Increasing TMRM concentration was associated with increasing red fluorescence, due to increased mitochondrial uptake of dye.

Figure 5.5. Contour plots of C_2C_{12} blasts incubated with increasing concentrations of TMRM for 30 minutes. An increase in TMRM red fluorescence (FL-2) is seen on the logarithmic y-axis. (N=10 000 per plot).



5.1.2.2. The effect of mClCCP on cellular TMRM fluorescence

C_2C_{12} blasts were incubated with increasing doses of TMRM, followed by trypsinisation and resuspension in Kreb's Buffer containing the mitochondrial respiratory uncoupler mClCCP at a final concentration of 20 nM. This resulted in a mean loss of FL-2 fluorescence at lower concentrations of TMRM (e.g. -47± 4% at 1.0 μM TMRM; $P < 0.003$; Figure 5.6). This trend of loss of TMRM fluorescence was, however, reversed at higher concentrations of TMRM, with a net gain in cellular fluorescence, at doses greater than 2 μM (Figures 5.7 & 5.8), due to the phenomenon of quenching (Duchen *et al.* 2003).

Figure 5.6. Flow cytometric analysis of TMRM stained C_2C_{12} cells. Control (coupled) cells are shown in the red histogram. Treatment with the mitochondrial respiratory uncoupler mClCCP (blue histogram) results in a left shift in fluorescence.

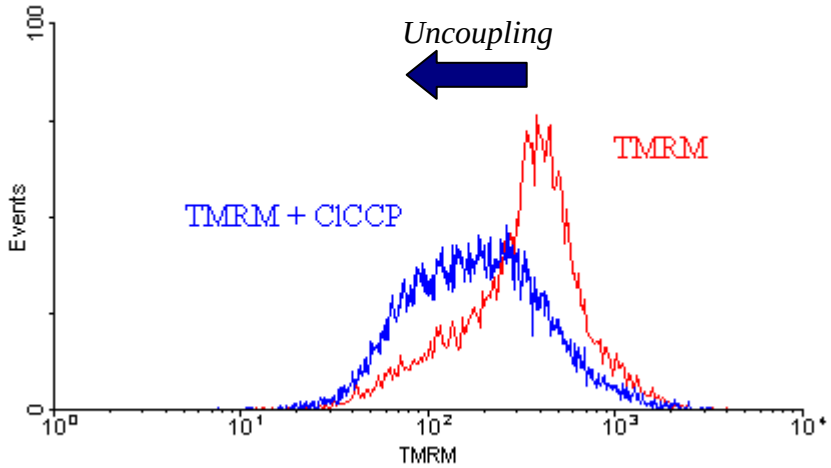


Figure 5.7. Flow cytometric measurement of TMRM fluorescence in untreated (coupled) and mClCCP-treated (uncoupled) C_2C_{12} myoblasts. (N=3 at each point)

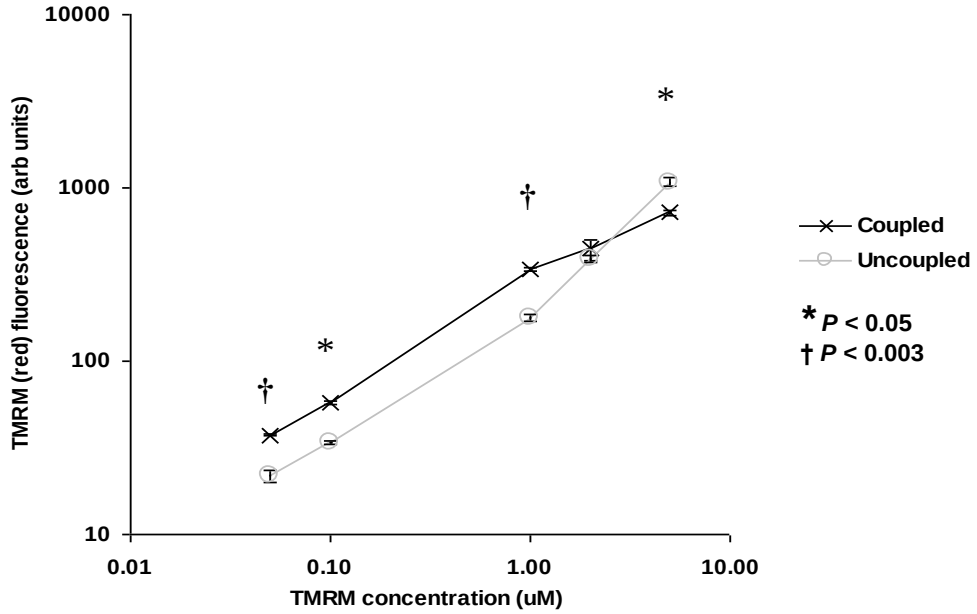
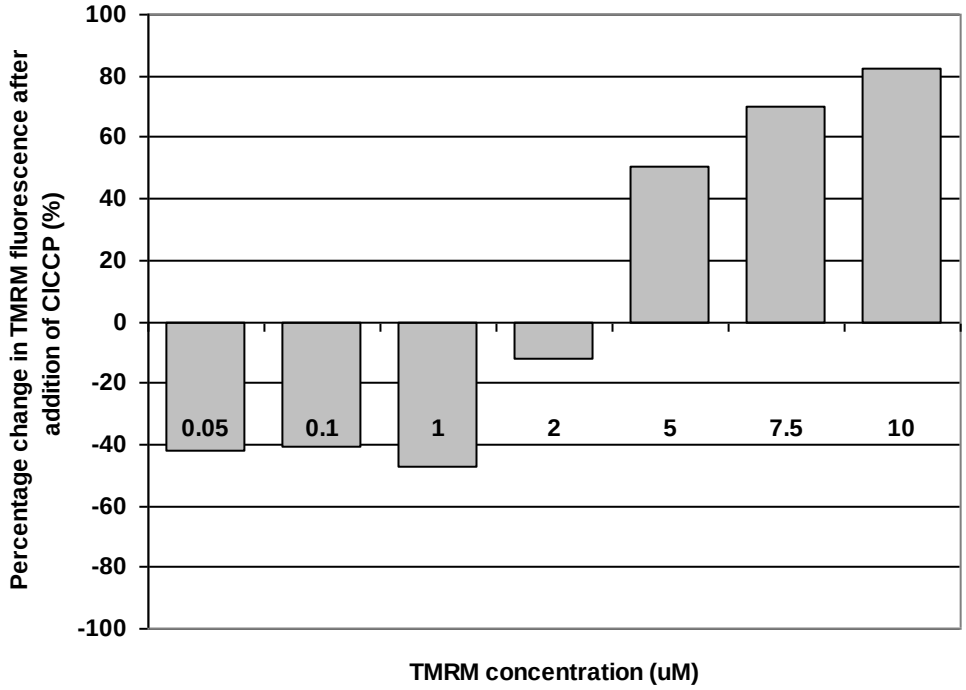


Figure 5.8. Relative change in TMRM fluorescence in C_2C_{12} myoblasts after treatment with 20 nM mClCCP (10000 cells). At lower loading doses of TMRM, uncoupling results in a reduction of fluorescence, whereas at higher doses, uncoupling induces an *increase* in fluorescence due to quenching.



5.1.3. JC-1 mitochondrial probe

5.1.3.1. Dose titrations

JC-1 exists as a monomer at low concentrations (or at low $\Delta\psi_m$) and forms "J-aggregates" at higher concentrations (in aqueous solutions above 0.1μM or at higher $\Delta\psi_m$). The monomer absorption/emission maxima in aqueous solutions are 510nm/527nm. Monomer fluorescence was therefore detected in the flow cytometer green (FL-1) channel following excitation at 488nm with its Argon laser. J-aggregates are excitable over a wide range from 485nm to its absorption maxima at 593nm, with emission maxima at 595nm and can therefore be detected on the FL-2 (red) channel. There was spectral overlap between the emission wavelengths of the JC-1 monomers and aggregates, so fluorescence compensations were carefully set to minimise 'bleed-through'.

Preliminary work with a preadipocyte cell line demonstrated that the degree of both aggregate (orange) and monomer (green) fluorescence was a factor of the concentration of JC-1 probe and of the incubation time with the probe (Figures 5.9-5.12). At low JC-1 concentrations (<0.5-1.0 μM), cells predominantly exhibited monomer fluorescence, and the proportion of cells that took up the dye (and fluoresced green) increased according to concentration and duration of incubation with dye. At higher probe concentrations, more cells achieved aggregate fluorescence at the expense of monomer fluorescence, and this effect was more pronounced the longer the dye was left to reach equilibrium. The ratio of JC-1 aggregate to monomer fluorescence (JC-1 A:M) was more reproducible and reflected the overall dye fluorescence characteristics.

Figure 5.9. Proportion of pre-adipocytes demonstrating green and orange fluorescence when incubated with increasing concentrations of JC-1 for 15 minutes (10000 cells at each concentration).

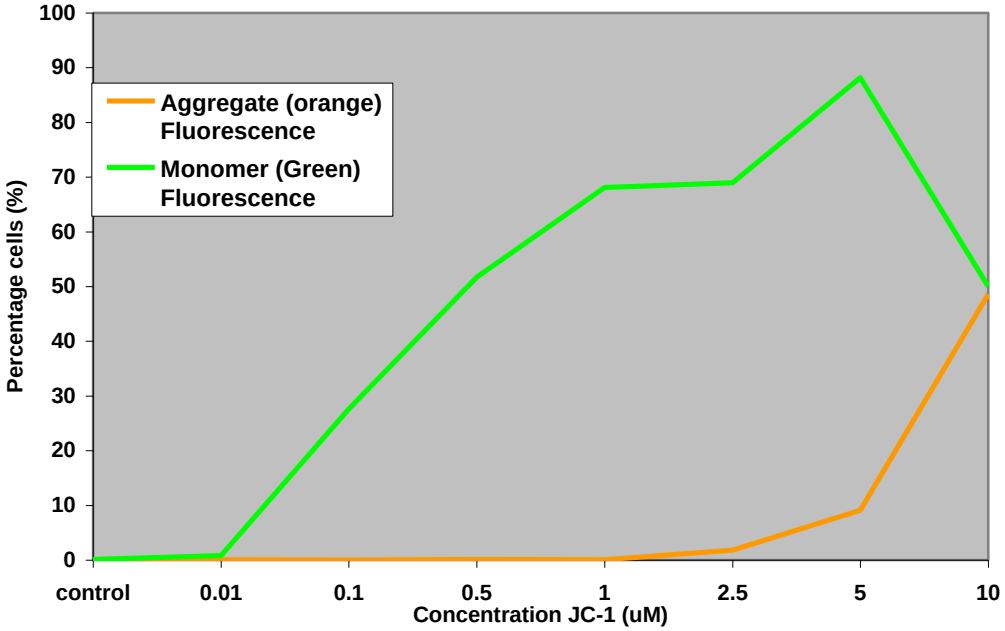


Figure 5.10. Proportion of pre-adipocytes demonstrating green and orange fluorescence when incubated with increasing concentrations of JC-1 for 60 minutes (N=10000 cells at each concentration).

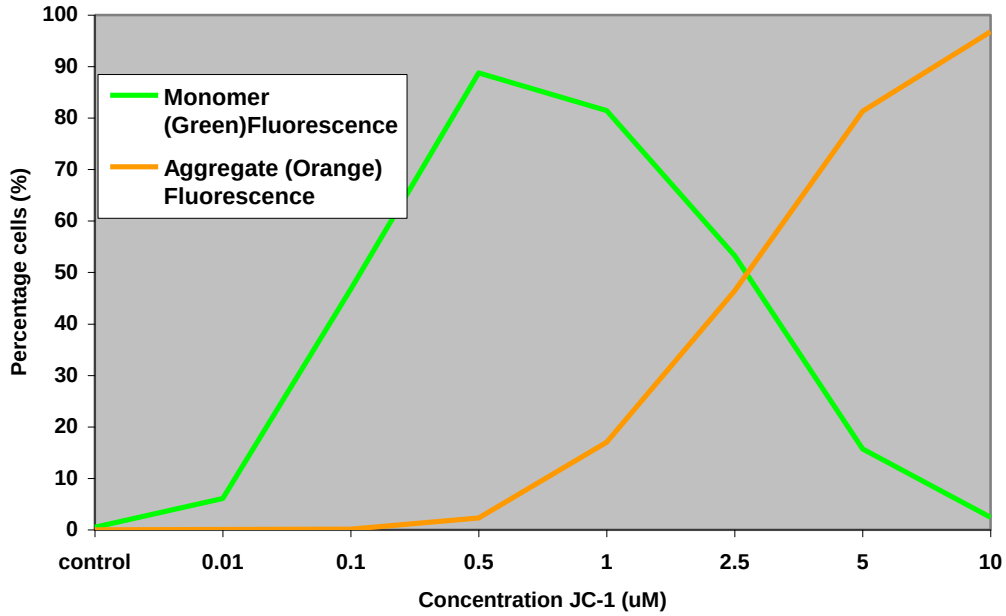


Figure 5.11. **Proportion of preadipocytes (total N=10000) exhibiting green monomer fluorescence incubated with increasing dose of JC-1 (varying time and concentrations).**

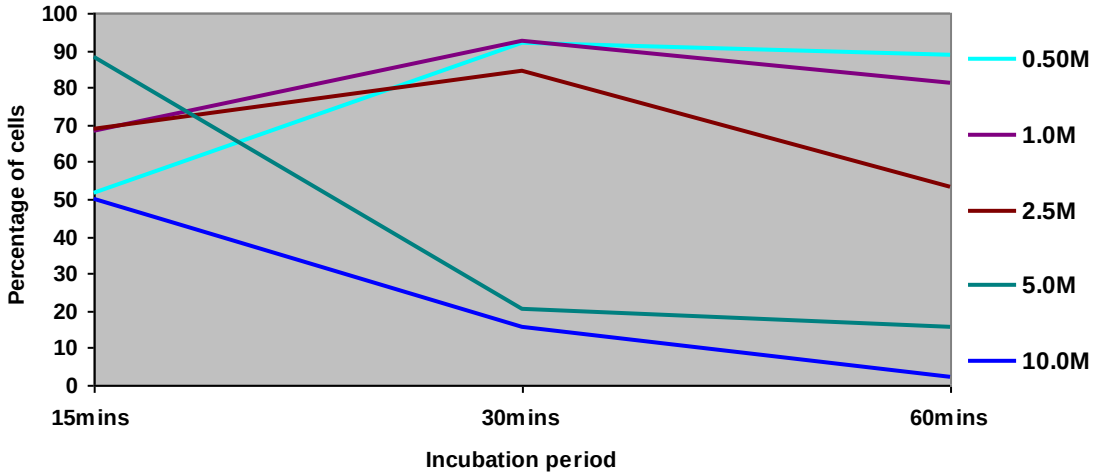
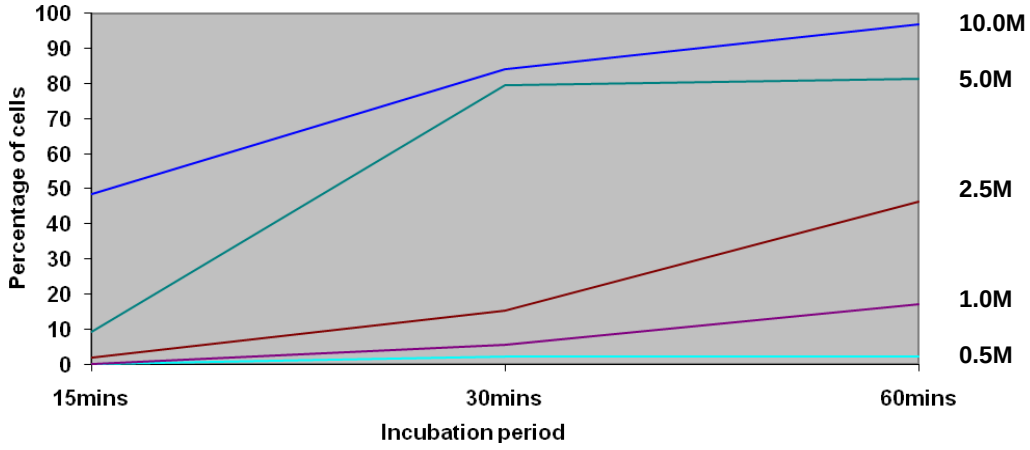


Figure 5.12. **Proportion of preadipocytes (total N=10000) exhibiting orange aggregate fluorescence incubated with increasing dose of JC-1 (varying time and concentrations).**



5.1.3.2. JC-1 and manipulation of $\Delta\psi_m$

It was found that the addition of uncoupler after incubating the cells with JC-1 resulted in very little change in aggregate fluorescence, in stark contrast to the uncoupler-induced changes seen in TMRM fluorescence. This difference is probably due to aggregates being slow to disassociate following acute changes in $\Delta\psi_m$. It was therefore decided to add uncoupler for 15 minutes before addition of JC-1.

Figure 5.13 shows a series of dot plot representations of flow cytometric analysis of C_2C_{12} myotubes incubated with increasing concentrations of JC-1 (37°C, 30 minutes), with the comparative plots below of C_2C_{12} myotubes pre-treated with 20 nM mClCCP. Again, the FL1 and FL2 channels have been optimised by compensating to reduce spectral overlap. As can be seen, the control population (no dye) predominates in the first log decade. Thereafter, increasing doses of JC-1 result in increasing monomer fluorescence (FL-1 green channel, x-axis), followed by increasing aggregate fluorescence (FL-2 orange, y-axis). Uncoupling with mClCCP prior to JC-1 cell loading resulted in greater monomer (green) fluorescence at the expense of aggregate fluorescence signal and also of the total number of cells exhibiting either fluorescence. The uncoupler-induced collapse of $\Delta\psi_m$ therefore prevents subsequent mitochondrial loading and concentrating of JC-1. In C_2C_{12} myotubes, addition of a low concentration of mClCCP (5 µM) resulted in complete loss of $\Delta\psi_m$ (99.2 ±0.2% reduction in JC-1 A:M fluorescence, $P < 0.0000001$, N=3; Figure 5.15). H9C2 cardiac blasts showed greater aggregate formation at a lower concentration of JC-1 (Figure 5.14). Again, pre-treatment with uncoupler (20 µl mClCCP) resulted in an increase in monomer fluorescence and a decrease in aggregate fluorescence. The proportion of cells achieving aggregate fluorescence began to plateau at 2.5-5.0 µM JC-1 (graph Figure 5.14).

Figure 5.13. Dot plot series. Upper panel: murine C_2C_{12} myotubes incubated for 30 mins at 37°C with increasing concentrations of the mitochondrial probe JC-1 followed by 2 channel flow cytometric analysis. Monomer fluorescence emission was detected in the FL-1 channel (x-axis) and aggregate fluorescence emission in the Fl-2 channel (y-axis). Quadrants have been drawn which best reflect monomer emission and aggregate emission. The percentage of cells within each quadrant is shown in their respective corners. As the concentration of JC-1 increased, the monomer fluorescence increased, with the development of aggregate fluorescence above 0.5 µM JC-1. A greater proportion of cells achieve aggregate fluorescence at higher JC-1 concentrations. Lower panel: pre-treatment with 20 µM mClCCP results in an increase in monomer fluorescence at the expense of both a decrease in aggregate fluorescence and number of cells with aggregate fluorescence (N=10000 each panel).

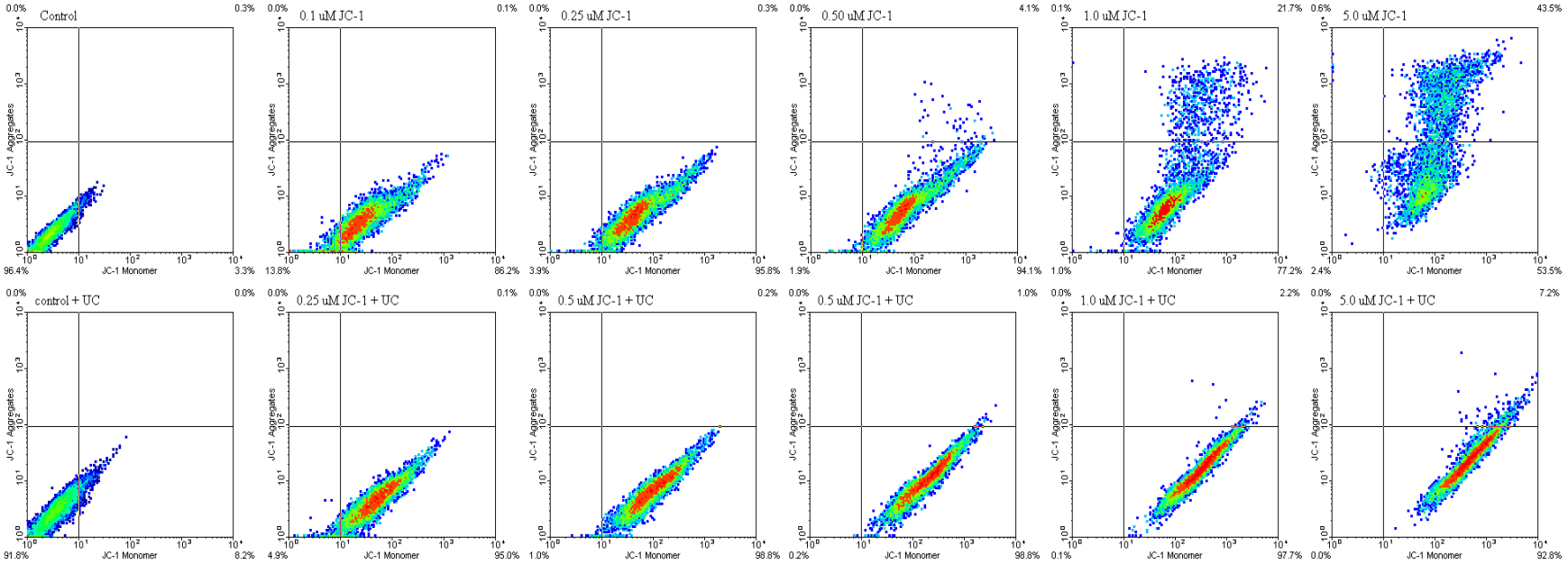
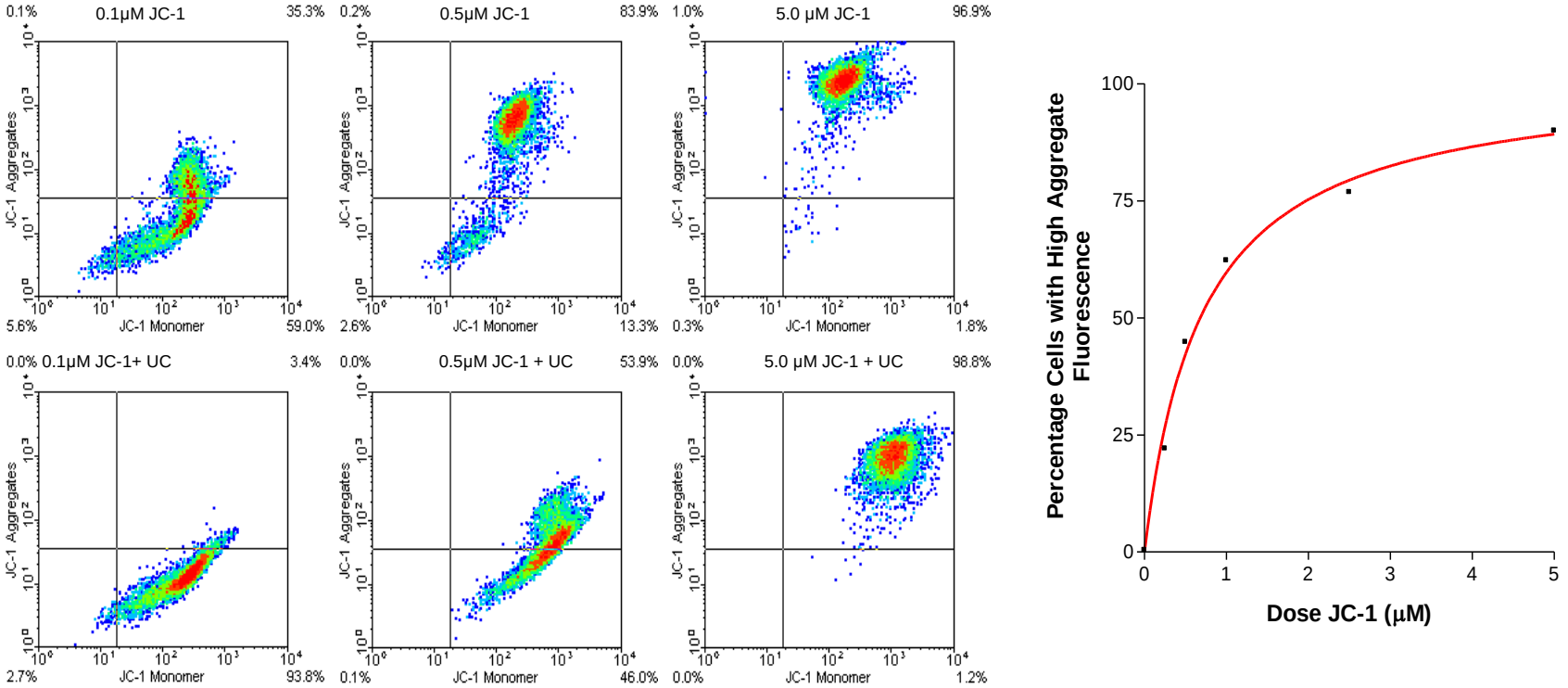
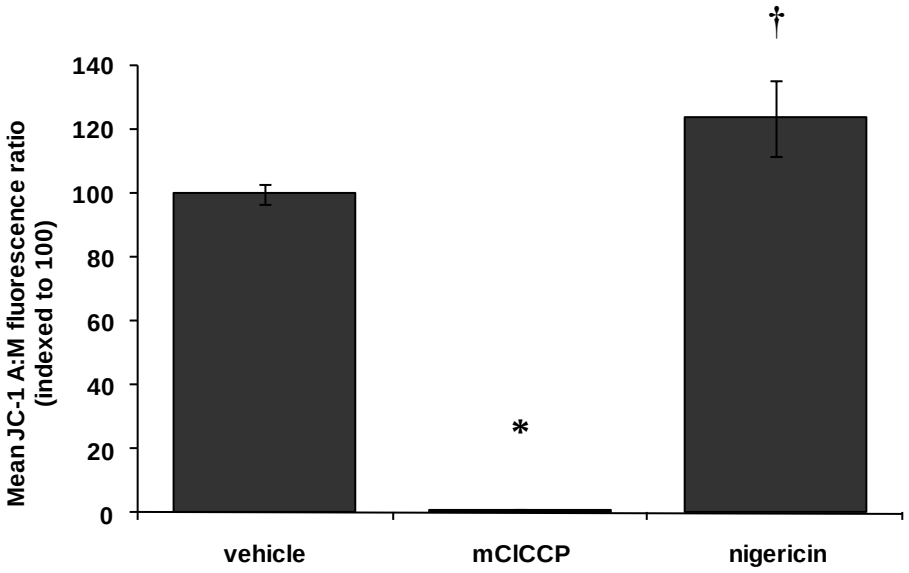


Figure 5.14. Dot plot series showing (upper panels) rat H9C2 cardiac blasts incubated for 30 mins at 37C with increasing concentrations of the mitochondrial probe JC-1 followed by 2 channel flow cytometric analysis. Monomer fluorescence emission was detected in the FL-1 channel (x-axis) and aggregate fluorescence emission in the Fl-2 channel (y-axis). H9C2 cells appear to form JC-1 aggregates more readily than myotubes. The lower panels show the result of pretreatment with the uncoupler mClCCP. The graph demonstrates the proportion of cells with aggregate fluorescence with increasing concentration of JC-1 (N=10000 for each panel).



The ionophore nigericin was used to induce a slight inner mitochondrial membrane hyperpolarisation. It catalyses the electroneutral exchange of K^+ for H^+, equalising their respective gradients across the membrane, thereby increasing $\Delta\psi_m$ (Section 1.5.2). Addition of a 10μM nigericin for 1 hour to C_2C_{12} myotubes resulted in a 24 ±12% increase in JC-1 A:M fluorescence (P = 0.03; N=3; Figure 5.15).

Figure 5.15. JC-1 aggregate/monomer (A:G) fluorescence ratio in C_2C_{12} myotubes incubated with vehicle, mClCCP or nigericin (10000 cells, N=3 repeats), * $P < 10^{-7}$; † $P = 0.03$



5.1.4. ACE inhibitor effects on $\Delta\psi_m$ assessed in whole cells by flow cytometry

5.1.4.1. TMRM

$\Delta\psi_m$ was measured in C_2C_{12} skeletal myotubes using a low dose (0.1 μM) of TMRM for 30 minutes. Treatment of C_2C_{12} myotubes with 10 μM ramiprilat for 24h resulted in a significant increase in TMRM fluorescence relative to control cells as measured by flow cytometry (117 ± 11u vs. 72 ± 6u for ramiprilat *vs.* control; n=3, P=0.004; mean 62% increase in fluorescence; Figures 5.16 & 5.17), i.e. *hyperpolarisation* of the inner

mitochondrial membrane. There was no difference in $\Delta\psi_m$ assessed after 48 h incubation (with no further addition of ramiprilat). C_2C_{12} cells were also incubated with 5 μM of the ACEi imidiprilat and perindoprilat (Figure 5.17). Lipid solubilities of these ACEi were ramiprilat >> imidiprilat > perindoprilat, which concurs with previously reported tissue affinity and lipophilicity data (Dzau *et al.* 2001). $\Delta\psi_m$ at 24 hour followed this trend, with a mean percentage increase in TMRM fluorescence of 12.8% and -0.3% for imidiprilat and perindoprilat respectively. Again, by 48 h, there was no difference in $\Delta\psi_m$ compared with vehicle treated cells.

Figure 5.16. Flow cytometric histogram plot demonstrating TMRM stained C_2C_{12} myotubes which have been untreated or ramiprilat (10^{-5}M) treated for 24hours.

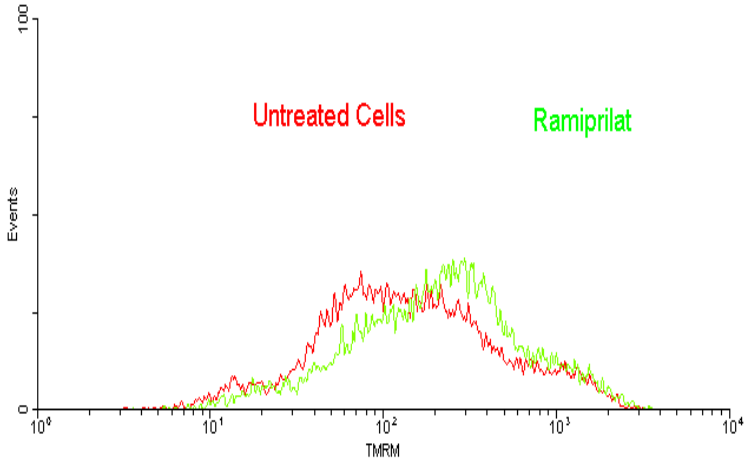
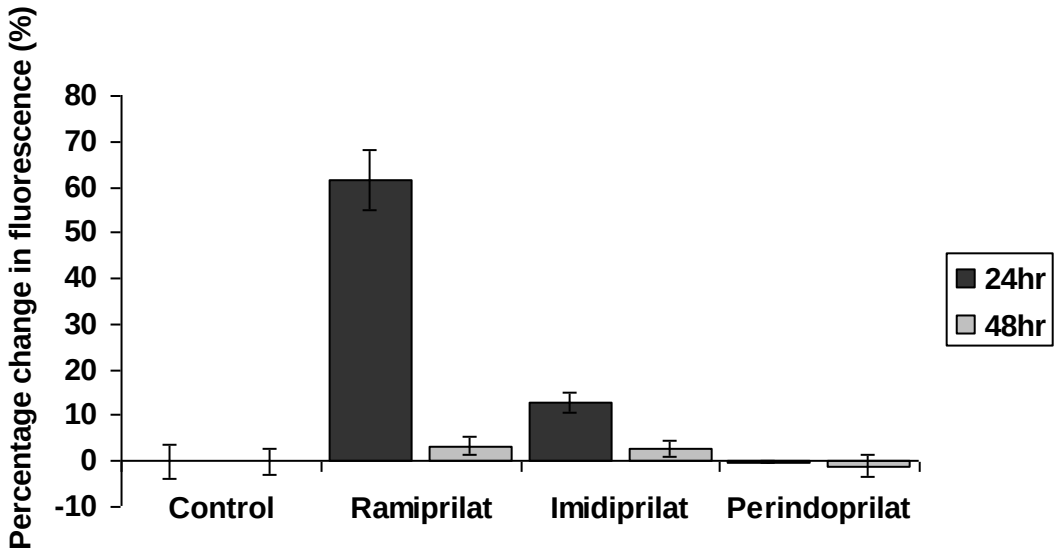


Figure 5.17. Mean change in TMRM dye fluorescence following incubation of C_2C_{12} myotubes with different ACE inhibitors at 10^{-5}M for 24 - 48 hours (N=3x 10000 cells)

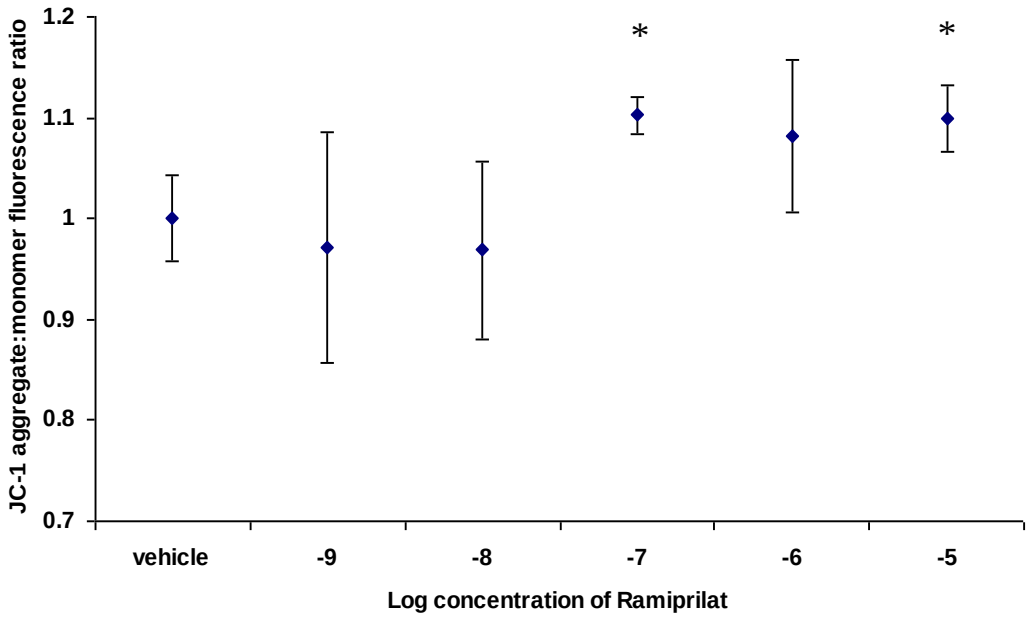


5.1.4.2. JC-1

Membrane potential assessed by JC-1 in C_2C_{12} myotubes confirmed dose-dependent $\Delta\psi_m$ hyperpolarisation after 24h treatment with Ramiprilat, increasing at concentrations of Ramiprilat greater than 10^{-7}M (10±0.3% at 10^{-7}M; mean ±SD; N=3; P<0.05; Figure 5.18). H9C2 cardiac blast cells showed a similar, dose-dependent hyperpolarisation of $\Delta\psi_m$ following incubation with 24hr ramiprilat.

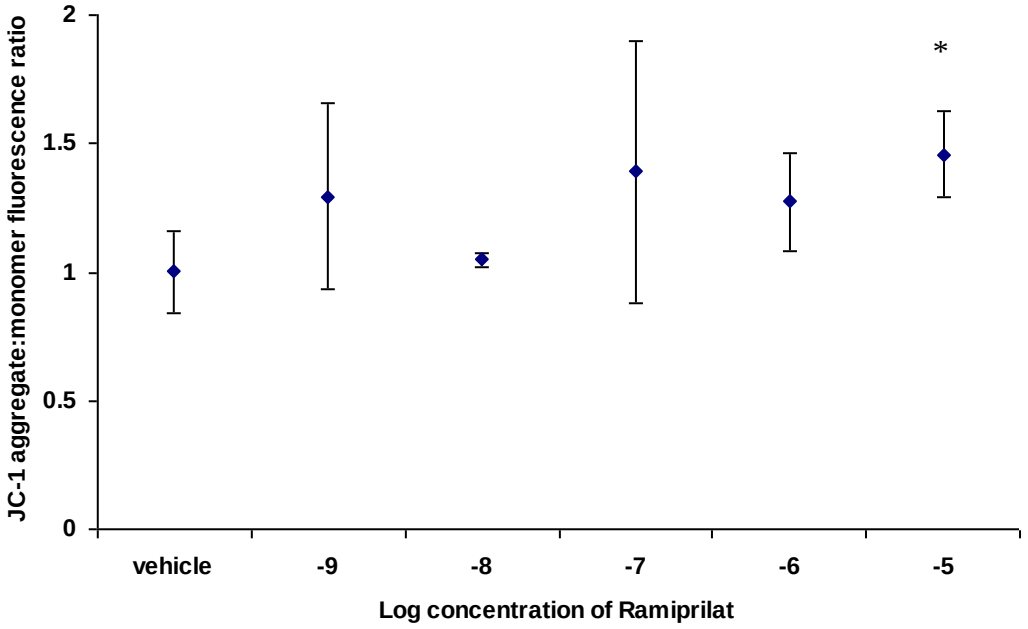
Adult rat primary cardiomyocytes were not ideally studied by flow cytometry as they tended to clump in the suspension.

Figure 5.18. Ratio of JC-1 aggregate to monomer fluorescence of C_2C_{12} myotubes incubated with increasing dose of ACEi Ramiprilat for 24 hours (n=3x 10000 cells).



* $P<0.05$ *vs.* vehicle

Figure 5.19. Ratio of JC-1 aggregate to monomer fluorescence of H9C2 cardiac blast cells incubated with increasing dose of ACEi Ramiprilat for 24 hours (n=2x 10000 cells)



* $P=0.01$ *vs.* vehicle

5.2 CONFOCAL ANALYSIS

Cells were seeded at low density into several types of culture slides (Perspex and glass Petri dishes and chamber slides) to attempt their visualisation on an inverted microscope (Nikon Eclipse T100) which was attached to the confocal system (Bio-Rad Radiance2000). Iwake round chamber slides had too large a surface area, allowing eddying of added drugs with incomplete admixture. The fluorescence properties of Perspex chamber slides did not allow good immunofluorescence imaging. Quartz two-chamber slides designed for immunofluorescence studies (Nunc™, Fisher Scientific, UK) were found to give the clearest imaging with the confocal microscope and also enabled sided-by-side treatment analyses.

C_2C_{12} myoblasts were seeded at low density (10^3 cells per well) into 2-well chamber slides. Cells were allowed to adhere overnight and then serum deprived for at least 3 days to stimulate differentiation into myotubes which were subsequently studied.

Dose titrations with potentiometric dyes were carried out to obtain optimal staining characteristics. For TMRM, concentrations were used in the lower range in the linear phase of dye uptake according to Nernstian principles to prevent quenching (Section 5.1.1.2). Several experiments were performed to obtain optimal laser scanning conditions (see Table 5.1). The settings were stored and the identical settings used for each comparative experiment.

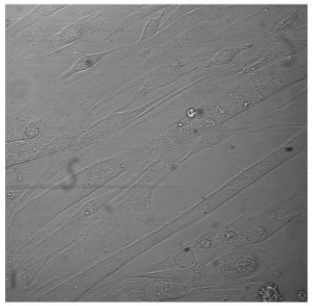
Treatment of C_2C_{12} myotubes with 0.1 μM TMRM for 30 minutes resulted in characteristic orange-red staining in fibrillar structures (Figure 5.20). Images were stored and transferred to a workstation where cellular fluorescence was then quantified by posthoc analysis using LaserPix software (Bio-Rad, UK). A region of interest (ROI) was drawn around each individual cell (first bluntly by edge recognition software, then optimised by dragging in the outline to the plasmamembrane edge). Mean fluorescence signal for the ROI (mean pixel brightness) was calculated on a 256 grey-scale. Treatment with the uncoupler mClCCP caused a significant, immediate 58% reduction of mean cellular fluorescent signal (P = 0.0002 measured at 20 seconds; Figure 5.20d). Following the initial drop, the cellular fluorescence continued to reduce gradually for the next 280 seconds down to a 74.7% mean loss of TMRM fluorescence (P = 0.00002).

Table 5.1. Typical settings for confocal imaging.

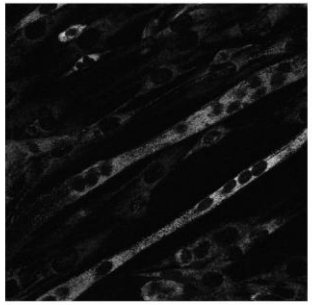
Probe	Laser	Power	Iris	Gain	Detection filter
TMRM	Argon	5%	1	29%	515/30
DCF	HeNe	30%	1	50%	590/70
JC-1	Argon	15%	1	13%	515/30
					(using 560 dichroiic mirror)
	HeNe	13%	1	50%	590/70

Figure 5.20. <u>Confocal analysis of mitochondrial TMRM fluorescence in C2C12 myotubes (x20 magnification).</u>

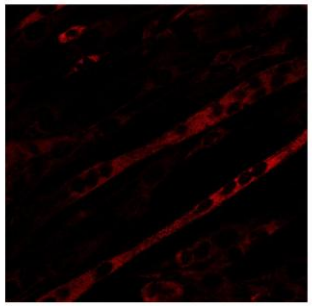
A. Transmission image



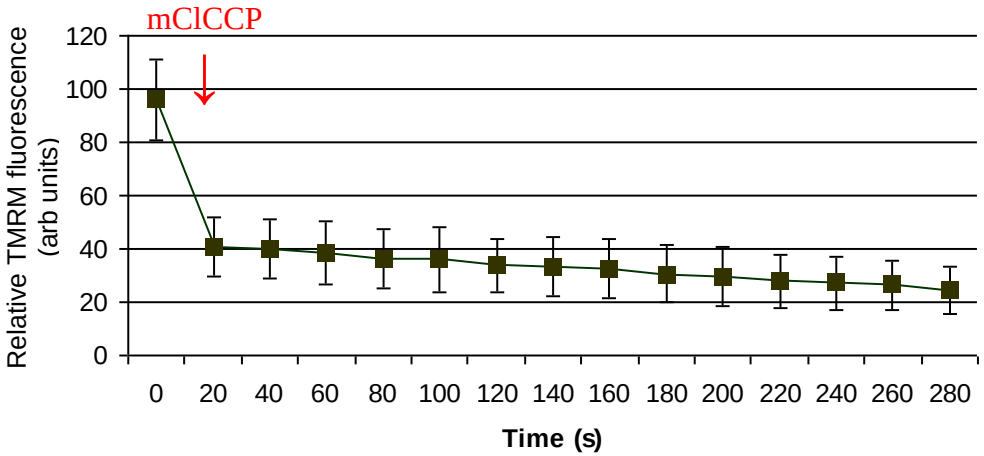
B. Grey-scale image of TMRM fluorescence



C. Red 'look-up' table applied to grey-scale



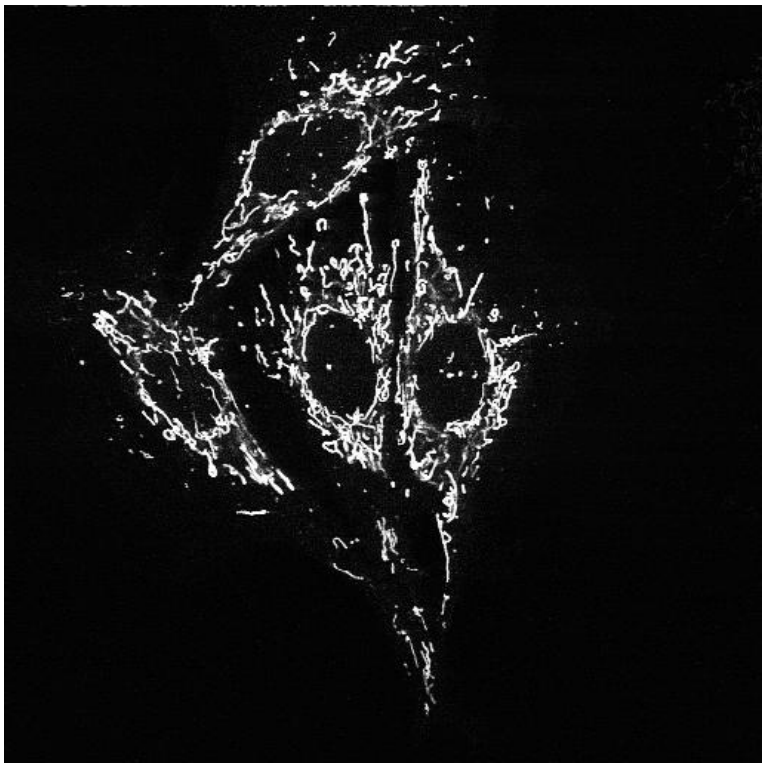
D. Time series of mean TMRM fluorescencence in C2C12 myotubes (N=5 myotubes) following addition of 20μM mClCCP (mean ± SD)



P<0.0002 for all time points after mClCCP addition when compared with starting TMRM fluorescence

H9C2 cardiac blasts were seeded at $1x10^4$ cells per chamber in quartz chamber-slides. Incubation with TMRM (0.1 μM for 30 minutes) showed dye uptake in mitochondria which were sinuous cytoplasmic structures (Figure 5.21) similar to the mitochondrial structures previously described in astrocytes (Duchen 1999) unlike the longitudinal arrangement in mature C_2C_{12} myotubes or cardiomyocytes.

Figure 5.21. Grey scale confocal image of a cluster of H9C2 blasts which have been stained with TMRM, a potentiometric dye which is taken up preferentially in mitochondria according to $\Delta\psi_m$. Note the dye dropout in the nucleus. (x40 magnification).



5.2.1. The effect of ACE inhibition on $\Delta\psi_m$ assessed by CLSM

5.2.1.1. C_2C_{12} skeletal myotubes

C_2C_{12} myotubes were incubated with 0.1 μM TMRM for 30 minutes. Prior treatment with 10^{-5}M ramiprilat for 24 hours resulted in a significant increase in TMRM fluorescence compared to vehicle treatment (Figure 5.22). Treatment of cells with 20 μM mClCCP resulted in a loss of fluorescence signal in both vehicle and ramiprilat treated cells, indicating that fluorescence was indeed located to mitochondria. Further analyses were performed on low power fields (x20) taken from separate cultures of vehicle and ramiprilat-treated C_2C_{12} cells (n = 3). As can be seen (Figure 5.23) ramiprilat treated cells again demonstrated an increase in $\Delta\psi_m$, marked by a relative increase in TMRM fluorescence (P = 0.01; whole field analysis). As can be seen, there was relative heterogeneity in cell morphology and cell response.

5.2.1.2. Cardiac cells

TMRM fluorescence of H9C2 cardiac blasts was also significantly increased by ramiprilat treatment (P = 0.008, n = 4 cells; Figure 5.24). As can be seen, there was heterogeneity in cell morphology. Adult ventricular cardiomyocytes (primaries) were isolated from the rat as described in Methods 2.4.4.1 using collagenase digestion in a Langendorff perfusion. Calcium tolerant cells were allowed to adhere to laminin-coated quartz chamber slides. Non-adherent cells were subsequently removed by washing. Cells in one chamber were treated with ramiprilat 10^{-5}M and the contra-lateral chamber treated with vehicle as an internal control.

Figure 5.22. C_2C_{12} myotubes stained with TMRM and analysed by confocal microscopy (magnification x40)

i. Vehicle 24 hours

ii. Ramiprilat 10^{-5}M 24 hours

iii. Relative TMRM Fluorescence measured by CLSM of ramiprilat and vehicle treated C2C12 myotubes (n=6)

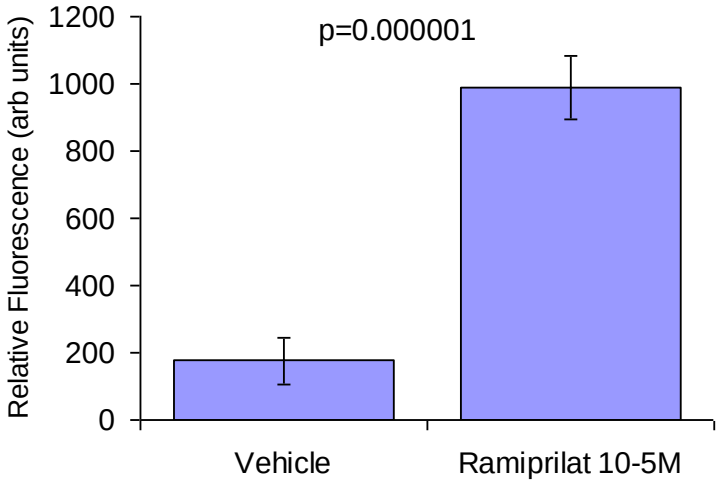
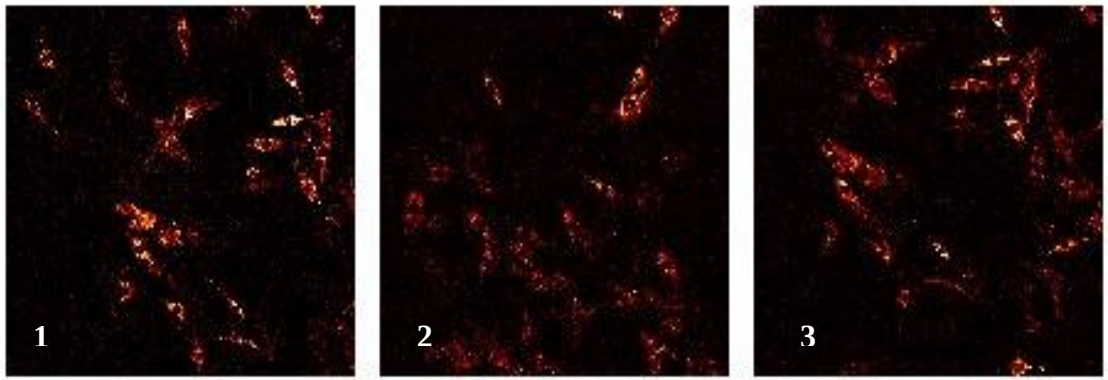
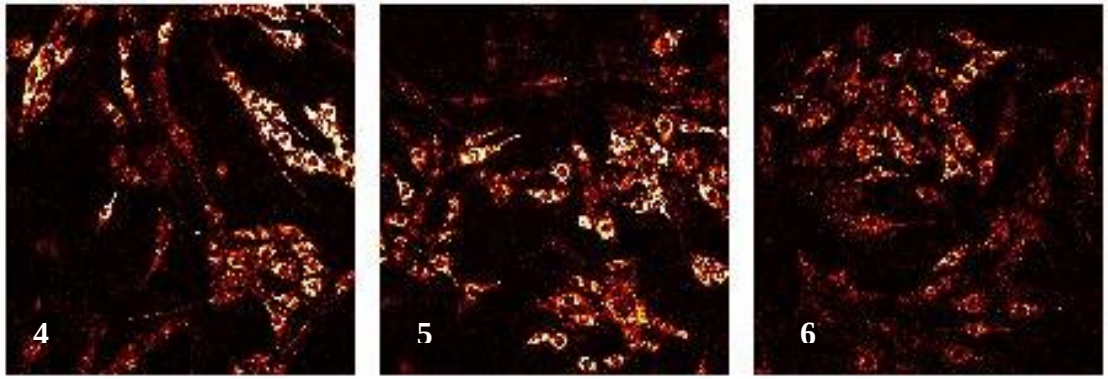


Figure 5.23. Fields of C_2C_{12} cells stained with TMRM and imaged with CLSM

i. Vehicle treated 24 hours



ii. Ramiprilat 10^{-5}M for 24 hr



iii. Relative TMRM fluorescence of fields of C_2C_{12} cells treated with either vehicle or ramiprilat for 24hours (n=3)

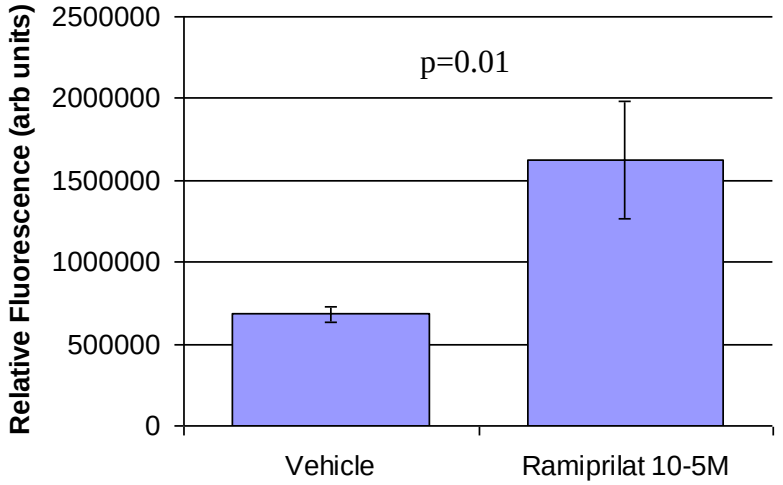
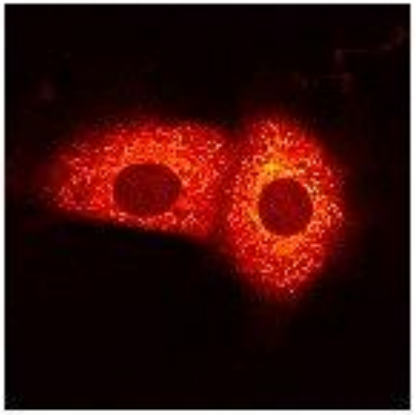
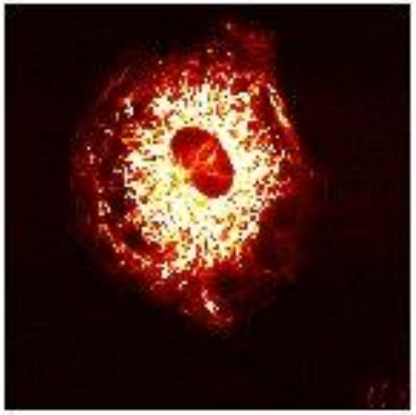


Figure 5.24. <u>H9C2 cardiac blasts stained with TMRM and analysed by confocal microscopy</u>

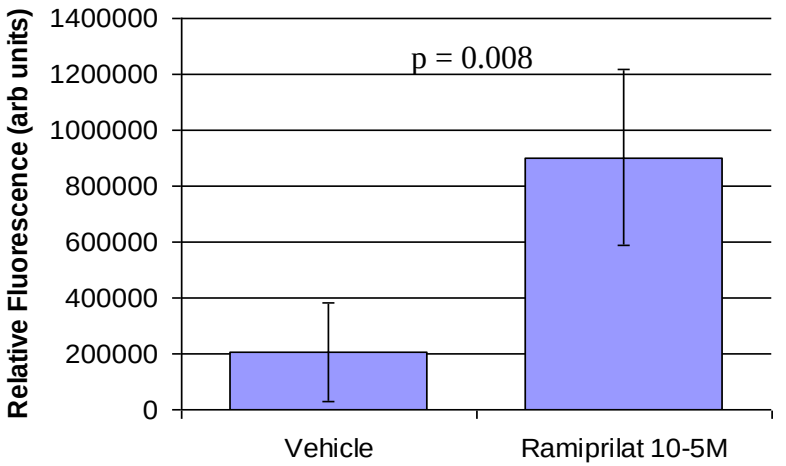
i. vehicle treated



ii. ramiprilat 10^{-5}M



iii. Relative TMRM fluorescence of fields of H9C2 blasts treated with either vehicle or ramiprilat for 24hours (n=3)



A single rod-shaped cell was recorded from each of 10 fields, as depicted round each chamber (Figure 5.25a). Experiments were repeated 3 times, giving 30 cells per treatment.

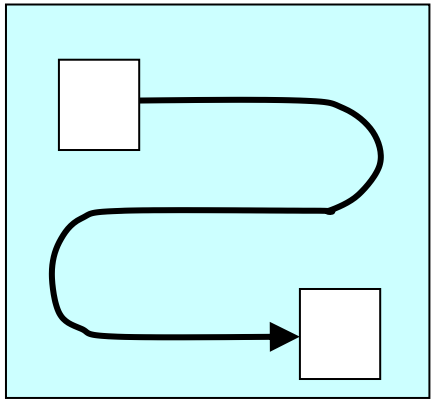
Cells treated with 100 nM TMRM displayed the characteristic banding pattern (Duchen *et al.* 2003), as mitochondria are arrayed along the cardiomyocyte striations. Fluorescence quantification was adjusted to cell cross-sectional area to give relative fluorescence per unit area, and vehicle treated cells indexed to a mean relative fluorescence of 100 (Figure 5.25c).

A time series of vehicle *vs.* ramiprilat (10^{-5}M) was conducted with time points at 1, 6, 12 and 24 hours. There was no difference between treatments at 1 or 6 hours. Ramiprilat treatment after 12 hours resulted in a significant increase in TMRM fluorescence (100 nM), with relative increases of 50% and 38% for 12h and 24 h respectively (both $P < 0.000001$).

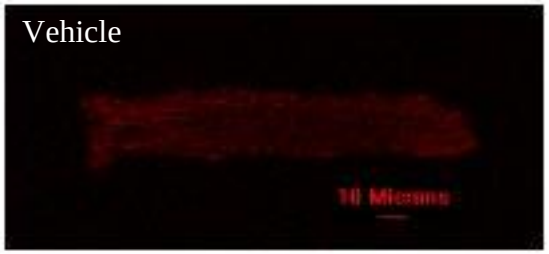
Analyses were possible on 1 sample of human adult right ventricular cardiomyocytes. The cells were isolated from the right ventricle of a 69 year old male patient with dilated cardiomyopathy undergoing cardiac transplantation. The patient had been on long-term ACEi maintenance therapy. Again, cardiomyocytes stained with 100 nM TMRM exhibited the typical striated patterning (Figure 5.26A). Addition of 5 μM mClCCP resulted in slow loss of TMRM fluorescence (Figure 5.26A, Figure 5.27). There was no difference in TMRM fluorescence between ACEi therapy for 24 hours with ramiprilat 10^{-5}M and vehicle treated cells (Figure 5.28).

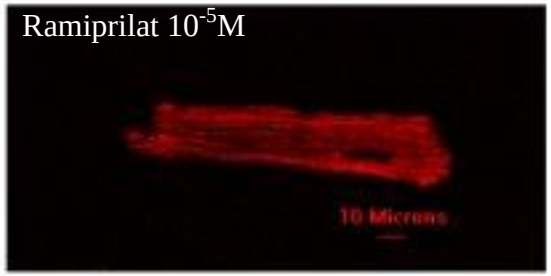
Figure 5.25. CLSM analysis of $\Delta\psi_m$ in rat adult ventricular primaries

a) 10 fields of view were chosen from each chamber slide in the direction indicated

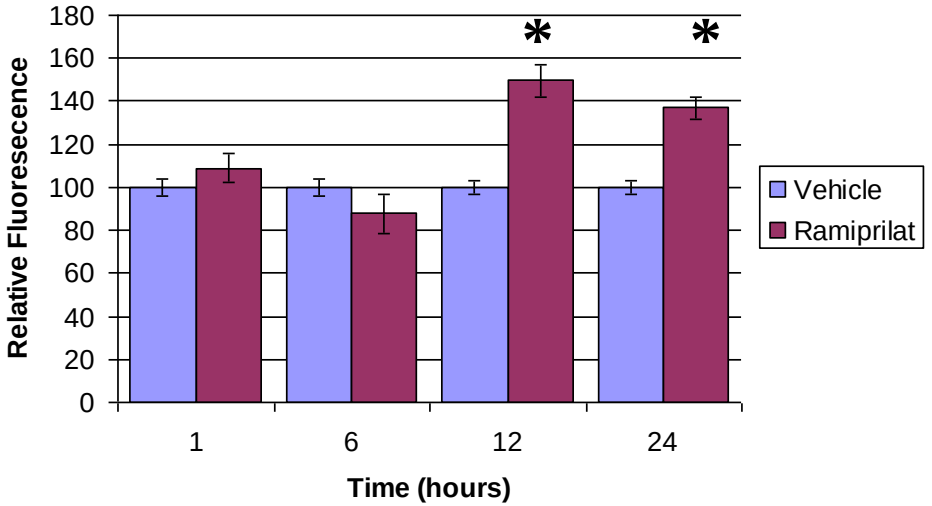


b) CLSM images of TMRM treated cells





c) Time course of relative TMRM fluorescence of ramiprilat *vs*. vehicle treated cardiomyocytes

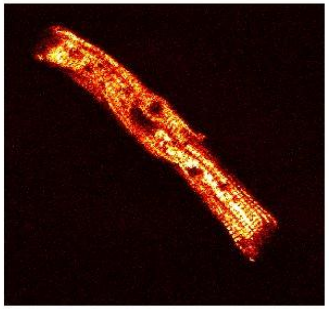


* $P < 0.00001$

Figures 5.28-28. <u>TMRM fluorescence in human adult ventricular cardiomyocytes</u>

Figure 5.26. Confocal images of mycotes stained with TMRM

A. Vehicle



B. mClCCP (5µM)

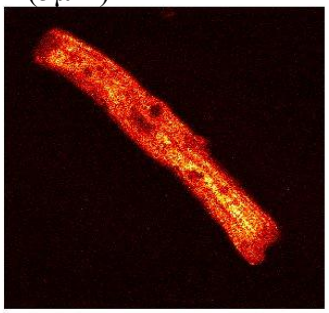


Figure 5.27. Time series in human cardiomyocyte after addition of 5µM mClCCP

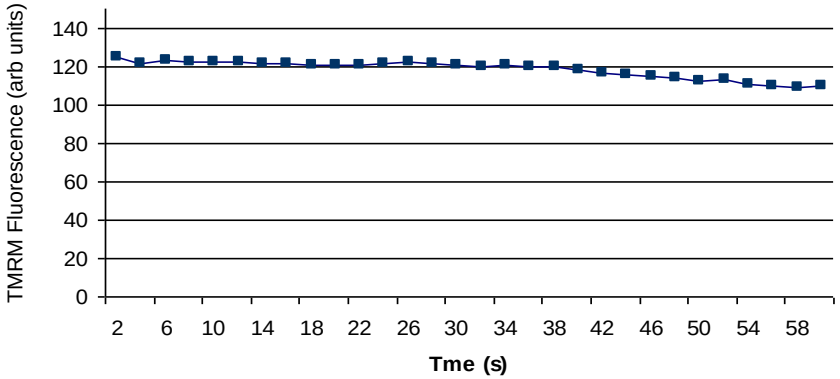
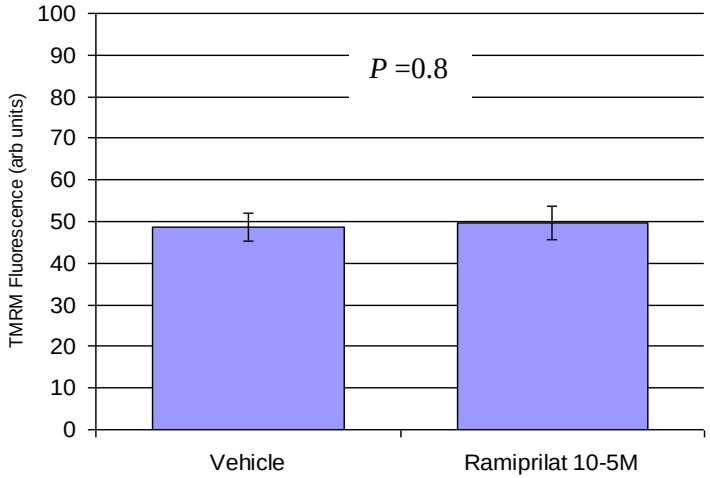


Figure 5.28 TMRM fluorescence in human right ventricular cardiomyocytes treated with either vehicle or ramiprilat (10^{-5}M) for 24 hours.



5.2.2. CLSM measurement of ROS in cardiomyocytes treated with ACEi

TMRM has been shown to induce photodynamic damage with repetitive laser scanning (Zhang *et al.* 2001). To test whether pre-treatment with the ACEi, ramiprilat had a beneficial effect on cellular reactive oxygen species (ROS) generation, adherent adult rat cardiomyocytes were incubated with either vehicle or Ramiprilat 10^{-5}M for 24 hours in chambers slides. The cells were then incubated with TMRM and DCF-DA to simultaneously measure mitochondrial membrane potential and ROS (NO and peroxynitrite). The cells underwent sequential laser scanning every 1 minute for 15 minutes using both Argon (488nm) and HeNe lasers and fluorescence signals detected at 560-650nm (560nm and 650nm dichroiic longpass mirrors and 590±70nm emission filter) for TMRM (red), and 515±30nm for DCF-DA green fluorescence (Figure 5.29). Cells treated with vehicle showed no significant change in TMRM fluorescence during this period (percentage change in fluorescence 2±2%) indicating there was no significant photobleaching effect over this time. However, there was an increase in DCF-DA fluorescence (12±3%). Pretreatment with ramiprilat resulted in a significant difference in DCF-DA fluorescence compared to vehicle (ramiprilat cells -9±8%, P = 0.009 *vs.* vehicle) and a reduction in TMRM fluorescence(-25±13%, P=0.009 compared with vehicle; Figure 5.29; N = 3).

Figure 5.29. Changes in TMRM and DCF-DA fluorescence in vehicle and Ramiprilat treated cardiomyocytes after sequential laser scanning.

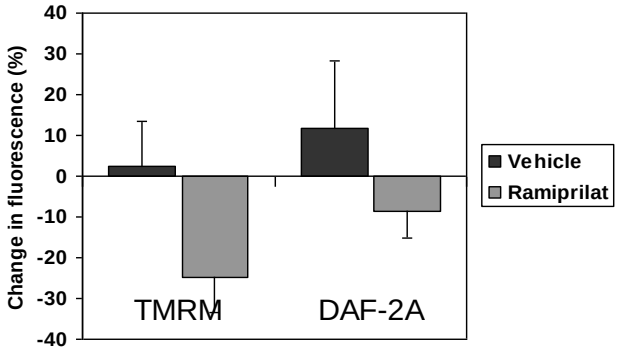
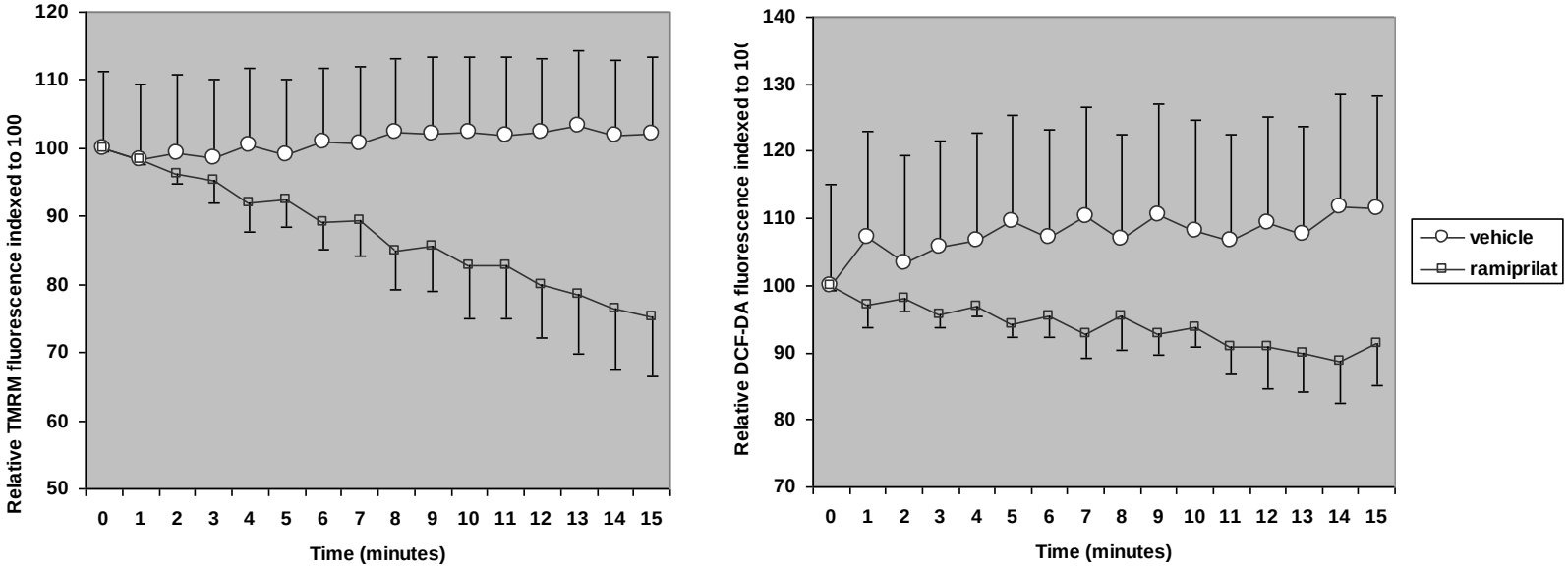


Figure 5.30. Relative TMRM and DCF-2A fluorescence of adult rat cardiomyocytes during repetitive laser scanning confocal microscopy. Cells were pretreated with either vehicle or ramiprilat 10^{-5}M for 24hr (n=2-4 cells)



5.3 CELLULAR OXYGEN CONSUMPTION

C_2C_{12} murine myoblasts were grown to confluency in 175cm^2 culture flasks and differentiated into myotubes as described (Methods 2.1.2) and then maintained in culture for 5-7 days. Cells were then treated with either vehicle or ramiprilat (10^{-5}M), LPS (1 µg.ml^{-1}) or Ang II (10^{-7}M) for 24 hour prior to resuspension in aerated Kreb's buffer. Oxygen consumption was measured using the OxySpot system (Methods 2.3).

Concentrations of C_2C_{12} myocytes below 2-3x10^6 cells.ml^{-1} did not measurably alter oxygen tension. A cell concentration at 4x10^6 cells.ml^{-1} gave a measurable reduction in oxygen tension with time (-7 ±0.6 mTorr.s^{-1}), but also tended to clump in solution. As can be seen in Figure 5.31, the addition of 5µM mClCCP resulted in the expected increase in oxygen consumption (uncoupled respiration -10 ±2 mTorr.s^{-1} *vs.* vehicle above, N = 3, *P*=0.03).

5.3.1. C_2C_{12} cells treated with LPS

Treatment with LPS (1µg.ml^{-1} for 24hr) resulted in a significant reduction in cellular respiration (-7 ±0.6 mTorr.s^{-1} *vs.* -2 ±0.7 mTorr.s^{-1} for vehicle *vs*. LPS treatment *P*=0.0008; Figure 5.29). Treatment with uncoupler increased respiration rate in LPS treated cells (-9 ± 5.0mTorr.s^{-1}; *P* = 0.04; N=3). There was no difference in uncoupled rates of respiration in vehicle and LPS treated cells (*P* = 0.9; Figure 5.29).

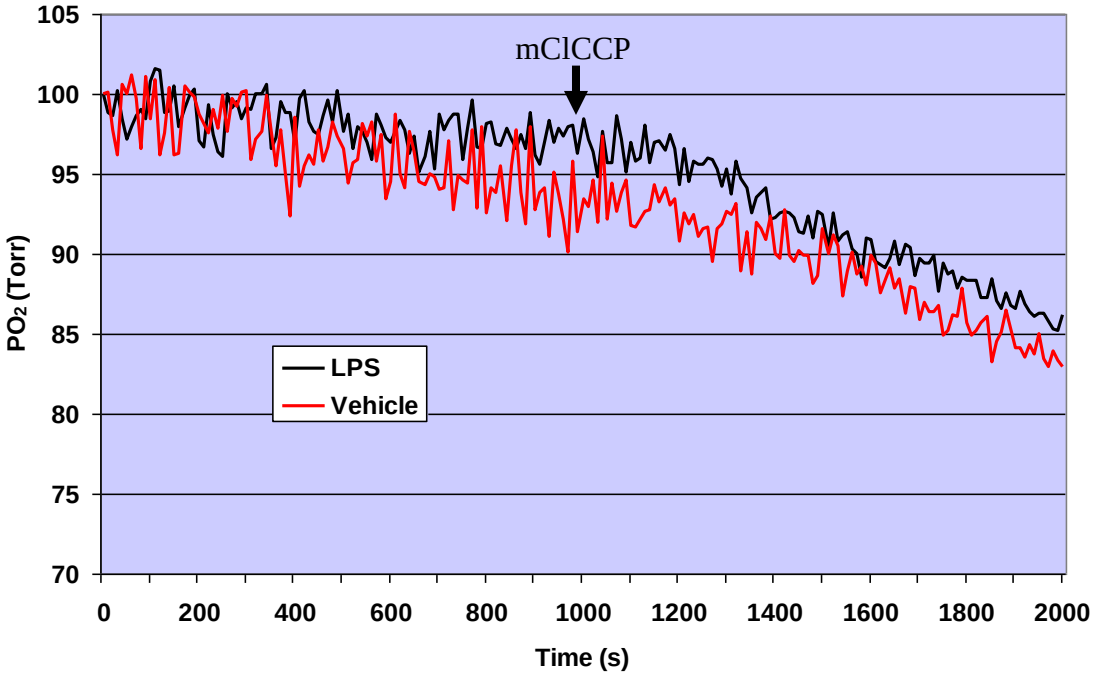
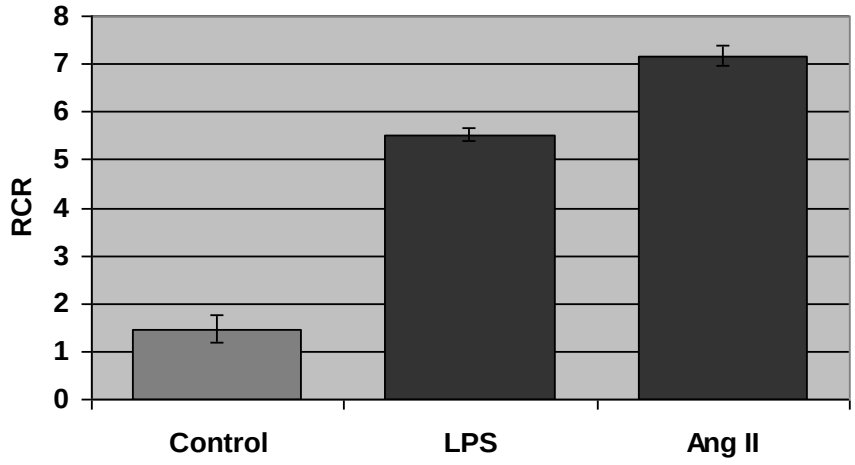


Figure 5.31. Oxygen consumption measured in C_2C_{12} myotubes pre-treated with vehicle or lipopolysaccharide (LPS; 1µg.ml^{-1}) for 24 hours. Oxygen consumption was lower in LPS treated cells. After addition of uncoupler (arrow) the oxygen tension curves run in parallel. (PO_2 indexed to 100).

Respiratory control ratio (RCR) was significantly higher in LPS treated cells compared to vehicle treated cells (2 ±0.1 *vs.* 6 ±0.1 for vehicle *vs.* LPS; $P = 0.0004$; Figure 5.32).

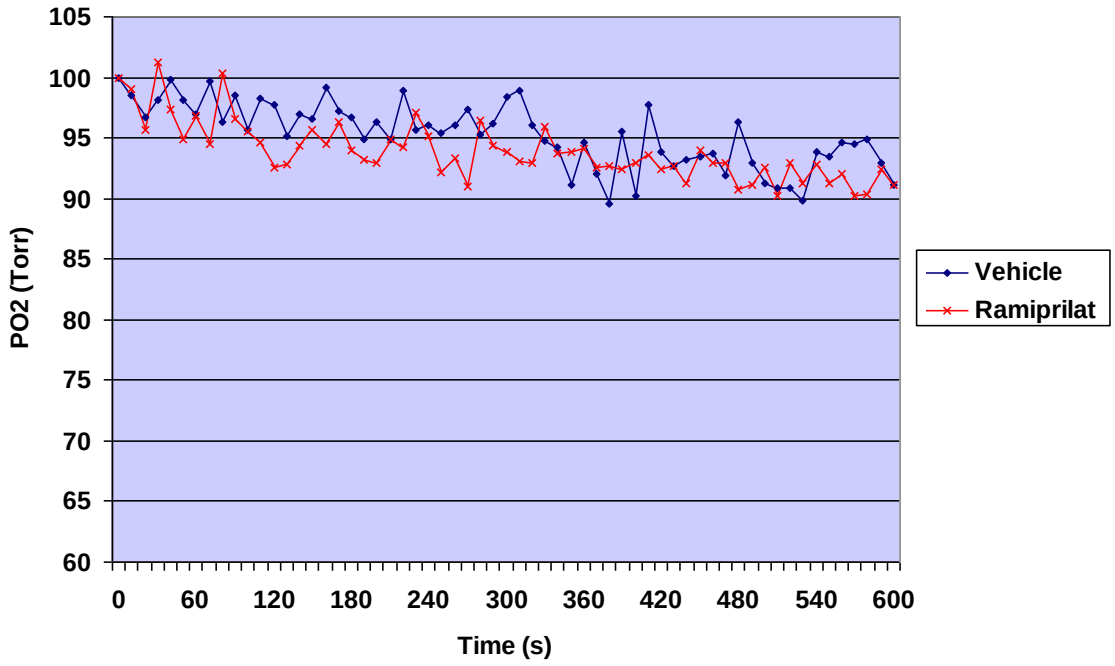
Figure 5.32. Respiratory control ratio (RCR) in vehicle, LPS and angiotensin II treated C_2C_{12} cells.



5.3.2. C_2C_{12} cells treated with ACE inhibitor

In preliminary experiments, no difference in basal oxygen consumption was observed between vehicle and ramiprilat (10^{-5} M) treated cells (-7 ±1.5 mTorr.s^{-1} *vs.* -5 ±2.3 mTorr.s^{-1}, respectively; *P*=NS; Figure 5.33).

Figure 5.33. OxySpot graph depicting oxygen consumption in C_2C_{12} myocytes treated with vehicle or Ramiprilat (10^{-5}M) for 24 hours (PO_2 indexed to 100)

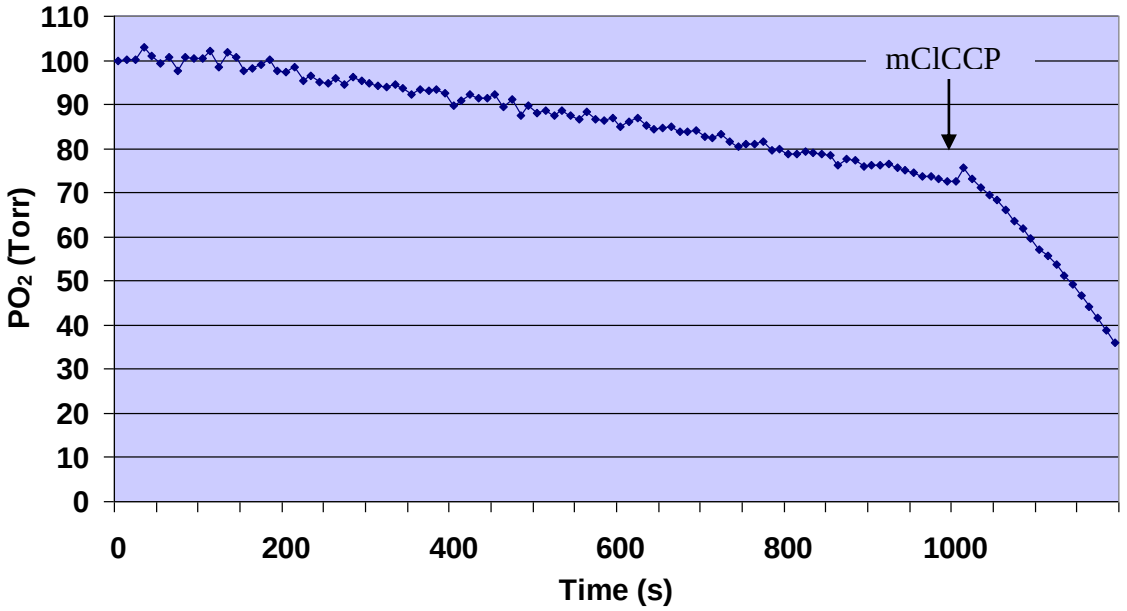


5.3.3. C_2C_{12} treatment with Angiotensin II

Pre-treatment of C_2C_{12} myocytes with Ang II (10^{-7}M) for 1 hour resulted in a significant increase in basal oxygen consumption in C_2C_{12} myotubes (-7 ±0.6 mTorr.s^{-1} *vs.* -23 ±8.2mTorr.s^{-1} for vehicle *vs.* Ang II treatment; *P*=0.03; n=3). Treatment with an uncoupler in Ang II treated cells resulted in a significant increase in oxygen consumption (-169 ±63 mTorr.s^{-1}; *P*=0.02).

The respiratory control ratio was significantly higher in Ang II cells than in vehicle treated cells (1 ± 0.3 *vs.* 7 ± 0.2 for vehicle *vs.* Ang II; $P = 0.0002$; Figure 5.34).

Figure 5.34. Graph depicting oxygen consumption in C_2C_{12} myocytes pre-treated with Angiotensin II (10^{-7}M) for 1 hour (PO_2 indexed to 100)



5.4 UCP3 mRNA EXPRESSION IN C_2C_{12} MYCOCYTES.

Primers were designed as previously described (Table 5.2) with a melting temperature (T_m) of 60ºC. Primers were designed to either include an intronic sequence within the amplified sequence (thereby yielding a longer amplicon if DNA contamination were present and thus allowing differentiation from amplified RNA) or designed to straddle exon-exon boundaries (preventing contaminant DNA from being amplified altogether). PCR conditions were optimised using magnesium titrations in the range 3.0-5.0 mM to enable the same primers to be used for PCR amplification with the LightCycler™ if subsequently required. PCR products were run on 2% agarose gels stained with

ethidium bromide against a 1 kB DNA ladder (GibCo). The mouse UCP3 cDNA was particularly difficult to amplify. Four sets of primers were tried before amplification was achieved.

In keeping with previous reports, the mRNA signal for UCP3 was greater than that for UCP2 in C_2C_{12} myocytes. The effect of 24 hours of ACEi treatment on UCP3 mRNA was assessed. There were no gross differences in the UCP3 mRNA signal when PCR product was run on 2% agarose gels. For a more accurate assessment, quantitative analysis by real-time RT-PCR was carried out.

The ratio of UCP3:GAPDH was quantified using RelQuant software as previously described (Methods 3.4.5.) and was similar to previous reports (Shimokawa *et al.* 1998).

Table 5.2. Forward and reverse primers for RT-PCR and amplicon size

Gene	Forward Primer	Reverse Primer	Amplicon size (bp)
mGAPDH	TGCATCCTGCAGCACCAACTG	CACAGCTTTCCAGAGGGGCCA	141
mUCP3	CCCGATACATGAACGCT	AGATTCCCGCAGTACC	203
mUCP2	GGTCCGCTTCCAGGCTCAGG	GCATTACGGGCAACATTG	138

Incubation of C_2C_{12} myotubes with increasing concentrations of ramiprilat for 24 hour resulted in no significant changes in UCP3 mRNA expression (Figure 5.33, n=3). To test whether ACE inhibition would modify the effect of cell stress on myocyte UCP3 mRNA expression, C_2C_{12} cells were incubated within a hypoxic (37C, 5%CO_2, 1% O_2, 94% N_2) environment for 6 hours, with or without pre-treatment with ramiprilat 10^{-5}M for 24 hours (Figure 5.34). Hypoxia resulted in an 11.6 fold decrease in UCP3 mRNA

expression at 6 hours (P=0.03; n=4) in both vehicle and ramiprilat pre-treated cells. There was no effect of ramiprilat treatment on the hypoxia induced suppression of UCP3 mRNA (P=0.5).

Figure 5.35. **Quantitative RT-PCR analysis of UCP3:GAPDH mRNA transcript ratio in C_2C_{12} myotubes treated with increasing concentration of the ACE inhibitor ramiprilat for 24 hours (mean ± SD of 3 experiments).**

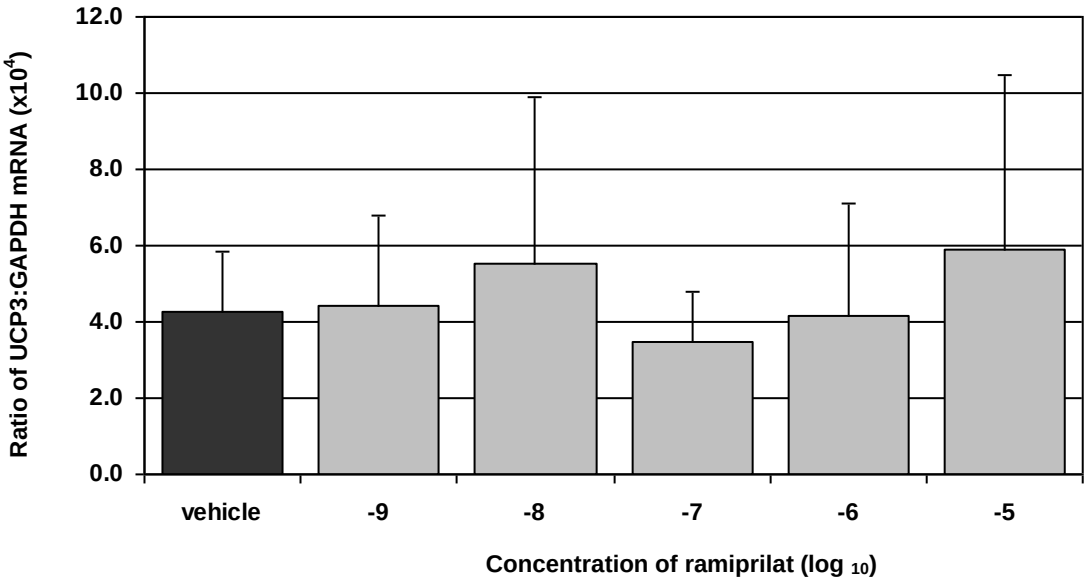
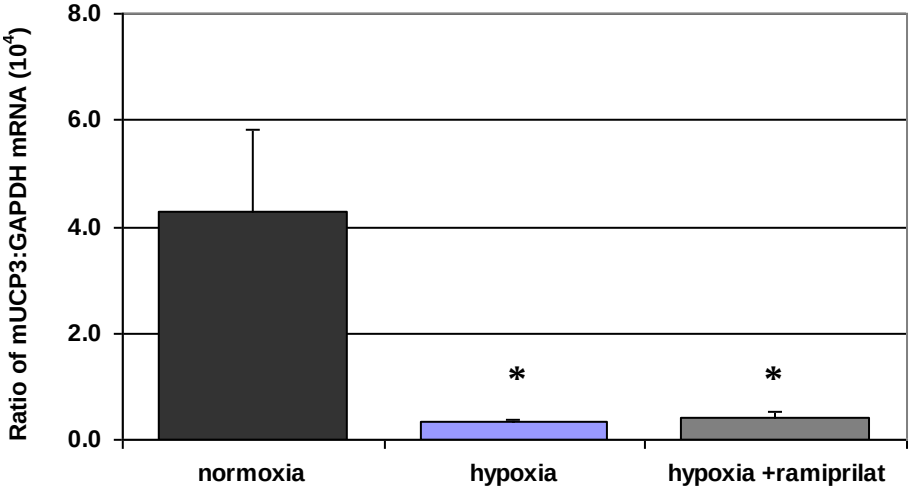


Figure 5.36 **The effects of 6 hours of hypoxia and co-treatment with the ACE inhibitor ramiprilat on the UCP3:GAPDH mRNA transcript ratio in C_2C_{12} myotubes analysed by quantitative RT-PCR (mean ± SD of 3-4 experiments)**



* $P < 0.05$ *vs.* normoxia

5.5 DISCUSSION

Preliminary observations were carried out using cell lines which have the benefit of ease of culture and rapid growth. However, cell lines may not match the physiological characteristics of the original source tissue and have the potential to transform or subclone. Primary cells were therefore preferred in later experiments examining $\Delta\psi_m$. Myotubular cells (differentiated C_2C_{12} cells) and, in particular, adult ventricular myocytes tended to clump and run slowly through the flow cytometer's fluidic system. In the case of C_2C_{12} tubes, this problem was overcome with frequent rinsing of the fluidics with FACSafe liquid between samples. Dye loading was carried out in Kreb's Buffer containing HEPES which is able to buffer fluctuations in environmental carbon dioxide as cells are moved from incubator for analysis. This is particularly important since mitochondrial probe loading is pH dependent.

The mitochondrial probe TMRM exhibited quenching above 1-2μM, with plateauing of the fluorescence-dose response curve. Treatment with the uncoupler mClCCP below this threshold resulted in a characteristic loss of fluorescent signal, whereas, above this threshold, uncoupling resulted in an increase in signal. This is consistent with observations by others (Duchen *et al.* 1998; Jacobson *et al.* 2001; Jacobson *et al.* 2002; Jacobson *et al.* 2002; Krieger *et al.* 2002; Duchen *et al.* 2003). In order to use TMRM in a semi-quantitative manner for measuring alterations in was decided to use a low concentration of dye where the increase in fluorescence signal was proportional to an increase in dye uptake, which is directly related to $\Delta\psi_m$ according to the Nernst equation (Ehrenberg *et al.* 1988; Loew *et al.* 1993; Scaduto *et al.* 1999).

The use of JC-1 as an indicator of changes in $\Delta\psi_m$ was also examined. JC-1 is known to form aggregates at high concentration, which have different spectral properties to monomer JC-1. In contrast to TMRM, addition of uncoupler after incubation with JC-1 resulted in no significant change in fluorescence characteristics. This supports the reported findings that aggregates tend to disassemble slowly in response to rapid changes in $\Delta\psi_m$ (Nicholls & Ward 2000). To examine the alterations in JC-1 fluorescence associated with mitochondrial uncoupling, mClCCP was therefore added to cell suspensions for 15 minutes prior to dye loading. This approach demonstrated that relatively high concentrations of dye were required to attain rapid equilibrium. JC-1 characteristics were similar to those observed by others (Reers *et al.* 1991; Smiley *et al.* 1991; Cossarizza *et al.* 1993; Di Lisa *et al.* 1995; Salvioli *et al.* 1997; Nuydens *et al.* 1999; Collins *et al.* 2000; Mathur *et al.* 2000; Rakhit *et al.* 2001).

Flow cytometric assessment of $\Delta\psi_m$ allowed a large sample of cells to be analysed quickly. Without direct visualisation, however, the assumption is made that the fluorescent signal is within mitochondria. Loss of fluorescence signal with treatment with low dose of uncoupler implies that dye accumulation is dependent on $\Delta\psi_m$, although uncoupler can dissipate plasma membrane potential, albeit at higher concentrations. With flow cytometry, simultaneous assessments of fluorescent signals can be recorded. This is particularly advantageous in the case of JC-1, allowing ratiometric measurement of $\Delta\psi_m$. It may also be possible to measure other aspects of mitochondrial physiology, such as ROS generation with a second fluorochrome. CLSM is also flexible in this regard. Simultaneous fluorescent recordings can be made. However, as with flow cytometry, spectral bleed-through is a potential hurdle. The scanning software now allows sequential laser scanning from separate laser sources. This has the potential of reducing bleed-through, and correction for emission spectral

overlap can be made by using different dichroiic mirrors and bandwidth filters. CLSM is a powerful tool, allowing direct visualisation of mitochondrial signal and physiological changes. Four-dimensional imaging is possible, with superfusion time analysis and 3D reconstruction. However, fewer cells can be examined, analysis software is not 'user-friendly' and analysis, itself, time consuming.

Treatment of all cell types with uncoupler resulted in collapse of $\Delta\psi_m$ as recorded by a loss of mitochondrial probe fluorescence. This was seen as an increase in cellular respiration, as recorded by OxySpot measurements in C_2C_{12} cells. Following collapse of $\Delta\psi_m$, uncontrolled, futile cycling of the ETC occurs, with resultant increase in oxygen consumption. However, there is no PMF to drive ATP synthesis, so ATP is generated within the cytoplasm by anaerobic glycolysis, although this ATP may, in turn, be rapidly depleted by reversal of ATP synthase (Nicholls *et al.* 2002).

Treatment with the ACEi ramiprilat for 24 hours increased $\Delta\psi_m$ in both skeletal and cardiac blasts and myocytes as assessed by the two complementary techniques of flow cytometry and confocal microscopy. The relative increase in fluorescence signal was 38-50% measured in primary cardiomyocytes, peaking at 12 hours. This relative hyperpolarisation of the inner mitochondrial membrane would lead theoretically to a decrease in mitochondrial respiration rate (Nicholls *et al.* 2002), i.e. with an increase in $\Delta\psi_m$, the ETC would be near equilibrium with a presumed lowering of the ADP/ATP ratio, thereby slowing the rate of respiration. However, no alteration in basal respiratory rate was seen in C_2C_{12} cells treated with ramiprilat. This may be due to lack of sensitivity of the assay to measure small changes in respiratory rate.

The increase in $\Delta\psi_m$ following ACEi treatment suggests an increase in the coupling between mitochondrial respiration and ATP generation and the delayed increase suggested a gene-transcriptional event. UCPs may uncouple oxidative-phosphorylation. Therefore, ACEi may decrease UCP expression and therefore increase mitochondrial coupling, or ACE inhibition may cause an increase in $\Delta\psi_m$ through another mechanism, with resultant increase in ROS generation triggering a protective increase in UCP expression. The effect of ACE inhibition on UCP3 mRNA expression in skeletal myotubes was therefore tested. However, no significant change in UCP3 gene transcription was observed in C_2C_{12} myotubes treated with ramiprilat for 24 hours. There was a wide degree of variation of UCP3 mRNA transcript ratio in both treated and untreated myotubes. This may be a reflection of the variability in UCP3 mRNA seen with degree of differentiation in this cell type (Shimokawa *et al.* 1998). This may have prevented small changes in transcript ratio being observed. Moreover, UCP2 and UCP3 undergo extensive post-transcriptional changes, so it may be more important to measure protein levels, but measuring protein has been hindered, until recently, by the lack of specific antibodies available (Pecqueur *et al.* 2001). However, neither the amount of mRNA nor the protein may reflect the degree of activation of UCP, which depends on the presence of fatty acid or ROS (Echtay *et al.* 2002).

The effect of the ACEi tested on $\Delta\psi_m$ followed the differences in lipophilicity of the respective active metabolite, from most lipid soluble (ramiprilat) having a greater response than imidiprilat (intermediate) which was greater than the most water soluble (perindoprilat) (Dzau *et al.* 2001). This does suggest that the biological action of each ACEi may rely on its diffusion capacity into or across the lipid bilayer of either the plasma or mitochondrial membrane, to ultimately increase the bioavailability of bradykinin and NO•.

Could alterations in NO• explain the effects of ACEi on $\Delta\psi_m$? NO• is a competitive inhibitor of oxygen at cytochrome oxidase (Brown *et al.* 1994; Cleeter *et al.* 1994), and therefore may be able to exert metabolic control over mitochondrial respiration (Shen *et al.* 1995; Clementi *et al.* 1999; Loke *et al.* 1999). The concentration and source of NO• is critical (Kojda *et al.* 1999). ACEi reductions in oxygen consumption and improvements in contractility in cardiac tissue have been shown to be NO•-dependent (Zhang *et al.* 1997), but the source of NO• is likely to be eNOS and of a low concentration. Persistent inhibition of respiration and contractile dysfunction can occur with higher concentrations of NO•, either provided by exogenously administered NO• donors (Beltran *et al.* 2000) or potent inducers of iNOS, such as endotoxin (Rosser *et al.* 1998). In these situations, oxygen consumption at cytochrome oxidase is inhibited and cellular ATP demand met by cytoplasmic anaerobic glycolysis, and reversal of ATP synthase and the adenine nucleotide translocator use this ATP to maintain a relative increase in $\Delta\psi_m$ (Beltran *et al.* 2000; Moncada *et al.* 2002). In keeping with this, treatment of C_2C_{12} cells with endotoxin (LPS) for 24 hours significantly suppressed cellular oxygen consumption by 73%. LPS has been shown to reduce cellular oxygen consumption in other cell types (James *et al.* 1995; Borutaite *et al.* 2001) in an NO•-dependent manner (Borutaite *et al.* 2001). Respiratory uncoupling by mClCCP was unaffected by the presence of LPS. However, the RCR was significantly higher in LPS treated cells, therefore implying that LPS exerted significant metabolic control on mitochondrial respiration.

Surprisingly, direct incubation of C_2C_{12} cells with Ang II tended to also increase the RCR, but, in contrast to LPS, this was primarily due to an increase in uncoupled respiratory rate. This effect was seen following 1 hour treatment with Ang II and has not been previously reported in the literature, and appears to be distinct from the NO•

modulation of respiration. Chronic Ang II treatment in rats is known to alter resting oxygen consumption (Cassis *et al.* 2002) and result in cachexia (Brink *et al.* 1996). Ang II may be decreasing mitochondrial ETC coupling by increasing activity of UCPs, which are also known to be upregulated in skeletal muscle in experimental cancer cachexia (Bing *et al.* 2000). Alternatively, Ang II may be acting directly on the ETC complexes or via inhibition of NO• pathways.

※ RESULTS CHAPTER SIX ※

IS THERE AN ASSOCIATION BETWEEN VARIATION IN THE *UCP3/UCP2* LOCUS AND CARDIOVASCULAR OR PERFORMANCE PHENOTYPES?

I hypothesised that some of the observed associations between *ACE* genotype and *BDKRB2* genotype on LV growth, cardiovascular risk and on performance phenotypes may be due to metabolic alterations within cardiac and skeletal muscle. The data presented in Chapters 5 confirm that alteration of ACE activity can affect mitochondrial coupling in these cell types. If this association holds true, then could common genetic variation in mitochondrial uncoupling proteins alter the same human cardiovascular and performance phenotypes as has been described for variation in the *ACE* and *BDKRB2* genes? Any such association may strengthen the argument that mitochondrial function or dysfunction is the primary mover in these (patho)physiological states.

The UCPs are attractive candidates for modulating human metabolic rate and performance. Both UCP2 and UCP3 are also negative regulators of mitochondrial ROS generation *in-vitro* (Echtay *et al.* 2002) and in animal models of sepsis and inflammation (Pecqueur *et al.* 2001; Sun *et al.* 2003). The *UCP2/3* genetic locus has been associated with differences in basal metabolic rate (Bouchard *et al.* 1997) and body mass index in children (Yanovski *et al.* 2000) and in adults (Cassell *et al.* 1999; Esterbauer *et al.* 2001) but the associations have not been replicated in all studies (Dalgaard *et al.* 1999; Dalgaard *et al.* 2001). A common, functional promoter variant has been described (Esterbauer *et al.* 2001), UCP2-866G>A, which is at the junction between negative and positive cis-acting DNA regions, and within a region containing binding sites for hypoxia, inflammation and pancreatic β-cell-specific binding factors.

The rare (A) allele has been associated with lower gene transcription (repression) in somatic non-β cells (Krempler *et al.* 2002), but more effective gene transcription in pancreatic β cells with reduced markers of β cell function (Krempler *et al.* 2002) as well as measures of reduced GSIS (Sesti *et al.* 2003). The A allele has been associated with protection from obesity (Esterbauer *et al.* 2001), but is associated with the presence of diabetes in obese subjects (Krempler *et al.* 2002). A common promoter variant has also been described in the UCP3 gene (-55C>T) (Cassell *et al.* 2000). The variant allele has been associated with obesity in a recessive manner in several studies (Cassell *et al.* 2000; Otabe *et al.* 2000; Halsall *et al.* 2001).

It could be anticipated that the *UCP2*-866A allele would be associated with lower UCP2 activity in cardiac and skeletal muscle and thus both increased mitochondrial coupling (increased 'efficiency'), but as a result, increased mitochondrial ROS generation. Similarly, the *UCP3*-55T allele may also represent a thrifty genotype, with enhanced mitochondrial coupling and preservation of substrate supply. I hypothesised that these two functional variants might therefore be associated with prospective changes in cardiac muscle mass and indices of skeletal muscle performance, as well as prospective CHD risk, which itself may be related to excess mitochondrial ROS generation. As such, I hypothesised that genetic variation at the *UCP2/UCP3* locus might also be associated with systemic markers of oxidative stress amongst a cohort of diabetic patients and systemic markers of inflammation during strenuous exercise.

6.1 GENETIC VARIATION IN *UCP2* AND *UCP3* AND LV MASS

The baseline characteristics of the 141/212 study subjects who completed army training in the prospective Big Heart 2 (Bassingbourn 2) study of the effects training on LV mass as assessed by CMR were described in Table 3.1.

6.1.1 *UCP2*-866G>A genotype

Of the study subjects, 136/141 (97%) were successfully genotyped for the *UCP2*-866G>A gene variant. The genotype frequency (GG 36.0%, GA 50.7%, AA 13.2%) was consistent with the Hardy-Weinberg equilibrium (χ^2=0.67; *P*=0.41) and rare (A) allele frequency (0.386) was similar to previous reports (Esterbauer *et al.* 2000). *UCP2*-866AA homozygotes had higher systolic blood pressures (SBP) at baseline than G allele carriers (Table 6.1; ANOVA 3 way analysis *P*=0.04, AA *vs.* G allele ANOVA *P* = 0.015). There was no difference between *UCP2* genotypes and either BMI or lean body mass, but adipose tissue mass was significantly higher in *UCP2*AA homozygotes (3 way analyses ANOVA *P* = 0.03, linear trend *P* = 0.007; AA vs. G allele carriers *P* = 0.05).

Before the onset of training, LV mass was independent of *UCP2* genotype (Figure 6.1; Table 6.1). Following training, however, the AA carriers had significantly higher LV mass than G allele carriers (204 ± 29 g *vs.* 190 ± 25 g *vs.* 192 ± 23 g for AA *vs.* GA *vs.* GG, respectively; *P* = 0.13 ANOVA; *P* < 0.05 for AA *vs.* G allele; Table 6.1). The difference in LV mass persisted after correction for SBP, BSA, adipose and lean mass.

There was no significant association with training-related change in LV mass. All data suggested a recessive effect of the A allele.

6.1.2 *UCP3*-55C>T genotype

The genotype frequency (CC 51.5%, CT 38.5%, TT 10.4%) was consistent with Hardy-Weinberg equilibrium and rare allele frequency (0.296) was similar to previous reports (Cassell *et al.* 2000; Meirhaeghe *et al.* 2000). Baseline characteristics were independent of *UCP3*-55C>T genotype: in particular there was no association between BMI, lean mass or adipose mass and genotype (Table 6.2). LV mass indexed to lean body mass ($LVMI^{LM}$) was significantly higher in TT homozygotes after training.

There was no evidence of LD between UCP2-866G>A and *UCP3*-55C>T genotypes ($\Delta = 0.23$; $P = 0.14$). Haplotypic analysis (combining *UCP2*-866GG+GA and *UCP3*-55CC+CT) provided small groups for comparison. The *UCP2*-866G+/*UCP3*-55C+ haplotype had the smallest mean LV mass before training and this was significant after training ($P = 0.04$ linear trend; Table 3.3; Figure 3.1) and reached greater statistical significance when indexed to lean mass ($P = 0.03$ for ANOVA; $P = 0.01$ for linear trend).

Table 6.1. Baseline characteristics of Big Heart 2 Study sample by *UCP2*-866G>A and *UCP3*-55C>T variants

Trait	*UCP2* -866G>A Genotype			*UCP3* -55C>T Genotype		
	GG n = 49	**GA n = 69**	**AA n = 18**	**CC n = 69**	**CT n = 52**	**TT n = 14**
Age (years)	19.0 (1.7)	19.9 (2.7)	19.9 (3.1)	19.4 (2.3)	19.8 (2.7)	19.9 (2.8)
Systolic blood pressure (mmHg)	118 (12)	116 (10)	125 (14)*	119 (13)	117 (10)	118 (12)
Diastolic blood pressure (mmHg)	67 (13)	65 (9)	67 (11)	66 (11)	68 (11)	63 (9)
Body mass index ($kg.m^{-2}$)	22.9 (1.9)	23.2 (2.3)	23.5 (2.7)	23.3 (1.9)	22.9 (2.5)	22.9 (2.1)
Lean mass (kg)	59.2 (6.0)	58.5 (6.2)	59.0 (6.0)	58.9 (6.3)	58.8 (5.8)	58.3 (6.1)
Adipose tissue mass (kg)	11.1 (2.9)	12.5 (4.2)	14.0 (4.1)†	12.2 (3.5)	12.1 (4.5)	11.7 (3.0)

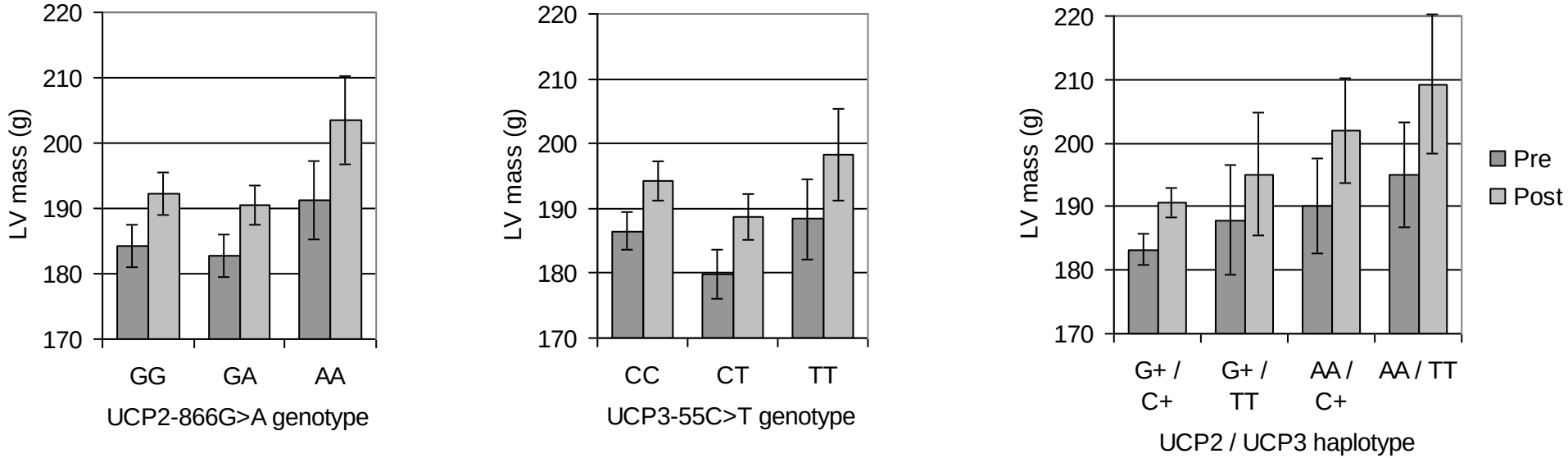
*P=0.04 for GG vs. GA vs. AA by ANOVA, P=0.015 for AA *vs.* G allele

†P=0.03 for GG vs. GA vs. AA by ANOVA, P=0.007 for linear trend

Table 6.2 Training related changes in cardiac parameters by *UCP2*-866G>A and *UCP3*-55C>T genotypes and haplotypes

Cardiac traits	*UCP2* -866G>A Genotype			P ANOVA	P G+ vs. AA
	GG	GA	AA		
LV mass pre-training (g)	184.2 (22.7)	182.7 (27.0)	191.2 (25.4)	0.45	0.67
$LVMI^{LM}$ pre-training ($x10^{-3}$)	3.11 (0.31)	3.17 (0.36)	3.29 (0.39)	0.19	0.13
LV mass post training (g)	192.3 (22.7)	190.4 (24.8)	203.6 (28.6)	0.13	0.04
$LVMI^{LM}$ post training ($x10^{-3}$)	3.17 (0.32)	3.21 (0.34)	3.37 (0.39)	0.12	0.05
Change in LV mass (%)	4.8 (8.8)	4.9 (7.9)	6.6 (6.7)	0.70	0.40
Change in LV stroke volume (%)	10.4 (31.8)	6.8 (26.7)	18.0 (31.3)	0.24	0.09
Change in RV stroke volume (%)	8.4 (19.6)	8.0 (22.7)	22.2 (25.9)	0.05	0.01
	***UCP3*-55C>T Genotype**			**P ANOVA**	**P C+ *vs.* TT**
	CC	**CT**	**TT**		
LV mass pre-training (g)	186.5 (24.3)	179.8 (27.4)	188.4 (23.2)	0.29	0.52
$LVMI^{LM}$ pre-training ($x10^{-3}$)	3.17 (0.34)	3.15 (0.38)	3.26 (0.27)	0.61	0.34
LV mass post training (g)	194.2 (24.8)	188.7 (24.7)	198.2 (26.3)	0.32	0.37
$LVMI^{LM}$ post training ($x10^{-3}$)	3.20 (0.35)	3.19 (0.33)	3.42 (0.27)	0.11	0.04
Change in LV mass (%)	4.5 (8.0)	5.7 (8.6)	5.2 (4.3)	0.29	0.9
Change in LV stroke volume (%)	13.0 (24.8)	7.7 (22.9)	3.9 (23.1)	0.71	0.3
Change in RV stroke volume (%)	11.4 (23.6)	9.0 (22.3)	6.5 (17.3)	0.05	0.5
	***UCP2*-886/*UCP3*-55 haplotypes**				**P ANOVA**	**P Linear trend**
	G+ / C+ N=106	**G+ / TT N=9**	**AA / C+ N=14**	**AA / TT N=4**		
LV mass pre-training (g)	183.2 (25.3)	187.9 (26.1)	190.1 (27.8)	195.0 (16.7)	0.62	0.19
$LVMI^{LM}$ pre-training ($x10^{-3}$)	3.15 (0.34)	3.31 (0.27)	3.32 (0.42)	3.20 (0.32)	0.25	0.11
LV mass post training (g)	190.7 (23.8)	195.1 (29.0)	202.0 (30.8)	209.3 (21.9)	0.22	0.04
$LVMI^{LM}$ post training ($x10^{-3}$)	3.17 (0.32)	3.50 (0.25)	3.38 (0.42)	3.34 (0.33)	0.03	0.01

Figure 6.1. Pre and post left ventricular (LV) mass measured by cardiac MRI in healthy young men according to *UCP2*-866G>A and *UCP3*-55C>T genotypes and combined haplotype.



G+ = *UCP2*-866G allele; C+ = *UCP3*-55C allele

6.2. PROSPECTIVE CARDIOVASCULAR RISK AND GENETIC VARIATION OF *UCP2* AND *UCP3*

Subjects were drawn from the NPHSII study of healthy UK men (Methods 2.1.2)

6.2.1 *UCP2*-866G>A (Dhamrait *et al.* 2004)

A total of 2695 from 2775 study subjects with DNA available (97.1%) were successfully genotyped, whose baseline characteristics by CHD event status (which did not differ from those in whom genotyping failed) are presented in Table 6.3. Genotype distribution was consistent with Hardy-Weinberg equilibrium (χ^2=0.04; *P*=0.84), and both genotype distribution and rare (A) allele frequency of 0.37 (0.35 - 0.38) were similar to that of healthy controls previously reported (Esterbauer *et al.* 2001; Sesti *et al.* 2003). Homozygosity for the A allele was more prevalent amongst obese subjects (64/375 obese vs. 297/2316 non-obese; *P*=0.03) and was associated with a significant elevation in baseline DBP. All other baseline characteristics were independent of *UCP2* genotype.

CHD risk was substantially elevated amongst those of *UCP2*-866AA genotype (HR 2.22 [1.53 - 3.22] for AA vs. GG genotype; *P* = 0.0002; Table 6.5). The hazard ratios demonstrated a recessive effect of the A allele (HR 2.08 [1.49 – 2.86]; *P* < 0.0001 for AA vs. GA+GG). The doubling in risk remained highly statistically significant even after adjustment for all baseline characteristics including BMI (HR 2.05 [1.28 – 3.26]; *P* = 0.003 for AA vs. GA+GG). CHD risk was doubled amongst AA homozygotes without traditional risk factors for CHD, for example non-obese subjects, non-diabetic or normotensive subjects (Figures 6.2 A-C; Table 6.6). Amongst the small group of diabetics, 38.5% (5/13) *UCP2*-866AA carriers had a CHD event (HR 4.00 [1.55 – 10.31]; *P* = 0.004 for diabetic AA vs. non-diabetic AA; Figure 6.2C).

Table 6.3. Baseline characteristics and genotype frequencies by coronary artery disease event status for 2695 men from the Second Northwick Park Heart Study (NPHSII) genotyped for the *UCP2*-866G>A gene variant. Data are mean (SD) unless otherwise stated.

Trait	Controls No CHD event n = 2491	Cases CHD event n = 204	Probability
Age (years)	56.0 (3.4)	56.6 (3.5)	0.04
Systolic Blood Pressure (mmHg)	138.0 (19.1)	143.8 (20.1)	<0.0001
Diastolic Blood Pressure (mmHg)	84.3 (11.3)	87.8 (11.6)	<0.0001
Body Mass Index ($kg.m^{-2}$)	26.4 (3.5)	27.1 (3.4)	0.004
Current smoking % (n)	27.5% (685)	38.2 % (78)	0.001
Diabetes % (n)	2.1% (51)	6.4% (13)	<0.0001
Cholesterol ($mmol.l^{-1}$)	5.70 (1.00)	6.07 (1.01)	<0.0001
Triglyceride ($mmol.l^{-1}$)*	1.77 (0.93)	2.09 (1.12)	<0.0001
CRP ($mg.l^{-1}$)* ; N=721	1.20 (1.33) N=623	4.65 (5.07) N=98	<0.0001
Fibrinogen ($g.l^{-1}$)*	2.70 (0.51)	2.84 (0.50)	0.0003

*geometric mean (approximate SD).

Table 6.4. Baseline characteristics in NPHS II by *UCP2*-866G>A genotype. Data are mean (SD) unless otherwise stated.

Trait	*UCP2* -866G>A Genotype				
	GG	**GA**	**AA**	**Probability**	**Probability**
	n = 1088	**n = 1245**	**n = 362**	**3 way**	**G+ vs. AA**
Age (years)	56.1 (3.4)	56.1 (3.4)	56.1 (3.5)	0.99	0.93
Systolic blood pressure (mmHg)	138.5 (19.2)	138.1 (18.9)	139.7 (20.3)	0.38	0.19
Diastolic blood pressure (mmHg)	84.2 (11.2)	84.4 (11.4)	86.2 (11.1)	**0.01**	**0.003**
Body mass index ($kg.m^{-2}$)	26.5 (3.5)	26.4 (3.5)	26.7 (3.4)	0.4	0.22
Current smoking % (n)	28.0% (305)	28.9% (360)	27.1% (98)	0.76	0.57
Diabetes n (%)	13 (0.012)	25 (0.020)	26 (0.024)	0.22	0.10
Cholesterol ($mmol.l^{-1}$)	5.71 (1.02)	5.72 (1.00)	5.78 (1.01)	0.59	0.34
Triglyceride ($mmol.l^{-1}$) *	1.78 (0.94)	1.78 (0.93)	1.88 (1.03)	0.2	0.07
CRP ($mg.l^{-1}$)*; n	1.25 (1.48); n=295	1.25 (1.37) n=321	1.53 (1.76) n=105	0.23	0.09
Fibrinogen ($g.l^{-1}$)	2.71 (0.52)	2.70 (0.51)	2.71 (0.51)	0.79	0.64

* geometric mean (approximate SD).

Table 6.5. CHD risk in NPHS II in subjects stratified by *UCP2*-866G>A genotype.

UCP2-866G>A Genotype	Number with CHD events / total number (%)	Hazard Ratio* (95% CI)	Fully Adjusted Hazard Ratio† (95% CI)
GG	69 / 1088 (6.3 %)	1	1
GA	87 / 1245 (7.0 %)	1.13 (0.82 – 1.55)	1.15 (0.71 – 1.87)
AA	48 / 362 (13.3 %)	2.22 (1.53 - 3.22)	2.22 (1.29 – 3.82)
Probability		0.0002	0.01
GG + GA	156 / 2333 (6.7%)	1	1
AA	48 / 362 (13.3%)	2.08 (1.49 – 2.86)	2.05 (1.28 – 3.26)
Probability		<0.0001	0.003

* Adjusted for age, practice.

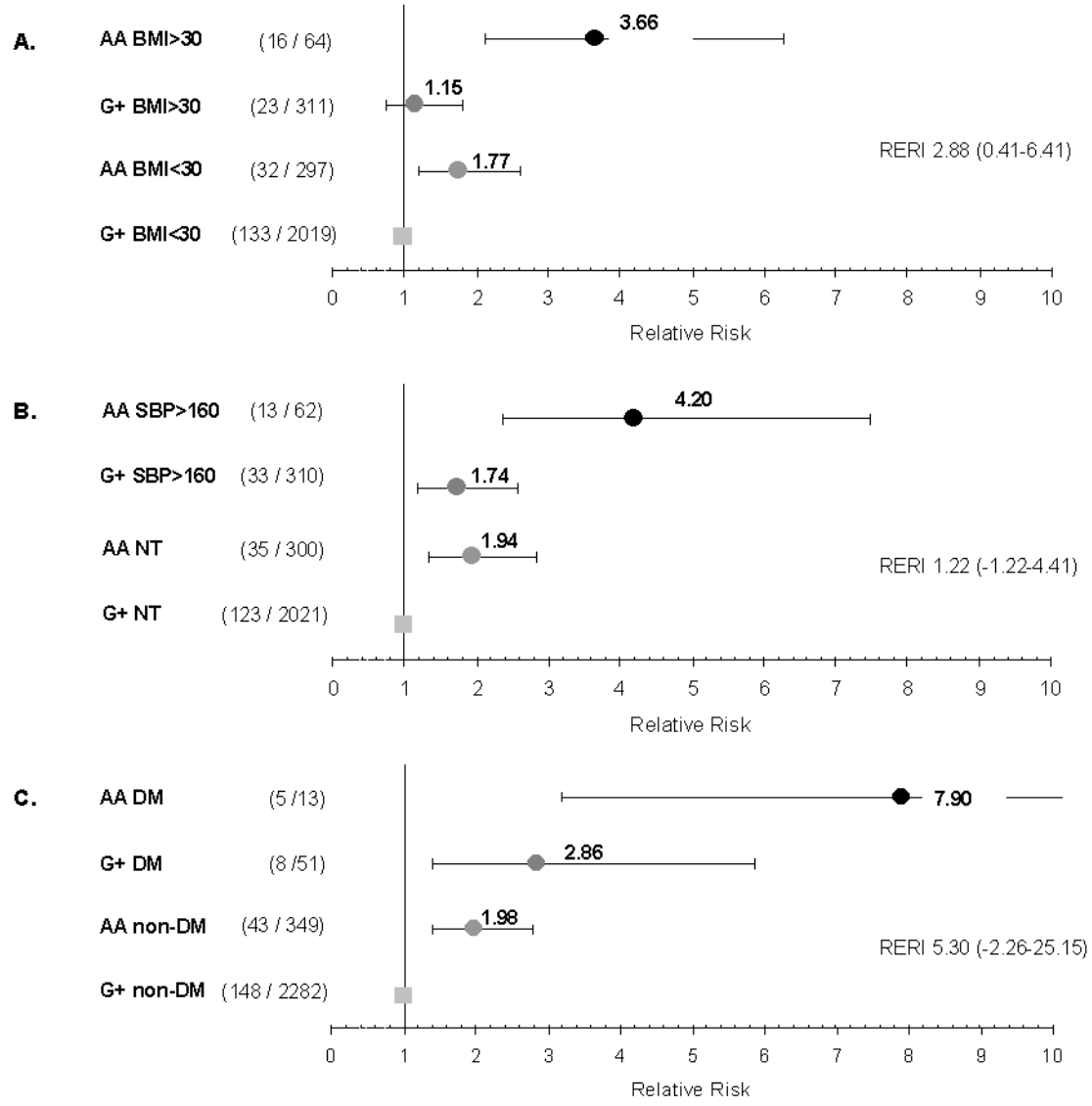
† Adjusted for age, practice, BMI, DBP, smoking, cholesterol, triglycerides, fibrinogen, diabetes and CRP.

Table 6.6. Relative risk of CHD event according to traditional risk factors of smoking, obesity, hypertension and diabetes for individuals stratified by *UCP2*-866G>A genotype.

Characteristic	*UCP2* AG+GG Events / Total No.	*UCP2* AA Events / Total No.	Relative Risk* [95% CI]	Probability
Non-smoker	90 / 1668 (5.4%)	36 / 264 (13.6%)	2.71 [1.83 – 4.01]	<0.001
Smoker	66 / 665 (9.9%)	12 / 98 (12.2%)	1.23 [0.66 -2.28]	0.51
BMI<30kg.m^{-2}	133 / 2019 (6.6%)	32 / 297 (10.8%)	1.77 [1.20 -2.61]	0.004
BMI≥30kg.m^{-2}	23 / 311 (7.4%)	16 / 64 (25.0%)	3.17 [1.65 – 6.10]	0.001
SBP<160mmHg	123 / 2021 (6.1%)	35 / 300 (11.7%)	1.94 [1.33 -2.84]	0.001
SBP≥160mmHg	33 / 310 (10.7%)	13 / 62 (21.0%)	2.41 [1.26 – 4.60]	0.008
DBP<95mmHg	110 / 1934 (5.7%)	35 / 283 (12.4%)	2.23 [1.52 -3.28]	<0.001
DBP≥95mmHg	46 / 396 (11.6%)	13 / 79 (16.5%)	1.57 [0.85 – 2.92]	0.15

* Adjusted for age & practice; SBP Systolic blood pressure, DBP Diastolic blood pressure, BMI Body mass index

Figure 6.2. Relative CHD risk in NPHSII by *UCP2*-866G>A genotype according to presence or absence of risk factors: A. obesity; B. Systolic hypertension; C. diabetes



BMI = Body mass index ($kg.m^{-2}$); G+ = G allele carriers

SBP>160 = systolic blood pressure>160mmHg; NT=normotensive

DM = diabetes mellitus

6.2.2 *UCP3*-55C>T

Of the 2775 study subjects with DNA available, 2694 (97%) were successfully genotyped. Genotype distribution was consistent with Hardy-Weinberg (χ^2 = 3.946, P = 0.05) and the rare (T) allele frequency of 0.23 (0.21 - 0.24) was similar to that in previous reports of European non-diabetic samples (Cassell *et al.* 2000; Meirhaeghe *et al.* 2000).

All baseline characteristics were independent of *UCP3* genotype (Table 6.7). Contrary to a previous report (Meirhaeghe *et al.* 2000), there was no association with obesity, lipid parameters nor with presence of diabetes

There was no association between *UCP3*-55C>T genotype and CHD risk (Table 6.8). In the small number of obese or diabetic TT homozygotes there did appear to be an elevated CHD risk, but there was no significant evidence of statistical interaction between genotype and either presence of obesity or diabetes.

Table 6.7. Baseline characteristics of study subjects in NPHS II by *UCP3*-55C>T genotype.

Trait	*UCP3* -55C>T Genotype			*P*
	CC	**CT**	**TT**	
	n = 1088	**n = 1245**	**n = 362**	
Age (years)	56.0 (3.5)	56.2 (3.4)	55.7 (3.3)	0.17
Systolic blood pressure (mmHg)	137.1 (18.8)	136.9 (18.8)	138.0(19.0)	0.78
Diastolic blood pressure (mmHg)	84.4 (10.9)	84.6 (11.9)	85.2 (11.6)	0.67
Body mass index ($kg.m^{-2}$)	26.3 (3.4)	26.2 (3.4)	25.9 (3.3)	0.38
Current smoking % (n)	29.0% (473)	27.9% (252)	22.6% (35)	0.23
Diabetes n (%)	2.5% (40)	2.2% (20)	3.2% (5)	0.74
Cholesterol ($mmol.l^{-1}$)	5.70 (1.01)	5.77 (1.01)	5.79 (1.01)	0.22
Triglyceride ($mmol.l^{-1}$) *	1.79 (0.95)	1.81 (0.94)	1.77 (0.96)	0.90
CRP ($mg.l^{-1}$)*; n	1.25 (1.48); n=295	1.25 (1.37) n=321	1.53 (1.76) n=105	0.23
Fibrinogen ($g.l^{-1}$)	2.73 (0.53)	2.69 (0.49)	2.67 (0.49)	0.08

Data are mean (SD) unless otherwise stated. * Geometric means (approximate SD)

Table 6.8. CHD risk in NPHS II by *UCP3*-55C>T genotype

UCP3-55C>T Genotype	Number with CHD events / total number (%)	Hazard Ratio* (95% CI)	Fully Adjusted Hazard Ratio† (95% CI)
CC	119 / 1634 (7.3 %)	1	1
CT	78 / 905 (8.6%)	1.20 (0.90 – 1.59)	1.21 (0.91 – 1.62)
TT	14 / 155 (9.0 %)	1.09 (0.62 – 1.91)	1.08 (0.61 – 1.91)
Probability		0.48	0.43

* Adjusted for age, practice.

† Adjusted for age, practice, BMI, DBP, smoking, cholesterol, triglycerides, fibrinogen, diabetes and CRP.

6.2.3 *UCP3*/2 haplotypes

There was no evidence of LD between the *UCP3*-55C>T and *UCP2*-866G>A variants (Table 6.9; $\Delta = 0.20$, $P < 0.001$) and haplotype frequencies were similar to those from young male British army recruits from the Big Heart Study (section 6.1). Those individuals who were *UCP3*-55TT/*UCP2*-866AA homozygotes had the highest CHD risk (compared to the common homozygote for both). However, there was no evidence of interaction between genotypes in determining CHD risk ($P = 0.2$), although risk appeared to rise more steeply according to *UCP2*-866G>A genotype amongst TT homozygotes (Figure 6.3). When the risk conferred by particular haplotypes was considered amongst cases and controls (Table 6.10; Figure 6.4), a highly significant effect was observed overall (χ^2 test $P < 0.00005$). The risk haplotype (more common amongst cases than controls) was *UCP3*-55T/UCP-866A ($P = 0.001$) with the converse true of the common *UCP3*-55C/*UCP2*-866G haplotype ($P = 0.005$).

Table 6.9. *UCP3*/2 haplotype distribution in NPHSII

NPHS2		***UCP2*-866G>A**		
		GG	GA	AA
***UCP3* -55C>T**	CC	759	678	170
	CT	273	473	144
	TT	34	72	45

Figure 6.3. Relative CHD risk in NPHS II according to UCP3/UCP2 haplotypes. The relative risk (± 2 standard deviations) is shown for each haplotype.

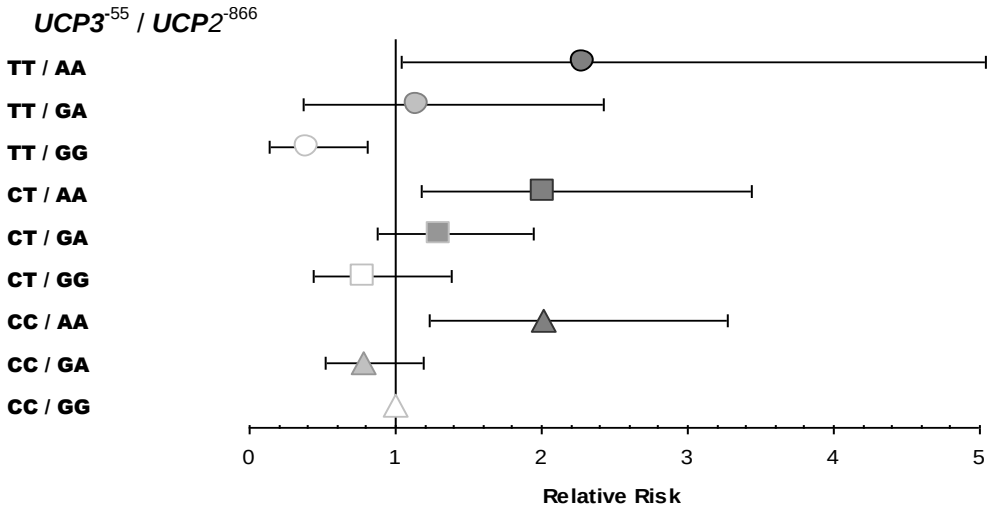
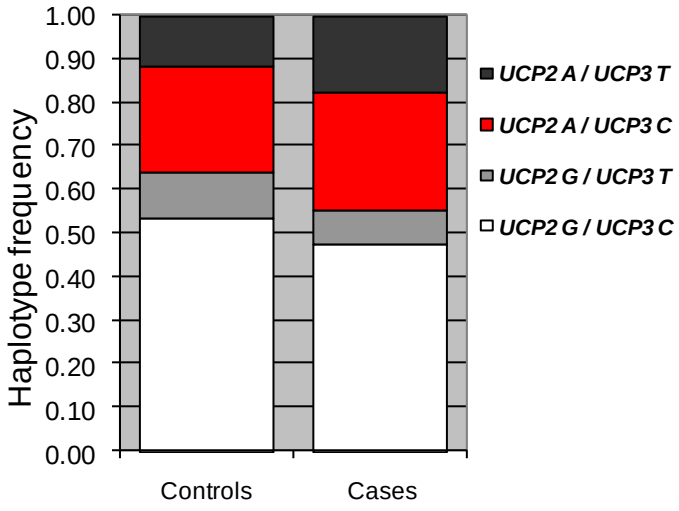


Table 6.10. *UCP3*/2 haplotype frequencies in cases and controls from NPHSII

Haplotype		Frequency			*P*
UCP3-55	*UCP2*-866	All	Controls	Cases	
C	G	0.531	0.535	0.472	0.005
C	A	0.244	0.242	0.271	0.19
T	G	0.103	0.105	0.078	0.08
T	A	0.122	0.117	0.177	0.001

Figure 6.4. *UCP3*/*UCP2* haplotype frequencies in cases and controls from NPHS II



6.3. SKELETAL MUSCLE PERFORMANCE AND *UCP* GENOTYPES.

The skeletal muscle efficiency study subjects were healthy male British army recruits undergoing basic army training and healthy female volunteers from the University of Staffordshire who underwent an endurance training programme (Methods 2.1.4). Genotype and haplotype frequencies were also sought in elite runners drawn from the British Olympic Athletes study (Methods 2.1.5).

6.3.1. Skeletal muscle efficiency

Data on those who had completed training and who were successfully genotyped for *UCP2*-866G>A (58/85; 68%) and *UCP3*-55C>T (61/85; 72%) are shown in Table 6.11. The low genotyping rate was due to degradation of DNA from the original delta efficiency study. There was no difference in baseline characteristics between those with and without genotype data. There was no frequency difference in either *UCP2*-866 G>A or *UCP3*-55C>T genotype between those who did and did not complete training. In subjects with complete data, both *UCP2* and *UCP3* genotypes were consistent with predicted Hardy Weinberg frequencies, with the rare allele frequencies similar to previous reports (Cassell *et al.* 2000; Esterbauer *et al.* 2001) and to the genotype frequency found in Bassingbourn 2 and NPHSII studies.

There were no significant associations between *UCP3* genotype and any baseline measurements including BMI and DE (Table 6.12).

There were no significant associations between *UCP2* genotype and baseline measurements, including DE (Table 6.12). *UCP2*-866A allele carriers had significantly higher DE after training (Table 6.12) due to a greater increase in DE associated with

training (-0.2 ±3.6% *vs.* 1.7 ±2.8% *vs.* 2.3 ±3.7% for GG *vs.* GA *vs.* AA, respectively; *P* = 0.07 ANOVA; *P* = 0.03 by linear trend; *P* = 0.02 for A allele carriers *vs.* GG homozygotes; Figure 6.5A). In univariate analysis, *UCP2*-866 genotype and presence or absence of the *UCP2*-866A allele accounted for 8.4% and 7.4% (adjusted R^2, respectively) of the interindividual variability in the absolute change in DE associated with endurance training. Of note, in multivariate analysis there was a significant interaction between *UCP2*-866G>A and *UCP3*-55C>T genotypes and their association with changes in DE with training (R^2 = 0.153; adjusted R^2 = 0.137, *P* for interaction = 0.003; Figure 6.5B) which was independent of the effect of either single polymorphism and also baseline characteristics of gender, height and mass. In a multivariate model, the *UCP2*-866A allele and the interaction between *UCP2* and *UCP3* genotypes accounted for 14.8% of the variation in training related change in DE (adjusted R^2).

Table 6.11. Baseline characteristics of the 131 subjects in the delta efficiency study

Trait	**Mean (SD)**
Age (years)	20.7 (4.4)
Gender (proportion male)	67%
Mass (kg)	70.4 (9.4)
Height (m)	1.74 (0.08)
Delta efficiency (%)	24.6 (2.6)
UCP3-55C>T CC / CT / TT (n)	29 / 27 / 5
T allele frequency (95% C.I.)	0.303 (0.222 -0.385)
UCP2-866G>A GG / GA / AA (n)	21 / 22 / 15
A allele frequency (95% C.I.)	0.448 (0.358-0.539)

C.I. = Confidence interval

Table 6.12. Training related changes in delta efficiency according to *UCP2*-866G>A and *UCP3*-55C>T genotypes

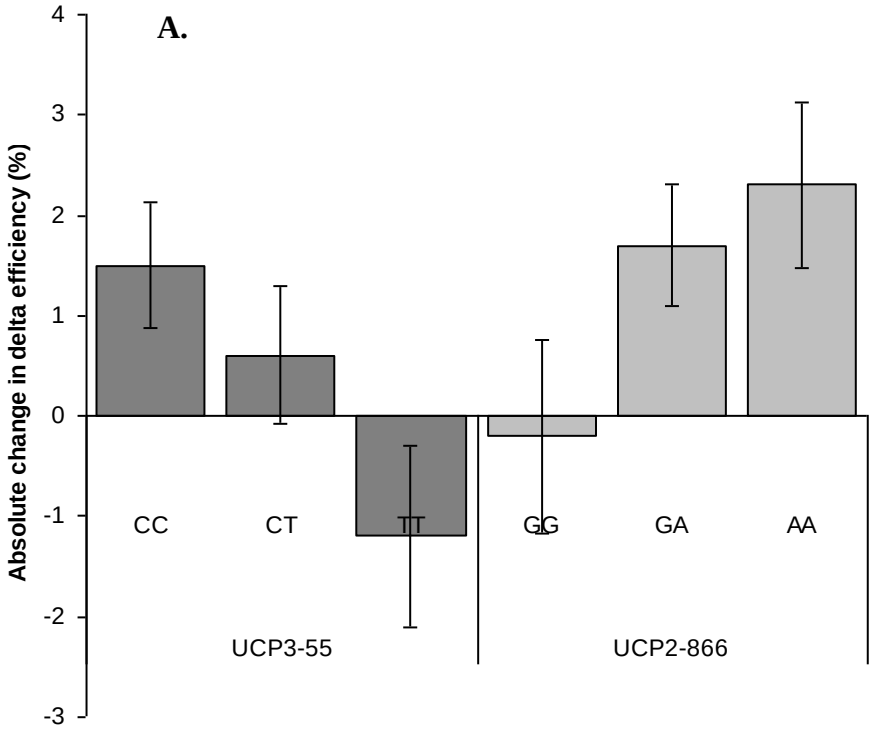
		Delta efficiency (%)			
UCP2-866G>A genotype		Pre	Post	Absolute change (%)	Proportional change (%)
UCP2-866	GG (21)	24.6 ± 2.6	24.4 ± 2.8	-0.2 ± 3.6	0.2 ± 14.6
	GA (22)	24.3 ± 3.0	26.1 ± 3.1	1.7 ± 2.8	7.9 ± 12.6
	AA (15)	24.0 ± 2.2	26.2 ± 3.3	2.3 ± 3.7	10.1 ± 15.1
	A allele	24.2 ± 2.7	26.1 ± 3.1	2.0 ± 3.1	8.8 ± 13.5
P ANOVA		0.8	0.1	0.07	0.08
P linear trend		0.9	0.07	0.03	0.03
P GG vs. A allele		0.5	0.04	0.02	0.03
UCP3-55	CC (29)	24.2 ± 2.8	25.8 ± 2.9	1.5 ± 3.3	7.1 ± 13.4
	CT (27)	24.6 ± 2.7	25.3 ± 3.3	0.6 ± 3.5	3.3 ± 14.7
	TT (5)	26.1 ± 1.1	24.9 ± 1.2	-1.2 ± 1.8	-4.3 ± 6.6
P ANOVA		0.4	0.8	0.2	0.2
P Linear trend		0.4	0.5	0.09	0.08

Data are expressed as mean **±** SD

Figure 6.5. **Training related changes in delta efficiency (mean ± SEM)**

A. by *UCP3*-55C>T and *UCP2*-866G>A genotypes

B. by *UCP3* and *UCP2* haplotypes



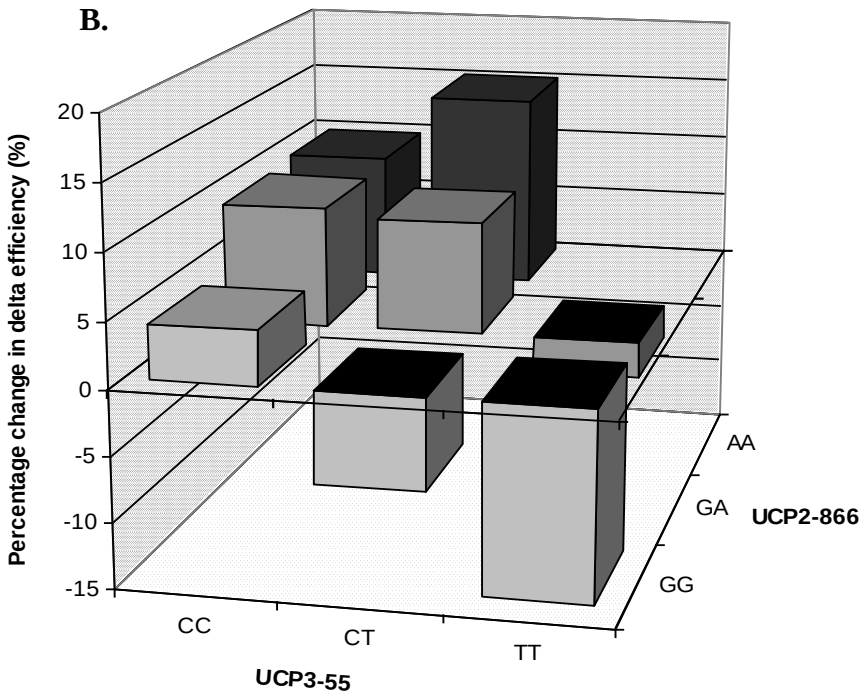


Table 6.13. *UCP3*-55C>T genotype distribution amongst 81 British Olympic athletes according to competitive distance event.

Running Distance	*UCP3*-55C>T			Total	T allele frequency
	CC	CT	TT		
≤200m	13	4		17	0.118 (0.009-0.226)
400-3000m	19	12	1	32	0.219 (0.117-0.320)
≥5000m	18	14		32	0.219 (0.117-0.320)
Total	50	30	1	81	0.198 (0.136-0.259)

6.3.2. British Olympic Athletes

Of those athletes with DNA available, 81/86 (94.1%) were successfully genotyped for the *UCP3*-55C>T gene variant. Genotype distribution was in Hardy-Weinberg equilibrium (χ^2= 2.29; *P*=0.13) and T allele frequency (0.198 [0.136-0.259]) were similar to previous reports. There was no difference in distance running event by genotype (*P*=0.2 by gene counting for ≤200m vs. ≥400m; Table 6.13).

UCP2-866G>A genotyping was successful in 81/86 (94.1%) of those subjects with DNA available. Genotype distribution was in Hardy-Weinberg equilibrium (χ^2= 0.195; *P*=0.66) and the A allele frequency was similar to previous reports. There was no difference in distance running event by genotype (*P*=0.15 for ≤200m vs. ≥400m; Table 6.14).

Table 6.14. *UCP2*-866G>A genotype distribution amongst 81 British Olympic athletes according to competitive distance event.

Running Distance	*UCP2*-866G>A			Total	A allele frequency
	GG	GA	AA		
≤200m	12	5	1	18	0.194 (0.065-0.324)
400-3000m	14	17	1	32	0.297 (0.185-0.409)
≥5000m	14	13	4	31	0.339 (0.221-0.457)
Total	40	35	6	81	0.290 (0.220-0.360)

UCP3/2 haplotype distributions are shown in Tables 6.15 and 6.16. The common haplotype was *UCP3*-55C/*UCP2*-866G. There were no *UCP3*-55T/*UCP2*-866A carriers amongst the small number of Afro-Caribbean athletes. There was no difference in haplotype frequencies between athletes whose competed above or below 400m (Figure 6.6; *P*=0.08). The common haplotype (*UCP3*-55C/*UCP2*-866G) frequency decreased with distance run and the *UCP3*-55C/*UCP2*-866A haplotype increased with distance run, i.e. variation at the *UCP2*-866G>A locus in *UCP3*-55C allele carriers. Interestingly, the haplotype frequencies amongst the Caucasian and Afro-Caribbean sprinters were similar (*P*=0.74 for difference).

Table 6.15. *UCP3/2* haplotype distribution in the BOA study, both overall and amongst the greater number of Caucasian subjects.

Overall		*UCP2*-866G>A		
		GG	GA	AA
UCP3	CC	27	20	1
-55C>T	CT	12	13	4
	TT		1	

Caucasian		*UCP2*-866G>A		
		GG	GA	AA
UCP3	CC	22	17	1
-55C>T	CT	10	13	4
	TT		1	

Figure 6.6. *UCP3*-55C>T and *UCP2*-866G>A haplotype distribution according to running distance and ethnicity of British Olympic athletes.

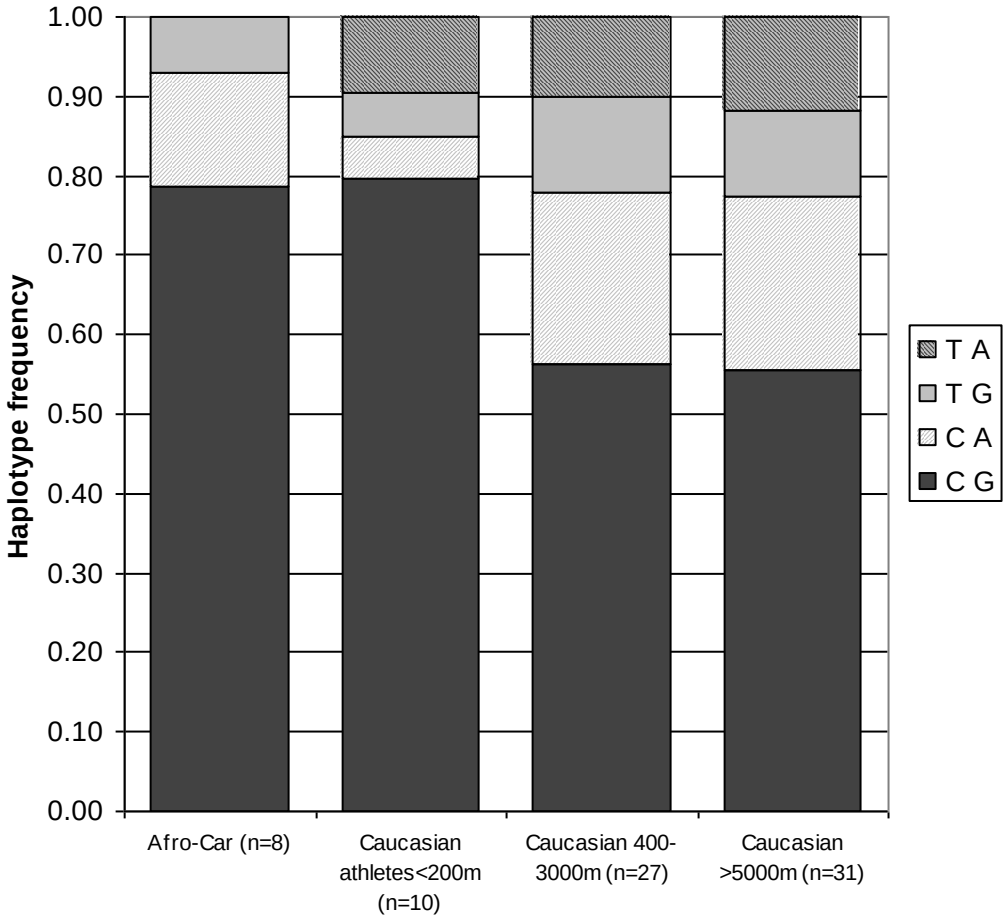


Table 6.16. *UCP3*/2 haplotype frequencies according to ethnicity and preferred running discipline amongst British Olympic track athletes. Haplotype frequencies from the Bassingbourn 3 study are included for comparison.

Haplotype		Haplotype frequency								
						≤ 200 m			≥ 400m	
UCP3-55	*UCP2*-866	BH3	Overall	Cauc	Afro-Car	Overall	Cauc	Afro-Car	Overall	Cauc
C	G	0.509	0.613	0.595	0.75	0.787	0.796	0.786	0.563	0.560
C	A	0.258	0.188	0.191	0.188	0.096	0.054	0.143	0.215	0.216
T	G	0.126	0.105	0.103	0.063	0.066	0.054	0.071	0.117	0.113
T	A	0.107	0.094	0.110	0	0.051	0.096	0	0.105	0.111

BH3, Bassingbourn 3 study; Cauc, Caucasian: Afro-Car, Afro-Caribbean

6.4 DIABETIC STUDY, OXIDATIVE STRESS AND *UCP* GENOTYPES (UDACS) (DHAMRAIT *ET AL.* 2004)

CHD was present in 105 out of the 465 men (22.5%), whose mean age, plasma triglyceride and CRP levels were higher, and plasma TAOS significantly lower, than those without CHD (Table 6.17). A significantly higher proportion of subjects with CHD were taking ACEi, statins, insulin and metformin- potentially accounting for their lower total cholesterol and BP.

Of the 485 Caucasian diabetic men from UDACS, 465 (95.9%) were successfully genotyped for the *UCP2*-866G>A variant (Table 6.17). The rare (A) allele frequency was 0.34 (0.31-0.37) and similar to that of healthy male cohorts from Big Heart 2 and NPHSII studies. There was no genotype or allelic association with the presence of CHD (A allele frequency 0.34 in both those with and without CHD, P=0.99).

The laboratory work regarding plasma markers of oxidative stress was conducted by Dr Jeffrey Stephens and is therefore shown in Appendix 4. Of note, there was a significant association between plasma TAOS and *UCP2*-866G>A genotype, which remained significant after adjustment for age, triglyceride, HDL-C, glucose, HbA_{1c} and proteinuria, and with evidence of interaction with CHD status. Diabetic men with CHD who had the *UCP2*-866AA genotype had the highest level of plasma ROS of all groups tested, as measured by plasma TAOS and F_2-isoprostanes. There was no difference in any other baseline characteristics (including treatment or duration of diabetes) by *UCP2* genotype (data not shown).

Table 6.17. Baseline characteristics of diabetic Caucasian men recruited in UDACS.

Trait	No CHD (n=360)	CHD (n=105)	*P*
Age (years)	59.2 (13.9)	67.5 (11.2)	<0.0001
Systolic blood pressure (mmHg)*	130 (128-149)	136.5 (123-151)	0.49
Diastolic blood pressure (mmHg)*	80 (75-87)	78 (70-84)	0.007
Body mass index ($kg.m^{-2}$)*	30 (25.2-30.9)	29.1 (24.7-32.2)	0.03
Current smoking % (n)	11.4 (41)	21.9 (23)	0.02
Cholesterol ($mmol.l^{-1}$)*	5.0 (1.0)	4.5 (1.0)	<0.0001
TC:HDL*	3.7 (3.1-4.7)	3.8 (3.1-4.6)	0.77
Triglycerides ($mmol.l^{-1}$)*	1.5 (1.0 -2.3)	1.8 (1.2-2.7)	0.03
CRP ($mg.l^{-1}$)*	1.41 (0.83 – 2.38)	2.02 (1.04-3.30)	0.004
TAOS (%)	43.3 (13.2)	40.3 (13.7)	0.04
Type 2 diabetes % (n)	70.6 (254)	95.2 (100)	<0.0001
Duration of diabetes (years)†	11 (5-20)	12 (6-18)	0.67
HbA_{1c} (%)*	7.7 (6.7 -7.8)	7.5 (6.5-8.8)	0.62
Creatinine ($mmol.l^{-1}$)*	93 (84 -109)	105 (88-124)	0.001
Proteinuria (%)	10%	17%	0.05
UCP2-866G>A Genotype frequency (GG/GA/AA)	154/165/41	44/51/10	0.820
UCP2-866A allele frequency (95%CI)	0.34 (0.31-0.38)	0.34 (27-0.40)	0.99
Drug Therapy			
Insulin (%)	34.6	47.3	0.05
Sulphonylureas (%)	29.6	40.3	0.04
Metformin (%)	42.4	63.6	<0.0001
Aspirin (%)	39.6	77.1	<0.0001
ACEI (%)	43.1	62.6	<0.0001
Statins (%)	14.6	62.6	<0.0001

Data are Mean (SD) or Median (IQR), Data transformed (*log, †square root)

6.5 ACUTE INFLAMMATORY RESPONSE AND CHANGES IN SERUM ACE ACTIVITY DURING INTENSE PHYSICAL EXERCISE

Extreme exercise drives an acute phase response similar to that seen in sepsis (Castell *et al.* 1997), but more readily quantified and prospectively studied (Pedersen *et al.* 2000). This response is associated with a rise in C reactive protein (CRP) (Weight *et al.* 1991; Brull *et al.* 2003) and interleukin-6 (IL6) levels (Ostrowski *et al.* 1998) driving the associated and well-defined rise in fibrinogen concentration, which have been associated with CHD (Ridker *et al.* 1997; Biasucci *et al.* 1999; Ridker *et al.* 2000; Lindmark *et al.* 2001; Danesh *et al.* 2005). Just as in sepsis, the magnitude of this rise depends upon both the magnitude of environmental stimulus and upon individual genetic variation.

The effect of an 11 week exercise training programme on serum ACE activity and its dependence on *ACE* was examined. Any association between *UCP2*-866G>A or *UCP3*-55C>T genotypes and plasma markers of inflammation (IL-6, CRP, fibrinogen) during this training period was examined.

Male army recruits were selected at random for the study (Brull *et al.* 2002). Of these, DNA was available on 223/250 individuals, whose baseline characteristics are shown in Table 6.18.

Table 6.18. Baseline characteristics of subjects from the Bassingbourn 3 Study

Trait	Mean (SD)
Age (years)	19.4 (2.2)
Systolic blood pressure (mmHg)	122.7 (11.2)
Diastolic blood pressure (mmHg)	71.4 (9.1)
Body mass index ($kg.m^{-2}$)	22.5 (2.6)
Current smokers (%)	48.9%
C-reactive protein ($mg.l^{-1}$)*	0.45 (0.20 - 1.26)
Interleukin-6 ($pg.ml^{-1}$)*	0.65 (0.40 – 1.04)
Fibrinogen ($g.l^{-1}$)	2.61 (0.56)
Serum ACE activity (nmol his-leu.ml^{-1}.min^{-1})	29.7 (9.6)
UCP3-55C>T CC / CT / TT (n) T allele frequency (95% C.I.)	132 / 76 / 13 0.23 (0.191 – 0.270)
UCP2-866G>A GG / GA / AA (n) A allele frequency (95% C.I.)	85 / 110 / 25 0.36 (0.319 – 0.409)

6.5.1 *ACE* genotype and serum ACE activity during training

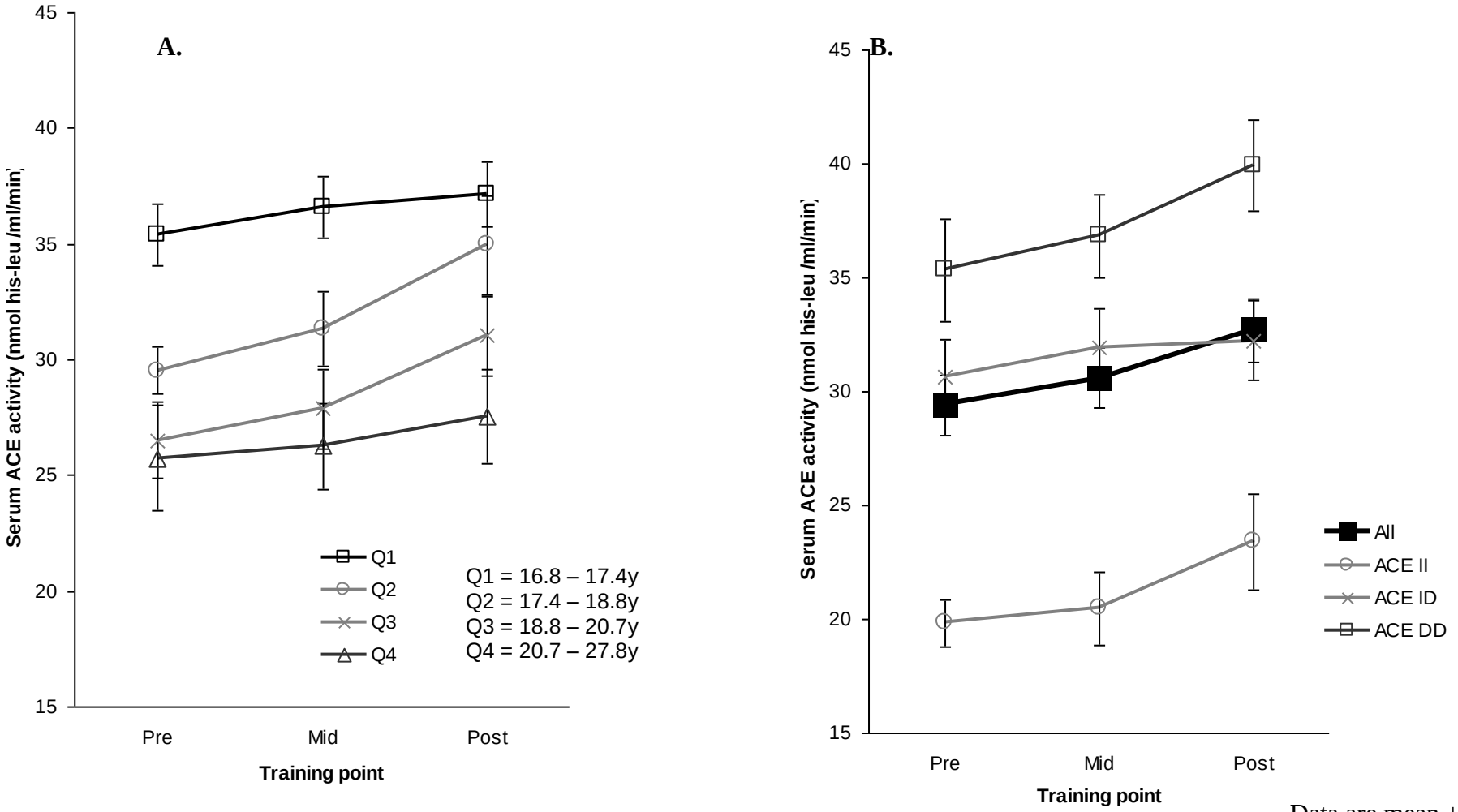
The objective was to select twenty subjects of each *ACE* genotype followed during training. Due to prospective recruit drop-out, the final genotype distribution was *ACE* II 17, *ACE* ID 21 and *ACE* DD 23 (total N=61). Serum ACE activity was normally distributed for all three time points. As anticipated, there were highly significant linear correlations in serum ACE activity between each time point (pre-mid, pre-post, mid-post; (correlation coefficients > 0.68; all $P<10^{-8}$). There was a significant inverse

relationship between age and serum ACE activity (correlation coefficient -0.33; $P<0.01$ for all time points; Figure 6.7A). There were no correlations between blood pressure, BMI or smoking status and ACE activity. There was no significant association between either absolute levels or changes in levels of IL-6, CRP or fibrinogen levels and serum ACE activity at any time point.

Mean serum ACE activity increased significantly during training (Figure 6.7B; activity was 29 ±10nmol his-leu.ml^{-1}.min^{-1} pre-training, 31 ±10 nmol his-leu.ml^{-1}.min^{-1} mid-training and 33 ±11 nmol his-leu.ml^{-1}.min^{-1} end of training; repeated measures analysis of effect of training on serum ACE activity $P < 0.004$). There was a gradual increase in serum ACE activity, reaching statistical significance between the mid-point and end of training (mean ±SEM increase of 2 ±0.7 nmol his-leu.ml^{-1}.min^{-1}; $P< 0.004$) and between the beginning and end of training (mean ±SEM increase of 3 ±1.1 nmol his-leu.ml^{-1}.min^{-1}; $P < 0.004$).

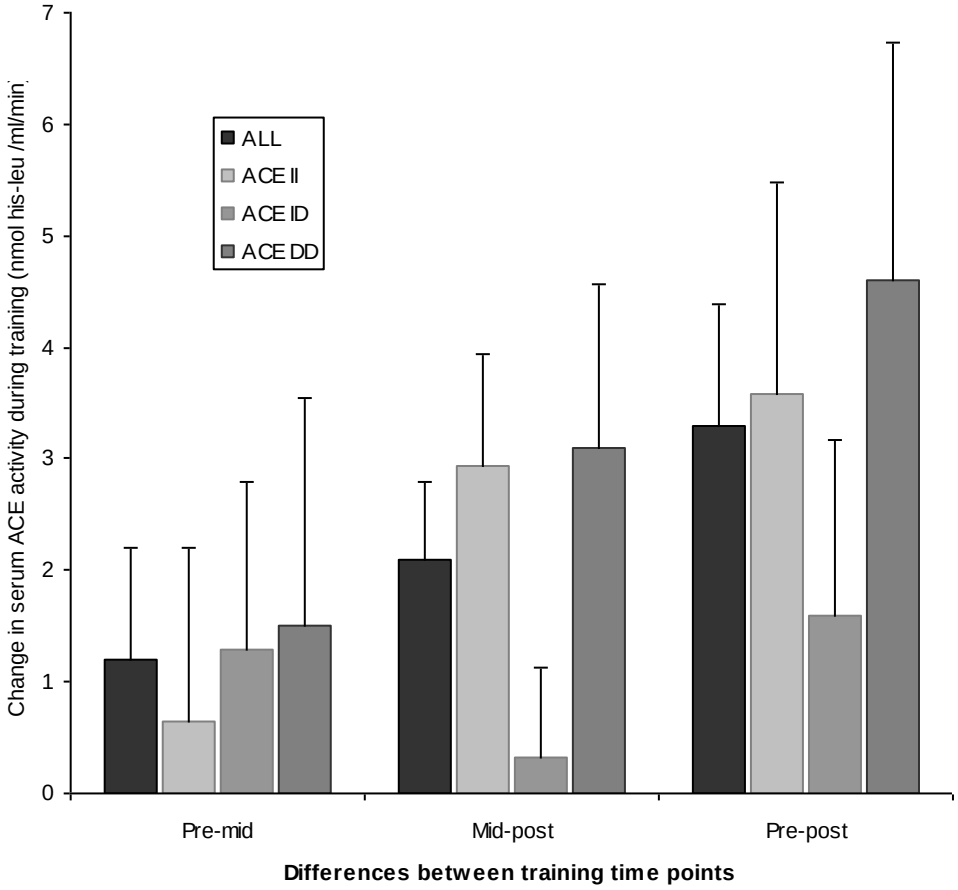
Serum ACE activity was highly *ACE* I/D genotype dependent at all time points (Figure 6.8; all $P<10^{-6}$). The training related increases were greatest in the order of *ACE* DD>*ACE* II>*ACE* ID, although repeated measures analysis suggested no significant interaction between time and *ACE* I/D genotype.

Figure 6.7. Serum ACE activity by quartile of age and by *ACE* I/D genotype during basic army training in 61 recruits.



Data are mean ± SEM

Figure 6.8. Absolute changes in serum ACE activity during basic army training according to *ACE* genotype.



6.5.2 Serum markers and *UCP3*-55C>T gene variant

Of the subjects, 221/223 (99.1%) were genotyped for the *UCP3*-55C>T variant (Table 4.9). The genotype frequency (CC 52.8%, CT 30.4% and TT 5.2%) was consistent with Hardy-Weinberg equilibrium (χ^2= 0.22; *P*=0.64) and the rare (T) allele frequency (0.23) was similar to previous reports in European non-diabetic samples (Cassell *et al.* 2000; Meirhaeghe *et al.* 2000). BMI was lower amongst TT individuals in a recessive manner (CC 22.5 ± 2.7 kg.m^{-2}, CT 22.4 ± 2.4 kg.m^{-2}, TT 20.9 ± 2.1 kg.m^{-2}; *P*=0.10 by ANOVA; *P* = 0.03 for C allele *vs.* TT). There was no other association between *UCP3* genotype and other baseline characteristics.

Amongst the cohort of 61 individuals with serum ACE activity measured at all time points, serum ACE activity was lower at all timepoints amongst *UCP3*-55 TT homozygotes. Serum ACE activity was therefore assayed retrospectively on stored samples to increase the sample size to see whether there was a significant association between serum ACE activity and *UCP3* genotype. Further samples were only available at the pre and post time points (total pre n=154, total post n=114). *UCP3*-55TT genotype was significantly associated with lower mean serum ACE activity throughout training on repeated measures analysis (P = 0.03; Figure 6.9A).

There was no significant differences in serum markers of inflammation by *UCP3*-55C>T genotype: CRP median [IQR]: 0.44 [0.23 - 1.58] $mg.l^{-1}$ *vs.* 0.44 [0.18 – 0.88] $mg.l^{-1}$ *vs.* 0.32 [0.18 – 1.24] $mg.l^{-1}$ for CC *vs.* CT *vs.* TT; P=0.33 and IL-6 median [IQR]: 0.65 [0.43 – 1.07] $pg.ml^{-1}$ *vs.* 0.70 [0.40 – 1.04] $pg.ml^{-1}$ *vs.* 0.39 [0.17 – 0.81] $pg.ml^{-1}$ for CC *vs.* CT *vs.* TT; P=0.35).

6.5.3 Serum markers and *UCP2*-866G>A gene variant

Of the study subjects, 220/223 (98.7%) were genotyped for the *UCP2*-866G>A variant (Table 6.19). The genotype frequency (GG 34%, GA 44%, AA 10%) was consistent with Hardy-Weinberg equilibrium (χ^2 = 1.42; P = 0.23) and the rare (A) allele frequency (0.36) was similar to the previous report in European non-diabetic samples (Esterbauer *et al.* 2001). There were no significant differences in baseline characteristics or serum ACE activity by *UCP2*-866G>A genotype (Figure 6.9B).

There was no evidence of linkage disequilibrium between *UCP2*-866G>A and *UCP3*-55C>T variants (Δ = 0.11; P = 0.63; Table 6.19). *UCP3*/*UCP2* combined genotype

analysis demonstrated that *UCP2*-866GG/*UCP3*-55TT homozygotes had the lowest mean serum ACE activity at all time points (Figure 6.9C), significant after training (ANOVA $P = 0.05$; linear trend $P = 0.03$).

Table 6.19. UCP haplotype distribution and frequency in the two Bassingbourn studies

Bassingbourn 3		*UCP2*-866G>A		
		GG	GA	AA
UCP3	CC	54	66	10
-55C>T	CT	27	35	14
	TT	3	9	1

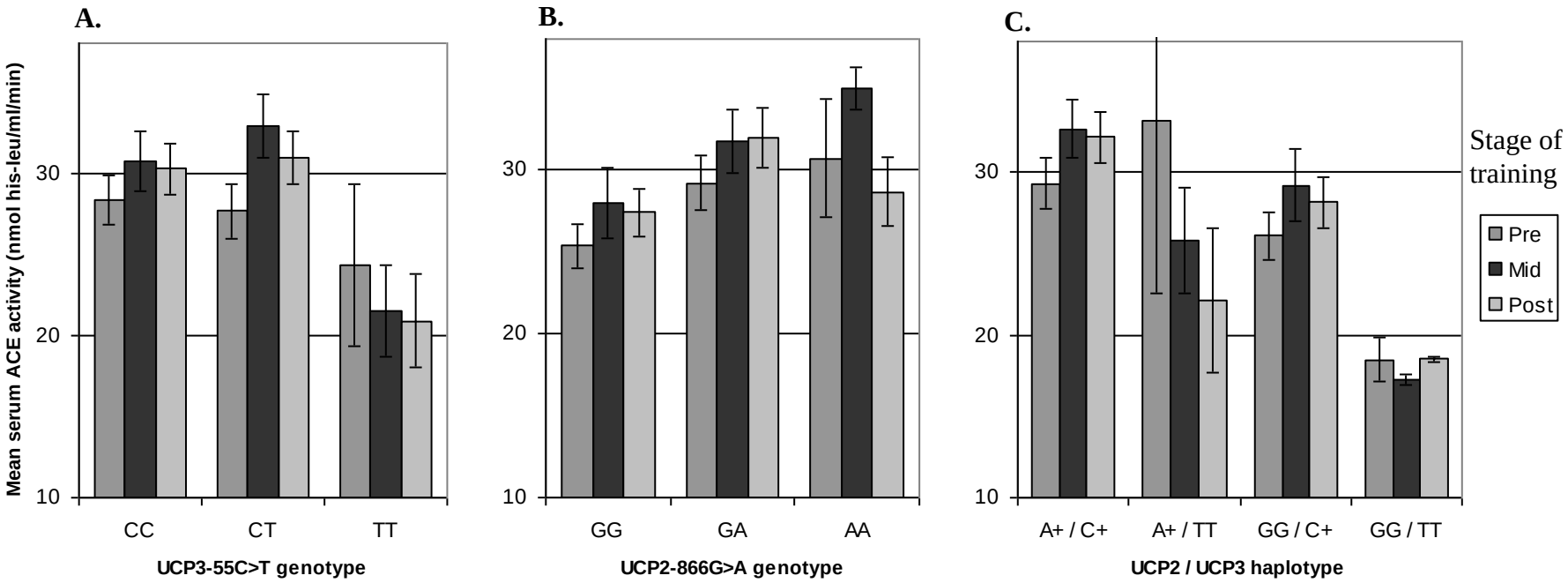
Bassingbourn (Big Heart) 2		*UCP2*-866G>A		
		GG	GA	AA
UCP3-	CC	31	28	9
55C>T	CT	26	31	5
	TT	1	8	4

Haplotype		Haplotype frequency	
UCP3-55	*UCP2*-866	Bassingbourn 3	Bassingbourn 2
C	G	0.509	0.499
C	A	0.258	0.245
T	G	0.126	0.127
T	A	0.107	0.128

P=NS for difference

Figure 6.9. Mean serum ACE activity during basic training stratified by:

A. *UCP3*-55C>T
B. *UCP2*-86G>A genotypes
C. *UCP2*/*UCP3* haplotypes.



A+ = *UCP2*-866A allele carriers
C+ = *UCP3*-55C allele carriers

6.6 DISCUSSION

6.6.1 LV mass

The *UCP2*-866G>A variant was associated with CMR-determined LV mass after basic army training in the BH2 study. The magnitude of the effect was smaller than that for the *BDKRB2* (+9/-9) gene variant. LV mass was higher in the AA homozygotes at baseline, and this difference reached statistical significance after training, matched by greater changes in stroke volumes with training. *UCP3*-55TT genotype was also associated with higher LV mass after training after indexing to lean body mass. This may in part be due to the presence of UCP3 in skeletal muscle and possible associations of this genotype with skeletal muscle mass. The *UCP2*-866AA/*UCP3*-55TT haplotype was associated with the highest left ventricular mass after training.

This is the first report of an association between either *UCP2* or *UCP3* genotype and LV mass. It is unclear whether the 'risk' haplotype (*UCP2*-866AA/*UCP3*-55TT) is directly causal in the development of LV growth or facilitates its development. The *UCP2*-866A allele has been associated with lower UCP2 mRNA expression in somatic cells (Krempler *et al.* 2002). The *UCP2*-866A/*UCP3*-55T haplotype may therefore be associated with lower mitochondrial UCP2 and/or UCP3 protein expression, leading to decreased uncoupling and therefore enhanced mitochondrial ROS production. ROS are known to mediate left ventricular growth (Amin *et al.* 2001; Bendall *et al.* 2002) and cardiac dysfunction (Ide *et al.* 2001; Suematsu *et al.* 2003). This may explain the non-significant differences observed at baseline, as ROS induced cardiac growth might be expected to accumulate over a life-time and accelerate during a period of physiological stress. Alternatively, genetically determined high ROS levels may decrease aortic

compliance (Delles *et al.* 2008), and this will result in LVH over time (Safar *et al.* 1987).

Alternatively, UCP2/3 phenotype may be associated with an alteration in the balance between cardiac glucose and fatty acid utilisation. There is increasing evidence that cardiac substrate utilisation is critical for cardiac function. Indeed, defects in enzymes involved in fatty acid oxidation cause childhood cardiomyopathies, and pharmacological inhibition of cardiac fatty acid import induces cardiac hypertrophy and causes rapid death in PPARa-knockout mice. Transgenic mice that overexpress long-chain acyl-CoA synthetase and take up excess long chain fatty acids initially exhibit cardiac hypertrophy, followed by LV dysfunction and death. Fatty acids generate more ATP per mol of substrate but at the expense of a greater oxygen requirement per mol of ATP than either glucose or lactate (Nicholls *et al.* 2002) and also induce a proton leak across the inner mitochondrial membrane (Borst *et al.* 1962), increasing cardiac oxygen consumption for the same amount of cardiac external work in both the isolated (Challoner *et al.* 1966) and whole animal model (Mjos 1971), resulting in an even lower actual ratio of ATP:oxygen consumed than that predicted (Brand *et al.* 1994). In vitro administration of fatty acids induce UCP2 mRNA expression in isolated neonatal cardiomyocytes and both tri-iodothyronine and phenylephrine induced UCP2 mRNA expression in a fatty acid dependent manner (Van Der Lee *et al.* 2000). However, elevation of serum free fatty acids (by high fat feeding, fasting or by induction of diabetes) all induce cardiac UCP3mRNA in the rat but do not alter cardiac UCP2 mRNA (Depre *et al.* 2000; Van Der Lee *et al.* 2000; Van der Lee *et al.* 2001).

Both UCP2 and UCP3 mRNA and protein expression are either increased or decreased in the heart depending on the animal model of LVH or heart failure used (Fukunaga *et*

al. 2000; Boehm *et al.* 2001; Langdown *et al.* 2001; Noma *et al.* 2001; Young *et al.* 2001). UCP2, but not UCP3, mRNA is significantly reduced in the left ventricle of patients with dilated cardiomyopathy together with a switch to a foetal metabolic gene profile (Razeghi *et al.* 2001) in keeping with a switch away from cardiac lipid metabolism to more energy efficient glucose metabolism but this may be at the expense of increased cardiac ROS generation.

6.6.2 Prospective CHD risk & oxidative stress

This is the first report to demonstrate that a common functional variant in the *UCP2* gene is associated with both increased oxidative stress and with prospective CHD risk. Such data support a role for UCP2 (and hence the mitochondrial electron transport chain) in the regulation of ROS generation, and highlights its potential impact upon CHD risk. The oxidation of vulnerable cell membrane unsaturated lipids by ROS (Evans *et al.* 2002) modulates diverse signal transduction pathways (Suzuki *et al.* 1997; Harrison *et al.* 2003), leading to increased expression of cell adhesion molecules, induction of pro-inflammatory pathways, activation of matrix metalloproteinase, vascular smooth muscle cell proliferation and death, and endothelial dysfunction and lipid peroxidation (Chisolm *et al.* 2000) - factors implicated in atherogenesis, to which the formation of oxidized LDL (OxLDL) may contribute (Suzuki *et al.* 1997; St-Pierre *et al.* 2001; Witztum *et al.* 2001; Navab *et al.* 2002; Harrison *et al.* 2003). Elevated OxLDL is independently associated with increased atherosclerotic burden and increased CHD risk (Toshima *et al.* 2000; Ehara *et al.* 2001; Weinbrenner *et al.* 2003).

In vitro, UCP2 is activated by ROS (Echtay *et al.* 2002), whilst selective down-regulation of UCP2 increases endothelial cell ROS generation (Duval *et al.* 2002).

Thus, under conditions of oxidative stress (OS), increased UCP2 expression should prove vasculo-protective (Arsenijevic *et al.* 2000; Echtay *et al.* 2002), and in support, UCP2 protects against atherosclerosis in LDL-receptor deficient mice (Blanc *et al.* 2003).

Diabetes is associated with increased OS (Cai *et al.* 2000; Brownlee 2001; Evans *et al.* 2002), and thus a fall in TAOS (Sampson *et al.* 2002). In keeping with a causal role for ROS in atherogenesis, we found that plasma TAOS was significantly lower in diabetic men with CHD than those without. In diabetic men, the *UCP2*-866A allele was independently associated with lower TAOS, suggesting a modulating influence of *UCP2* genotype on OS burden, although it was not clear whether this was a recessive or dominant effect. Patients with the *UCP2*-866AA genotype who also had CHD, demonstrated the lowest levels of plasma TAOS of all groups tested, with TAOS 30% lower than those non-CHD AA subjects and 33% lower than non-CHD G allele carriers. In a subset of diabetic men with CHD matched for baseline characteristics (including age and treatment), *UCP2*AA homozygosity was associated with 40% lower TAOS mirrored by a 100% increase in F_2-isoprostane concentrations. UCP2 activity and expression is induced by OS (Pecqueur *et al.* 2001; Echtay *et al.* 2002) (which is itself induced by CHD and its risk factors), thus protecting from further mitochondrial ROS generation. This may explain the dependence of the observed genotypic effect on the presence /absence of CHD. These data therefore suggest the *UCP2*-866A allele to be strongly associated with increased ROS burden. Given the putative role of ROS in atherogenesis, we predicted a similar genotype association with prospective CHD risk, which was confirmed. In prospectively studied middle-aged men, CHD risk was doubled amongst those homozygous for the *UCP2*-866AA allele, even amongst the normotensive, lean, non-smokers and non-diabetics. However, the risk associated with

genotype was substantially increased by the presence of conventional CHD risk factors known to be associated with increased OS, such as hypertension, obesity and diabetes (Cai *et al.* 2000; Chisolm *et al.* 2000; Brownlee 2001; Witztum *et al.* 2001; Evans *et al.* 2002; Harrison *et al.* 2003). Indeed, the risk of CHD was elevated nearly eight-fold amongst the small number of diabetic subjects of *UCP2*-866AA genotype, when compared to non-diabetic G allele carriers and almost four-fold in obese AA compared to non-obese G allele carriers.

The association of the *UCP2*-866A allele with reduced TAOS, increased esterified F_2-isoprostanes and with prospective CHD risk is mechanistically consistent, supporting the validity of our findings. The *UCP2*-866G>A promoter variant is located within a multifunctional *cis* regulatory site, involving putative binding sites for pancreatic and hypoxia-induced transcription factors (Esterbauer *et al.* 2001). The *UCP2*-866G>A polymorphism has been shown to be functional *in vitro* and *in vivo*. The *UCP2*-866A allele has been associated with insulin resistance (Krempler *et al.* 2002; Sesti *et al.* 2003) and with type 2 diabetes (Krempler *et al.* 2002), both being conditions associated with increased OS and CHD risk. Although promoter constructs of the -866A allele are associated with greater transcriptional activity in pancreatic β cells, they are associated with greater *repression* of transcription in somatic non-β cells (Krempler *et al.* 2002). It is likely, therefore, that the *UCP2*-866A allele is related to lower inducible UCP2 expression within the vasculature or circulating immune cells. As such, one would anticipate the A allele to be associated with increased OS and higher risk of CHD as demonstrated in these studies. Furthermore, the -866G>A variant appears to be strongly associated with functionality across the gene cluster (Esterbauer *et al.* 2001).

At first sight, the lack of any significant difference in genotype distribution between those with and without CHD in the diabetic subjects would appear to conflict with the prospectively-derived data. However, this is not the case. Firstly, prospective gene-association studies are more powerful than case-control studies (Humphries *et al.* 2003). Secondly, increased obesity, increased OS, inflammation, and hyperglycaemia might all overwhelm the UCP genotype 'strength of signal' in diabetics. Thirdly, case-control cross-sectional studies are prone to intrinsic bias, for example due to possible altered rates of disease progression, subsequent progression of secondary phenotypes, or genotype associations with death or treatment changes. Indeed, the presence of the A allele might be associated with both earlier disease presentation and earlier death in some, subsequently balanced by more aggressive secondary prevention strategies. Such influences are well-recognised confounders (Risch 2000; Humphries *et al.* 2003; Sing *et al.* 2003).

The number of diabetic men recruited to NPHSII is small, and confirmation of these findings should be sought in other diabetic groups. However, the substantially-increased CHD risk amongst diabetic men of *UCP2*-866AA genotype in NPHSII is congruent with the finding of increased markers of OS found in the plasma of diabetic men from UDACS. The association of *UCP2* genotype with altered markers of OS also requires examination in other 'high ROS' groups and these observations should also be extended to those of other races, and to women. In addition, further *in vitro* functional studies are required. However, the conclusions from these two independent studies are consistent and statistically robust, and, if confirmed, will have important implications. There is a global epidemic of diabetes in which CHD is the major cause of mortality (Amos *et al.* 1997), and diabetes is one of the major risk factors for CHD (Castelli *et al.* 1986). However, no more than 25% of the excess CHD risk in diabetes can be accounted for by

modulation of established risk factors (Pyorala *et al.* 1987), and a search for mechanistic understanding may thus have profound implications for the development of novel therapeutic options. These data suggest that modulation of UCP2 expression may be one such important mechanistic target.

Larger scale studies are also required to extend our observations from middle-aged Caucasian males to those of other racial origins and age ranges, as well as to women. Although case-control studies may be confounded by population stratification, the prospective study design of NPHSII is much more robust, and it is extremely unlikely that the association with risk we see here could be explained by such an effect. Population stratification could be ruled out by genotyping the samples for a group of randomly distributed single nucleotide polymorphisms (SNPs) but, to date, of the more than 40 SNPs where we have genotype data in NPHSII no statistically significant evidence of population stratification has been obtained.

These data extend our understanding of the mechanisms through which LVH and CHD are associated. LVH is an independent cardiovascular risk factor (Levy *et al.* 1990). Extensive animal data support a role for BDKRB2 activation in diminishing this hypertrophic response (Linz *et al.* 1992) and implicate perturbations in both UCP2 and UCP3 in the abnormal metabolism associated with LVH (Fukunaga *et al.* 2000; Boehm *et al.* 2001; Langdown *et al.* 2001; Noma *et al.* 2001; Young *et al.* 2001). I have presented novel associations between the *BDKRB2*(+9) allele (associated with reduced receptor mRNA expression) and both an enhanced *prospective* human left ventricular hypertrophic response and with *prospective* coronary artery disease only amongst hypertensives, as well as novel associations between a new risk haplotype (*UCP3-55T/UCP2-866A*) and both LV mass and *prospective* CHD risk as well as the mechanistic association between the *UCP2*-866A allele and plasma markers of

oxidative stress in high risk diabetic men. These data suggest common mechanisms through which the deleterious effects of hypertension on left ventricular hypertrophy and CHD may be mediated and adds credence to the hypothesis that RAAS and UCP activity may be linked at the cellular level.

6.6.3 Skeletal muscle efficiency & endurance phenotypes

There were significant associations between genetic variation at the *UCP2*/3 locus and the efficiency of contraction of human skeletal muscle: there were no associations with DE at baseline in healthy sedentary young adults, but there were significant exercise training related changes. The *UCP2*-866A allele carriers had significantly increases in DE (absolute increase of 2% DE) after training, whereas GG homozygotes had no change in DE after training. The small number of *UCP3*-55TT homozygotes tended to have lower DE after training. There was significant evidence of interaction between the *UCP2*-866 and UCP-55 loci in determining changes in DE.

The *UCP2*/3 locus was also associated with competitive distance running event amongst a small sample of elite, Olympic standard athletes. *UCP2*-866A allele frequency tended to increase with running distance and this was reflected in an increased prevalence of *UCP3*-55C/*UCP2*-866A haplotype carriers in those athletes competing in predominantly endurance events and an increased prevalence of *UCP3*-55C/*UCP2*-866G haplotype carriers in sprinters. There were very few *UCP3*-55T allele carriers amongst Afro-Caribbean athletes and this may have explained the overall increase in *UCP3*-55T allele frequency seen according to competing distance, as there was a shift towards shorter competing distance amongst Afro-Caribbean elite athletes, with no such athlete studied competing at longer distances. There was not enough power in this

study to test whether there were significant allelic differences between Caucasian and Afro-Caribbean study subjects. This will be looked at in more detail in Chapter 7.

The exact role of UCP2 and UCP3 in myocyte function remains unknown. The *UCP2*/*UCP3* locus has been associated with basal metabolic rate in adult humans (Bouchard *et al.* 1997). These data support an association between the *UCP2*/*UCP3* gene locus and skeletal muscle efficiency. Variation at the two SNPs studied accounted for 14.8% of the interindividual variation in exercise related gains in skeletal muscle efficiency in this study. It is however difficult to delineate which gene product is responsible for the association as the *UCP2* and *UCP3* genes are separated by only 7kB in chromosomal region 11q13 (Solanes *et al.* 1997). Much larger sample sizes and more SNPs would be required to decipher any association further.

Skeletal muscle is a major determinant of resting and exercising metabolic rate (Zurlo *et al.* 1990). UCP3 expression is highly tissue specific, being expressed predominantly in human skeletal muscle and, to a lesser extent, in cardiac muscle (Boss *et al.* 1997), whereas UCP2 mRNA expression is ubiquitous, being expressed at a lower level than UCP3 in human skeletal muscle, and predominating in cardiac muscle (Boss *et al.* 1997).

The close sequence homology between UCP1 and both UCP2 and UCP3 has naturally led to the hypothesis that both UCP3 and UCP2 are able to increase skeletal muscle oxygen consumption by increasing mitochondrial proton conductance. The evidence is still divergent. Proton leak (state 4 respiration) was reduced in skeletal muscle mitochondria isolated from the *UCP3*(-/-) knockout mouse in some (Gong *et al.* 2000; Vidal-Puig *et al.* 2000) but not all studies (Cadenas *et al.* 2002). In whole animal

studies using NMR spectroscopy, *UCP3* knockout mice had a doubling of the ATP synthesis rate in skeletal muscle without any increase in TCA cycle flux rate in the fasting state implying increased mitochondrial coupling (Cline *et al.* 2001). *UCP3* overexpression in yeast (Gong *et al.* 1997; Zhang *et al.* 1999; Harper *et al.* 2002), proteoliposomes (Echtay *et al.* 2001) and mammalian cells (Boss *et al.* 1998) leads to artifactual uncoupling of mitochondrial respiration (Cadenas *et al.* 2002), possibly because of differences in insertion, folding or interaction within the inner mitochondrial membrane compromising membrane integrity, and do not aid in understanding the physiological function of UCP3.

Both UCP2 and UCP3 mRNA are reduced between 40-70% by an 8 week endurance treadmill training program in rats in both fast twitch and slow twitch skeletal muscle fibres (Boss *et al.* 1998). UCP3 protein expression does vary according to muscle fibre type in humans, being most abundant in type 2b fast-glycolytic fibres, to a lesser extent in type 2a fast-oxidative-glycolytic fibres and only moderately in type 1 slow-oxidative fibres (Hesselink *et al.* 2001). UCP3 protein is lower in the skeletal muscle of endurance trained individuals compared to untrained subjects (Russell 2003) and is lower in all fibre types compared to untrained muscle, although the same hierarchy exists.

This data suggests that UCP2 and UCP3 may be associated with endurance training effects in skeletal muscle. There is some debate as to the exact function of UCP3 in human skeletal muscle. In an important paper, Schrauwen's group tested whether physiological elevation of skeletal muscle UCP3 protein content in 6 human subjects altered the PCr resynthesis rate after PCr had been depleted by high-intensity exercise with limb blood flow occlusion (Hesselink *et al.* 2003). The authors concluded that an

increase in UCP3 content within the physiological range did not affect mitochondrial coupling *in vivo* in human skeletal muscle. There was a positive association between UCP3 protein content and PCr resynthesis rate overall, suggesting that the authors were measuring a surrogate marker of total mitochondrial protein or volume rather than the relative amount of UCP3. Indeed, the relationship between UCP3 protein content and PCr resynthesis rate appeared to change during high fat feeding but the study was not powered to detect such a difference. In support of this argument, it has been shown that during 6 weeks of endurance training, total UCP3 protein remains unchanged in human skeletal muscle, but when indexed to a marker of mitochondrial volume (citrate synthase), the relative amount of UCP3 protein decreased significantly by 53% (Fernstrom *et al.* 2004).

Most of the training and endurance associations in this study were due to variation at the *UCP2*-866 locus with sedentary *UCP2*-866A allele carriers benefiting from greater efficiency after training and elite athletes who were *UCP2*-866A carriers, more likely to compete at endurance distances. The *UCP2*-866G>A polymorphism has been shown to be functional *in vivo* (Krempler *et al.* 2002; Sesti *et al.* 2003) and *in vitro*. Promoter constructs of the -866A allele are associated with greater *repression* of transcription in somatic cells (Krempler *et al.* 2002). It is tempting to postulate that the *UCP2*-866A allele is associated with lower inducible UCP2 expression within skeletal muscle and therefore greater mitochondrial coupling. As such, one would anticipate the A allele to be associated with the endurance phenotypes described. Furthermore, the -866G>A variant appears to be strongly associated with functionality across the gene cluster (Esterbauer *et al.* 2001).

6.6.4 The effects of endurance training on serum ACE activity

These are the first data to show that 11 weeks of an exercise training programme is associated with a small but significant increase in serum ACE activity (mean increase of 3.3±1.10 nmol his-leu.ml^{-1}.min^{-1}). Serum ACE increased to similar degrees in all *ACE* I/D genotype groups examined, with the relative differences between genotype groups remaining highly statistically significant at all time points. This has important implications. Firstly, many of the phenotypes relating the *ACE* I/D polymorphism to endurance training phenotypes (such as DE) have relied on *ACE* I/D genotype related ACE activity remaining in the order of *ACE* DD>ID>II during training. These data show that this hierarchy remains. Secondly the inter-individual differences in ACE activity remained throughout training, permitting any causal differences in phenotypic expression, such as DE, to persist.

It is unclear why serum ACE activity increased during training. Very little is known about the regulation of ACE. Serum ACE activity increases temporarily during adolescence (Cambien *et al.* 1988). In this study group, there was a negative relationship between increasing age and serum ACE activity as the subjects were recruited in late adolescence and early adulthood during which time serum ACE activity drops back to adult range. Thereafter, there is little change in serum ACE activity during adulthood. This would imply that time itself could not act as a confounder and that any changes observed would be related to the training itself. Shear stress upregulates endothelial ACE (Gosgnach *et al.* 2000) and may release more endothelial bound ACE into the circulation during exercise resulting in a transient rise in serum ACE activity. Against this notion, the mid-point ACE activity was measured during a period of convalescence. Further studies are required to test whether the levels of serum

ACE change during acute exercise and to examine the time course of any changes. It would also be important to test if there were serial related changes in tissue ACE activity in metabolically active tissues such as skeletal muscle.

There was no association between serum ACE activity and any other baseline characteristic measured, including blood pressure. Importantly, there was also no association between markers of systemic inflammation, such as CRP, IL-6 and fibrinogen, measured at any time point with absolute or relative changes in serum ACE.

It is intriguing that there was a significant association between serum ACE activity and *UCP3*-55C>T genotype and *UCP2*/*UCP3* haplotypes amongst the recruits, with *UCP3*-55TT and *UCP2*-866GG homozygotes achieving the lowest serum ACE activity. This is the first example of genetic variation in an unrelated gene effecting regulation of the human *ACE* gene. The *UCP3*/*UCP2* gene cluster and *ACE* gene are coded on different chromosomes, implying that direct gene-gene interaction would be unlikely. It is plausible that the *UCP2* or *UCP3* gene product is influencing *ACE* gene transcription or ACE enzyme activity. How might this occur? Both ACE and the UCPs are thought to influence cell metabolism and UCP-induced mitochondrial uncoupling may provide a negative feedback loop to ACE. UCPs negatively regulate mitochondrial ROS generation, and ROS have been shown to inhibit ACE activity (Chen *et al.* 1993; Michel *et al.* 2001). The interaction between UCP and ACE will be the focus of more work in chapter 7.

※ RESULTS CHAPTER SEVEN ※

IS THERE AN ASSOCIATION BETWEEN THE UCP3/UCP2 GENETIC LOCUS AND CIRCULATING ACE ACTIVITY?

A novel association between genetic variation in the UCP3 gene and serum ACE activity was described in chapter 4. ACE is an ectoenzyme, bound to the cell surface at the C-terminus. Endothelial-bound ACE is cleaved at the juxtamembrane stalk region by an unknown 'ACE secretase' to yield circulating catalytically-active ACE (Ching *et al.* 1983). Little is known about the regulation of somatic ACE, and no cohesive pathway of its induction has been delineated. Serum ACE activity rises rapidly during adolescence and then remains remarkably stable within adult individuals, rising only slowly with age (Cambien *et al.* 1988). Circulating ACE is a highly heritable trait, with the majority of interindividual variability in ACE levels due to genetic variation in the *ACE* gene (Cambien *et al.* 1988; Keavney *et al.* 1998). The *ACE* gene is polymorphic, with 78 varying sites found during whole gene sequencing in 11 individuals (Rieder *et al.* 1999). In common with other genes, the sequence variation in *ACE* is greater in African populations (Rieder *et al.* 1999; Cox *et al.* 2002). European populations can be divided into 3 major *ACE* clades (closely related haplotype branches). The two most common (A and B), have contrasting alleles at many polymorphic sites which are in complete LD, including the *Alu* I/D variant. It appears that the *ACE Alu* insertion occurred, along with 17 other substitutions, some time after the human/chimpanzee split and is very old (> 1 million years) (Rieder *et al.* 1999). The next two most common clades (C and D) probably occurred due to distinct ancestral recombination between clades A and B, between intron 5 and exon 6 and approximately 18kb away from this point, 3' of the last exon. Approximately 36% of the variation in serum ACE

activity in Europeans can be assigned by this haplotype/clade approach (Keavney *et al.* 1998). It is suggested that the majority of the variation in the *ACE* QTL is associated with variation within the 18kb region between the ancestral break points, but the extraordinary degree of LD between these two points in Europeans has made it impossible to detect which variants are functional (Cox *et al.* 2002). Recently, studying the greater sequence and haplotype diversity in a Nigerian sample, allowed the identification of four functional variants in the *ACE* gene, three within this 18kb region (A23495G, 31839insC and A31958G) and one in the promoter (A6138C) accounting for approximately 20% and 5% of the interindividual variation in serum ACE activity (Cox *et al.* 2002). There have been no other reports of association between serum ACE activity and variation in any other gene.

To test whether genetic variation at the *UCP3/2* locus is associated with serum ACE activity, I extended the observed association in the BH3 study by retrospectively increasing the sample size and, not only genotyping the subjects for the *UCP3*-55C>T and *UCP2*-866G>A, but also for the *UCP2* 3'UTR (exon 8) 45bp deletion/insertion (D/I) variant. The *UCP2* D/I variant is associated with alteration in UCP2 mRNA stability (Esterbauer *et al.* 2001), and has been associated with BMI (Cassell *et al.* 1999) (Yanovski *et al.* 2000) and variably with basal metabolic rate (Walder *et al.* 1998; Yanovski *et al.* 2000). Moreover, the *UCP2*-866G>A and *UCP2* D/I variants were found to be in strong LD, and variation at the promoter SNP accounted for 71% of the variability in D/I mRNA transcript ratio in gene-promoter constructs (Esterbauer *et al.* 2001). Similarly, genetic association between these *UCP3/2* genetic variants and serum ACE activity was sought in a healthy cohort of men drawn from a single tribe from the Republic of South Africa (RSA), and then differences in association between the two ethnic groups compared. In addition, similar associations between *UCP3/2* genetic

variants and serum ACE activity were sought in two samples of diabetic patients (UDACS and a sample of type 1 diabetic patients from Denmark) in whom OS is elevated (Brownlee 2001), Chapter 3). Any association between plasma TAOS (an inverse marker of OS) and ACE activity was also tested in UDACS.

7.1 HEALTHY CAUCASIAN AND NATIVE SOUTH AFRICANS

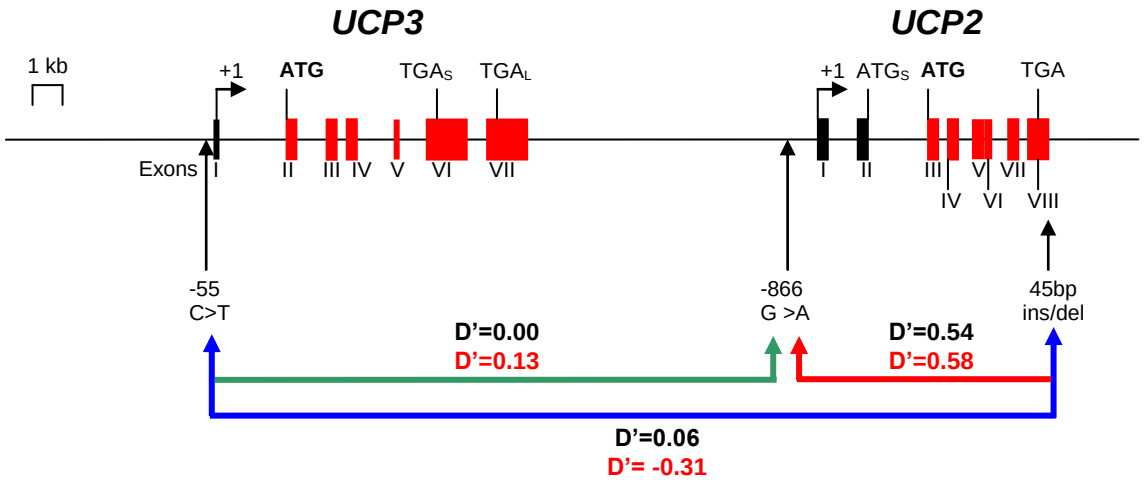
Of the 200 men recruited to the RSA study, 190 (95%) were successfully genotyped for the *UCP3*-55C>T variant, 191 (95.5%) for the *UCP2*-866G>A variant and 187 (93.5%) for the *UCP2* Del/Ins variant. Results from the RSA study were compared with analysis from the BH3 study, a Caucasian sample (Methods 2.16, Results 6.5). The number of subjects with complete data in the BH3 study was enlarged by retrospectively enriching the number of subjects in whom the baseline ACE was measured. Of the BH3 study subjects, 154 out of the 250 had baseline serum stored which could be analysed for post-hoc analysis of serum ACE activity. The genotype distributions and rare allele frequencies for both RSA and BH3 are shown in Table 7.1. All genotypes were within Hardy-Weinberg equilibrium. The rare allele for all 3 genotypes was found less frequently in the RSA study sample than in the BH3 study sample (P=0.0006, P=0.04, P=0.0000002 for *UCP3*-55T allele, *UCP2*-866A allele and *UCP2* Ins allele, respectively).

There was no significant LD between *UCP3*-55C>T and *UCP2*-866G>A genotypes in either ethnic group (Table 7.2). However, there was highly significant and similar LD between the *UCP2*-866G>A and *UCP2* Del/Ins in both ethnic samples (D'0.54 – 0.58; P<0.0001; Figure 7.1), although both results were less than the LD (approx 0.75) between these two loci described previously for a Caucasian population (Esterbauer *et al.* 2001). There was negative LD (-0.31; P=0.0005) between the *UCP3*-55C>T and

Table 7.1. ***UCP3*-55C>T, *UCP2*-866G>A and *UCP2* Del/Ins genotype distributions for the RSA and BH3 studies.**

Genotype	RSA		BH3		Comparison (X^2)
	Genotype distribution	Rare allele frequency (95% CI)	Genotype distribution	Rare allele frequency (95% CI)	*P*
UCP3-55C>T	146 / 40 / 4	0.126 (0.093 – 0.160)	132 / 76 / 13	0.231 (0.191 – 0.270)	0.0006
UCP2-866G>A	97 / 81 /13	0.280 (0.235 – 0.325)	85 / 110 / 25	0.364 (0.319 – 0.409)	0.04
UCP2 Del/Ins	140 / 45 / 2	0.131 (0.097 – 0.165)	105 / 93 / 16	0.292 (0.249 – 0.335)	0.0000002

Figure 7.1. **Linkage disequilibrium between common variants in the *UCP3/2* gene cluster in the RSA (D' black) and BH3 (D'red) studies.**



UCP2 Del/Ins genotypes in BH3, as previously described in a cohort from Chenai, South India (Cassell *et al.* 2000), but no LD between these 2 loci in the RSA study sample (Figure 7.1).

These data suggest that there has been significant recombination between the *UCP3*-55 and *UCP2*-866 sites in the evolutionary older African population, as has been reported for other genetic loci (Gabriel *et al.* 2002), but not between the two neighbouring *UCP2* polymorphisms. In the European sample, the *UCP3*-55C allele showed allelic association with the *UCP2* I allele, and the latter with the *UCP2*-866A allele.

Table 7.2. Distribution and linkage disequilibrium (D') between *UCP* genotypes in the RSA and Bassingbourn (Big Heart) 3 studies

RSA		*UCP2*-866G>A		
		GG	GA	AA
UCP3	CC	75	59	10
-55C>T	CT	16	21	3
	TT	4	0	0

D' = 0; *P*=1.0

Bassingbourn (Big Heart) 3		*UCP2*-866G>A		
		GG	GA	AA
UCP3	CC	54	56	10
-55C>T	CT	27	35	14
	TT	3	9	1

D'=0.13; *P*=0.49

RSA		*UCP2* Del/Ins		
		DD	DI	I I
UCP3	CC	107	30	2
-55C>T	CT	24	14	0
	TT	4	0	0

D' = 0.06; *P*=0.95

Bassingbourn (Big Heart) 3		*UCP2* Del/Ins		
		DD	DI	I I
UCP3	CC	47	67	15
-55C>T	CT	45	25	1
	TT	12	1	0

D'= -0.31; *P*=0.0005

RSA		*UCP2* Del/Ins		
		DD	DI	I I
UCP2	GG	91	2	0
-866G>A	GA	38	37	2
	AA	7	5	0

D' = 0.54; *P*<0.0001

Bassingbourn (Big Heart) 3		*UCP2* Del/Ins		
		DD	DI	I I
UCP2	GG	68	15	0
-866G>A	CT	34	67	7
	TT	3	10	9

D' = 0.58; *P*<0.0001

There was a highly significant difference in *UCP3/2* haplotype distribution between the RSA and BH3 studies ($P<0.0005$; Table 7.3). The observed difference between the two haplotype distributions was driven primarily by differences in LD between the *UCP3* promoter and *UCP2* Del/Ins variants.

ACE activity in serum was measured by a kinetic assay (Sigma Diagnostics, St Louis, MO, USA; Methods 2.1.9.). The within-assay and between-assay variabilities were 13% and 11%. Serum ACE activity was weakly associated with increasing age in the RSA study (r = 0.16; $P = 0.03$), but with decreasing age in the BH3 study whose participants were of a younger age (r = -0.19; $P = 0.02$; Figure 7.2). There was a highly statistically significant difference in mean serum ACE activity, despite correction for age, between the RSA and BH3 study samples (47.2 ±12.1 nmol His-Leu.ml^{-1}.min^{-1} and 27.4 ±9.0 nmol His-Leu.ml^{-1}.min^{-1}, respectively; $P = 2x10^{-48}$).

Age-adjusted ACE activity amongst *UCP3*-55TT homozygotes was lower in both studies by a mean difference of approximately 6 nmol His-Leu.ml^{-1}.min^{-1} (Figure 7.3). Serum ACE activity was higher in the *UCP2*-866A allele carriers in the BH3 study. However, there appeared to be differential regulation of serum ACE activity in both studies according to *UCP2* genotypes (Figure 7.3), but only significant differences by *UCP2*D/I genotypes in the BH3 study (linear trend $P=0.04$; I allele *vs.* DD homozygotes $P=0.02$). The statistical interaction between *UCP2* genotypes accounted for up to 4.1% and 2.3% of the interindividual variation in serum ACE activity in the BH3 and RSA studies, respectively.

Table 7.3. Comparison of *UCP* haplotype distribution in the RSA and BH3 studies (graphic depiction of same on right)

Haplotype			Haplotype frequency		*P*
UCP3 -55	*UCP2* -866	*UCP2*	RSA	BH3	difference
C	G	Del	0.612	0.463	<0.0001
C	A	Del	0.152	0.024	0.0002
T	G	Del	0.094	0.120	0.11
C	A	Ins	0.092	0.225	<0.0001
T	A	Ins	0.023	0.009	0.62
C	G	Ins	0.015	0.058	0.001
T	A	Del	0.011	0.100	0.001
T	G	Ins	0	0	N/A

Overall *P*<0.0005 for comparison of haplotype distribution in the 2 samples

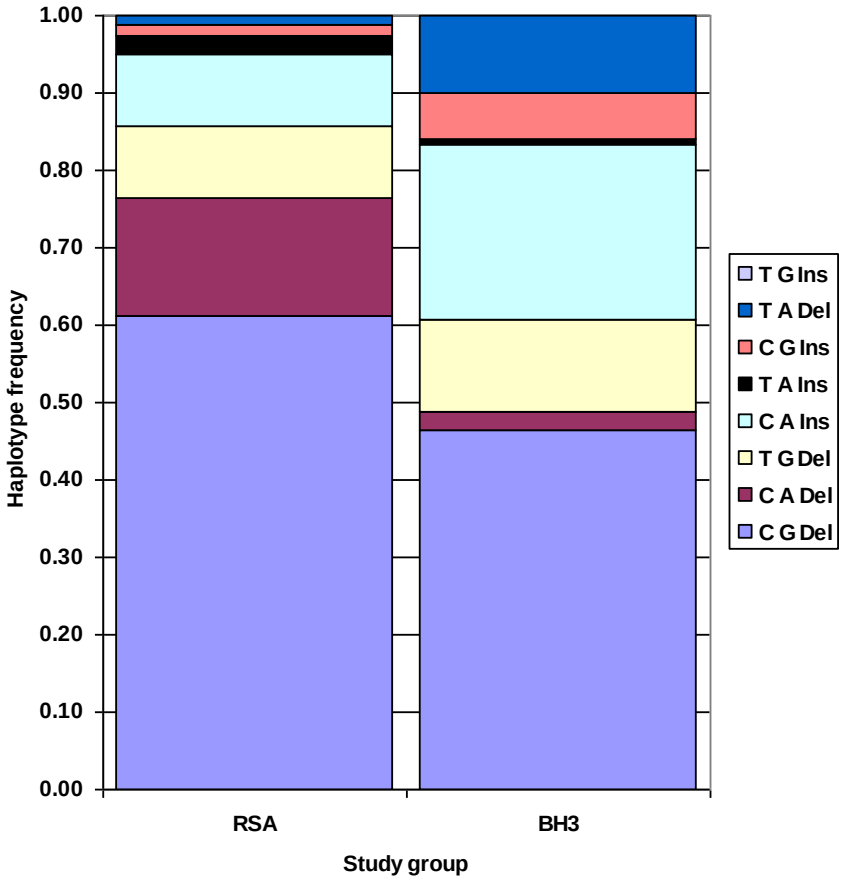


Figure 7.2. **Scatter plots of serum ACE activity *vs.* age in the BH3 (left) and RSA (right). Best fit correlation curves are shown with 95% confidence intervals.**

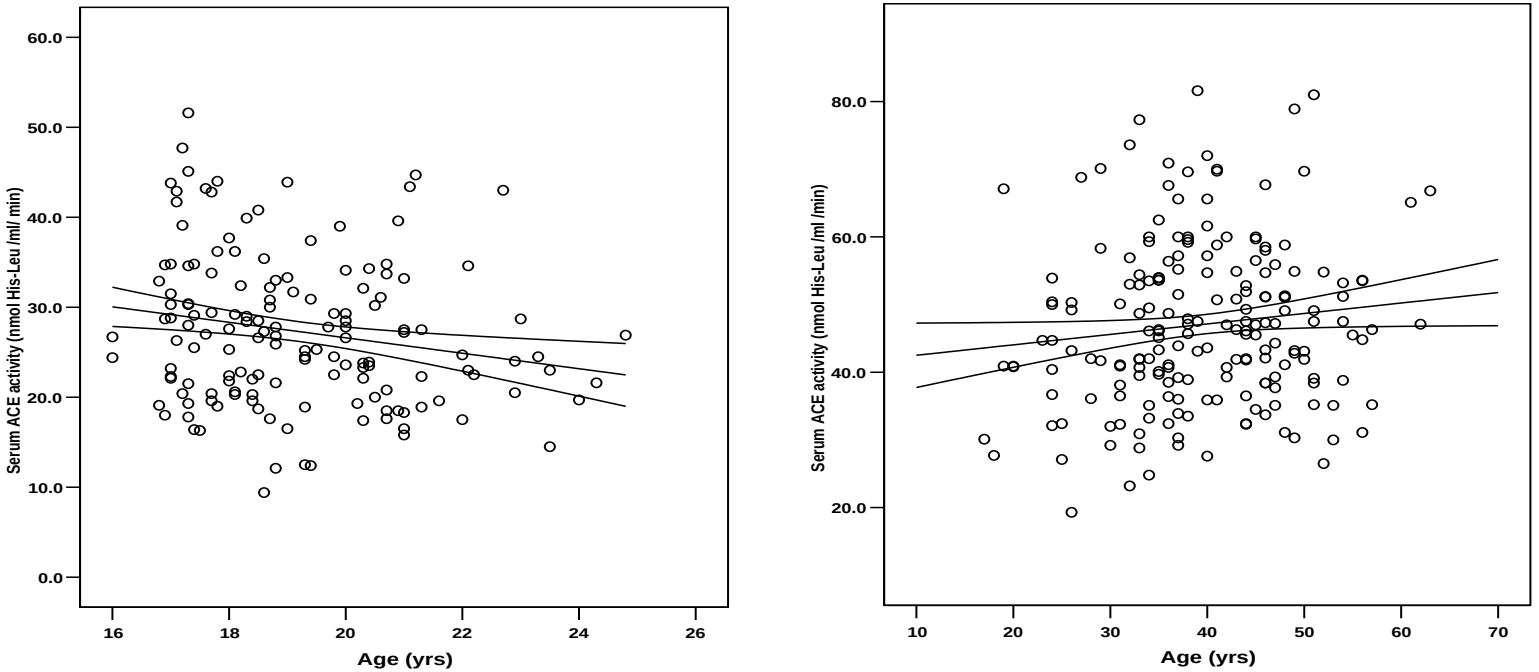
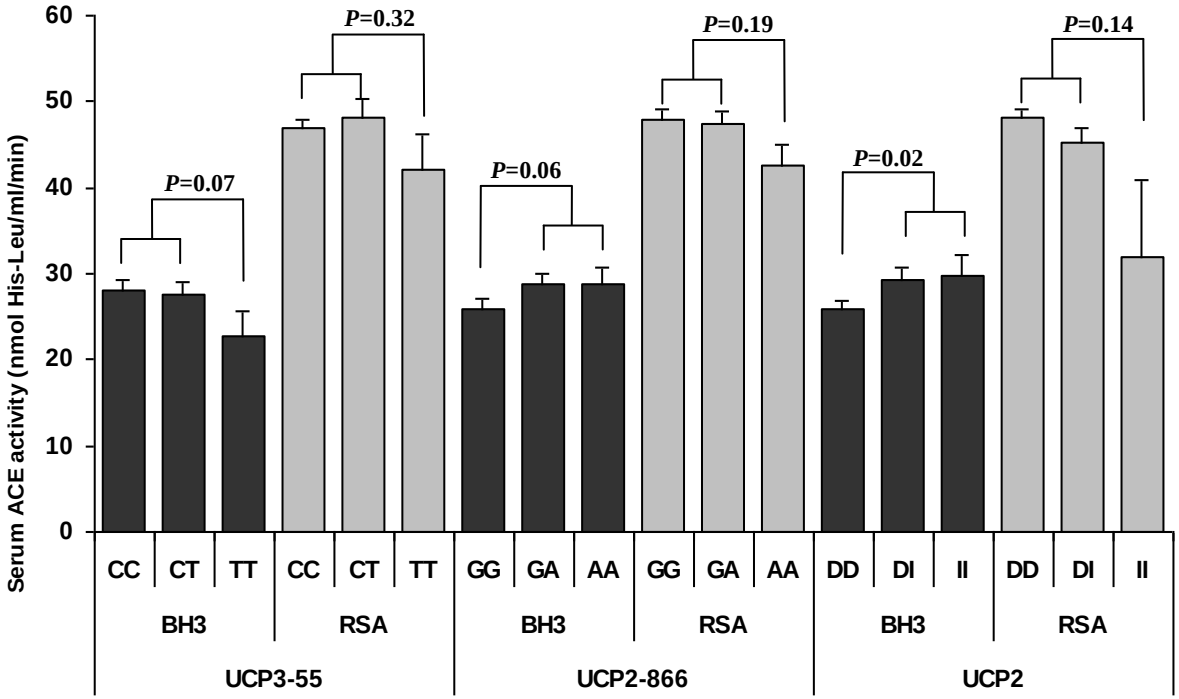
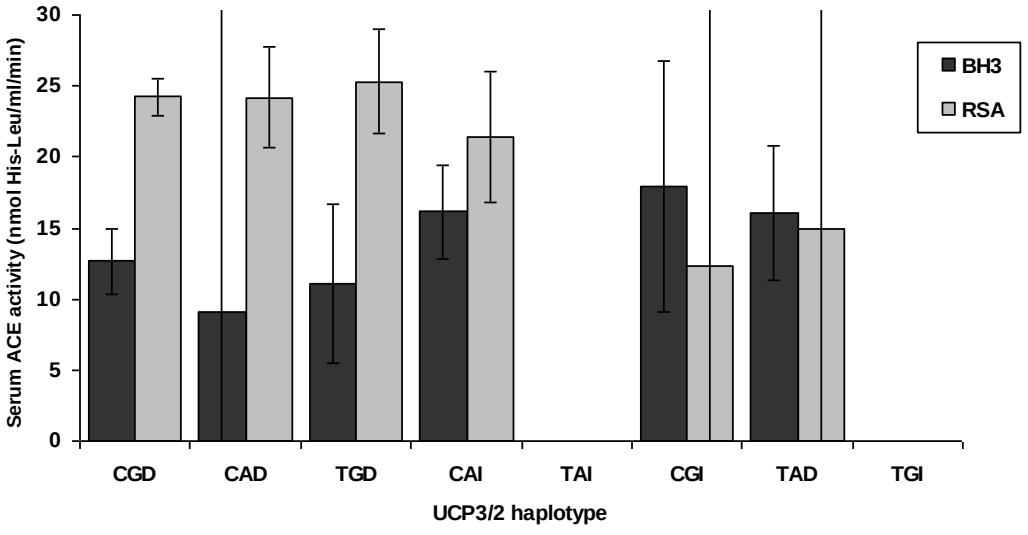


Figure 7.3. Serum ACE activity by UCP3 and UCP2 genotypes in the RSA and BH3 studies (*P* values are age-adjusted).



The expected phenotypic means according to estimated haplotypes for the two studies are shown in Figure 7.4. The *UCP3*-55T/*UCP2*-866A/*UCP2*I and the *UCP3*-55T/*UCP2*-866G/*UCP2*I haplotype groups were too small for analysis. There was not enough statistical power to detect a haplotypic effect in predicting serum ACE activity in either study (P = 0.24 for BH3 and P = 0.61 for RSA). There was a significant difference in the haplotype effects in predicting serum ACE activity between the two groups (once adjusted for effect of study group on ACE activity P = 0.02), but statistical significance was lost if the smaller *UCP3*-55C/*UCP2*-866G/*UCP2*I haplotype group was excluded. A SNP change from the *UCP3/2* CG**D** haplotype to CG**I** led to a mean increase in serum ACE activity of 5.2 nmol His-Leu.ml^{-1}.min^{-1} in BH3 but to a mean decrease in RSA of 11.9 nmol His-Leu.ml^{-1}.min^{-1}. Again, a SNP change from the *UCP3/2* CA**D** haplotype to CA**I** led to a mean increase in serum ACE activity of 7.1 nmol His-Leu.ml^{-1}.min^{-1} in BH3 but to a mean decrease in RSA of 2.7 nmol His-Leu.ml^{-1}.min^{-1}.

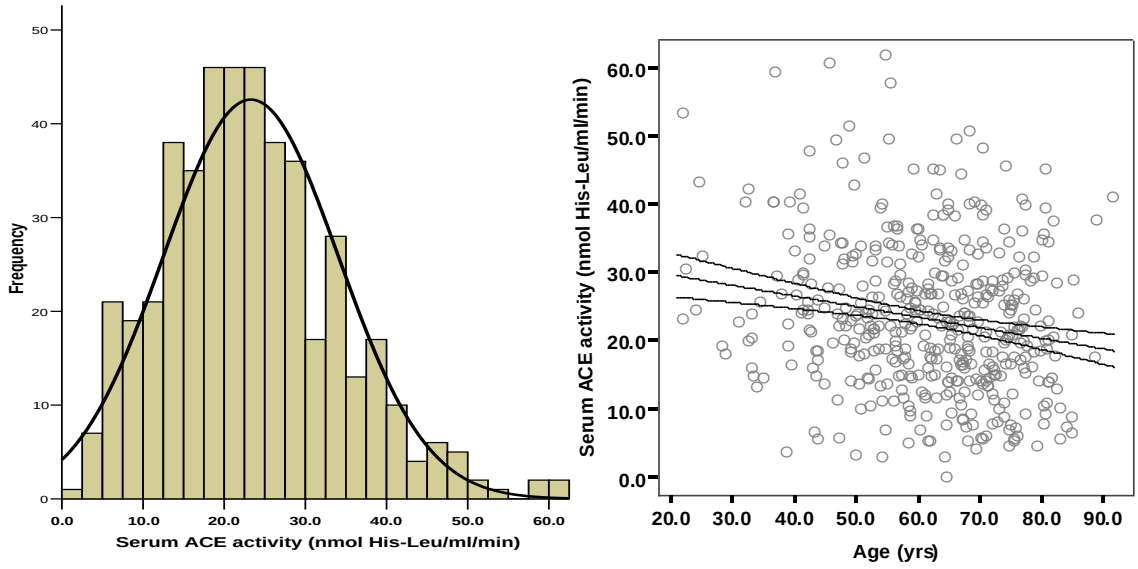
Figure 7.4. Predicted *UCP3*-55C>T, *UCP2*-866G>A and *UCP2*D/I haplotype effects on serum ACE activity in the BH3 and RSA study samples. Data are mean ± 95% confidence intervals.



7.2 DIABETIC PATIENTS (UDACS)

Of the 465 Caucasian male patients (mean age 61.1±13.7 years), 441 had serum ACE activity measured and this was normally distributed (mean serum ACE 23.1±10.7 nmol His-Leu.ml^{-1}.min^{-1}; Figure 7.5). Amongst diabetics, serum ACE activity was *inversely* related to increasing age (correlation coefficient r= -0.19; P<0.0001; Figure 7.5). No other baseline characteristic was associated with ACE activity, including duration of diabetes and HbA_{1c}.

Figure 7.5. Distribution of serum ACE activity in male Caucasians drawn from UDACS and negative correlation with age (linear regression curve with 95% confidence intervals; serum ACE =32.8 -0.15 * age nmol His-Leu.ml^{-1}.min^{-1})



Of this sample, 99/441 (22.4%) were type 1 diabetics. There was a significant difference in serum ACE activity according to type of diabetes: 26.2 ±11.8 nmol His-Leu.ml^{-1}.min^{-1} *vs.* 22.2±10.2 nmol His-Leu.ml^{-1}.min^{-1}, for type 1 and type 2 diabetics respectively; P = 0.001. This difference was accounted for by the significant difference in mean age between the groups (mean age 48.1 ±12.1 yrs and 65.9 ± 10.8yrs for type 1 and type 2 diabetics respectively; $P < 1x10^{-35}$). After adjustment for age, there was no difference in serum ACE activity between type of diabetes (mean ACE activity ± SD

type 1 diabetics 23.8 ±11.7 nmol His-Leu.ml^{-1}.min^{-1} and type 2 diabetics 22.9 ± 10.2 nmol His-Leu.ml^{-1}.min^{-1}; *P* = 0.45).

Less than half (48%) of the study group were treated with ACEi drug therapy (Figure 7.6). As expected, ACEi therapy was associated with lower mean serum ACE activity (no ACEi 24.7±10.8 nmol His-Leu.ml^{-1}.min^{-1} *vs.* ACEi 21.8 ±10.2 nmol His-Leu.ml^{-1}.min^{-1} *P* = 0.003 for age adjusted data). There was no significant difference in mean serum ACE activity in the 7% of patients taking ARBs (no ARB 23.0 ±10.4 nmol His-Leu.ml^{-1}.min^{-1} *vs.* ARB 25.1 ±11.8 nmol His-Leu.ml^{-1}.min^{-1}; *P*=0.3). The 2 patients taking both an ACEi and ARB had the lowest serum ACE activity of all (Figure 7.6). There was a significant statistical interaction between ACEi and ARB medication in determining serum ACE activity (interaction term *P* = 0.002; 2% variability in levels).

In patients who were not taking either ACEi or ARB, there was no relationship between TAOS and serum ACE activity overall, in type 1 or in type 2 diabetics (correlation coefficients r =0.058, 0.042, 0.125, respectively for age-adjusted data; Figure 7.8).

All *UCP3/2* genotype distributions tested were in Hardy-Weinberg equilibrium. Rare allele frequencies are shown in Figure 7.7. There was no statistical difference in any genotype distribution between UDACS type 1 and type 2 diabetics and between either diabetic subgroup and the BH3 control sample.

Figure 7.6. Mean serum ACE activity in male Caucasian subjects from UDACS according to drug treatment with an ACE inhibitor (ACEi) or angiotensin type 1 receptor blocker (ARB). Data are mean ± SEM.

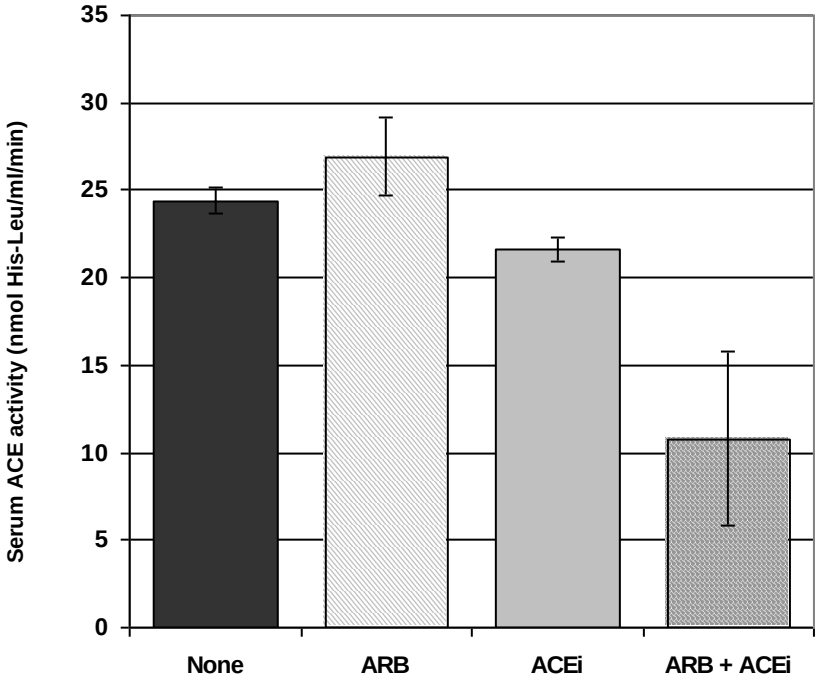


Figure 7.7. Rare allele frequencies (±SD) for *UCP3*-55C>T, *UCP2*-866G>A and *UCP2* I/D gene variants in the BH3 study and in the UDACS male Caucasian type 1 and type 2 sub-groups.

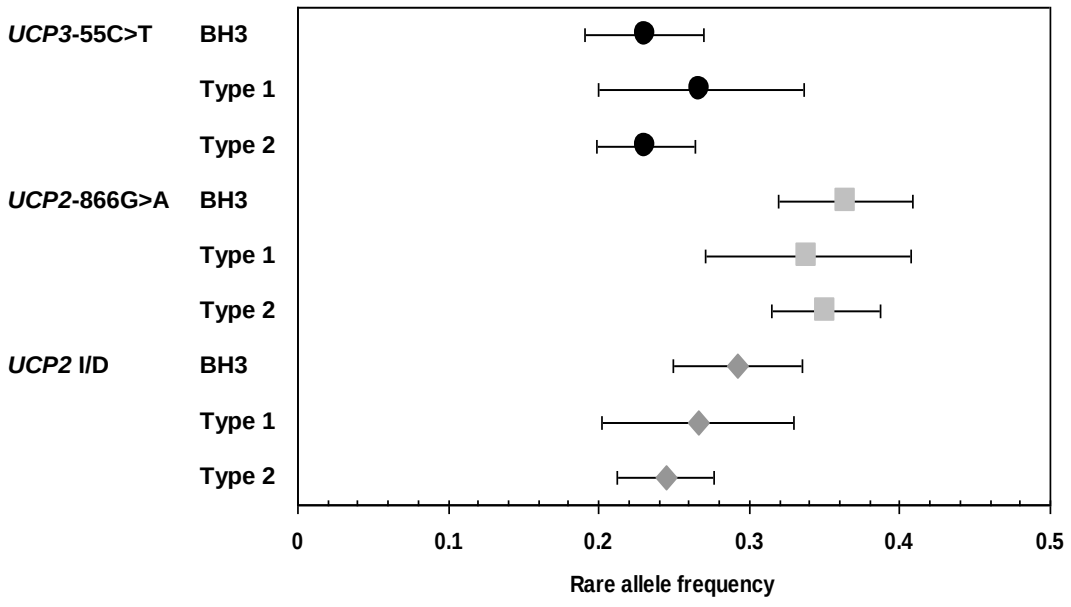
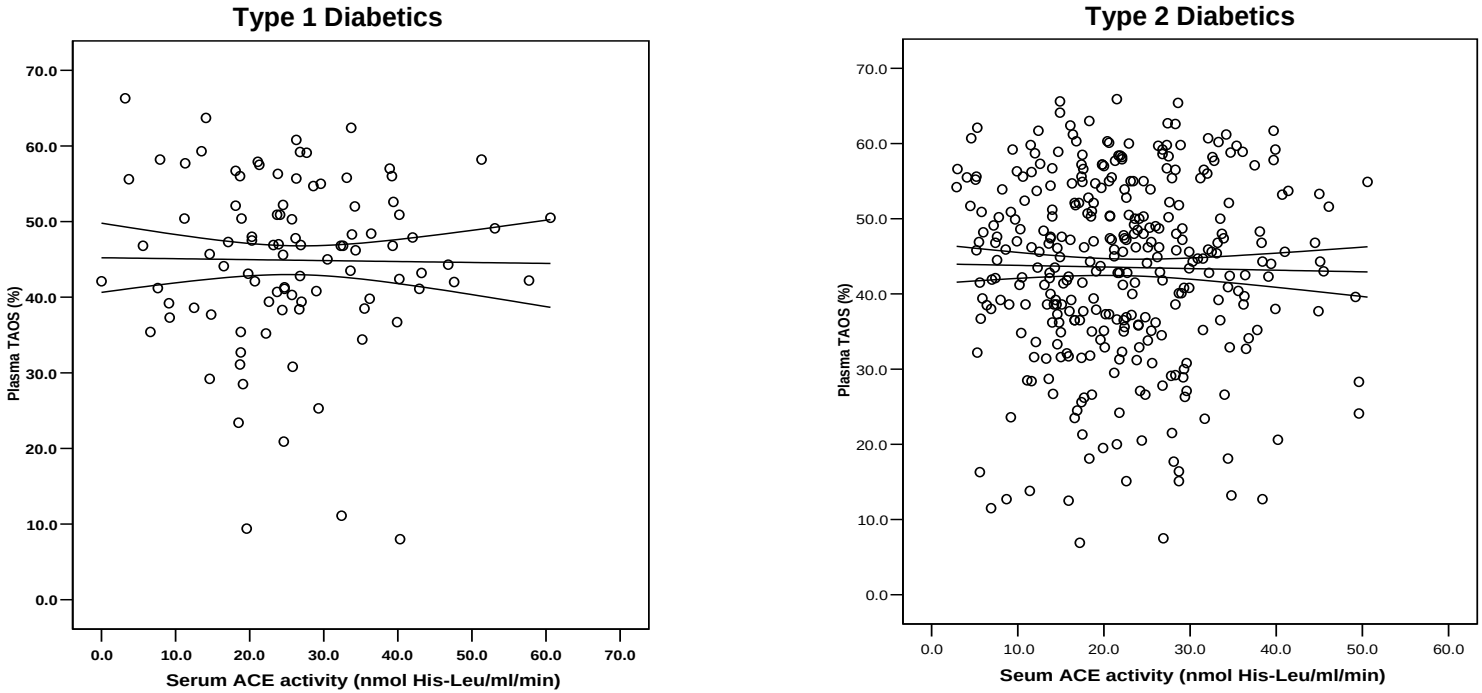


Figure 7.8. **The lack of correlation between serum ACE activity and TAOS in ACEi naive Caucasian men drawn from UDACS divided by type of diabetes (best fit correlation curves are shown with 95% confidence intervals).**



There was significant LD across the *UCP3/2* locus in the larger type 2 diabetics and there was similar association in the smaller type 1 diabetic sub-group, being significant only between the *UCP2*-866G>A and *UCP2*I/D polymorphisms (Table 7.4). The LD was similar to that observed in the BH3 Caucasian study sample (Section 7.1; Figures 7.1 & 7.9).

There were no genotype associations with any baseline characteristic. Genotype relationships with age-adjusted serum ACE activity were therefore tested in the study sample overall and also according to type of diabetes, but only in ACEi- and ARB naive patients (Figure 7.10). In these diabetic patients, there were no clear patterns of associations between UCP genotypes and serum ACE activity. The only statistically significant genotype association was between the UCP2-866G>A variant and serum ACE activity overall and specifically in type 1 diabetics (age-adjusted serum ACE activity was 22.6 ±8.7 nmol His-Leu.ml^{-1}.min^{-1} *vs.* 26.4 ±12.6 nmol His-Leu.ml^{-1}.min^{-1} *vs.* 22.6 ±8.7 nmol His-Leu.ml^{-1}.min^{-1} for GG *vs.* GA *vs.* AA overall; P = 0.04 ANOVA).

Table 7.4. *UCP* haplotype distribution and linkage disequilibrium (D') in UDACS.

Caucasian type 2 diabetics		*UCP2*-866G>A GG	GA	AA
UCP3 -55C>T	CC	164	156	27
	CT	57	92	35
	TT	1	18	7

D' = 0.23; *P* <0.0001

Caucasian type 1 diabetics		*UCP2*-866G>A GG	GA	AA
UCP3 -55C>T	CC	47	37	5
	CT	16	26	9
	TT	4	4	2

D'= 0.21; *P* = 0.16

Caucasian type 2 diabetics		*UCP2* Del/Ins DD	DI	I I
UCP3 -55C>T	CC	159	158	30
	CT	102	75	6
	TT	23	2	1

D' = -0.19; *P*=0.0003

Caucasian type 1 diabetics		*UCP2* Del/Ins DD	DI	I I
UCP3 -55C>T	CC	47	37	4
	CT	30	19	2
	TT	7	2	1

D'= -0.06; *P* = 0.97

Caucasian type 2 diabetics		*UCP2* Del/Ins DD	DI	I I
UCP2 -866G>A	GG	198	33	3
	GA	84	178	10
	AA	12	36	24

D' = 0.60; *P*<0.0001

Caucasian type 1 diabetics		*UCP2* Del/Ins DD	DI	I I
UCP2 -866G>A	GG	60	10	3
	CT	26	48	1
	TT	4	8	4

D' = 0.52; *P*<0.0001

Figure 7.9. Linkage disequilibrium between common variants in the *UCP3/2* gene cluster in UDACS type 1 (Black) and type 2 (red) diabetics.

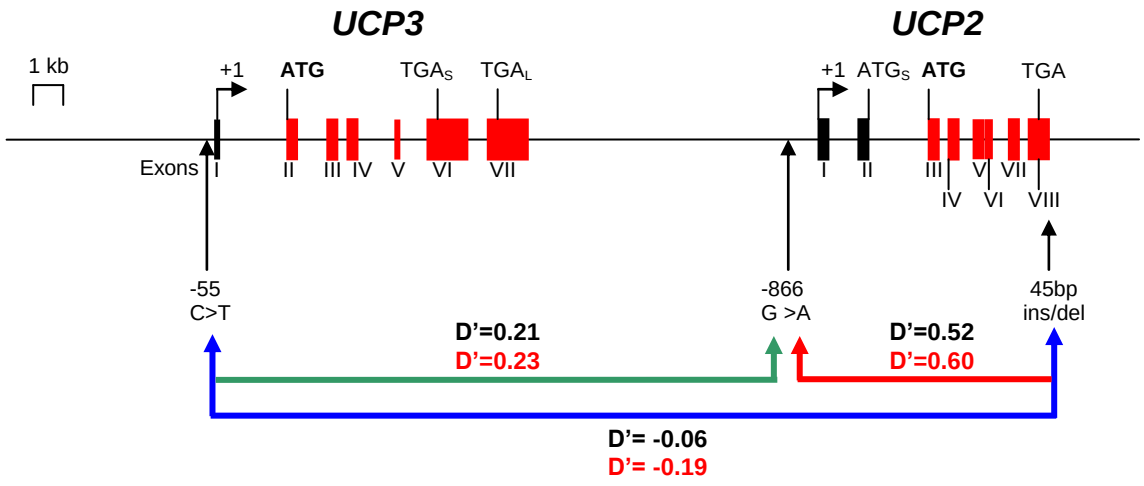
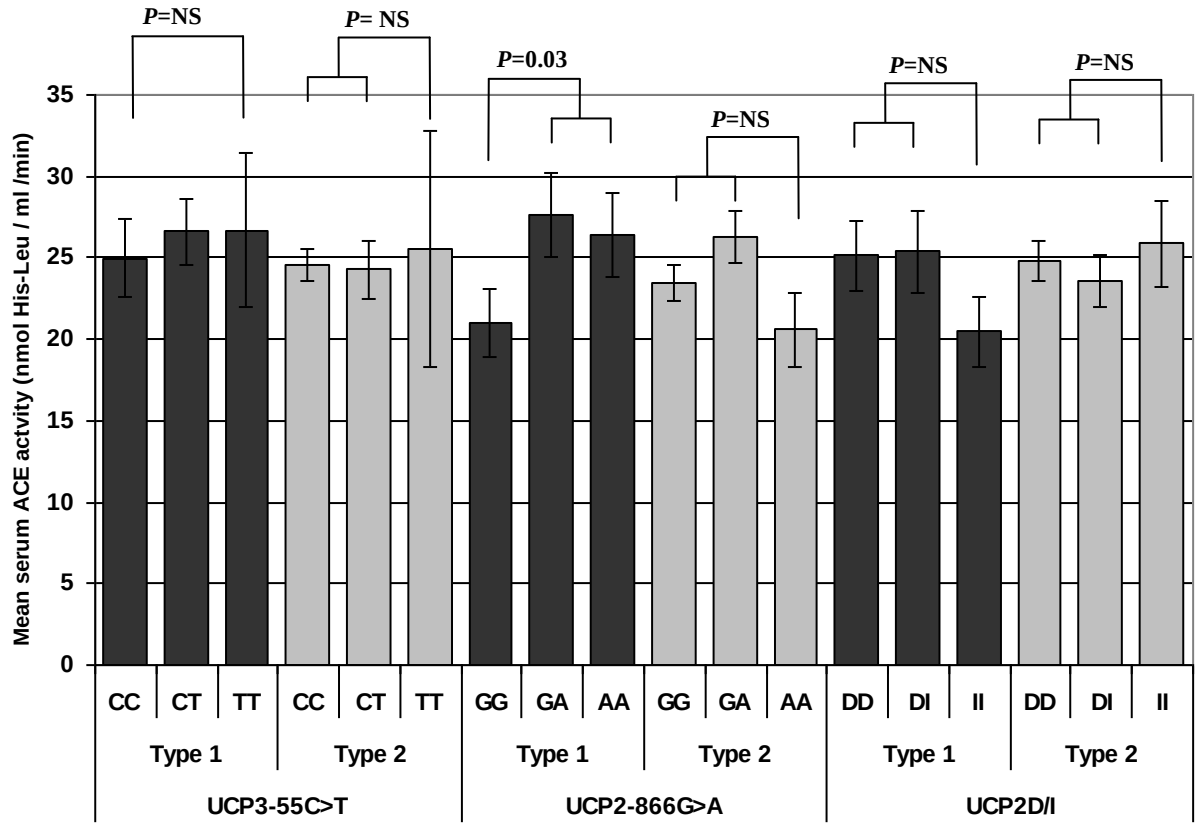


Figure 7.10. **Mean serum ACE activity by *UCP*3 and *UCP*2 genotypes in ACEi- and ARB-naïve subjects from UDACS according to type of diabetes (data are age-adjusted).**



7.3 TYPE 1 DANISH DIABETICS

The study consisted of 287 consecutive adult (≥ 18 years old; mean age 45.5±13.7 years; 117 female) type 1 diabetic patients drawn from the outpatients department Hillerød Hospital, Denmark by Pedersen-Bjergaard and colleagues. These patients had had diabetes for more than 2 years, with type 1 diabetes defined as need for insulin treatment from the time of diagnosis and unstimulated C-peptide concentrations below 300 pmol.L^{-1} or stimulated (venous blood glucose concentration > 12mmol.L^{-1}) C-peptide concentrations below 600 pmol.L^{-1} (Pedersen-Bjergaard *et al.* 2001).

Serum ACE activity was normally distributed (mean serum ACE 41.2 ±19.6 nmol His-Leu.ml^{-1}.min^{-1}; Figure 7.11). There was no significant difference in ACE activity between men and women (mean serum ACE 45.9 ±21.0 nmol His-Leu.ml^{-1}.min^{-1} and 45.0 ±17.1 nmol His-Leu.ml^{-1}.min^{-1}, respectively; P = 0.75). As opposed to the Caucasian samples from BH3 and UDACS, serum ACE activity was weakly associated with increasing age (correlation coefficient r = 0.12; P = 0.04).

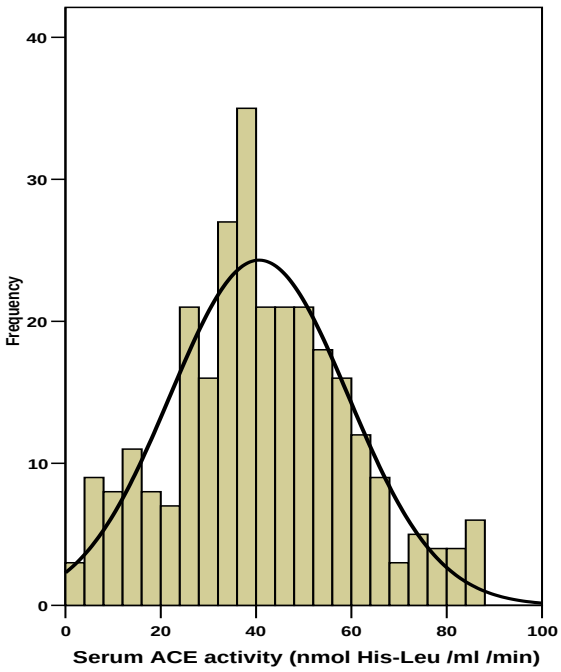


Figure 7.11. The distribution of serum ACE activity amongst a sample of Danish type 1 diabetics (curve fit represents normal distribution).

Those subjects treated with ACEi (n = 60) had significantly lower serum ACE activity (no ACEi 50.7 ±17.3 nmol His-Leu /ml /min and ACEi-treated 25.8 ±14.3 nmol His-Leu /ml /min; $P < 1x10^{-21}$ age-adjusted; Figure 7.12). There was no significant difference in serum ACE activity in the smaller number of patients taking ARBs alone or in combination with ACEi (n = 8 and 3, respectively) and no significant statistical interaction between ACEi and ARB treatment in determining serum ACE activity.

UCP3-55C>T, *UCP2*-866G>A and *UCP2*D/I genotypes were in Hardy Weinberg equilibrium and rare allele frequencies were similar to those from a healthy Caucasian sample (Bassingbourn 3, NPHSII) and to those from UDACS, and, as with those samples, there was significant positive LD between *UCP3*-55C>T and *UCP2*-866G>A and between *UCP2*-866G>A and *UCP2*D/I and significant negative LD between *UCP3*-55C>T and *UCP2*D/I loci (Table 7.5, Figure 7.13). There were no genotype associations with any baseline characteristic.

With analyses restricted to those subjects who were both ACEi- and ARB-naïve, there were significant genotype associations with serum ACE activity, which followed the same pattern as those associations in the BH3 study (Figures 7.2 & 7.14): *UCP3*-55TT homozygotes had significantly lower serum ACE activity, *UCP2*-866AA carriers tended to have higher serum ACE activity and *UCP2*II homozygotes had significantly higher serum ACE activity. In multivariate analysis, statistical interaction between all three genotypes accounted for 5% of the interindividual variability in (age-adjusted) serum ACE activity, with the majority of this accounted for by the statistical interaction between *UCP2*-866G>A and *UCP2*D/I genotypes (P=0.001). This effect is demonstrated in Figure 7.15.

Figure 7.12. **Mean serum ACE activity in Danish type 1 diabetics according to drug treatment with an ACE inhibitor (ACEi) or angiotensin type 1 receptor blocker (ARB). Data are mean ± SEM.**

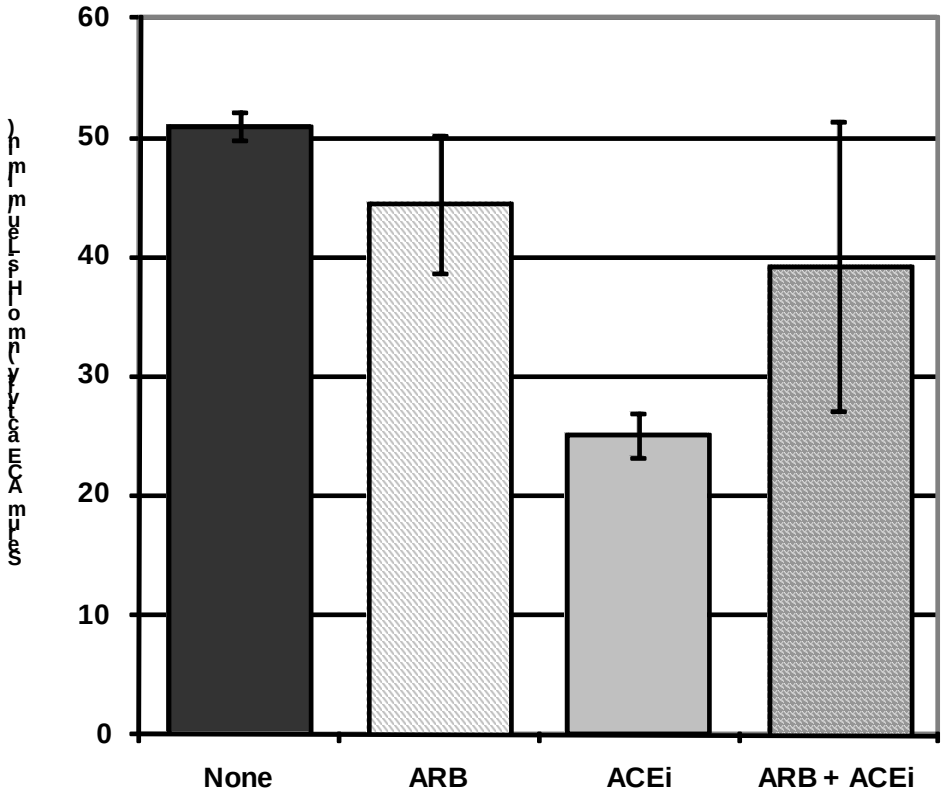


Table 7.5. ***UCP*** **haplotype distribution and linkage disequilibrium (D') in the Danish type 1 diabetic patients.**

Danish type 1 DM		*UCP2*-866G>A GG	GA	AA
UCP3 -55C>T	CC	74	60	17
	CT	35	57	12
	TT	4	10	9

D'= 0.23; *P* =0.005

Danish type 1 DM		*UCP2* Del/Ins DD	DI	I I
UCP3 -55C>T	CC	67	63	21
	CT	68	32	4
	TT	19	3	0

D'= - 0.26; *P*=0.001

Danish type 1 DM		*UCP2* Del/Ins DD	DI	I I
UCP2 -866G>A	GG	88	25	3
	CT	57	59	12
	TT	12	14	12

D' = 0.38; *P*<0.0001

Figure 7.13. **Linkage disequilibrium between common variants in the *UCP3/2* gene cluster in the Danish type 1 diabetics**

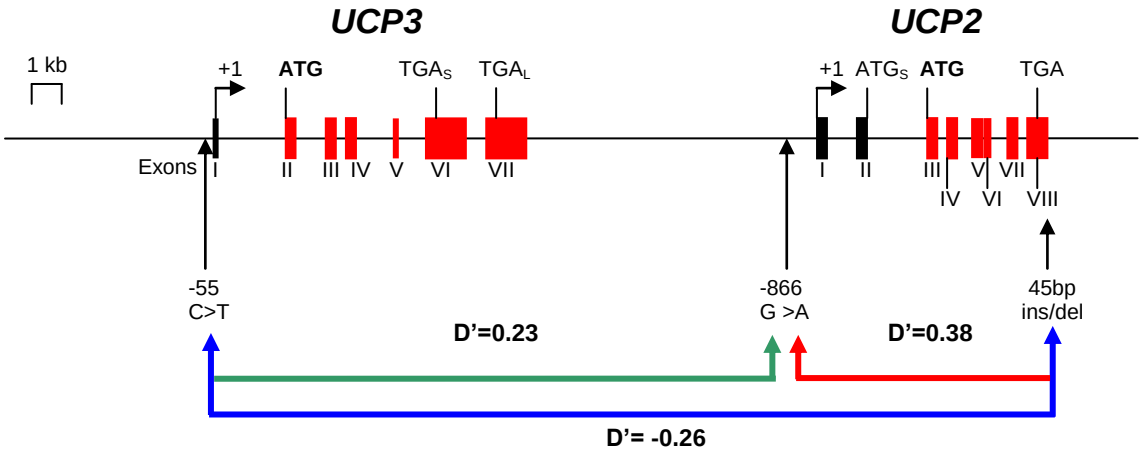
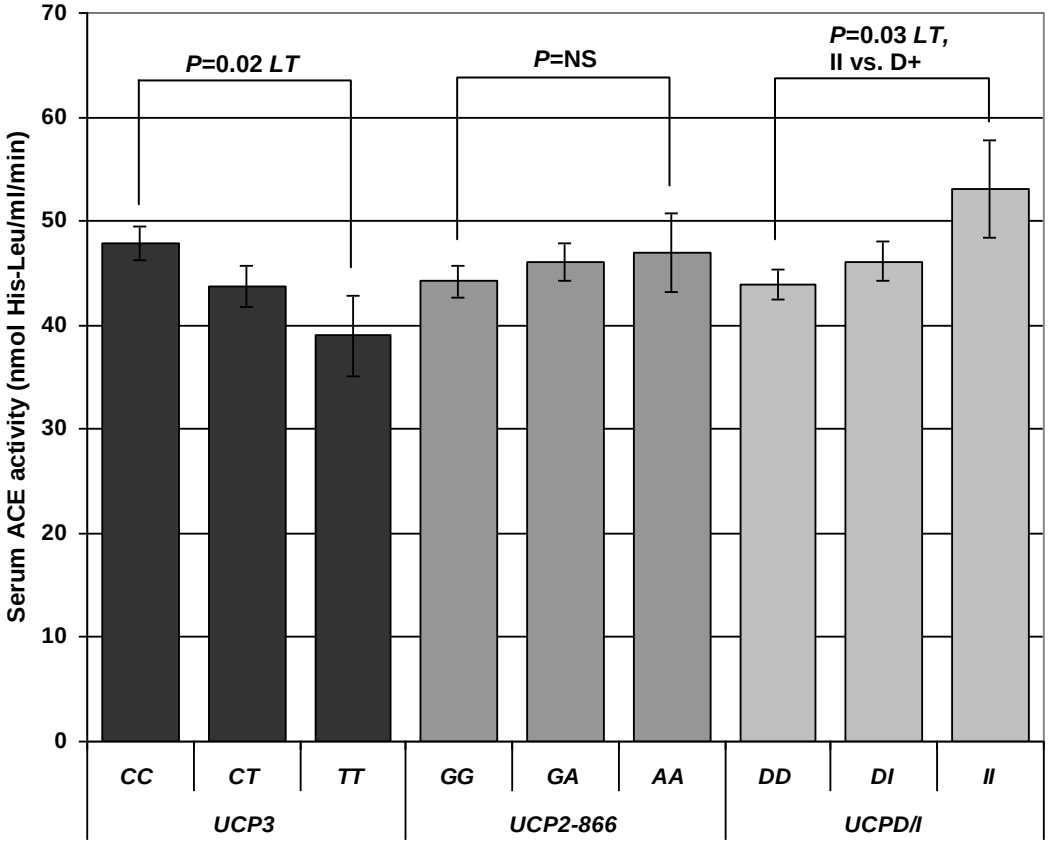


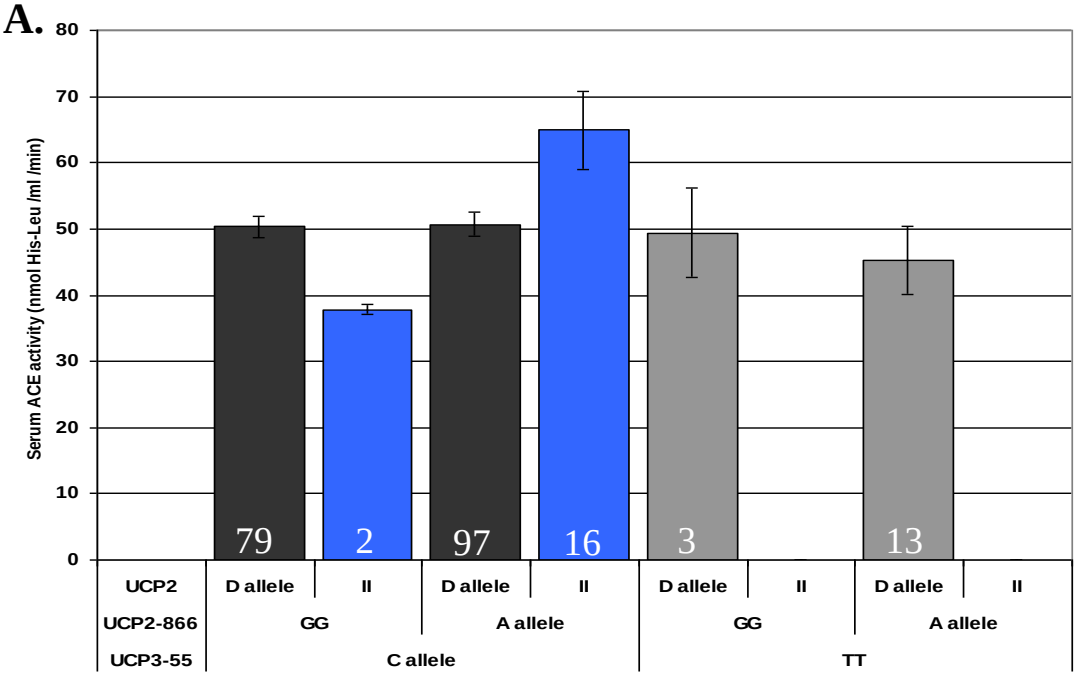
Figure 7.14. Serum ACE activity (adjusted for age) in ACEi/ARB naïve Danish type 1 diabetic patients according to *UCP3*-55C>T, *UCP2*-866G>A and *UCP2*D/I genotypes.



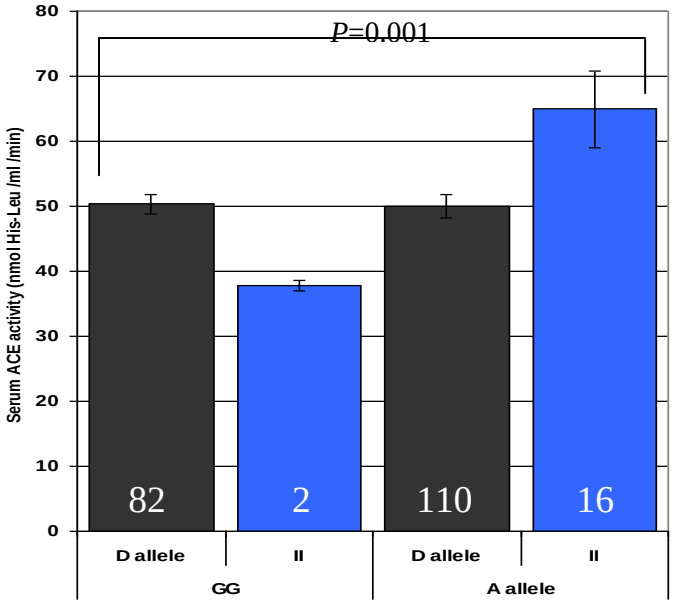
Data are mean ±SEM;
LT= linear trend analysis, II vs. D+ = ANOVA of II vs. D allele carriers

Figure 7.15. <u>The statistical interaction between *UCP3*-55C>T, *UCP2*-866G>A and *UCP2*D/I genotypes in determining serum ACE activity (adjusted for age) in Danish type 1 diabetic patients.</u> All 3 genotypes are shown in Figure A and data for just *UCP2*-866G>A and *UCP2*D/I haplotypes (number in each group shown at the base of each column).

A.



B.



7.4 DISCUSSION

The *UCP3*-55C>T, *UCP2*-866G>A and *UCP2*D/I genotype distributions in the Bassingbourn 3 study was similar to previous reports in Caucasians (Cassell *et al.* 1999; Dalgaard *et al.* 1999; Cassell *et al.* 2000; Meirhaeghe *et al.* 2000; Otabe *et al.* 2000; Dalgaard *et al.* 2001; Esterbauer *et al.* 2001; Kimm *et al.* 2002). However, there have been few studies to date in African populations (Argyropoulos *et al.* 1998), Afro-Caribbean (Chapter 4) and in African-American groups (Argyropoulos *et al.* 1998; Kimm *et al.* 2002). The *UCP3*-55C>T and *UCP2*-866G>A genotype distributions from the RSA study were not dissimilar to the much smaller group of Afro-Caribbean athletes in Chapter 4. The RSA study consisted of 200 consecutive male recruits from the Xhosa ethnic group. This sample appeared to be older genetically than the Caucasian sample, with evidence of greater recombination: the rare allele frequency was significantly less for each genotype in the RSA group than those from the Bassingbourn 3 study. Furthermore, there were significant differences in LD across the locus between the RSA and Bassingbourn 3 studies, exemplified by negative LD (D'=0.31) between *UCP3*-55C>T and *UCP2*D/I in Caucasians and no LD between these 2 sites in South Africans (the negative degree of LD between *UCP3*-55C>T and *UCP2*D/I had not been previously reported in Caucasians (Kimm *et al.* 2002)). This resulted in highly significant differences in haplotype distribution between the two ethnic groups. It has been demonstrated recently that African-origin populations exhibit substantially greater haplotype diversity than do Caucasian-origin populations (Reich *et al.* 2001; Gabriel *et al.* 2002).

The resolution to detect functional polymorphisms in a fine mapping study is limited by the size of the population and the evolutionary history of the population (Cox *et al.*

2002) as well as the strength of association with the phenotype or complex trait. The study of the *ACE* gene and its association with serum ACE activity has been described as one of the best human models to assess the genetics of a complex trait (Cox *et al.* 2002), but the extraordinary degree of LD in the *ACE* gene in Europeans had made it impossible to detect which variants are functional (Cox *et al.* 2002). Cox *et al* studied the greater sequence and haplotype diversity in a Nigerian sample to improve the identification of functional variants within the *ACE* gene (Cox *et al.* 2002).

As was to be expected, serum ACE activity was much higher in the RSA group than the Caucasians, despite correction for confounders. There were significant but weak associations between *UCP*3/2 genotypes and serum ACE activity in both the RSA and BH3 studies. This is the first report of an association between common variation in a gene other than *ACE* and serum ACE activity. Variation at the *UCP3* promoter SNP resulted in a mean, age-adjusted difference of approximately 6 nmol His-Leu.ml^{-1}.min^{-1} in ACE activity in both Caucasians and South Africans Xhosans. However, variation in *UCP2* genotypes appeared to have opposite effects on serum ACE activity in the two ethnic groups. The sample sizes and the strength of association were too small to detect overall haplotypic associations with serum ACE activity. However, there was a significant difference in haplotype effect *between* the ethnic groups, mostly explained by differential effects at the *UCP2*D/I variant.

There were similar associations between serum ACE activity and *UCP3/2* genotypes in the Danish type 1 diabetic cohort. In this Caucasian sample, variation at the *UCP3/2* locus accounted for 5% of the variability in serum ACE activity between individuals. There was only weak association in the smaller male type 1 diabetic group drawn from UDACS drawn from UDACS, with the *UCP2*-866GG homozygotes having

significantly lower serum ACE activity than A allele carriers. There was a similar, but borderline significant association in the BH3 study. There were no such associations between any *UCP* genotype and ACE activity in the male type 2 diabetic group from UDACS.

These data suggest that variation at the *UCP3/2* genetic locus has a small but significant impact on serum ACE activity. The differential association in the two ethnic groups studied could be a result of genetic recombination with resultant differences in haplotype structure. Only three genetic variants were examined, so it is difficult to draw firm conclusions as to which (if any) of these is functional or is associated with the greatest influence on ACE activity. As previously discussed, the genetic variants or haplotypes examined may not be functional themselves, but may mark functional variation elsewhere in the cluster. In order to unravel the association further, the sample sizes would need to be increased and more gene variants across the locus would need to be tested. However, this process is limited by the strength of the association between *UCP3/2* genotypes and serum ACE activity which reduces the power of any such study.

The lack of association between serum ACE activity and *UCP3* and *UCP2* genotypes in the cross-section of type 2 diabetic men from UDACS may be due to the phenotypic complexity of this group, such that any true association might be diluted by confounders, such as drug therapy, duration of diabetes and presence of hypertension.

The *UCP3/UCP2* locus has been linked to resting metabolic rate (Bouchard *et al.* 1997) and is in a region that has been linked to diabetes and obesity (Fleury *et al.* 1997). However, I found no association between the *UCP3* or *UCP2* genotypes tested and the presence of diabetes or obesity. This agrees broadly with most previous cross-sectional

studies in Caucasians, with no association between the *UCP2D/I* variant and obesity reported in 744 obese Danish men (Dalgaard *et al.* 1999) nor in British women (Cassell *et al.* 1999). In contrast, association was found between this variant and fat accumulation in a small prospective study of Caucasian patients on peritoneal dialysis (Nordfors *et al.* 2000), childhood obesity (Yanovski *et al.* 2000), older (more than 45 years old) Pima Indians (Walder *et al.* 1998) and a larger cross-sectional study of South Indian women (Cassell *et al.* 1999). No association between *UCP2*D/I variant and the presence of type 2 diabetes was previously found (Cassell *et al.* 1999), but positive association was found between the *UCP3*-55T allele and diabetes in French Caucasians (Meirhaeghe *et al.* 2000) and with BMI in morbidly obese French Caucasians (Otabe *et al.* 2000), as well as the *UCP2*-866G allele and obesity (Esterbauer *et al.* 2001) and protection against diabetes (Krempler *et al.* 2002). Differences in reported association may have been caused by differences in ethnic group studied, sample size (smaller genetic studies may be more likely to report false positive association with a possibility of publication bias (Colhoun *et al.* 2003), or differences in the severity of the phenotype in the study group (for example morbid obesity rather than comparison of BMI or childhood onset obesity rather than adult-onset).

In summary, a significant genetic association between the *UCP3/UCP2* locus and serum ACE activity has been described. There were significant ethnic differences in *UCP3/UCP2* haplotype distribution and significant haplotypic associations with serum ACE activity. The majority of this association appeared to be due to variation in the *UCP2* gene.

Further work is required to delineate the proposed *UCP3/UCP2* genetic association with ACE activity, with a larger sample size and an increase in the number of gene variants examined.

※ RESULTS CHAPTER EIGHT ※

DO UNCOUPLING PROTEINS 2 OR 3 ALTER ACE EXPRESSION IN HUMAN ENDOTHELIAL CELLS?

8.1 UNCOUPLING PROTEINS ARE EXPRESSED AT THE MRNA LEVEL IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVECS)

HUVECs were grown to sub-confluency in 175ml culture flasks and RNA harvested as described in Chapter 2. Messenger RNA was isolated and target sequences amplified using forward and reverse primers (Table 8.1) as previously described. Abundant UCP2 and ACE mRNA were both identified, whereas UCP3 mRNA expression appeared to be low (Figure 8.1). This is the first report of UCP expression in human endothelial cells. Shortly after this discovery, Duval *et al* published data in murine endothelial cells using antisense to *UCP2* to increase cellular ROS generation; RT-PCR was not performed, and *UCP3* antisense had no effect on endothelial ROS (Duval *et al.* 2002).

8.2 MANIPULATION OF $\Delta\psi_m$ IN HUVECS

Mitochondrial membrane potential ($\Delta\psi_m$) was measured in HUVECs using flow cytometry. Cells were pre-treated with either 5 μM mClCCP for 30 minutes or 10 μM rotenone for 1 hour or 10 μM nigericin for 1 hour and then loaded with 5 μM JC-1 for 1 hour prior to analysis of $\Delta\psi_m$ (Figure 8.2). Uncoupling of mitochondrial respiration with the protonphore mClCCP resulted in collapse of $\Delta\psi_m$ (mean 95 ±0.1% reduction, $P < 0.01$).

Figure 8.1. RT-PCR of mRNA isolated from 4 samples of HUVECs. PCR for *GAPDH*, *UCP2*, *ACE* and *UCP3* were performed and products run on a 2% agarose gel against a 1kb DNA ladder.

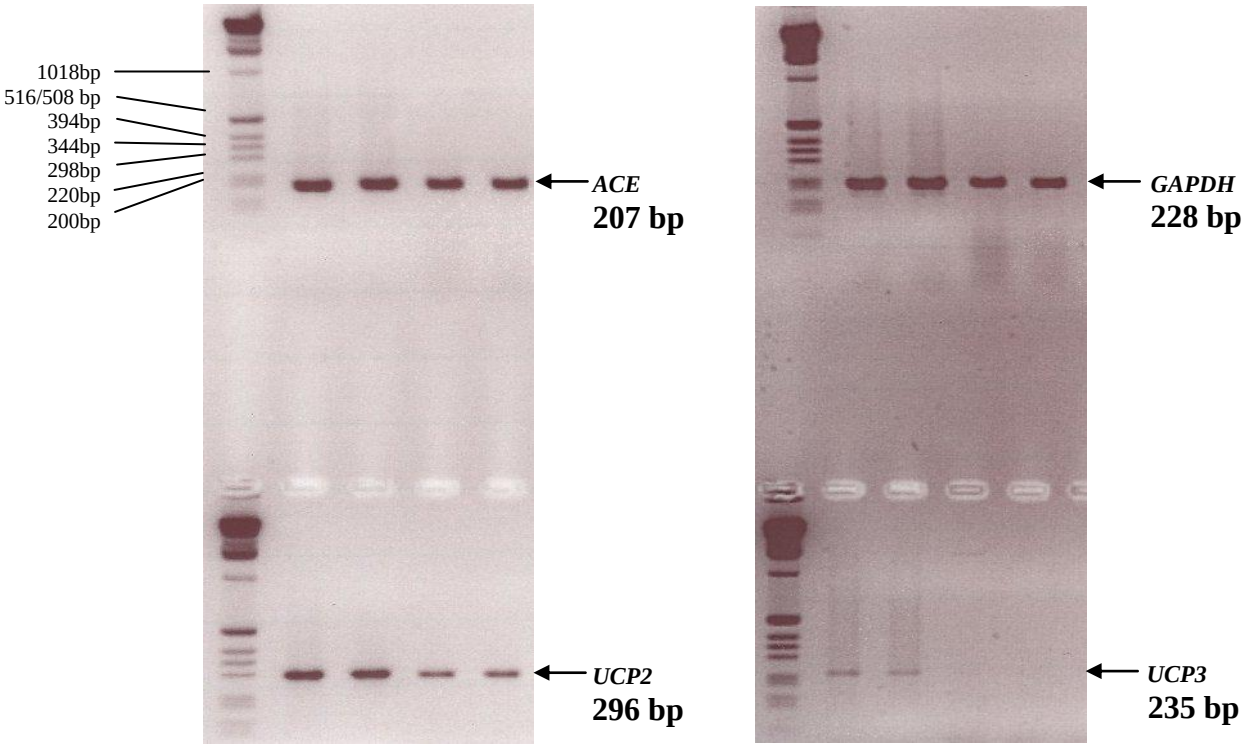
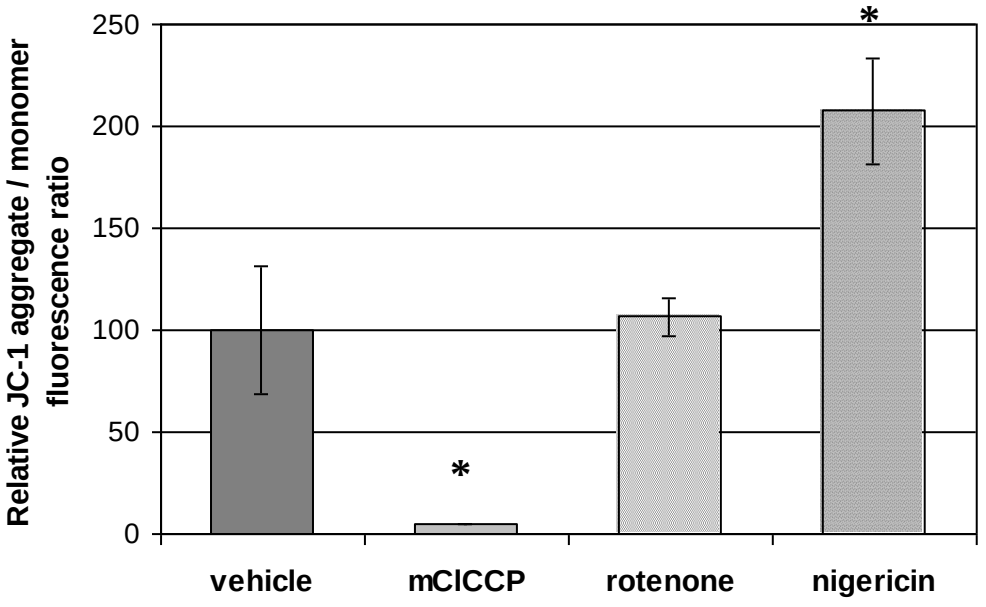


Table 8.1. Forward and reverse primers for RT-PCR and amplicon size. Primers were designed to either include an intronic sequence within the amplified sequence (if genomic DNA were present as contaminant) or designed to straddle exon-exon boundaries.

Gene	cDNA accession no.	Primers	Amplicon size, bp
h*GAPDH*	J04038	F: GGGGAAGGTGAAGGTCGGAGT R: CCTGGAAGATGGTGATGGGAT	228
h*UCP3*	U84763 / AF050113	F: CCTCACTACCCGGATT R: GTTGACGATAGCATTCCT	235
h*UCP2*	NM_003355 / AF019409	F: GCTTTGAAGAACGGGAC R: CTGTAACCGGACTTTAGCA	296
hACE	J04144	F: ACCAATGACACGGAAAG R: GTGGGTTTCGTTTCGG	207

The ionophore nigericin caused the expected hyperpolarisation of $\Delta\psi_m$ (mean 107 ±26.1% increase $P<0.01$). The complex 1 inhibitor rotenone had no effect on $\Delta\psi_m$, confirming that the cells were maintaining their membrane potential through glycolytic metabolism, as they were in Krebs' solution with glucose as substrate.

Figure 8.2. <u>Relative JC-1 aggregate to monomer (A:M) fluorescence in HUVECs pretreated with mClCCP, rotenone and nigericin.</u>

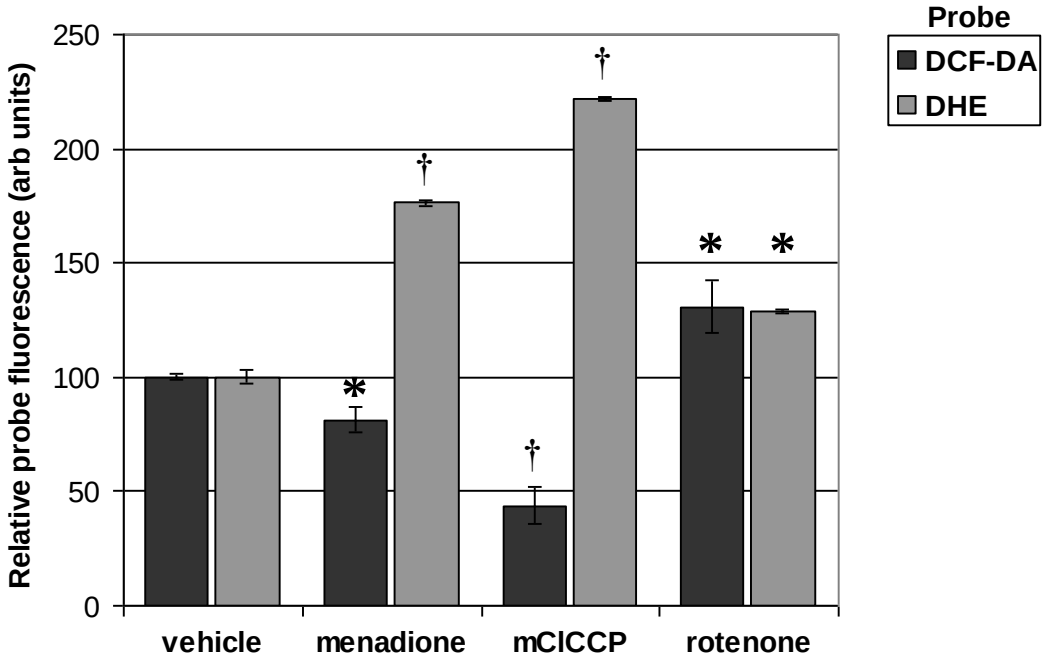


Data are mean of 2 experiments ± SD; for vehicle ratio indexed to 100
***** P <0.01 compared to vehicle

8.3 ROS GENERATION IN HUVECS

The redox cycler menadione (1 mM for 1 hour) caused a significant increase in mitochondrial superoxide production as measured with DHE fluorescence in HUVECs (76 ±2.6% increase, P = 0.009; Figure 8.3). This appeared to be at the expense of mitochondrial peroxynitrite, as demonstrated by a significant reduction in DCF-DA fluorescence (19 ±11.2% reduction P = 0.02). This pattern was repeated with mClCCP (5 μM for 30 minutes) treatment, although the changes in fluorescence were greater (122 ±2.2% increase in DHE fluorescence, P = 0.001 and 56 ±16.4% reduction in DCF-DA, P < 0.0001). In contrast, rotenone (10 μM for 1 hour) led to a modest increase in both DHE and DCF fluorescence (29 ±1.3% increase, P = 0.01 and 31 ±22.9% increase, P = 0.04, respectively).

Figure 8.3. Effect of addition of menadione, mClCCP and rotenone on the flow cytometric measurement of intracellular DCF-DA (5μM) and DHE (10μM) fluorescence in HUVECs.



Data are mean ± SEM of 3-4 experiments (each N=10000 cells)
* P<0.05
† P<0.01, compared to vehicle

8.4 THE EFFECTS OF PROLONGED HYPOXIA, UNCOUPLING AND HIGH GLUCOSE ON $\Delta\psi_m$ AND SUPEROXIDE GENERATION IN CULTURED HUVECS

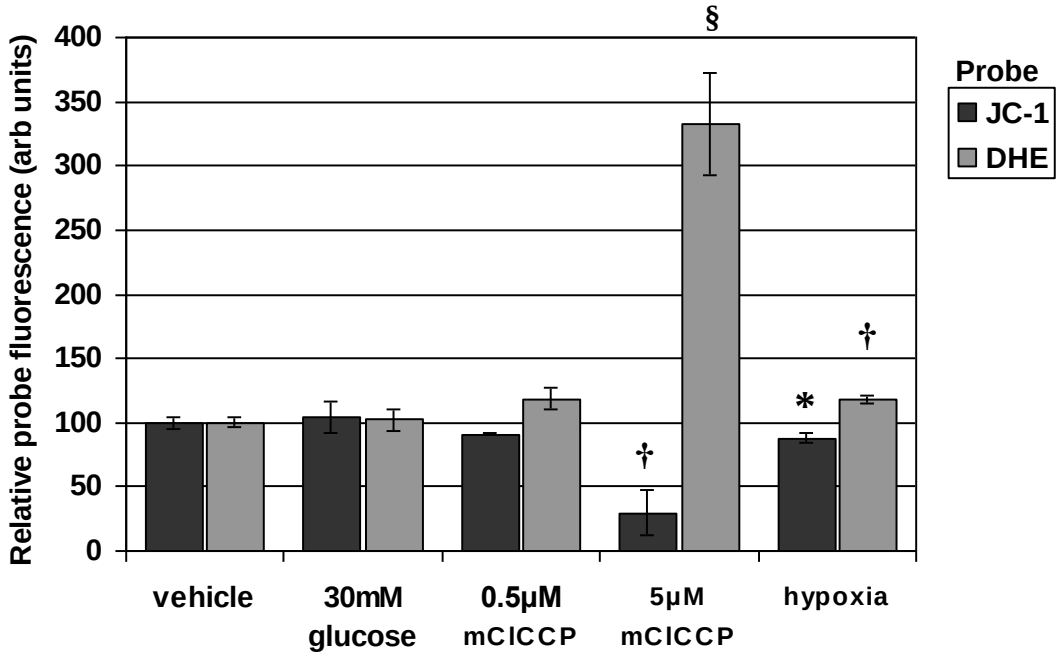
HUVECs were incubated for 24 hours in different experimental conditions to induce cell stress. Superoxide generation and $\Delta\psi_m$ were measured by flow cytometric measurement of DHE and JC-1 cell fluorescence, respectively (Figure 8.4). Control (vehicle-treated) cells were incubated in standard EBM media containing 5.6 mM glucose at 20% O_2 and 5% CO_2.

In contrast to a previous report (Nishikawa *et al.* 2000), exposure to a high glucose concentration (30 mM) for 24 hours resulted in no change in either $\Delta\psi_m$ or superoxide generation (N = 5; Figure 8.4).

Treatment with the respiratory uncoupler mClCCP for 24 hours (N = 3) resulted in a dose dependent decrease in $\Delta\psi_m$ (5 μM mClCCP: mean 71 ±17.5% reduction in JC-1 A:M fluorescence; $P = 0.003$). This profound, persistent uncoupling of respiration was also associated with a significant dose-dependent production of superoxide (5 μM mClCCP: mean 232 ±40.0% increase in DHE fluorescence; $P = 0.00002$; Figure 8.4).

HUVECs incubated in hypoxic conditions for 24 hours (5% O_2; N = 3) exhibited significant cell stress with a reduction in $\Delta\psi_m$ (mean 12 ±3.6% reduction in JC-1 A:M fluorescence; $P = 0.04$) and increase in superoxide (mean 18 ±3.4% increase in DHE fluorescence; $P = 0.006$; Figure 8.4).

Figure 8.4. Effects of 24 hours of high glucose, protonphore mClCCP and hypoxia (5% O2) on HUVEC $\Delta\psi_m$ and superoxide production (measured by JC-1 aggregate to monomer fluorescence ratio and DHE fluorescence, respectively)



Data are mean of 2-6 experiments (n=10000 cells each) ± SD

***** *P* <0.05

† *P* <0.01

§ *P* <0.001

8.5 THE EFFECTS OF UCP ANTISENSE ON HUVEC MITOCHONDRIAL FUNCTION

An antisense strategy was employed to selectively downregulate UCP2 and UCP3 mRNA expression in HUVECs, using a similar approach in murine endothelial cells as Duval *et al* (Duval *et al.* 2002). Complementary oligonucleotide sequences (21mer) were designed to straddle the published transcription start sites of *UCP1*, *UCP2* and *UCP3* (Figure 8.5). The sequences were entered into a Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information website http://www.ncbi.nlm.nih.gov/BLAST/) to rule out close sequence homology with other

human genomic sequences. A randomly scrambled *UCP2* antisense sequence which did not recognise any published human genomic sequence was also designed (Figure 8.5).

Cells were then incubated with 2 ml serum-free media supplemented with 1-10 μM antisense oligonucleotide for a total of 48hours, with fresh media changed at 24 hours to maintain a more even concentration of oligonucleotide. After this time, the supernatant was aspirated and stored at -80°C for subsequent analysis. The cells were immediately either lysed for isolation of mRNA or washed in Krebs solution prior to assessment of $\Delta\psi_m$ or ROS status.

Figure 8.5. Nucleotide sequence of 21mer antisense strands designed to bind across *UCP1*, *UCP2* and *UCP3* transcription start sites and of a *UCP2* 'scrambled' antisense oligonucleotide

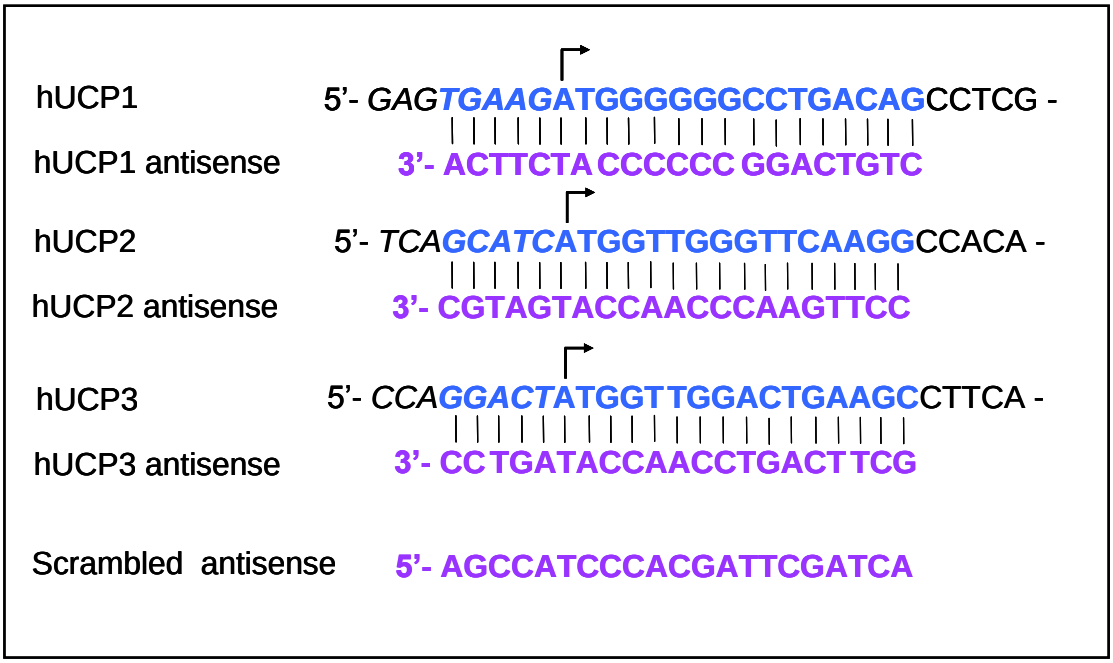
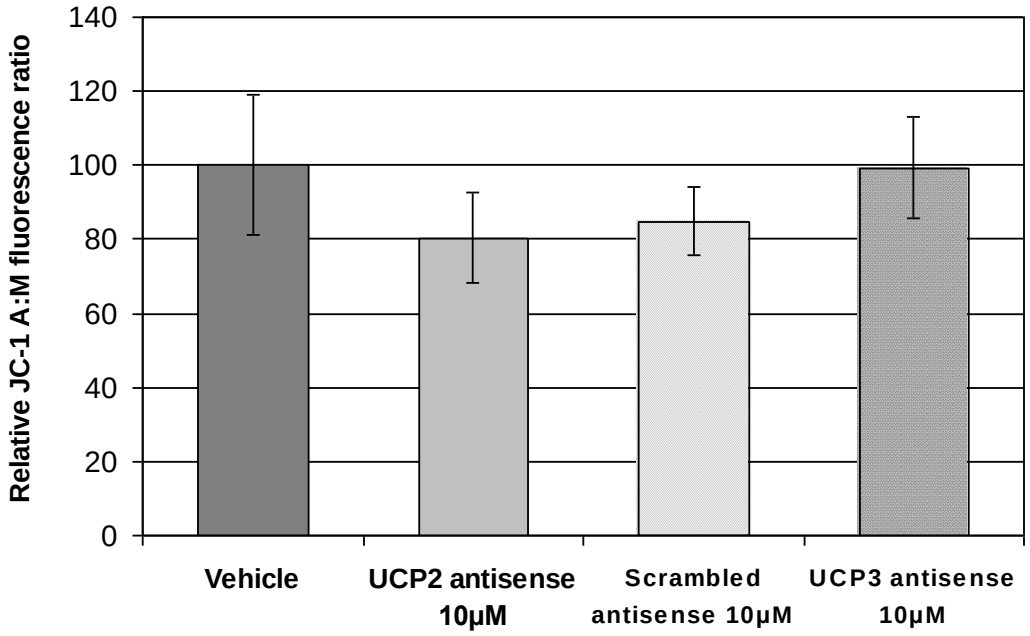
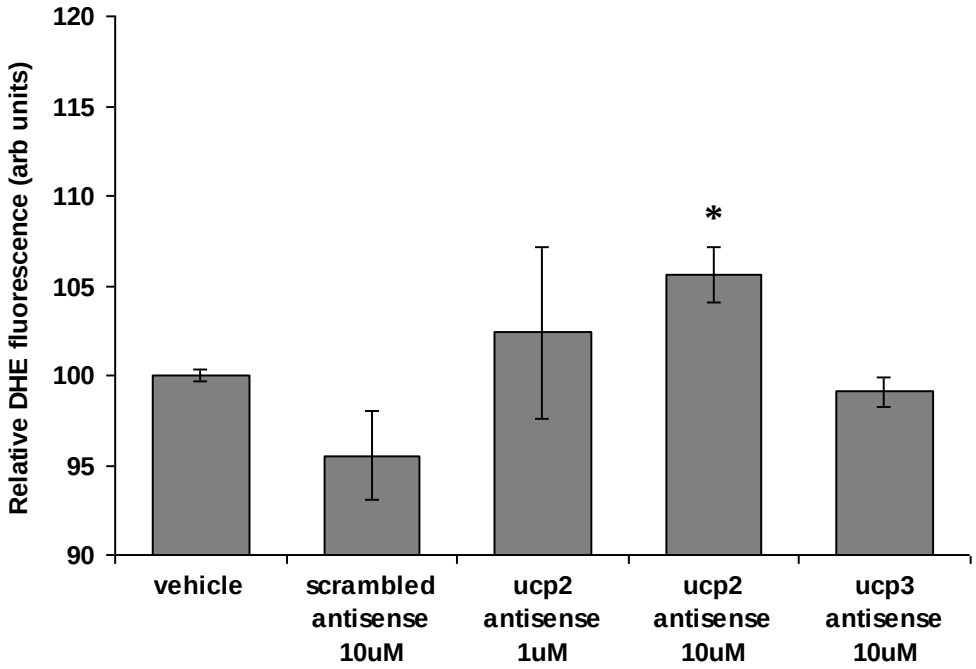


Figure 8.6. **Mitochondrial membrane potential of HUVECs treated with antisense to UCP2 and UCP3 using flow cytometric assessment of cellular JC-1 (5µM) fluorescence.**



Data are mean of 3 experiments ± SEM
Vehicle ratio indexed to 100

Figure 8.7. **Dihydroethidium fluorescence in HUVECs incubated with antisense to uncoupling proteins for 48 hours.**



Data are mean of 4 experiments ± SD

* P <0.05 compared to vehicle

There was no significant effect of any antisense oligonucleotide on resting $\Delta\psi_m$ in cultured HUVECs as assessed by JC-1 A:M fluorescence (Figure 8.6).

UCP2 antisense led to small, but significant increase in superoxide generation in HUVECs (7 ±3.5% increase in DHE fluorescence; $P = 0.01$; Figure 8.7). There was no effect of *UCP3* or scrambled *UCP2* antisense on DHE fluorescence compared to vehicle.

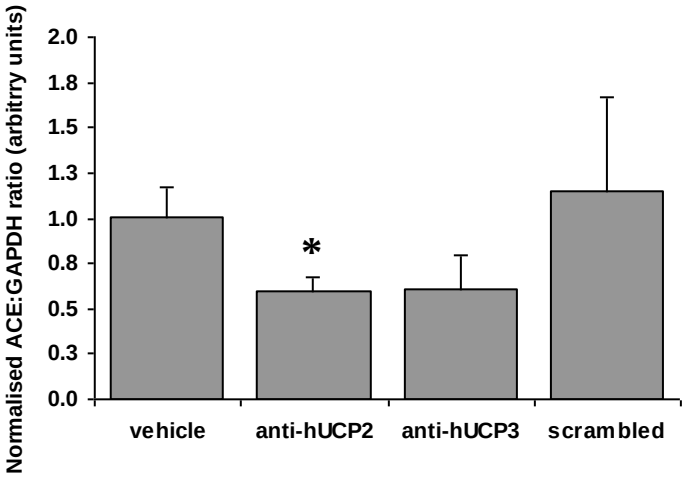
8.6 THE EFFECT OF *UCP* ANTISENSE ON ACE EXPRESSION IN ENDOTHELIAL CELLS

HUVEC monolayers in 6 well culture plates were treated with vehicle, *UCP2*, *UCP3* and scrambled (*UCP2*) antisense for 48 hours. After this time, total cellular mRNA was extracted and ACE mRNA was quantified using the LightCycler. ACE activity was also measured in the supernatant as previously described.

Treatment with *UCP2* antisense resulted in a significant 40 ± 8.0% decrease in ACE mRNA expression (N = 4; $P < 0.05$ ANOVA; Figure 8.8). There was no effect of scrambled antisense on ACE expression.

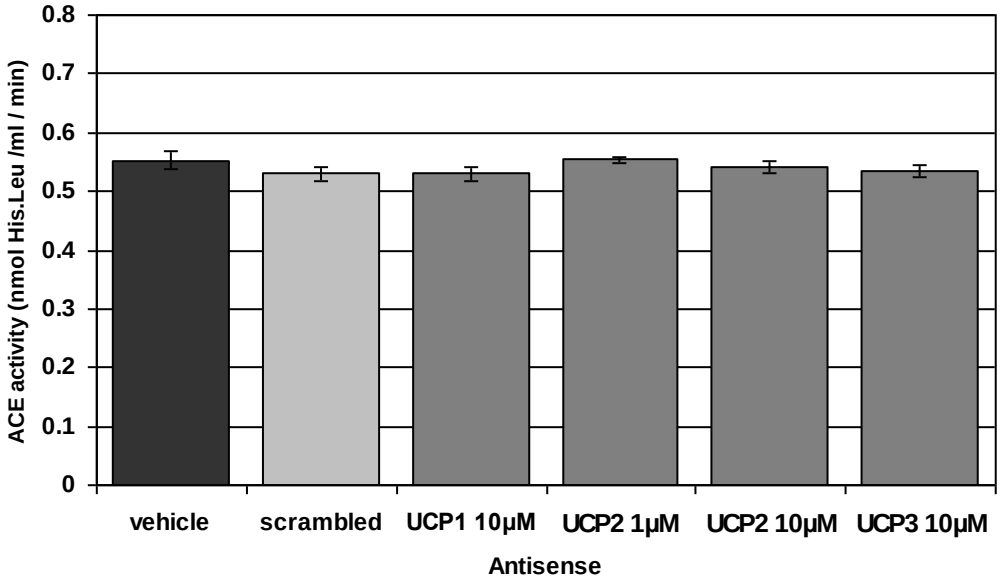
There were no measurable alterations in ACE activity in the cell culture media following antisense treatments (Figure 8.9).

Figure 8.8. ACE mRNA expression in cultured HUVECs following treatment for 48hr with 10mM *UCP* antisense (data are mean ± SEM).



P<0.05 vs. vehicle;
N=4 repeats

Figure 8.9. ACE activity measured in the culture media of HUVECs following treatment with *UCP* antisense for 48 hours (N=7, data are mean ± SEM)



8.7 DISCUSSION

This is the first study to show expression of any UCP in human endothelial cells. Such expression was alluded to, but not specifically sought, in one study of rat tissue (Prunet-Marcassus *et al.* 1999). UCP2 and UCP3 have now been localised, together with ACE, to a cell type that is central to the pathophysiology of atherosclerosis. ACE protein was previously incidentally found within adrenal mitochondria (Peters *et al.* 1996) on the inner membrane, and this novel data may make regulatory or feedback control between ACE and uncoupling proteins plausible.

Using flow cytometric assays, it was possible to measure and manipulate $\Delta\psi_m$ in HUVECs and also measure intracellular ROS generation. Pre-incubation of HUVECs with the protonphore mClCCP led to collapse of $\Delta\psi_m$ with subsequent futile cycling of the ETC and generation of $O_2\bullet^-$ (increased DHE fluorescence) probably from reverse electron transport and $O_2\bullet^-$ generation from complexes I and III (Nicholls *et al.* 2002). This is in contrast to previous reports (Nishikawa *et al.* 2000). There was a concomitant reduction in DCF-DA fluorescence, a measure of $ONOO^-$ production (Possel *et al.* 2002; Roychowdhury *et al.* 2002). Production of mitochondrial superoxide by HUVEC treatment with the redox cycler menadione led to a similar, but less extreme pattern of probe fluorescence, as did treatment with mClCCP. This may be as a result of upregulation of Mn-SOD (as has been previously reported in glial cells (Roychowdhury *et al.* 2002)) with superoxide rapidly dismutased to H_2O_2 (and this reduced to H_2O by glutathione reductase) rather than used to generate $ONOO^-$ from NO•. It is possible that respiratory uncoupling by mClCCP or mitochondrial superoxide generation leads to cellular stress and a reduction in cellular NO• production and thus a reduction of $ONOO^-$ and DCF-DA fluorescence. A further possibility is that mitochondrial

superoxide production in endothelial cells reduces the bioavailability of molecular oxygen at complex IV, which is replaced by NO• (Brown *et al.* 1994; Cleeter *et al.* 1994) and thus reducing NO• bioavailability for $ONOO^-$ generation.

The addition of rotenone to HUVEC cultures led to modest increases in both mitochondrial $O_2•^-$ and $ONOO^-$ production. Rotenone is a complex 1 inhibitor, binding to complex I downstream from its $O_2•^-$ production site, therefore generating $O_2•^-$ when electron transport through this complex is from NAD^+-linked substrates such as glucose (Nicholls *et al.* 2002). It was suggested that rotenone inhibition of complex I could reduce $O_2•^-$ production from complex I (Duranteau *et al.* 1998; Pearlstein *et al.* 2002). However, in those studies, cells were exposed to hypoxia, during which time glycolytically formed ATP is further depleted by reversal of the ATP synthase which maintains $\Delta\psi_m$ but generates $O_2•^-$ from complexes I and III by reverse electron transport. Rotenone treatment during cellular hypoxia therefore binds complex I upstream from the site of $O_2•^-$ production, thereby reducing $O_2•^-$. (Parthasarathi *et al.* 2002). In ductus arteriosus endothelial cells, which are exquisitely sensitive to oxygen tension, rotenone has been shown to *decrease* cellular ROS production (Michelakis *et al.* 2002). Furthermore, these cells have been shown to be insensitive to respiratory uncouplers such as cyanide (Michelakis *et al.* 2002). This may demonstrate a difference in mitochondria and mitochondrial function between endothelial sub-types, but also that care must be taken in interpretation of these data as mitochondrial function varies considerably according to the experimental condition (Nishikawa *et al.* 2000).

Chronic exposure of HUVECs to respiratory uncoupling led to a dose dependant loss of $\Delta\psi_m$ and a dose dependant increase in superoxide generation. Similar patterns of change were demonstrated with chronic hypoxia (Pearlstein *et al.* 2002). However,

high glucose concentration for 24 hours led to no change in either $\Delta\psi_m$ or superoxide generation, in contrast to previous findings (Nishikawa *et al.* 2000), although this study did not report the duration of incubation in these conditions. It may be that more than 24 hours of relative hyperglycaemia is required in order to cause an alteration of cellular ROS generation. Of note, amongst diabetics, there was no association between TAOS and both HbA_{1c} and random plasma glucose.

Duval and colleagues subsequently reported the presence of UCP2 mRNA in murine endothelial cells and demonstrated its negative regulation using antisense (Duval *et al.* 2002). I therefore utilised a similar approach in *human* endothelial cells. Treatment of HUVECs with antisense to *UCP2* for 48 hours led to a significant dose dependent increase in cellular $O_2\bullet^-$ generation as measured by DHE fluorescence, without a measurable change in $\Delta\psi_m$. There were no significant changes associated with treatment of HUVECs with *UCP3* antisense. These data support the RT-PCR data that UCP2 is the predominant UCP in HUVECs.

The same dose of *UCP2* antisense led to a significant 40% decrease in ACE mRNA expression in cultured HUVECs without a measurable change in ACE activity in the culture media at 48 hours. The assay for ACE activity may not have been sensitive enough to discriminate small changes of ACE activity *in vitro*. *UCP3* antisense had an almost identical effect on ACE mRNA, but without reaching statistical significance, which may have reflected the small sample size. This borderline effect on ACE mRNA may have been directly as a result of a reduction in UCP3 protein activity or possibly as a result of inhibition of RNA polymerase binding 5' upstream at the *UCP3/2* locus to generate *UCP2*.

These data confirm that UCP2 is involved in mitochondrial ROS generation in HUVECs and that UCP2 is involved in regulation of endothelial ACE expression. It is tempting to speculate that the two are linked by UCP2-mediated mitochondrial $O_2\bullet^-$ generation. ROS are mediators of many cell physiological processes, including cell growth (Nishio *et al.* 1997), angiotensin II- induced hypertrophy (Ushio-Fukai *et al.* 1998; Zafari *et al.* 1998) and oxygen sensing (Michelakis *et al.* 2002). There is accumulating evidence that UCPs may be negative regulators of oxidative stress in a variety of cell types (Negre-Salvayre *et al.* 1997; Lee *et al.* 1999; Arsenijevic *et al.* 2000), now including endothelial cells (Duval *et al.* 2002). In fact, $O_2\bullet^-$ directly activates mitochondrial proton conductance via UCP1, 2 and 3, thereby reducing mitochondrial ROS generation (Echtay *et al.* 2002). However, against this hypothesis, there was no correlation between circulating ACE activity and TAOS in diabetic men.

In summary, it has been shown that ACE, UCP2 and, to a lesser extent, UCP3 were expressed at the mRNA level in HUVECs. Specific antisense to *UCP2*, but not *UCP3*, led to a significant increase in endothelial (mitochondrial) $O_2\bullet^-$ generation and to a decrease in HUVEC ACE mRNA expression. Further *in vitro* work will be required to dissect the putative role of UCP2 or UCP3 in mitochondrial ROS generation and HUVEC ACE expression.

※ CHAPTER NINE ※

CONCLUSIONS

A genetic approach was used to test the hypothesis that kinins may be responsible for some of the observed associations between variation in the *ACE* gene and effects on both the cardiovascular system and skeletal muscle efficiency. Such effects may rely on alterations in mitochondrial function. *In vitro* assays were therefore established to test the hypothesis that ACE can influence mitochondrial coupling. Novel mitochondrial uncoupling proteins (UCPs) have been discovered whose functions may include the regulation of mitochondrial respiratory chain coupling, mitochondrial ROS generation and mitochondrial substrate utilisation, and, as such, these proteins may be a subcellular target of ACE. A genetic approach was used to test associations between common variation in the *UCP3/2* locus and both cardiovascular pathophysiology and skeletal muscle efficiency. These genetic studies suggested a novel regulatory pathway between circulating ACE protein activity and mitochondrial UCPs. *In vitro* assays were used to explore such potential interactions between ACE and UCP within human endothelial cells.

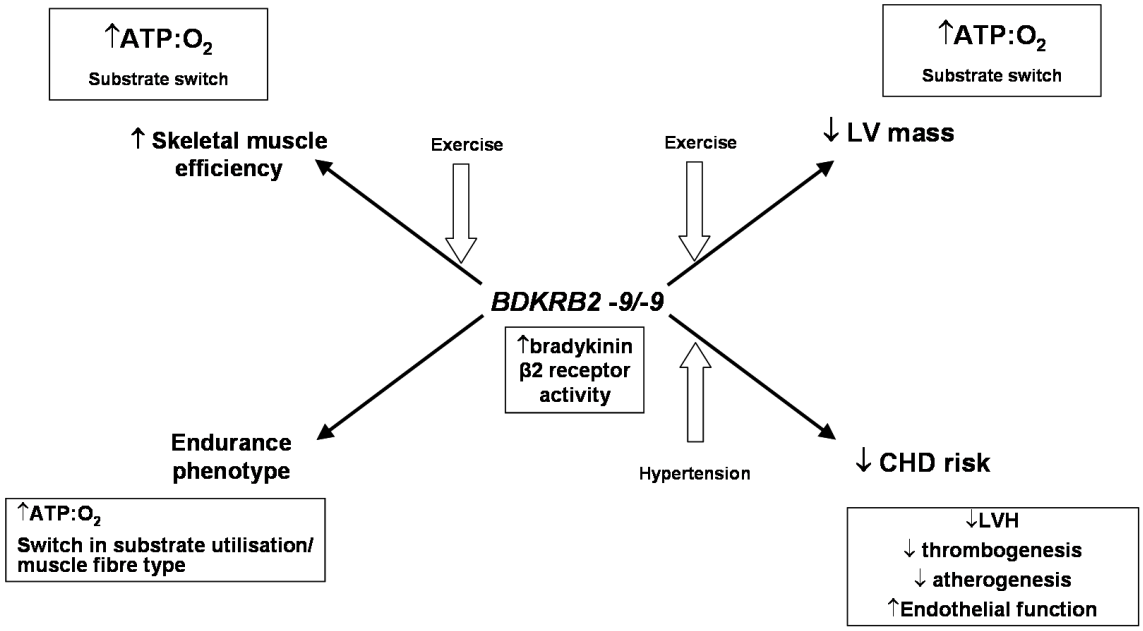
9.1. GENETIC VARIATION IN THE BRADYKININ β_2 RECEPTOR (*BDKRB2*) GENE

In vitro work has previously shown that the *BDKRB2* -9 allele is associated with higher gene transcriptional activity (Braun *et al.* 1996), higher mRNA expression (Lung *et al.* 1997) and increased likelihood of angioedema in C1-esterase deficiency, a disease

characterised by high kinin activity (Lung *et al.* 1997). In this study, there were significant associations between the *BDKRB2* -9 allele and lower prospective LV growth as well as a protective effect from prospective hypertensive CHD risk. Prospective gene-environment studies are a useful tool in providing indirect answers to studies which have proved difficult to conduct *in vivo* in humans and are also useful in further hypothesis generation. These first data in humans add credence to the long-held suspicions that the observed benefits of ACE inhibition on vascular risk may be, in part, due to reduced kinin degradation (Linz *et al.* 1995), as kinetic studies have shown that bradykinin is the preferred substrate of ACE (Jaspard *et al.* 1993). In this study, individuals with the highest predicted kinin activity (*ACE I BDKRB2-9* homozygotes) demonstrated no change in LV mass during the physiological challenge of 12 weeks of basic army training, whereas those individuals with the lowest predicted kinin activity (*ACE D BDKRB2+9* homozygotes) had the highest gain in LV mass (15.7 ± 14.2g) during training. Statistical analysis suggested that variation at the *BDKRB2*+9/-9 locus accounted for 4% of the interindividual variability in prospective LV growth.

Could the beneficial (antitrophic) effects of kinins on LV growth and on hypertensive CHD risk be related? Extensive animal and *in vitro* data support a role for BDKRB2 activation in diminishing blood pressure (Wang *et al.* 1997) and cardiac hypertrophic responses (Linz *et al.* 1993; Linz *et al.* 1995; Linz *et al.* 1996; Rosenkranz *et al.* 1999). Conversely, *BDKRB2* gene knockout mice develop hypertension and LVH with exaggerated responses to salt and Ang II (Madeddu *et al.* 1997; Emanueli *et al.* 1999).

Figure 9.1. Schematic summary of gene-environment studies conducting in this thesis. Published data suggests that the *BDKRB2-9* allele is associated with high β2 receptor expression. The stressor for each study is depicted with an open arrow. The associated phenotype for each study is depicted alongside the proposed mechanism (boxed).



Hypertension is the commonest cause of LVH and is associated with a systemic (Koenig *et al.* 1999) and local vascular (Parissis *et al.* 2000) inflammatory response which, through interleukin-driven activation of NF-κβ, induces (potentially protective) vascular BDKRB1 expression (Ni *et al.* 1998). Whether BDKRB2 expression may be similarly modulated is not known. Both kinin receptor subtypes are expressed in the atheromatous plaque (Raidoo *et al.* 1997) and the *BDKRB2*-58C rather than -58T allele has been previously reported in excess amongst hypertensive (rather than normotensive) sufferers of acute MI (Aoki *et al.* 2001). The beneficial effects of ACEi and ARBs on LV remodelling and function post MI are reduced in BDKRB2 knockout mice (Yang *et al.* 2001). More broadly speaking, the KKS may afford vascular protection through a number of other potential mechanisms, including BDKRB2-mediated local NO• synthesis (Kichuk *et al.* 1996), coronary vasodilatation (Su *et al.* 2000) and inhibition of

vascular smooth muscle cell growth (Murakami *et al.* 1999), as well as potent antithrombotic/antiplatelet effects (Colman *et al.* 1999; Schmaier 2000).

Physiological cardiac hypertrophy in response to regular physical exercise (so called "athletes' heart") is associated with preserved or enhanced cardiac function and is distinct from pathological cardiac hypertrophy secondary to pressure or volume overload, which is associated with depressed cardiac function and an increase in morbidity and mortality. At the molecular level, only pathological hypertrophy is associated with an excess of cardiac fibrosis and extracellular matrix deposition and the two processes may therefore differ in their subcellular molecular pathways. The association of the *ACE* I/D variant and now the *BDKRB2* +9/-9 variant with both physiological LV growth and pathological states (*e.g*. hypertensive CHD risk) is highly suggestive of some commonality in the signalling pathways underlying both forms of LVH.

A highly significant positive association between the *BDKRB2* -9 allele and both human skeletal muscle metabolic efficiency and endurance athletic performance, and, as above, these associations were greatest amongst those individuals in whom one could predict the highest kinin activity (*ACE* I *BDKRB2* -9 homozygotes). Several genetic studies from this laboratory have shown a clear association between the *ACE* I-allele and markers of metabolic efficiency, such as increased efficiency of skeletal muscle contraction (Williams *et al.* 2000) and endurance performance (Montgomery *et al.* 1998; Myerson *et al.* 1999), but these are the first data to implicate a specific and plausible underlying mechanism. Skeletal muscle contains a complete KKS with functional BDKRB2 (Figueroa *et al.* 1996; Mayfield *et al.* 1996; Rabito *et al.* 1996). Muscle blood flow and skeletal muscle glucose uptake may be influenced by bradykinin

generated *in situ* (Wicklmayr *et al.* 1979; Langberg *et al.* 2002), levels of which are under genetic influence (Murphey *et al.* 2000). Bradykinin, acting through the BDKRB2, has metabolic effects on skeletal muscle, including enhanced insulin mediated GLUT-4 translocation (Taguchi *et al.* 2000), activation of inositol 1,4,5-triphosphate/calcium induced excitation-coupling (Foster 1994; Rabito *et al.* 1996) and alteration of mitochondrial oxygen utilisation via endogenous NO production (Shen *et al.* 1995; Zhang *et al.* 1997; Moncada *et al.* 2002). *ACE* I/D genotype is associated with fibre type distribution (Zhang *et al.* 2003) and this may be caused by chronic differences in kinin activity influencing trophic changes in skeletal muscle.

Skeletal and cardiac muscle share many of the described metabolic pathways, and it is possible that the cardioprotective effects of kinins are in large part metabolic in origin. ACEi and kininogen both increase NO• production and reduce oxygen consumption in isolated myocardium, in a BDKRB2-dependent manner (Zhang *et al.* 1997). Interestingly, these effects are preserved in explanted failing human heart (Loke *et al.* 1999). Bradykinin perfusion of ischaemic, isolated, beating rat hearts results in a reduction in LDH and CK activity and in myocardial lactate production, with preservation of high energy phosphates and glycogen with concomitant improvements in cardiac haemodynamics (Schoelkens *et al.* 1992) suggesting a protective metabolic switch towards glucose metabolism, as well as more fundamental changes in mitochondrial function. It is possible, therefore, that the observed association between the low ACE/low kinin haplotype (*ACE I/BDKRB2-9*) and low prospective cardiac growth is due to alterations in cardiac metabolism.

9.2. ACE & MITOCHONDRIA

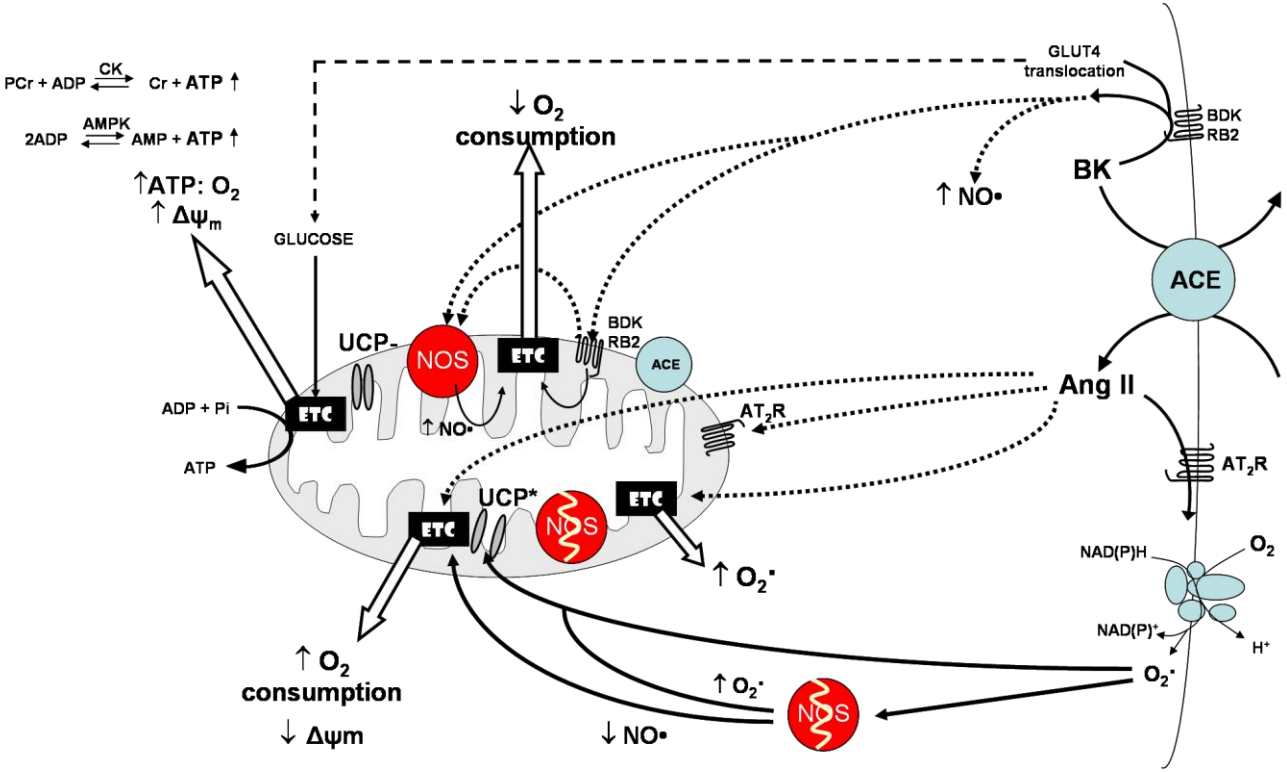
Flow cytometric and confocal microscopic assays were developed using fluorescent potentiometric dyes to indirectly measure $\Delta\psi_m$ in cultured live cells. Treatment with the ACEi ramiprilat for 24 hours lead to a highly significant increase in $\Delta\psi_m$ in both skeletal and cardiac blasts and myocytes as assessed by the two complementary techniques. This relative hyperpolarisation of the inner mitochondrial membrane suggests increased coupling between mitochondrial respiration and ATP generation. This would lead, theoretically, to a decrease in mitochondrial respiration rate (Nicholls *et al.* 2002), *i.e.* a reduction in proton leak in the basal state would lead to a lowering of the ADP/ATP ratio, which would slow the rate of oxygen consumption. However, no alteration in basal respiratory rate was seen in C_2C_{12} myocytes pretreated with ramiprilat. This may be due to lack of sensitivity of the OxySpot assay to measure small differences particularly in the resting state. An increase in cellular activity such as with mechanical work may produce more pronounced and measurable differences in respiratory rate.

Ramiprilat treatment resulted in a delayed increase in $\Delta\psi_m$ between 6 and 12 hours suggesting a gene-transcriptional event might be responsible for the putative increase in mitochondrial coupling. However, no significant change in the dominant uncoupling protein, UCP3, gene transcription was observed in C_2C_{12} myotubes treated with ramiprilat for 24 hours. There was a wide degree of variation of UCP3 mRNA transcript ratio in both treated and untreated myotubes, possibly explained by the degree of cultured myocyte differentiation (Shimokawa *et al.* 1998), and this may have prevented small changes in transcript ratio being observed. Moreover, UCP3 and UCP2 undergo extensive post-transcriptional changes, so it may be more important to measure absolute protein levels (Pecqueur *et al.* 2001), although neither the amount of mRNA or

protein reflect the degree of activation of UCP, which can be altered by the presence of fatty acid or ROS (Echtay *et al.* 2002).

ACEi increase the bioavailability of NO• which is thought to exert metabolic control over mitochondrial respiration (Shen *et al.* 1995; Clementi *et al.* 1999; Loke *et al.* 1999). The concentration and source of NO• is critical (Kojda *et al.* 1999). A biphasic inotropic response to NO• and cGMP is seen in cardiac myocytes with low (submicromolar) concentrations resulting in positive inotropy and higher (submillimolar) concentrations resulting in negative inotropy (Mohan *et al.* 1996). Similar effects seem apparent in relation to mitochondrial function (Kojda *et al.* 1999), with physiologically relevant submillimolar concentrations of NO• being associated with a reduction in cardiac oxygen consumption without a reduction in ATP flux through the CK-PCr system (Decking *et al.* 2001). ACEi-associated reductions in O_2 consumption and improvements in contractility in cardiac tissue have been shown to be NO•-dependent (Zhang *et al.* 1997), but the source of NO• is likely to be eNOS or mtNOS and of a much lower concentration that that induced by LPS activation of iNOS. It has been suggested that cytochrome c, modulated by the NO•:O_2 ratio, acts as an O_2 sensor, and that constitutive low concentrations of NO• produced by eNOS or mtNOS is required by cells to exhibit control over O_2 consumption, particularly as O_2 tension decreases (Clementi *et al.* 1999). Regions with relatively reduced blood flow and oxygenation would have enhanced mitochondrial complex III inhibition, as NO•-dependent mitochondrial respiratory inhibition is greater at lower O_2 tension. It may be that the benefits in tissue contractile function and reductions in O_2 consumption from ACEi treatment are due to improvements in tissue perfusion / consumption mismatch. ACEi induced vasodilatation would result in both a local increase in oxygen supply as well as an increase in NO• generation.

Figure 9.2. Cartoon depiction of the putative mitochondrial actions of ACE and its downstream effectors, bradykinin (BK) and angiotensin II (Ang II). BK acts via cell surface β2 receptors (BDKRB2) to increase intracellular NO· activity via NOS and also increases cellular glucose uptake, switching mitochondrial metabolism to more energetically favourable glucose metabolism, resulting in preservation of high energy phosphate bonds. Similarly a mitochondrial RAAS or mitochondrial NOS may exist which may also directly or indirectly influence mitochondrial respiration (ETC) to decrease mitochondrial O_2 utilisation at complex IV and increase $\Delta\psi_m$. There may be direct actions to limit UCP activity or indirect via reduction in mitochondrial ROS generation. Ang II may have indirect, via NAPDH-oxidase ROS generation, or direct mitochondrial effects. Mitochondrial AngII receptors may exist (ATR) which may mediate a direct increase in ETC O_2 utilisation or ETC ROS generation. NADPH-oxidase related ROS may uncouple NOS leading to a vicious cycle of ROS generation and reduction in NO· which would result in an increase in complex IV O_2 consumption, reduction in $\Delta\psi_m$ and compensatory activation of UCP (UCP*) to limit further ROS generation.



Pretreatment of C_2C_{12} myotubes with LPS for 24 hours significantly suppressed cellular O_2 consumption by 73%. Maximal respiratory cycling caused by treatment with the uncoupler *m*ClCCP was unaffected by pre-treatment with LPS, suggesting an intact ETC. LPS has been shown to reduce cellular O_2 consumption in other cell types (James *et al.* 1995; Borutaite *et al.* 2001) in an NO•-dependent manner (Borutaite *et al.* 2001). In these situations, maximal competitive inhibition of complex IV and of O_2 consumption at cytochrome oxidase results in a reduction of mitochondrial ATP synthesis (Brookes *et al.* 1999), so cellular ATP demand is met by cytoplasmic anaerobic glycolysis. The cessation of flow through the primary proton pump (complexes I-IV) results in reversal of the secondary proton pump ATP synthase ($F_1.F_o$-ATPase) to maintain $\Delta\psi_m$ (Section 1.5.1). Glycolytic ATP enters the mitochondrial matrix through 'reversal' of the adenine nucleotide translocator (ANT) and this matrix ATP is hydrolysed by ATP synthase, now a consumer of ATP, to pump H^+ into the intermembrane space to maintain a proton gradient (Beltran *et al.* 2000; Moncada *et al.* 2002). The initial block in electron transport at cytochrome c leads to a transition from state 3 to state 4 respiration and an increase in $\Delta\psi_m$, and may lead to O_2 utilisation at complexes I and III to generate superoxide ($O_2\bullet^-$). After persistent inhibition of respiration by NO•, the depletion of glycolytic ATP and/or oxidative damage leads to a collapse in $\Delta\psi_m$ and cell death (Beltran *et al.* 2000).

How does *m*ClCCP drive cellular O_2 consumption during maximal inhibition of mitochondrial respiration by NO•? This effect has not been previously reported. It is possible that sudden collapse of $\Delta\psi_m$ with a low dose of *m*ClCCP, leads to futile electron cycling through complexes I to III and consumption of O_2 at complexes I and III to produce $O_2\bullet^-$, as demonstrated in HUVECs (*vide infra).* Alternatively, *m*ClCCP

may decrease the bioavailability of NO•, possibly by ROS induced uncoupling of iNOS, and therefore allow O_2 to compete at complex IV.

An exciting novel finding was that Ang II directly increased the resting O_2 consumption of C_2C_{12} myocytes *in vitro*, which was distinct and opposite to the NO• modulation of respiration. Chronic Ang II infusion in rats is known to alter resting O_2 consumption (Cassis *et al.* 2002) and result in cachexia (Brink *et al.* 1996), and this may be through increased activity of mitochondrial UCPs which are also upregulated in skeletal muscle in experimental cancer cachexia (Bing *et al.* 2000). Alternatively, Ang II may be acting directly on the ETC complexes through other novel mechanisms. Ang II induction of cellular ROS via NADPH oxidase may result in 'uncoupling' of NOS (eNOS or mtNOS) and therefore inhibition of endogenous NO• bioavailability at cytochrome oxidase with a subsequent increase in mitochondrial O_2 consumption (Brown *et al.* 1994; Cleeter *et al.* 1994). The extra-mitochondrial NADPH oxidase pathway relies on activation of the AT_1R; could any direct mitochondrial effect of Ang II rely on mitochondrial angiotensin receptors?

9.3. UCP2 AND UCP3 AND THE CARDIOVASCULAR SYSTEM

9.3.1. UCPs and prospective cardiac growth

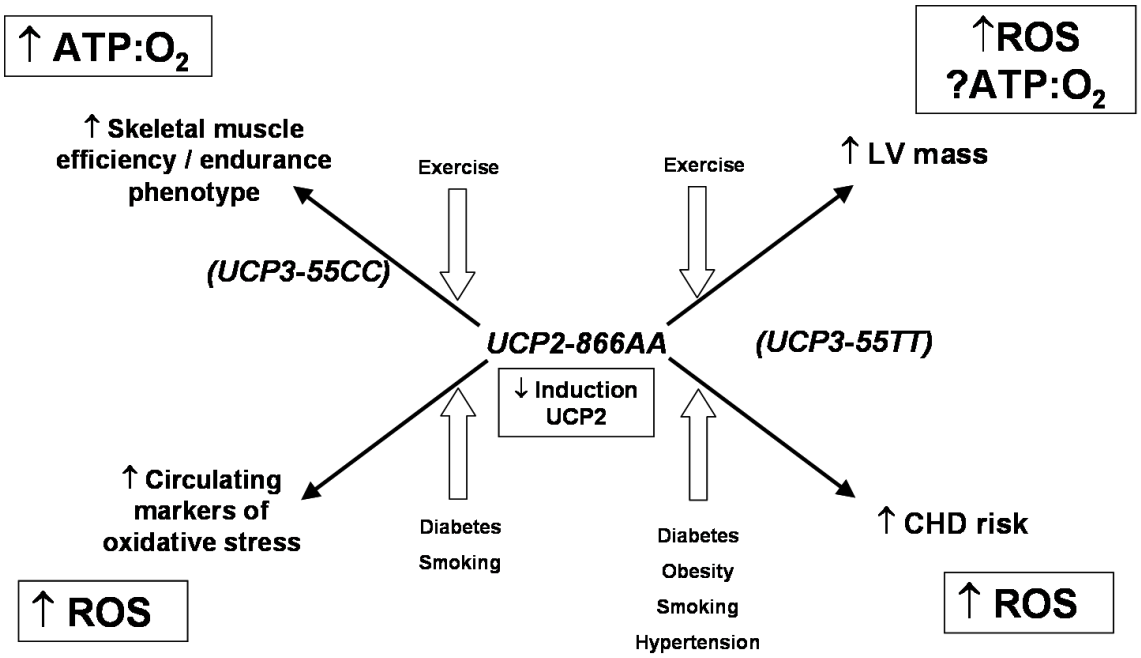
The mammalian UCP1 homologues UCP2 and UCP3 were discovered by reverse cloning (Boss *et al.* 1997; Fleury *et al.* 1997) and researchers have since struggled to find a plausible function for either of these proteins. Both are expressed in the human myocardium at the mRNA and protein level, but their precise function in the

cardiovascular system was unknown at the onset of this work. I used gene-environment studies to test the hypotheses that UCP2 and UCP3 are integral components of normal cardiovascular physiology, being involved in cardiomyocyte mitochondrial function, substrate utilisation and oxidative stress and, as such, may be important in determining LV growth and prospective cardiovascular risk.

This was the first report of an association of variation at the *hUCP3/UCP2* locus and LV mass: significant associations were found between both the *UCP2*-866G>A and *UCP3*-55TT genotypes and CMR-determined LV mass in the BH2 study. The rare *UCP2*-866AA/*UCP3*-55TT haplotype was associated with the highest LV mass after training, being a mean 19 g greater than in subjects who were *UCP2*-866G allele/*UCP3*-55C allele carriers. It is unclear whether this 'risk' haplotype (*UCP2*-866AA/*UCP3*-55TT) is directly causal in the development of LV growth, with higher UCP2 or UCP3 activity being deleterious, or whether it relates to lower UCP2 or UCP3 activity which results in a permissive phenotype, facilitating the development of LVH.

UCP2 mRNA is expressed at a higher level than UCP3 mRNA in the myocardium (Fleury *et al.* 1997; Razeghi *et al.* 2001; Young *et al.* 2001), but whether either UCP is expressed at the protein level (in rodents) is controversial (Boehm *et al.* 2001; Langdown *et al.* 2001; Pecqueur *et al.* 2001). Both UCP2 and UCP3 mRNA and protein expression can be increased or decreased in the heart depending on the animal model of LVH or heart failure studied (Fukunaga *et al.* 2000; Boehm *et al.* 2001; Langdown *et al.* 2001; Noma *et al.* 2001; Young *et al.* 2001).

Figure 9.3. Schematic summary of gene-environment studies conducting in this thesis and in appendix 4. It has been postulated that the UCP2-866A allele is associated with repression of induction of UCP2 expression. The associated phenotype for each study is depicted alongside the proposed mechanism (boxed). Haplotypic association is shown with the UCP3-55C>T variant as shown.



More recently, both UCP2 and UCP3 protein have been detected in human cardiac tissue explanted at the time of coronary surgery, with significant positive correlations between both UCPs and circulating free fatty acid concentrations (Murray *et al.* 2004). UCP2/3 phenotype may therefore be associated with an alteration in the balance between cardiac glucose and fatty acid utilisation. Fatty acids induce UCP2 mRNA in isolated rat neonatal cardiomyocytes (Van Der Lee *et al.* 2000) and induce cardiac UCP3 mRNA in the rat (Depre *et al.* 2000; Van Der Lee *et al.* 2000; Van der Lee *et al.* 2001) via PPAR dependent and independent mechanisms (Van Der Lee *et al.* 2000; Young *et al.* 2001).

UCP induction may itself result in increased proton leak and O_2 consumption at the expense of reduced ATP production (reduced mitochondrial coupling). It has been postulated that UCP2 and 3 may be directly involved in fatty acid metabolism, facilitating cycling of fatty acid anions through the inner mitochondrial membrane (Boss *et al.* 2000). But, although endurance exercise is associated with enhanced muscle fat oxidative capacity, UCP2 and UCP3 mRNA expression is downregulated in cardiac and skeletal muscle, respectively (Boss *et al.* 1998). Mitochondrial fatty acid oxidation, itself, is less metabolically efficient as it results in a lower ratio of ATP:O_2 consumed than pyruvate oxidation (Brand *et al.* 1994), thereby increasing cardiac O_2 consumption for the same amount of cardiac external work (Challoner *et al.* 1966; Mjos 1971). This energy deficient cardiac state may result in compensatory cardiac hypertrophy and fibrosis. A similar inefficient state is also seen in CCF, with catecholamine induced lipolysis favouring cardiac fatty acid oxidation, together with impaired carbohydrate metabolism due to a reduction of GLUT4 expression, inhibition of pyruvate oxidation via fatty acid inhibition of the PDH complex (Stanley *et al.* 2002), insulin resistance and downregulation of glycolytic enzymes (Kalsi *et al.* 1999; Razeghi *et al.* 2001; Murray *et al.* 2004).

Conversely, UCP upregulation in the heart may be protective. It has been argued that UCP2 and UCP3 are upregulated in cardiac and skeletal muscle during acute rises in circulating free fatty acids, such as acute exercise, refeeding and acute cold exposure, as a protective measure during a surplus of free fatty acid supply to the myocyte (Schrauwen *et al.* 2002). In this model, excess free fatty acids can enter the mitochondrial matrix, rather than using the CPT system, 'flip-flopping' across the inner membrane where they may become protonated. Neither neutral nor anionic fatty acids can be metabolised within the matrix. UCP2 and UCP3 may therefore act as fatty acid

anion exporters to remove excess protonated fatty acid whose mitochondrial accumulation may result in toxicity (Schrauwen *et al.* 2002). This mechanism may be particularly important in CCF.

A more straightforward hypothesis, given that UCP2 and UCP3 protect against myocardial ROS production, and the association of the *UCP2*-866AA/*UCP3*-55TT haplotype with LVH risk, is that the haplotype is associated with lower mitochondrial UCP2 and/or UCP3 protein expression, and that this leads to either decreased uncoupling and thus enhanced mitochondrial ROS production, or decreased protection from ROS. The UCP2 -866A allele has been associated with lower dynamic UCP2 mRNA expression in somatic cells (Krempler *et al.* 2002). Redox-sensitive pathways are known to mediate LV growth (Amin *et al.* 2001; Bendall *et al.* 2002), cardiac dysfunction (Ide *et al.* 2001; Suematsu *et al.* 2003) and fibrosis (Bendall *et al.* 2002). ROS-mediated activation of mitogen activated protein kinases and of nuclear factor-κB is involved in neurohormonal (*e.g.* angiotensin II, norepinephrine) and stretch-induced cardiomyocyte hypertrophy and can be inhibited by the administration of antioxidants *in vitro* (Griendling *et al.* 2000; Hirotani *et al.* 2002). This may explain the non-significant differences observed at baseline, as ROS induced cardiac growth might be expected to accumulate over a life-time and accelerate during a period of physiological stress.

9.3.2. UCPs and CHD risk

This was the first report of a highly significant association between a common functional variant in the *UCP2* gene and prospective CHD risk (Dhamrait *et al.* 2004). CHD risk was more than doubled amongst men of *UCP2-866AA* genotype (HR 2.22

[1.53 - 3.22] for AA vs. GG genotype; *P*=0.0002) and this risk was amplified by the presence of conventional risk factors which are associated with increased oxidative stress, such as hypertension (x2), smoking (x2), obesity (x2) and diabetes (x4). Although, there were no such significant associations with the *UCP3-55C>T* gene variant, there were highly significant associations between haplotype and prospective CHD risk when haplotype was assessed at the *UCP3-55C>T* and *UCP2-866G>A* loci. As was the case for prospective LV growth, the 'risk' haplotype was the rare *UCP3-55T / UCP2-866A* haplotype, which was found in 12.2% of men studied. Perturbations in both UCP2 and UCP3 are implicated in the abnormal metabolism associated with LVH (Fukunaga *et al.* 2000; Boehm *et al.* 2001; Langdown *et al.* 2001; Noma *et al.* 2001; Young *et al.* 2001) and now may also be important in CHD risk. This again suggests common mechanisms through which the deleterious effects of hypertension on left ventricular hypertrophy and CHD may be mediated.

Contributory mechanistic data from joint work with Dr JW Stephens (Appendix 4.) examined the association between plasma TAOS and *UCP2-866G>A* genotype. The presence of diabetes is associated with increased oxidative stress (Cai *et al.* 2000; Brownlee 2001; Evans *et al.* 2002), and thus a fall in TAOS (Sampson *et al.* 2002). In keeping with a role for ROS in atherogenesis, plasma TAOS was significantly lower in diabetic men with CHD than those without, an effect that was confirmed in health men in the NPHSII study (Stephens *et al.* 2006). In the UDACS cohort, the risk *UCP2*-866A allele was independently associated with lower TAOS, suggesting a modulating influence of *UCP2* genotype on ROS burden. In a smaller subset of diabetic men with CHD matched for baseline characteristics, *UCP2*AA homozygosity was associated with highly significant increases in markers of oxidative stress, with 40% lower TAOS mirrored by a 100% increase in F_2-isoprostane concentrations (Dhamrait *et al.* 2004).

These data support a role for UCP2 (and hence the mitochondrial electron transport chain) in the regulation of ROS generation, and highlights its potential impact upon CHD risk. *In vitro*, UCP2 activity and expression is induced by oxidative stress (Pecqueur *et al.* 2001; Echtay *et al.* 2002), whilst selective down-regulation of UCP2 increases murine endothelial cell ROS generation (Duval *et al.* 2002). $O_2\bullet^-$ does not directly activate UCP, but releases iron from intramitochondrial iron-sulphur centred proteins which then generates carbon-centered radicals on phospholipids that initiate lipid peroxidation, yielding breakdown products that activate UCPs (Murphy *et al.* 2003). Under conditions of oxidative stress, upregulation of UCP2 expression would decrease $\Delta\psi_m$, thereby protecting from further mitochondrial ROS generation and should prove vasculo-protective (Arsenijevic *et al.* 2000; Echtay *et al.* 2002). In support, UCP2 overexpression protects against atherosclerosis in LDL-receptor deficient mice (Blanc *et al.* 2003).

9.3.3. UCPs and oxidative stress

UCPs have not yet been localised to human atherosclerotic plaque. This is the first report of UCP2 and UCP3 mRNA expression in human endothelial cells (HUVECs). $\Delta\psi_m$ and mitochondrial ROS were successfully manipulated in HUVECs and measured using flow cytometric assays. Chronic exposure of HUVECs to respiratory uncoupling with mClCCP led to a dose-dependent loss of $\Delta\psi_m$ and a dose-dependent increase in $O_2\bullet^-$ generation. Similar patterns of change were demonstrated with chronic hypoxia as previously described (Pearlstein *et al.* 2002). However, high glucose concentration for 24 hours led to no change in either $\Delta\psi_m$ or superoxide generation, in contrast to previous findings (Nishikawa *et al.* 2000), although the previous study by Nishikawa *et al.* did not report the duration of incubation and this may have lead to the observed

differences. Of note, amongst diabetics in UDACS, there was also no association between a marker of circulating redox state (TAOS) and either HbA_{1c} or random plasma glucose (Appendix 4).

Treatment of HUVECs with antisense to *UCP2* for 48 hours led to a significant dose dependent increase in cellular $O_2\bullet^-$ generation as measured by DHE fluorescence, *without* a measurable change in $\Delta\psi_m$. There were no significant changes associated with treatment of HUVECs with *UCP3* antisense or negative controls. These data support the RT-PCR data that UCP2 is the predominant UCP in HUVECs. A lack of a measurable change in $\Delta\psi_m$ may be because of a lack of sensitivity of the assay or because UCPs may not be true uncouplers of respiration as has been suggested.

The hypothesis that UCP2 is functional in endothelial cell mitochondria and that it negatively regulates mitochondrial ROS generation is supported by these data, which are in agreement with previous reports in murine endothelial cells (Duval *et al.* 2002). It would be important to repeat these experiments, including the use of UCP activators, and to measure UCP2 and UCP3 protein levels. Moreover, UCP2 has also been localised within macrophages (Kizaki *et al.* 2002) where it negatively regulates ROS (Arsenijevic *et al.* 2000) and NO• production (Kizaki *et al.* 2002). These data have important implications on disease mechanisms and provides a putative role for UCP2 in atherogenesis as well as a potential novel therapeutic target.

9.3.4. UCPs and skeletal muscle efficiency

There were significant associations between genetic variation at the *UCP2*/3 locus and endurance-training related changes in the efficiency of contraction of human skeletal

muscle (DE). The *UCP2*-866A allele carriers had significant gains in DE after training, whereas the small number of *UCP3*-55TT homozygotes tended to have lower DE after training. There was evidence of significant statistical interaction between the *UCP2*-866G>A and *UCP*-55C>T genotypes in determining changes in DE, explaining up to 14.8% of the interindividual training related changes in DE. It is however difficult with the small sample size to delineate which gene product is responsible for this association as the *UCP2* and *UCP3* genes are separated by only 7kB in chromosomal region 11q13 (Solanes *et al.* 1997), and the two SNPs show strong LD. Much larger sample sizes and more SNPs would be required to disentangle any association further.

There were no significant associations between *UCP2*/3 haplotypes and competitive distance running event amongst a sample of elite, Olympic standard UK athletes, probably partly due to the small sample size and the low frequency of the rare alleles. The *UCP2*-866A allele frequency tended to increase with running distance, reflected in an increased prevalence of *UCP3*-55C/*UCP2*-866A haplotype carriers amongst endurance events athletes coupled with an increased prevalence of *UCP3*-55C/*UCP2*-866G haplotype carriers in sprinters. In this small sample of athletes, there were no Afro-Caribbean elite athletes competing at longer distances. There were very few *UCP3*-55T allele carriers amongst Afro-Caribbean athletes and this may have explained the overall increase in *UCP3*-55T allele frequency seen according to competing distance. The *UCP3*-55C>T and *UCP2*-866G>A genotype distributions in the small group of Afro-Caribbean athletes was similar to that found in the RSA Study sample, suggesting that sampling error was unlikely.

The exact role of UCP2 and UCP3 in skeletal myocyte function is controversial. The close sequence homology between UCP1 and both UCP2 and UCP3 has naturally led to

the hypothesis that both UCP3 and UCP2 are able to increase skeletal muscle O_2 consumption by increasing mitochondrial proton conductance. The evidence is still divergent. In favour of an uncoupling effect, the *UCP2*/*UCP3* locus has been linked to basal metabolic rate in adult humans (Bouchard *et al.* 1997) and both UCP2 and UCP3 mRNA is reduced significantly in all skeletal muscle fibre types in endurance training rats (Boss *et al.* 1998). There is a positive association between UCP3 protein expression and degree of training and muscle fibre fatiguability in humans: UCP3 protein content is lower in endurance trained individuals compared to untrained subjects (Russell 2003) and is most abundant in type 2b fast-glycolytic fibres (Hesselink *et al.* 2001). Proton leak (state 4 respiration) is reduced in skeletal muscle mitochondria isolated from the *UCP3*(-/-) knockout mouse in some (Gong *et al.* 2000; Vidal-Puig *et al.* 2000) but not all studies (Cadenas *et al.* 2002). In whole animal studies using NMR spectroscopy, *UCP3* knockout mice had a doubling of the ATP synthesis rate in skeletal muscle in the fasting state, without any increase in TCA cycle flux rate, implying an increase in mitochondrial coupling (Cline *et al.* 2001). Conversely, *UCP3* overexpression studies in yeast (Gong *et al.* 1997; Zhang *et al.* 1999; Harper *et al.* 2002), proteoliposomes (Echtay *et al.* 2001) and mammalian cells (Boss *et al.* 1998) leads to artifactual uncoupling of mitochondrial respiration and add little to the understanding of the physiological function of UCPs (Cadenas *et al.* 2002).

In an important but small study, Schrauwen's group used high-intensity exercise with limb blood flow occlusion as a model to deplete skeletal muscle of the energy reserve compound PCr in 9 healthy men on a low fat diet and then measured the rate of PCr resynthesis during subsequent recovery once limb blood flow was reinstated (Hesselink *et al.* 2003). The rate of PCr resynthesis (from mitochondrial generated ATP) is a surrogate marker of mitochondrial respiratory coupling. The study was repeated after

one week ingestion of a high fat diet to induce a significant 'physiological' increase in skeletal muscle UCP3 protein. There was no statistical difference in PCr content measured over time between the two groups, so the authors concluded that a high fat diet-induced *physiological* increase in UCP3 content did not affect mitochondrial coupling *in vivo* in human skeletal muscle. However, this study has been criticised by others (Fernstrom *et al.* 2004). Looking at the data more closely, there was a positive linear association between UCP3 protein content and PCr resynthesis rate overall, suggesting that the authors were in fact measuring a surrogate marker of total mitochondrial mass per unit of muscle rather than the relative amount of UCP3. Of note, this linear relationship was steeper during low fat feeding, *i.e.* for any given mitochondrial mass there was more rapid PCr resynthesis, suggesting tighter coupling of mitochondrial respiration in the low fat fed state. In support of this argument, it has been shown that absolute UCP3 protein content per amount of tissue apparently remained unchanged in human skeletal muscle after 6 weeks of endurance training, but when taking into account the training-induced increase in mitochondrial mass by indexing to a marker of mitochondrial volume (citrate synthase), the *relative* mitochondrial content of UCP3 protein in fact decreased significantly by 53% (Fernstrom *et al.* 2004), thereby potentially contributing to training related increases in mitochondrial efficiency.

Most of the training and endurance associations in this study were due to variation at the *UCP2*-866 locus with sedentary *UCP2*-866A allele carriers benefiting from greater efficiency after training and elite athletes who were *UCP2*-866A carriers, more likely to compete at endurance distances. It is tempting to postulate that the *UCP2*-866A allele is associated with lower inducible UCP2 expression within skeletal muscle and

therefore greater mitochondrial coupling. As such, one would anticipate the A allele to be associated with the endurance phenotypes described.

The *UCP3/UCP2* locus is within a region that has been linked to diabetes and obesity (Fleury *et al.* 1997). However, I found no association between any *UCP3* or *UCP2* genotypes tested and the presence of diabetes or obesity. This agrees with some (Cassell *et al.* 1999; Dalgaard *et al.* 1999), but not the majority of published studies (Walder *et al.* 1998; Cassell *et al.* 1999; Meirhaeghe *et al.* 2000; Nordfors *et al.* 2000; Otabe *et al.* 2000; Yanovski *et al.* 2000; Esterbauer *et al.* 2001; Krempler *et al.* 2002), although many of these studies examined extreme phenotypes or subsets of racial groups. Differences in reported association may therefore have been caused by differences in ethnic group studied, sample size, as smaller genetic studies may be more likely to report a false positive association with a possibility of publication bias (Colhoun *et al.* 2003), or differences in the severity of the phenotype in the study group, for example morbid obesity and childhood onset obesity rather than comparison of BMI in adults.

9.4. UCP AND ACE

9.4.1. Genetic studies

An intriguing, significant association between serum ACE activity and variation in the *UCP3/UCP2* locus was found amongst male army recruits in the Bassingbourn 3 study: *UCP3*-55TT and *UCP2*-866GG homozygotes were found to have the lowest serum ACE activity. This is the first example of genetic variation in an unrelated gene effecting regulation of the human *ACE* gene. There were similar associations between

serum ACE activity and *UCP3/2* genotypes in the Danish type 1 diabetic cohort, variation at the *UCP3/2* locus accounting for 5% of the interindividual variability in serum ACE activity.

The ACE-*UCP* phenotype-genotype interaction was examined further in Chapter 7, increasing the variants tested and extending the ethnic diversity in an attempt to tease out which gene variant was functional, in a process previously described for the *ACE* gene (Cox *et al.* 2002). The RSA study group sampled from the South African Xhosan Tribe appeared more ancient (genetically) than the Caucasian sample, with evidence of greater recombination and significant differences in LD across the locus between the two ethnic groups, exemplified by significant (negative) LD (D'=0.31) between *UCP3*-55C>T and *UCP2*D/I in Caucasians and no LD between these two sites in South African Xhosans. The greater observed haplotype diversity amongst the RSA sample is consistent with recent reports in black African samples (Reich *et al.* 2001; Gabriel *et al.* 2002). There were significant associations between *UCP3/2* genotypes and serum ACE activity in both the RSA and BH3 studies. Variation at the *UCP3* promoter SNP resulted in a mean, age-adjusted difference of approximately 6 nmol His-Leu.ml^{-1}.min^{-1} in ACE activity in both Caucasians and South Africans Xhosans. However, variation in *UCP2* genotypes appeared to have opposite effects on serum ACE activity in the two ethnic groups, mostly explained by differential effects at the *UCP2*D/I variant.

The data suggests that variation at the *UCP3/2* genetic locus has a small but significant impact on serum ACE activity. Only three genetic variants were examined, so it is difficult to draw firm conclusions as to which (if any) of these is functional or is associated with the greatest influence on ACE activity. The resolution to detect

functional polymorphisms is limited by the size of the population and the evolutionary history of the population. The differential association in the two ethnic groups studied could be a result of genetic recombination with resultant differences in haplotype structure. As previously discussed, the genetic variants or haplotypes examined may not be functional themselves, but may mark functional variation elsewhere in the cluster. In order to unravel the association further, the sample sizes would need to be increased and more gene variants across the locus would need to be tested. However, this process is limited by the strength of the association between *UCP3/2* genotypes and serum ACE activity which reduces the power of any such study.

9.4.2. *In vitro* work

In vitro work sought to examine the mechanism of the ACE-UCP association. It was first demonstrated that ACE, UCP2 and UCP3 were all co-expressed at the mRNA level in human endothelial cells (HUVECs). Incubation of HUVECs with *UCP2* antisense led to a significant 40% decrease in ACE mRNA expression as well as a significant increase in cellular ROS generation. There was no measurable change in ACE activity in the culture media, although the assay for ACE activity may not have been sensitive enough to discriminate small changes of ACE activity *in vitro*. *UCP3* antisense did not have a significant effect on ACE mRNA and ROS generation. These data confirm that UCP2 may regulate both mitochondrial ROS generation and *ACE* gene expression in endothelial cells, although there was no correlation between circulating ACE activity and TAOS measured in diabetic men.

9.5. STUDY LIMITATIONS & FUTURE DIRECTIONS

In general, the genetic studies presented here would benefit from an increase in sample size and further replication in independent studies, to confirm the observed associations. However, the prospective nature of the NPHSII, BH2 and BH3 studies tends to reduce the impact of confounders such as survivor selection and difficulties in control matching, which are inevitable in larger case-control cross-sectional genetic association studies (Humphries *et al.* 2003). Statistically robust small gene-environment studies are possible by matching a small number of carefully chosen individuals with or without a known common gene-variant, who are then challenged by an environmental stimulus and in whom pathophysiological responses are observed (Montgomery *et al.* 2003). This is exemplified by the effects of exercise on physiological LV growth in young male army recruits by ACE genotype (Montgomery *et al.* 1997; Brull *et al.* 2001; Myerson *et al.* 2001) and in the highly significant differences in two plasma markers of ROS which required only 20 closely matched *UCP2-866G>A* homozygotes from the UDACS study (Dhamrait *et al.* 2004).

Further genetic studies are required to confirm these observations and to extend our observations from middle-aged Caucasian males to those of other racial origins and age ranges, as well as to women. It would be interesting to extend the association studies to pathological LVH, including regression of LVH following surgical relief of aortic stenosis, as well as to patients with established CHD, such as survivors of myocardial infarction, including those with LV dysfunction. Following myocardial infarction, the vast majority of patients receive long-term treatment with either an ACEi or ARB, and any prospective pharmacogenomic interaction should be sought.

Ideally, both the *BDKRB2* gene and *UCP3/2* locus should be screened for further polymorphisms and the common haplotypes assessed in different racial groups, as some of the genetic variants so far tested may not be functional themselves, but may be in strong LD with other nearby functional variants. With this knowledge, the genetic association studies should be extended to include these haplotypes in order to test which variant(s) are functional or causal in LV growth, CHD risk and in the association between the *UCP3/2* locus and serum ACE activity. For such haplotype studies in search of functionality, larger sample sizes may well be required depending on the frequency of the rarer haplotypes.

Further assessment of the functionality of the gene variants under study should be made. For instance, HUVECs could be isolated according to homozygosity for the *UCP2*-866G>A variant and then studied *in vitro* for assessments of $\Delta\psi_m$, ROS, O_2 consumption and ATP content at baseline and in response to treatment with hydroxynonenal, fatty acids, ACEi, Ang II and LPS. Similar work could be carried out with isolated monocytes taken from volunteers of known genotype. In this case, manipulations could be undertaken to induce potential changes in gene expression, such as consumption of a high fat diet or vaccination to induce a systemic inflammatory reaction or euglycaemic hyperinsulinaemic clamp in diabetic patients.

It would be important to perform immunostaining of human atherosclerotic plaque to identify whether and where UCP2 and UCP3 are expressed, predicting expression within both endothelium and macrophage/foam cells. If such expression was confirmed, *in vivo* assessment of culprit coronary plaque temperature could be made according to *UCP2* genotype in patients presenting with unstable angina and acute MI using a thermography catheter (Stefanadis *et al.* 1999). Further ACE/UCP

colocalisation studies could be carried out in atherosclerotic plaque as well as more specifically in HUVECs in culture using immunofluorescence.

The major finding of this thesis was direct mitochondrial effects of ACE inhibition in cultured cells. There were differential effects according to the lipophilicity of the ACEi tested, suggesting an intracellular action. ACE protein has been previously localised to adrenal mitochondria in the rat by electron microscopy (Peters *et al.* 1996), and this study should be repeated in human cardiac and skeletal muscle, with co-localisation with UCP2 and UCP3. The direct activation of cellular O_2 consumption by Ang II, might suggest direct activation of *mitochondrial* angiotensin receptors, and their presence, as well as the presence of kinin receptors should be sought within mitochondria, both with electron microscopy and with protein isolation from mitochondrial subfractions. If the presence of a mitochondrial RAAS and their receptors is confirmed, then further work would be required to understand the post-translational trafficking and targeting of these components to the mitochondria. ACE protein does not have a mitochondrial targeting sequence.

Further studies are required on isolated skeletal and cardiac mitochondria to assess direct effects of ACEi, kinins, angiotensins and their agonists on mitochondrial respiratory rate. State 4 respiration could be assessed in the presence of GDP and palmitate to inactivate or activate UCPs respectively. Similar experiments could be carried out in tissues isolated from UCP2ko and UCP3ko animals as well as utilising both antisense and siRNA technology in isolated cells in order to tease out UCP dependent mechanisms of action.

9.6. SUMMARY

This thesis has used human genetic and *in vitro* studies to show novel mitochondrial effects of components of the RAAS and a novel regulatory system involving mitochondrial uncoupling proteins and ACE. This interaction suggests that many of the observed non-pressor effects of ACEi on cardiac pathophysiology might be metabolic in origin. Furthermore, genetic studies confirmed for the first time that both bradykinin and the uncoupling proteins have a significant role in left ventricular hypertrophy, cardiovascular risk and skeletal muscle performance. These studies suggest that UCP2 and UCP3 could be potential novel targets in cardiovascular biology.

※ CHAPTER TEN ※

APPENDICES

APPENDIX 1: Published manuscripts arising from this thesis

1. Bradykinin B2BKR receptor polymorphism and left-ventricular growth response.

Brull D, **Dhamrait S**, Myerson S, Erdmann J, Regitz-Zagrosek V, World M, Pennell D, Humphries SE, Montgomery H.

Lancet 2001; 358: 1155–1156

Angiotensin-converting-enzyme (ACE) activity regulates left ventricular growth. The deletion (D), rather than the insertion (I), ACE gene variant is associated with increased ACE activity and kinin degradation, and the absence (-) rather than the presence (+) of a 9 bp deletion in the gene encoding the bradykinin 2 receptor (B2BKR) is associated with greater gene expression. We determined the ACE and B2BKR genotype of 109 male army recruits, and measured their physiological left ventricular growth response to a 10-week physical training programme. Mean left-ventricular growth was 15.7 g (SE 3.5) in those with ACE genotype D/D and B2BKR genotype +9/+9, but -1.37 g (4.1) in those with ACE genotype I/I and B2BKR genotype -9/-9 (p=0.003 for trend across genotypes). These results suggest that kinins regulate left-ventricular growth, mediating some of the effects of ACE in this regard.

2. Variation in bradykinin receptor genes increases the cardiovascular risk associated with hypertension.

Dhamrait SS, Payne JR, Li P, Jones A, Toor IS, Cooper JA, Hawe E, Palmen JM, Wootton PTE, Miller GJ, Humphries SE, Montgomery HE.

European Heart Journal 2003; 24: 1672–1680

Aims. The contribution of kinins to the beneficial effects of angiotensin I converting enzyme (ACE) inhibition in cardiovascular risk reduction remains unclear. The genes for the kinin inducible B_1 receptor (B_1R) and constitutive B_2 receptor (B_2R) contain functional variants: the $B_1R-699C$ (rather than G) and the $B_2R(-9)$ (rather than +9) alleles are associated with greater mRNA expression and the $B_2R(-9)$ allele with reduced left ventricular hypertrophic responses. We tested whether these gene variants influenced hypertensive coronary risk in a large prospective study.

Methods and results. Two thousand, seven hundred and six previously healthy UK men (mean age at recruitment 56 years; median follow-up 10.8 years) were genotyped for the kinin receptor variants. The coronary risk attributable to systolic hypertension (SBP≥160 mmHg) was significantly higher only in $B_1R-699GG$ homozygotes (HR 2.14 [1.42–3.22]; P<0.0001) and $B_2R(+9,+9)$ individuals (HR 3.51 [1.69–7.28]; P=0.001) but not in $B_1R-699C$ allele carriers (HR 0.82 [0.28–2.42]; P=0.76) or in $B_2R(-9,-9)$ homozygotes (HR 1.25 [0.51-3.04]; P=0.63).

Conclusions. Common variation in the genes for the kinin B_1 and B_2 receptors influences prospective hypertensive coronary risk. These are the first reported human data to suggest a role for the B_1R in human coronary vascular disease, and the first prospective study to demonstrate a similar role for the B_2R.

3. Cardiovascular risk in healthy men and markers of oxidative stress in diabetic men are associated with common variation in the gene for uncoupling protein 2.

Dhamrait SS, Stephens JW, Cooper JA, Acharya J, Manic AR, Moore K, Miller GJ, Humphries SE, Hurel SJ, Montgomery HE.

European Heart Journal. 2004; 3: 1–8

Background. Oxidative stress reduces total antioxidant status (TAOS) and is implicated in atherogenesis. Mitochondrial uncoupling protein 2 (UCP2) negatively regulates reactive oxygen species generation. The UCP2 gene demonstrates a common functional promoter variant (-866G>A).

Methods and results. Amongst 465 diabetic men (age 61.7±13.3 years), an association of the UCP2-866A allele with significantly lower TAOS in those without CHD was even more pronounced in those with CHD (TAOS 30.1±16.1% vs. 41.6±12.4% for AA vs. GG; *P*=0.016). In a sample of 20 diabetic men selected for homozygosity for the UCP2-866G>A variant, matched for baseline characteristics, plasma markers of oxidative stress in those with CHD were significantly higher in AA genotype men (TAOS 31.7±7.3% vs. 52.6±6.3%; *P*=0.001 and F2-isoprostanes 220.6±37.2 $pg.ml^{-1}$ vs. 109.9±51.1 $pg.ml^{-1}$; *P*=0.005 for AA vs. GG). Amongst 2695 healthy men (age 56.1±3.5 years) prospectively studied for a median 10.2 years, AA homozygotes had a highly significant doubling in CHD risk after adjustment for established risk factors (HR 1.99 [1.37–2.90]; *P*=0.002). Risk associated with this genotype was substantially increased by the presence of other risk factors (obesity, hypertension and diabetes).

Conclusions. This study provides the first in vivo evidence of a role for UCP2 in modifying oxidative stress and CHD risk in humans.

4. Bradykinin receptor gene variant and human physical performance.

Williams AG, **Dhamrait SS**, Wootton PTE, Day SH, Hawe E, Payne JR, Myerson SG, World M, Budgett R, Humphries SE, Montgomery HE.

J Appl Physiol. 2004; 96: 938–942.

Accumulating evidence suggests that athletic performance is strongly influenced by genetic variation. One such locus of influence is the gene for angiotensin-I converting enzyme (ACE), which exhibits a common variant [ACE insertion (I)/deletion (D)]. ACE can drive formation of vasoconstrictor ANG II but preferentially degrades vasodilator bradykinin. The ACE I allele is associated with higher kinin activity. A common gene variant in the kinin β_2 receptor (B_2R) exists: the -9 as opposed to +9 allele is associated with higher receptor mRNA expression. We tested whether this variant was associated with the efficiency of muscular contraction [delta efficiency (DE)] in 115 healthy men and women, or with running distance among 81 Olympic standard track athletes. We further sought evidence of biological interaction with ACE I/D genotype. DE was highly significantly associated with B_2R genotype (23.84 ± 2.41 vs. 24.25 ± 2.81 vs. 26.05 ± 2.26% for those of +9/+9 vs. +9/-9 vs. -9/-9 genotype; n = 25, 61, and 29, respectively; P = 0.0008 for ANOVA adjusted for sex). There was evidence for interaction with ACE I/D genotype, with individuals who were ACE II, with B_2R -9/-9 having the highest DE at baseline. The ACE I/B_2R -9 "high kinin receptor activity" haplotype was significantly associated with endurance (predominantly aerobic) event among elite athletes (P = 0.003). These data suggest that common genetic variation in the B_2R is associated with efficiency of skeletal muscle contraction and with distance event of elite track athletes and that at least part of the associations of ACE and fitness phenotypes is through elevation of kinin activity.

APPENDIX 2: Published letters arising from this thesis

1. In search of genetic precision

Humphries SE, Hawe E, **Dhamrait SS**, Miller GJ, Talmud PJ
Lancet. 2003; 361: 1908-1909.

Sir—We have previously suggested the existence of an interaction between the apolipoprotein ε4 allele and cigarette smoking in determining risk of coronary heart disease.[1] In their Research letter, Bernard Keavney and colleagues (Feb 1, p 396)[2] dispute our finding, while your accompanying Editorial (p 357)[3] calls into question the value of association studies in biomedical research.

Keavney and colleagues, collaborators in the International Study of Infarct Survival (ISIS), misrepresent our data. We reported 146 (not 115, as stated) prospective cases of coronary heart disease and stated that smoking caused a significant increase in risk "in men of all genotypes but particularly in men carrying the ε4 allele", not, as Keavney and co-workers assert "only among carriers of the ε4 allele".

We question the genotyping methods reported by Keavney and colleagues, given that our Madge method[1] is referenced as being used in their study. Furthermore, their negative finding does not perhaps carry the weight with which it is credited. All candidate gene association studies are not the same, and cross-sectional case-control studies, such as ISIS,[4] have major weaknesses. More than half of all cases will have been unavailable for study in ISIS, since up to 40% of individuals with myocardial infarction do not survive to reach hospital,[5] and in addition, those ineligible for thrombolysis, and a further 14·5% of early deaths were all excluded from analysis in ISIS. If smokers carrying the ε4 allele were at increased risk of sudden or early death or less suitable for thrombolysis, this would make any further analysis unreliable. The same would hold true if smokers with the ε4 allele contributed significantly to the 18·1% who were excluded from ISIS because they did not respond to the questionnaire.

Such generic weaknesses of case-control gene-environment studies of candidate genes are further compounded by specific methodological flaws. Retrospective assessment of

the magnitude of the environmental stimulus (in Keavney and colleagues' study smoking history was sought 6 months after the event) may prove unreliable. Meanwhile, a proportion of the controls in Keavney and colleagues' study were first-degree relatives of the cases, thus sharing inherited factors and, potentially, environmental factors such as passive smoking. It is usual (and genetically appropriate) for individuals with the ε3/3 genotype to be used as the reference group, whereas Keavney and co-workers use those with the ε3/2 genotype. Keavney and colleagues also excluded, for no apparent reason, individuals with the ε2/2 genotype and those with the high risk ε4/4 genotype from their Table 1. By contrast, in Table 2 they include individuals homozygous for the ε3 and ε4 alleles.

We reanalysed data from Keavney and colleagues' Table 2 (we excluded individuals with the ε2/4 genotype) to calculate the risk of coronary heart disease by apolipoprotein E (APOE) genotype and smoking status in ISIS (Figure). Our reanalysis confirms that smokers with the ε4 allele have a significantly greater than additive risk of coronary heart disease, with a relative excess risk of interaction of 1·62 (95% CI 0·4–2·97). Furthermore, although Keavney and colleagues state that there was no difference in the proportion of survivors between the APOE genotypes, individuals with the ε3/4 genotype had a lower survival rate in the 6 months after myocardial infarction (p=0·06) and individuals with the ε4/4 genotype, with anticipated low survival rates, were inexplicably excluded from this analysis.

Bigger is not always better: smaller prospective studies of gene-environment interaction are generally more robust and reliable than somewhat larger case-control studies. The comments by Keavney and colleagues, which are affirmed in your Editorial, through confusion, risk throwing a healthy baby out due to the presence of some tainted bath water. We would counsel against such a response.

References

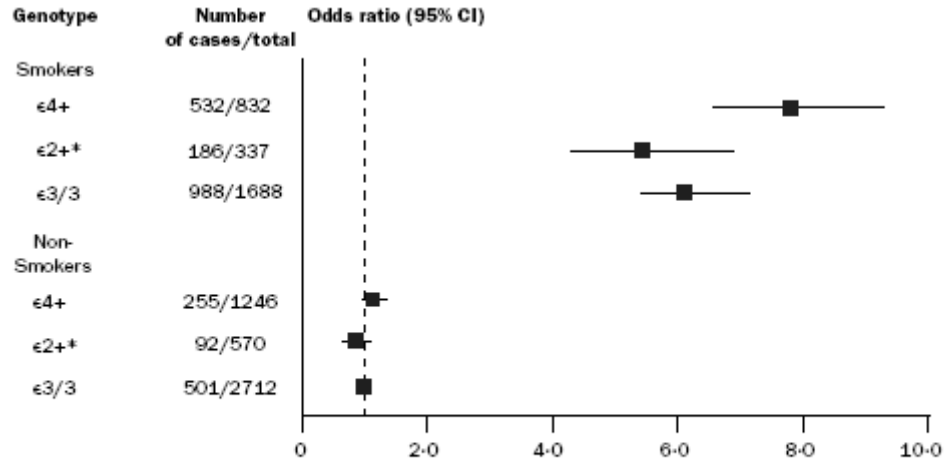
1 Humphries SE, Talmud PJ, Hawe E, Bolla M, Day IN, Miller GJ. Apolipoprotein E4 and coronary heart disease in middle-aged men who smoke: a prospective study. *Lancet* 2001; 358: 115–19.

2 Keavney B, Parish S, Palmer A, et al. Large-scale evidence that the cardiotoxicity of smoking is not significantly modified by the apolipoprotein E ε4/ε4 genotype. *Lancet* 2003; 361: 396–8.

3 Editorial. In search of genetic precision. *Lancet* 2003; 361: 357.

4 Parish S, Collins R, Peto R, et al, for the International Studies of Infarct Survival (ISIS) Collaborators. Cigarette smoking, tar yields, and non-fatal myocardial infarction: 14,000 cases and 32,000 controls in the United Kingdom. *BMJ* 1995; 311: 471–77.

5 Law MR, Watt HC, Wald NJ. The underlying risk of death after myocardial infarction in the absence of treatment. *Arch Intern Med* 2002; 162: 2405–10



Coronary heart disease risk according to *APOE* genotype and smoking status in ISIS
Recalculated from table 2 in reference 2. *E2/4 genotype excluded.

2. ACE Genotype and Performance

Montgomery HE, **Dhamrait SS.**
J Appl Physiol 2002; 92: 1774–1775.

To the Editor: The angiotensin-converting enzyme (ACE) insertion/deletion (I/D) polymorphism has previously been associated with measures of training response and human endurance performance. A recent article (4) concluded that such an association was weak or spurious. However, this conclusion is erroneous, being derived from flaws in the gene-environment model used. The application of four different training regimes to nine subgroups of race and sex in a sample of only 117 individuals will per force prevent the identification of any such association. The rationale for this contention is discussed.

Gene-environment interaction (GEI) studies relate the magnitude of physiological response to a uniform environmental stimulus with variation in a candidate gene. However, response magnitude depends on stimulus nature and scale and on the subject's physical and genetic characteristics. The ideal GEI study thus seeks 1) subject homogeneity (similar individuals of identical race and sex), 2) stimulus homogeneity (nature, magnitude, and duration), and 3) selection of an appropriate phenotype to assess.

Sonna et al. (4) report such a GEI study: candidate gene was ACE, variant was I/D polymorphism, subjects were army recruits, environmental stimulus was exercise, and phenotypic responses were performance measures. However, their conclusion that the ACE gene exerts minor or spurious effects on performance characteristics is not justified.

Subjects were not homogeneous. The use of nine combinations of race and sex is disadvantageous. 1) Associations of phenotype with race confound association with x genotype if polymorphism frequency also varies dramatically (as here) with race. 2) Phenotype is influenced by genes associated with race and gender. Left ventricular growth is greater in men and those of African descent (3), and some performance responses will also be influenced by race and sex. 3) The influence of a polymorphism on phenotypic response will, as the authors acknowledge, differ as a function of the genetic background of the population under study. Mixing such backgrounds by

inclusion of diverse race and sex combinations is thus detrimental. 4) Polymorphism functionality may itself vary with race. Fundamental to this study, the ACE I/D polymorphism is used as a marker of tissue ACE activity. Such an association is unexplored and unproven among non-Caucasians. However, its association with serum ACE activity in Caucasians may be absent among African-Americans (1), thus negating the very rationale for the use of the polymorphism as a marker of ACE activity in such groups entirely.

The complex effects of race and sex on the gene-environment interaction cannot be modeled when 117 subjects are divided into nine potential combinations of race and sex themselves spread over three genotypes. Furthermore, detecting a 20% difference in phenotypic response (far greater than that reported in any gene-environment association studies of mixed race-sex cohorts so far) with 80% power requires (they suggest) 30 subjects in each group, which is far more than is found for any race-sex combination here. Thus, with only 37 African-Americans starting training (3 of II genotype, roughly one-half likely to be women) and a reported cohort dropout rate of 20.4%, even fewer subjects are left for analysis.

The problem cannot be overcome by "lumping together" those of one sex (and different race) or those of different race, as performed in the Sonna et al. study (4) for the three non-African-American/Caucasian groups. Furthermore, conclusions cannot be drawn from the study of Caucasians alone. Data for only 117 (61 women) individuals were studied, of 147 "starters," and, of the original cohort, only 57% were Caucasian. If we assume no race and sex selection in passing training (an additional confounder that we cannot judge from the data presented), then there would have been only 29 male Caucasians of three genotypes in the final analysis, which is again far too few for meaningful comparisons with other race-sex groups.

Training stimulus was inhomogeneous. Training stimulus was inhomogeneous, differing according to four ability groups whose racial or gender composition, genotype mix, and "pass rate" may have also differed.

Choice of phenotype. An association of ACE genotype with training-related change in maximal O2 uptake has never been shown. Other measures (such as pressups and sit-ups) may require mixed strength and endurances. The association of the I allele with the latter and the D allele with the former (2) would confound allele association with such measures.

Furthermore, raw data were adjusted for age and sex to provide a "score" in a range of 0–100, the effect being to "reduce the statistical impact of outliers without excluding

them from analysis" (4), a major problem when the genetic variation being examined may account for such outliers.

In addition, lack of association of genotype with such measures (even if statistically valid) cannot be used to discount past association with different measures of performance in other more homogeneous groups. Nor can such data be used to infer anything of the validity of other studies of entirely other types (e.g., candidate gene-association studies of elite athletes).

Thus a heterogeneous (four group) environmental stimulus has been applied to nine combinations of race and sex with three genotypes among only 117 individuals. The conclusion that "the apparent association described by some is due to a minor effect of the ACE gene on physical performance that is important only under selected circumstances" (4) is thus questionable.

Putative race and sex dependence of the strength of association of a polymorphism with a given phenotypic trait remains an important issue to address. This study did not set out to (and could not) do this. In addition, analysis of mixed training in such small mixed-sex and race groups does not prove, or disprove, the impact of any given polymorphism in any population, mixed or otherwise.

References

1. Bloem LJ, Manatunga AK, and Pratt JH. Racial difference in the relationship of an angiotensin I-converting enzyme gene polymorphism to serum angiotensin I-converting enzyme activity. *Hypertension* 1996; 27: 62–66.

2. Folland J. Angiotensin-converting enzyme genotype affects the response of human skeletal muscle to functional overload. *Exp Physiol* 2000; 85: 575–579.

3. Schieken RM, Schwartz PF, and Goble MM. Tracking of left ventricular mass in children: race and sex comparisons: the MCVTwin Study. Medical College of Virginia. *Circulation* 1998; 97: 1901–1906.

4. Sonna LA, Sharp MA, Knapik JJ, Cullivan M, Angel KC, Patton JF, and Lilly CM. Angiotensin-converting enzyme genotype and physical performance during US Army basic training. *J Appl Physiol* 2001; 91: 1355–1363.

3. ACE gene, physical activity, and physical fitness

Williams AG, Day SH, **Dhamrait S**.
J Appl Physiol. 2002; 93(4): 1561-2

To the Editor: We read with interest the recent article on the topic of the angiotensin-converting enzyme (ACE) gene by Fuentes and colleagues (2). We see some value in aspects of their study, for example, as an investigation of the effect of the ACE insertion (I)/deletion (D) polymorphism on blood pressure in a specific population. However, we were disturbed at elements of both the introduction section and the discussion and conclusion.

An introduction section is traditionally used to outline the theoretical basis for the investigation being presented. In this case (2), a major focus of the study was the possible association of the ACE gene with self-reported moderate-intensity leisure time physical activity. The theoretical basis presented for such an association was weak in the extreme, for two reasons.

First, no literature was cited by Fuentes et al. (2) to support the premise that a greater fitness level and/or trainability will encourage greater physical activity in adulthood (regardless of any influences of specific genes). Yet this was the premise underpinning a major part of the study (see Table 2 in Ref. 2).

Second, even if appropriate literature supporting the premise that greater fitness and/or trainability produces greater activity in adulthood had been cited, there are strong reasons why seeking an effect of the ACE gene in this regard is likely to prove fruitless. Specifically, the research on the ACE gene has suggested possible beneficial effects of both alleles on the response to physical training. Growing evidence associates the D allele with the growth of muscle in humans (1, 6, 9) and elite performance in power events (8). Furthermore, evidence from a nonhuman model associates angiotensin II (the product of the action of ACE on angiotensin I) with skeletal muscle growth (4). Thus evidence suggests that the D allele might predispose an individual to successful performance in particular sporting events. Although we acknowledge that there is some important contradictory evidence (10,11), there is also considerable evidence associating the I allele with certain endurance phenotypes (5, 12, 13) and with elite endurance status (3, 7, 8). Hence, evidence suggests that the I allele, in addition to the D allele, might predispose an individual to successful performance in particular sporting events. Consequently, an effect of the ACE gene on activity in adulthood due to effects

on fitness and/or the response to training is highly unlikely. Even more importantly, the lack of association of the ACE I/D polymorphism with adulthood activity is used (2) to "confirm" reports of no association between ACE I/D and physical fitness in both the concluding paragraph of the article and the abstract. The lack of association reported by Fuentes et al. (2) does not confirm anything of the sort. Single-question self-reported moderate-intensity leisure time physical activity is not a recognized fitness phenotype suitable for investigating gene and gene-environment effects. Thus the data presented by Fuentes et al. add nothing to the debate on the effect of the ACE gene on fitness phenotypes and should not be used to "confirm" results of one sort or another in that field.

References

1. Folland J, Leach B, Little T, Hawker K, Myerson S, Montgomery H, and Jones D. Angiotensin-converting enzyme genotype affects the response of human skeletal muscle to functional overload. *Exp Physiol* 2000; 85: 575–579.

2. Fuentes RM, Perola M, Nissinen A, and Tuomilehto J. ACE gene and physical activity, blood pressure, and hypertension: a population study in Finland. *J Appl Physiol* 2002; 92: 2508–2512.

3. Gayagay G, Yu B, Hambly B, Boston T, Hahn A, Celermajer DS, and Trent RJ. Elite endurance athletes and the ACE I allele–the role of genes in athletic performance. *Hum Genet* 1998; 103: 48–50.

4. Gordon SE, Davis BS, Carlson CJ, and Booth FW. ANG II is required for optimal overload-induced skeletal muscle hypertrophy. *Am J Physiol Endocrinol Metab* 2001; 280: E150–E159.

5. Hagberg JM, McCole SD, Brown MD, Ferrell RE, Wilund KR, Huberty A, Douglass LW, and Moore GE. ACE insertion/ deletion polymorphism and submaximal exercise hemodynamics in postmenopausal women. *J Appl Physiol* 2002; 92: 1083– 1088.

6. Montgomery HE, Clarkson P, Dollery CM, Prasad K, Losi MA, Hemingway H, Statters D, Jubb M, Girvain M, Varnava A, World M, Deanfield J, Talmud P, McEwan JR, McKenna WJ, and Humphries S. Association of angiotensin converting enzyme gene I/D polymorphism with change in left ventricular mass in response to physical training. *Circulation* 1997; 96: 741–747.

7. Montgomery HE, Marshall R, Hemingway H, Myerson S, Clarkson P, Dollery C, Hayward M, Holliman DE, Jubb M, World M, Thomas EL, Brynes AE, Saeed N,

Barnard M, Bell JD, Prasad K, Rayson M, Talmud PJ, and Humphries SE. Human gene for physical performance. *Nature* 1998; 393: 221– 222.

8. Myerson S, Hemingway H, Budget R, Martin J, Humphries S, and Montgomery H. Human angiotensin I-converting enzyme gene and endurance performance. *J Appl Physiol* 1999; 87: 1313–1316.

9. Myerson SG, Montgomery HE, Whittingham M, Jubb M, World MJ, Humphries SE, and Pennell DJ. Left ventricular hypertrophy with exercise and ACE gene insertion/deletion polymorphism: a randomized controlled trial with losartan. *Circulation* 2001; 103: 226–230.

10. Rankinen T, Perusse L, Gagnon J, Chagnon YC, Leon AS, Skinner JS, Wilmore JH, Rao DC, and Bouchard C. Angiotensin- converting enzyme ID polymorphism and fitness phenotype in the HERITAGE Family Study. *J Appl Physiol* 2000; 88: 1029–1035.

11. Rankinen T, Wolfarth B, Simoneau J-A, Maier-Lenz D, Rauramaa R, Rivera MA, Boulay MR, Chagnon YC, Perusse L, Keul J, and Bouchard C. No association between the angiotensin-converting enzyme ID polymorphism and elite endurance athlete status. *J Appl Physiol* 2000; 88: 1571–1575.

12. Williams AG, Rayson MP, Jubb M, World M, Woods DR, Hayward M, Martin J, Humphries SE, and Montgomery HE. The ACE gene and muscle performance. *Nature* 2000; 403: 614.

13. Woods DR, World M, Rayson MP, Williams AG, Jubb M, Jamshidi Y, Hayward M, Mary DASG, Humphries SE, and Montgomery HE. Endurance enhancement related to the human angiotensin I-converting enzyme I-D polymorphism is not due to differences in the cardiorespiratory response to training. *Eur J Appl Physiol* 2002; 86: 240–244.

APPENDIX 3: Other papers arising during this thesis

1. Brull DJ, Montgomery HE, Sanders J, **Dhamrait S**, Luong L, Rumley A, Lowe GDO, Humphries SE, Montgomery HE. Interleukin-6 Gene –174G>C and –572G>C promoter polymorphisms are strong predictors of plasma IL6 levels after coronary artery bypass surgery. **Atheroscler Throm Vasc Biol**. 2001; 21: 1458-1463.

2. Harding D, **Dhamrait S,** Marlow N, Whitelaw A, Gupta S, Humphries S, Montgomery H. Angiotensin-converting enzyme DD genotype is associated with worse perinatal cardiorespiratory adaptation in preterm infants. *J Pediatr*. 2003; 143: 746-749.

3. Harding D, **Dhamrait S,** Millar A, Humphries S, Marlow N, Whitelaw A, Montgomery H. Does IL6 -174 GG genotype predict the development of septicaemia in preterm infants? *Paediatrics.* 2003; 112: 800–803.

4. **Dhamrait SS**, James L, Brull DJ, Myerson S, Hawe E, Pennell D, World M, Humphries SE, Haddad F, Montgomery HE. Cortical bone resorption during exercise is interleukin-6 genotype-dependent. *Eur J Appl Physiol.* 2003 Mar; 89(1): 21-25.

5. **Dhamrait SS,** Jones A, Payne JR, Hawe E, P Li, Toor IS, Luong L, Wootton PTE, Miller GJ, Humphries SE, Montgomery HE. The A allele of the angiotensin II type 2 receptor +1675 A>G polymorphism is associated with raised coronary artery disease morbidity in hypertensive males. *Hypertension.* 2003; 42: 500-506.

6. Payne JR, **Dhamrait SS**, Toor IS, Cooper J, Jones A, Miller GJ, Humphries SE, Montgomery HE. The -344T>C promoter variant of the gene for aldosterone synthase (CYP11B2) is not associated with cardiovascular risk in a prospective study of UK healthy men. *Atherosclerosis*. 2004; 174(1): 81-86.

7. Tsianos G, Sanders J, **Dhamrait S**, Humphries SE, Grant S, Montgomery HE. The ACE gene insertion/ deletion polymorphism and elite endurance swimming. *Eur J Appl Physiol*. 2004 ;92(3): 360-2.

8. Harding DR, **Dhamrait S**, Whitelaw A, Humphries SE, Marlow N, Montgomery HE. Does interleukin-6 genotype influence cerebral injury or developmental progress after preterm birth? *Pediatrics*. 2004 Oct; 114(4): 941-947.

9. Williams AG, Day SH, Folland JP, Gohlke P, **Dhamrait S**, Montgomery HE. Circulating Angiotensin Converting Enzyme Activity Is Correlated with Muscle Strength. *Med Sci Sports Exerc*. 2005 Jun;37(6): 944-948.

10. Boomsma F, Pedersen-Bjergaard U, Agerholm-Larsen B, Hut H, **Dhamrait SS**, Thorsteinsson B, van den Meiracker AH. Association between plasma activities of semicarbazide-sensitive amine oxidase and angiotensin-converting enzyme in patients with type 1 diabetes mellitus. *Diabetologia*. 2005 May;48(5): 1002-1007.

11. Harding DR, **Dhamrait S**, Devadason D, Humphries SE, Whitelaw A, Marlow N, Montgomery HE. Does angiotensin-1 converting enzyme genotype influence motor or cognitive development after pre-term birth? *J Neuroinflammation*. 2005 Feb 22;2(1):6.

12. Stephens JW, **Dhamrait SS**, Cooper JA, Acharya J, Miller GJ, Hurel SJ, Humphries SE. The D allele of the ACE I/D common gene variant is associated with Type 2 diabetes mellitus in Caucasian subjects. *Mol Genet Metab*. 2005 Jan;84(1): 83-9

13. Stephens JW, **Dhamrait SS**, Acharya J, Humphries SE, Hurel SJ. A common variant in the ACE gene is associated with peripheral neuropathy in women with type 2 diabetes mellitus. *J Diabetes Complications*. 2006 Sep-Oct;20(5): 317-21

14. Payne JR, **Dhamrait SS**, Gohlke P, Cooper J, Scott RA, Pitsiladis YP, Humphries SE, Rayner B, Montgomery HE. The impact of ACE genotype on serum ACE activity in a black South African male population. *Ann Hum Genet*. 2007 Jan;71(Pt 1): 1-7

15. Gable DR, Stephens JW, **Dhamrait SS**, Hawe E, Humphries SE; Hifmech Study Group. European differences in the association between the UCP2 -866G > A common gene variant and markers of body mass and fasting plasma insulin. *Diabetes Obes Metab*. 2007 Jan;9(1): 130-1

16. Day SH, Gohlke P, **Dhamrait SS**, Williams AG. No correlation between circulating ACE activity and VO2max or mechanical efficiency in women. *Eur J Appl Physiol*. 2007 Jan;99(1): 11-8.

17. Stephens JW, **Dhamrait SS**, Mani AR, Acharya J, Moore K, Hurel SJ, Humphries SE. Interaction between the uncoupling protein 2 -866G>A gene variant and cigarette smoking to increase oxidative stress in subjects with diabetes. *Nutr Metab Cardiovasc Dis.* 2008 Jan;18(1): 7-14.

18. Pedersen-Bjergaard U, **Dhamrait SS**, Sethi AA, Frandsen E, Nordestgaard BG, Montgomery HE, Pramming S, Hougaard P, Thorsteinsson B. Genetic variation and activity of the renin-angiotensin system and severe hypoglycaemia in type 1 diabetes. *Am J Med.* 2008 Mar;121(3): 246.e1-8

19. Palmer BR, Devereaux CL, **Dhamrait SS**, Mocatta TJ, Pilbrow AP, Frampton CM, Skelton L, Yandle TG, Winterbourn CC, Richards AM, Montgomery HE, Cameron VA. The common *G-866A* polymorphism of the *UCP2* gene and survival in diabetic patients following myocardial infarction. *Cardiovasc Diabetol*. 2009 Jun 15; 8: 31

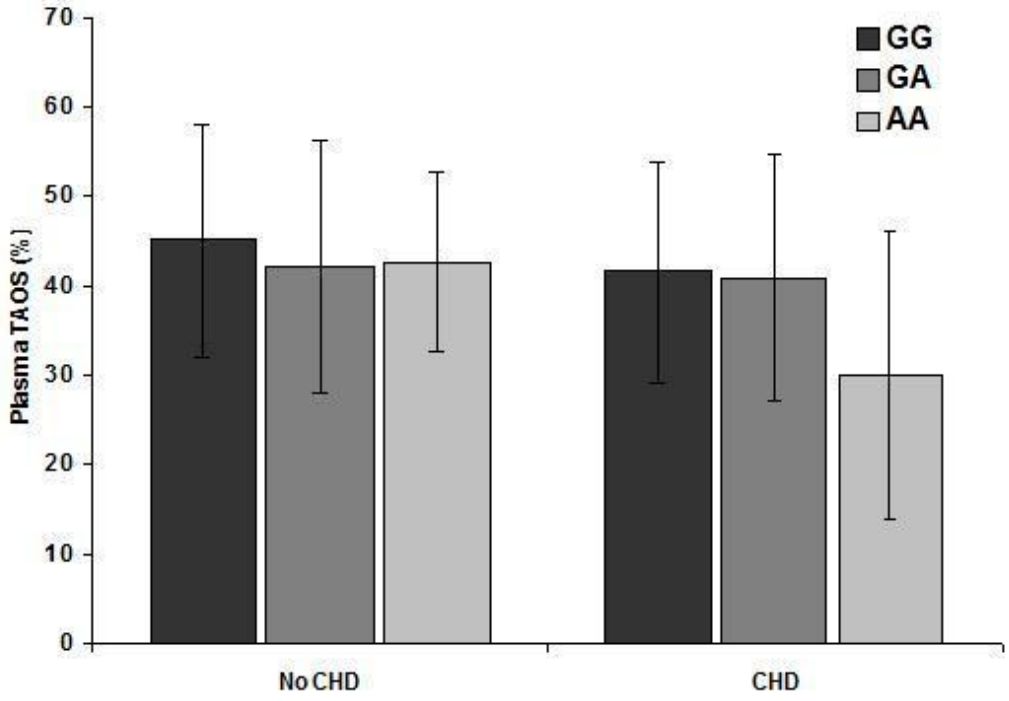
APPENDIX 4: Assessment of circulating oxidative stress in the University College Diabetes and Cardiovascular disease Study (UDACS) (Dhamrait *et al.* 2004)

Assays were performed by Dr JW Stephens. Plasma total anti-oxidant status (TAOS), which is inversely related to ROS generation, was measured by Sampson's modification of Laight's photometric microassay (Sampson *et al.* 2002), using 2.5µl citrated plasma samples in 96-well ELISA plates. Inter- and intra-assay coefficients of variation were 14.1% and 4.3% respectively. In addition, plasma esterified F_2-isoprostanes were measured using gas chromatography and mass spectroscopy as previously described (Morrow *et al.* 1994; Ferraro *et al.* 2003) in 20 *UCP2*-866G>A homozygous subjects (5 GG and 5 AA with CHD and 5 GG and 5AA without CHD), with the mean for each group closely matched for baseline characteristics including drug treatment as shown in Table 8.1. Results were calculated by reference to deuterated 8-iso-$PGF_2\alpha$ internal standards. Analyses were confined to Caucasian diabetic (type 1 and 2) men only (n=465; mean age 61.1±13.3 years). The relationships between baseline parameters and plasma TAOS were tested by Spearman rank correlation co-efficient. An ANOVA was also performed to test the association between genotype and TAOS after adjustment for the potential confounders using multiple regression analysis to obtain a residual.

Plasma TAOS was independent of pharmacotherapy, but correlated positively with plasma HDL-cholesterol, and negatively with triglyceride, glucose, HbA_{1c} and proteinuria (correlation co-efficient r =0.12, -0.15, -0.11, -0.10 and -0.07, respectively; all $P<0.05$). Plasma TAOS was significantly associated with *UCP2*-866G>A genotype, with subjects homozygous for the A allele having the lowest values (TAOS 44.3±12.7%, 41.8±14.0%, 40.2±12.4% for GG, GA, AA respectively; ANOVA $P=0.07$, GG *vs.* A allele, $P=0.03$). When results were stratified by CHD status (Figure 8.1),

there was a small but significant difference between genotype and plasma TAOS in men without CHD (45.1±12.8%, 42.1±14.1%, 42.7±10.0% for GG, GA, AA respectively;

Figure 10.1. Plasma TAOS in 465 diabetic Caucasian men recruited to the UDACS according to CHD status and stratified by *UCP2* -866G>A genotype.



Footnote: * *P*=0.04 for GG *vs.* A allele carriers; † *P*=0.01 for G allele *vs.* AA homozygotes; numbers of subjects are shown at the base of each column.

ANOVA *P*=0.13, GG *vs.* A allele, *P*=0.04), which remained significant after adjustment for age, triglyceride, HDL-C, glucose, HbA_{1c} and proteinuria (GG *vs.* A allele, *P*=0.04). These genotypic differences in plasma TAOS were more pronounced amongst men with CHD (41.6±12.4%, 41.03±13.9%, 30.1±16.1% for GG, GA, AA respectively; ANOVA *P*=0.049, G allele *vs.* AA *P*=0.01). Diabetic men with CHD who had the *UCP2*-866AA genotype had the lowest plasma TAOS level of all groups tested. There was evidence

of interaction between *UCP2*-866G>A genotype and CHD status in determining plasma TAOS (*P*=0.025 for G allele *vs.* AA).

To further corroborate these data, 20 men from UDACS were selected for homozygosity for the *UCP2*-866G>A variant and closely matched for baseline characteristics (Table 8.1). There were non-significant differences in plasma markers of OS between the presence/absence of CHD; with TAOS lower (CHD 42.2±12.7% *vs.* no-CHD 48.4±12.9%; *P*=0.22) and F_2-isoprostanes higher (CHD 168.3±74.2pg.ml^{-1} *vs.* no-CHD 111.7±79.1pg.ml^{-1}; *P*=0.05). However, there were highly significant differences in plasma TAOS between AA and GG homozygotes overall (AA 36.9±8.4% *vs.* GG 53.6±10.5%; *P*<0.0001*)*, in those without CHD (AA 42.1±6.1% *vs.* GG 54.7±7.0; *P*=0.016) and in those with CHD (AA 31.7±7.3% *vs.* GG 52.6±6.3%; *P*=0.001). AA homozygotes with CHD had the lowest TAOS of all groups (Figure 8.2a). In accordance with this, plasma F_2-isoprostane concentrations were highly significantly elevated in AA homozygotes both overall (AA 175.4±64.5pg.ml^{-1} *vs.* GG 104.6±72.8pg.ml^{-1}; *P*=0.011) and in those with CHD (AA 220.6±37.2pg.ml^{-1} *vs.* GG 109.9±51.1pg.ml^{-1}; *P*=0.005), but not significantly in AA homozygotes without CHD (AA 119.1±40.2pg.ml^{-1} *vs.* GG 105.9±27.5pg.ml^{-1}; *P*=0.58; Figure 8.2b). There was evidence of significant interaction between genotype and CHD status in determining F_2-isoprostanes (*P*=0.014) but not plasma TAOS (*P*=0.19).

Table 10.1. Plasma TAOS and esterified F_2-isoprostanes in relation to *UCP2*-866G>A genotype and CHD status in the UDACS substudy.

	No CHD			CHD		
	AA (n=5)	**GG (n=5)**	***P***	**AA (n=5)**	**GG (n=5)**	***P***
Age (years)	64.6 (4.4)	65.4 (5.3)	0.80	64.0 (6.2)	65.1 (7.2)	0.79
BMI ($kg.m^{-2}$)	32.7 (6.9)	29.6 (4.8)	0.43	30.34 (3.7)	36.2 (19.0)	0.52
Duration DM (years)	6 (0.5-20.5)	6 (4-12)	0.78	12 (7.5-21)	10 (8-17)	0.64
HbA1c (%)	6.5 (0.7)	7.5 (1.3)	0.19	8.4 (2.1)	8.6 (1.5)	0.88
Glucose ($mmol.l^{-1}$)	9.2 (5.0-15.7)	8.1 (7.2-10.1)	0.47	9.6 (6.8-16.6)	9.2 (7.5-16.2)	0.99
Cholesterol ($mmol.l^{-1}$)	5.1 (0.8)	5.5 (1.0)	0.50	4.5 (0.3)	4.6 (0.9)	0.90
LDL ($mmol.l^{-1}$)	2.8 (0.6)	3.4 (1.0)	0.28	2.2 (0.6)	2.4 (0.5)	0.65
HDL ($mmol.l^{-1}$)	1.3 (0.4)	1.4 (0.3)	0.93	1.1 (0.2)	1.2 (0.1)	0.27
Tg ($mmol.l^{-1}$)	2.1 (0.6)	2.3 (1.5)	0.81	3.3 (1.4)	2.2 (1.7)	0.30
TAOS (%)	**42.06 (6.12)***	**54.68 (6.99)**	**0.02**	**31.70 (7.27)***	**52.6 (6.25)**	**0.001**
F_2 isoprostanes ($pg.\ ml^{-1}$)	**119.1 (40.2)****	**105.9 (27.48)**	**0.58**	**220.6 (37.2)****	**109.9 (51.1)**	**0.005**
Systolic BP (mmHg)	141 (12)	145 (12)	0.63	136 (20)	148 (27)	0.46
Diastolic BP (mmHg)	81.2 (8.4)	80.4 (8.1)	0.88	81 (9)	82 (7)	0.57
% Proteinuria	30 % (n=3)	40% (n=4)	0.64	60 % (n=3)	60 % (n=3)	1.00
% Smokers	20 % (n=1)	20 % (n=1)	1.00	0 % (n=0)	20% (n=1)	0.29
% on insulin	20 % (n=1)	20 % (n=1)	1.00	40 % (n=2)	60 % (n=3)	0.53
% on Aspirin	20 % (n=1)	40 % (n=2)	0.49	60 % (n=3)	80 % (n=4)	0.49
% on Statin	20 % (n=1)	0 % (n=0)	0.29	100 % (n=5)	80 % (n=4)	0.29
% on ACEI	40 % (n=2)	20 % (n=1)	0.49	60 % (n=3)	80 % (n=4)	0.49

Mean (SD) shown or Median (IQR); Compared with Student's t-test/ Mann-Whitney/ Chi-squared test

Genotype groups were closely matched for baseline characteristics.

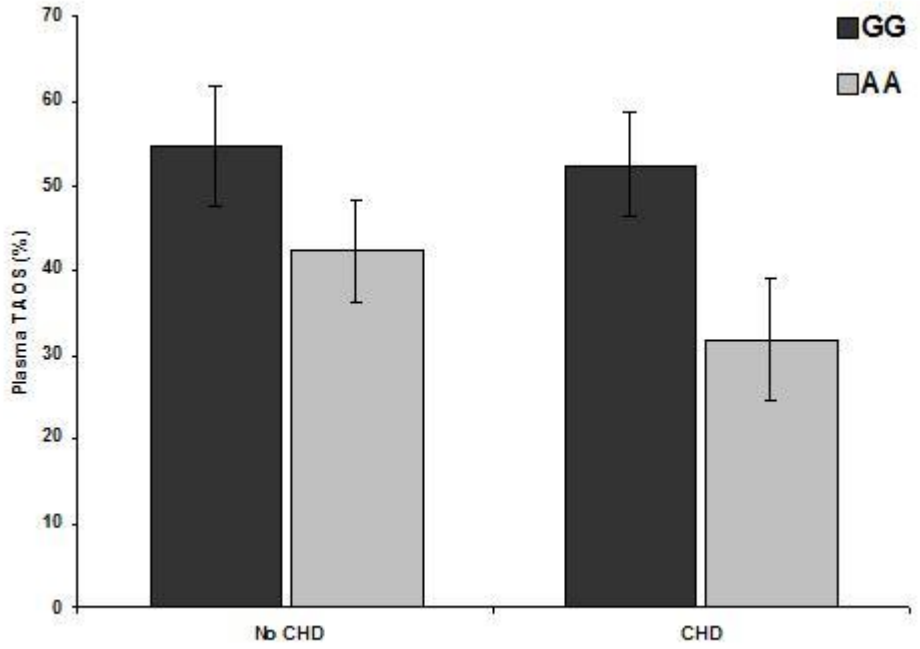
Comparing AA (No CHD v CHD)

*P<0.05

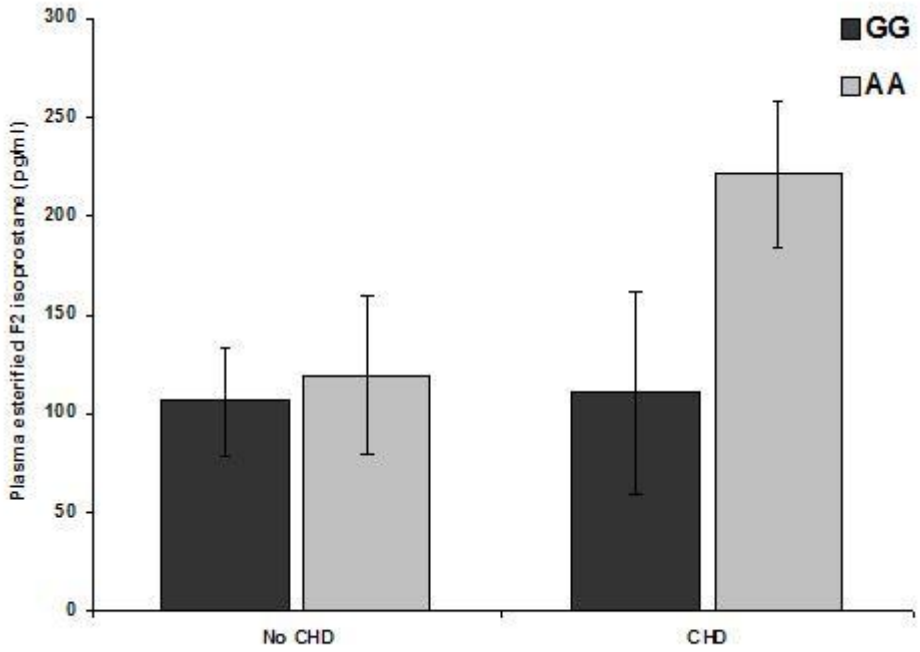
**P<0.01

Figure 10.2. Plasma TAOS and F_2-isoprostane in UDACS substudy of 20 diabetic closely matched men selected for homozygosity for *UCP2*-866G>A genotype and stratified by CHD status.

A. Plasma TAOS



B. Plasma esterified F2 isoprostanes



※ CHAPTER ELEVEN ※

BIBLIOGRAPHY

AbdAlla, S., Lother, H. and Quitterer, U. AT1-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. *Nature* (2000); **407**(6800): 94-98.

Abraham, W. T., Fisher, W. G., Smith, A. L., Delurgio, D. B., Leon, A. R., Loh, E., Kocovic, D. Z., Packer, M., Clavell, A. L., Hayes, D. L., Ellestad, M., Trupp, R. J., Underwood, J., Pickering, F., Truex, C., McAtee, P. and Messenger, J. Cardiac resynchronization in chronic heart failure. *N Engl J Med* (2002); **346**(24): 1845-1853.

Acin, A., Rodriguez, M., Rique, H., Canet, E., Boutin, J. A. and Galizzi, J. P. Cloning and characterization of the 5' flanking region of the human uncoupling protein 3 (UCP3) gene. *Biochem Biophys Res Commun* (1999); **258**(2): 278-283.

Adams, M. R., Jessup, W. and Celermajer, D. S. Cigarette smoking is associated with increased human monocyte adhesion to endothelial cells: reversibility with oral L-arginine but not vitamin C. *J Am Coll Cardiol* (1997); **29**(3): 491-497.

Afzal, A., Brawner, C. A. and Keteyian, S. J. Exercise training in heart failure. *Prog Cardiovasc Dis* (1998); **41**(3): 175-190.

AIRE Study Investigators. Effect of ramipril on mortality and morbidity of survivors of acute myocardial infarction with clinical evidence of heart failure. The Acute Infarction Ramipril Efficacy (AIRE) Study. *Lancet* (1993); **342**(8875): 821-828.

Alhenc-Gelas, F., Baussant, T., Hubert, C., Soubrier, F. and Corvol, P. The angiotensin converting enzyme in the kidney. *J Hypertens Suppl* (1989); **7**(7): S9-13; discussion S14.

Altshuler, D., Pollara, V. J., Cowles, C. R., Van Etten, W. J., Baldwin, J., Linton, L. and Lander, E. S. An SNP map of the human genome generated by reduced representation shotgun sequencing. *Nature* (2000); **407**(6803): 513-516.

Alvarez, R., Terrados, N., Ortolano, R., Iglesias-Cubero, G., Reguero, J. R., Batalla, A., Cortina, A., Fernandez-Garcia, B., Rodriguez, C., Braga, S., Alvarez, V. and Coto, E. Genetic variation in the renin-angiotensin system and athletic performance. *Eur J Appl Physiol* (2000); **82**(1-2): 117-120.

Amin, J. K., Xiao, L., Pimental, D. R., Pagano, P. J., Singh, K., Sawyer, D. B. and Colucci, W. S. Reactive oxygen species mediate alpha-adrenergic receptor-stimulated hypertrophy in adult rat ventricular myocytes. *J Mol Cell Cardiol* (2001); **33**(1): 131-139.

Amos, A. F., McCarty, D. J. and Zimmet, P. The rising global burden of diabetes and its complications: estimates and projections to the year 2010. *Diabet Med* (1997); **14 Suppl 5**: S1-85.

Antonicka, H., Floryk, D., Klement, P., Stratilova, L., Hermanska, J., Houstkova, H., Kalous, M., Drahota, Z., Zeman, J. and Houstek, J. Defective kinetics of cytochrome c oxidase and alteration of mitochondrial membrane potential in fibroblasts and cytoplasmic hybrid cells with the mutation for myoclonus epilepsy with ragged-red fibres ('MERRF') at position 8344 nt. *Biochem J* (1999); **342 Pt 3**: 537-44.

Anversa, P., Olivetti, G., Leri, A., Liu, Y. and Kajstura, J. Myocyte cell death and ventricular remodeling. *Curr Opin Nephrol Hypertens* (1997); **6**(2): 169-76.

Aoki, S., Mukae, S., Itoh, S., Sato, R., Nishio, K., Iwata, T. and Katagiri, T. The genetic factor in acute myocardial infarction with hypertension. *Jpn Circ J* (2001); **65**(7): 621-6.

Aoki, S., Mukae, S., Itoh, S., Sato, R., Nishio, K., Iwata, T. and Katagiri, T. The genetic factor in acute myocardial infarction with hypertension. *Jpn Circ J* (2001); **65**(7): 621-626.

Arbustini, E., Dal Bello, B., Morbini, P., Burke, A. P., Bocciarelli, M., Specchia, G. and Virmani, R. Plaque erosion is a major substrate for coronary thrombosis in acute myocardial infarction. *Heart* (1999); **82**(3): 269-272.

Arechaga, I., Ledesma, A. and Rial, E. The mitochondrial uncoupling protein UCP1: a gated pore. *IUBMB Life* (2001); **52**(3-5): 165-173.

Ares, M. P., Kallin, B., Eriksson, P. and Nilsson, J. Oxidized LDL induces transcription factor activator protein-1 but inhibits activation of nuclear factor-kappa B in human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* (1995); **15**(10): 1584-1590.

Argyropoulos, G., Brown, A. M., Willi, S. M., Zhu, J., He, Y., Reitman, M., Gevao, S. M., Spruill, I. and Garvey, W. T. Effects of mutations in the human uncoupling protein 3 gene on the respiratory quotient and fat oxidation in severe obesity and type 2 diabetes. *J Clin Invest* (1998); **102**(7): 1345-1351.

Arsenijevic, D., Onuma, H., Pecqueur, C., Raimbault, S., Manning, B. S., Miroux, B., Couplan, E., Alves-Guerra, M. C., Goubern, M., Surwit, R., Bouillaud, F., Richard, D., Collins, S. and Ricquier, D.

Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. *Nat Genet* (2000); **26**(4): 435-439.

Assmann, G., Schulte, H. and Cullen, P. New and classical risk factors--the Munster heart study (PROCAM). *Eur J Med Res* (1997); **2**(6): 237-242.

Assmann, S. F., Hosmer, D. W., Lemeshow, S. and Mundt, K. A. Confidence intervals for measures of interaction. *Epidemiology* (1996); **7**(3): 286-90.

Aubert, J., Champigny, O., Saint-Marc, P., Negrel, R., Collins, S., Ricquier, D. and Ailhaud, G. Up-regulation of UCP-2 gene expression by PPAR agonists in preadipose and adipose cells. *Biochem Biophys Res Commun* (1997); **238**(2): 606-611.

Austin, M. A., King, M. C., Bawol, R. D., Hulley, S. B. and Friedman, G. D. Risk factors for coronary heart disease in adult female twins. Genetic heritability and shared environmental influences. *Am J Epidemiol* (1987); **125**(2): 308-318.

Bak, M. I. and Ingwall, J. S. Acidosis during ischemia promotes adenosine triphosphate resynthesis in postischemic rat heart. In vivo regulation of 5'-nucleotidase. *J Clin Invest* (1994); **93**(1): 40-49.

Barbe, P., Larrouy, D., Boulanger, C., Chevillotte, E., Viguerie, N., Thalamas, C., Oliva Trastoy, M., Roques, M., Vidal, H. and Langin, D. Triiodothyronine-mediated up-regulation of UCP2 and UCP3 mRNA expression in human skeletal muscle without coordinated induction of mitochondrial respiratory chain genes. *Faseb J* (2001); **15**(1): 13-15.

Barua, R. S., Ambrose, J. A., Srivastava, S., DeVoe, M. C. and Eales-Reynolds, L. J. Reactive oxygen species are involved in smoking-induced dysfunction of nitric oxide biosynthesis and upregulation of endothelial nitric oxide synthase: an in vitro demonstration in human coronary artery endothelial cells. *Circulation* (2003); **107**(18): 2342-2347.

Beckman, J. A., Creager, M. A. and Libby, P. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *Jama* (2002); **287**(19): 2570-2581.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A. and Freeman, B. A. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* (1990); **87**(4): 1620-1624.

Beer, M., Seyfarth, T., Sandstede, J., Landschutz, W., Lipke, C., Kostler, H., von Kienlin, M., Harre, K., Hahn, D. and Neubauer, S. Absolute concentrations of high-energy phosphate metabolites in normal, hypertrophied, and failing human myocardium measured noninvasively with (31)P-SLOOP magnetic resonance spectroscopy. *J Am Coll Cardiol* (2002); **40**(7): 1267-1274.

Bellamy, M. F., McDowell, I. F., Ramsey, M. W., Brownlee, M., Bones, C., Newcombe, R. G. and Lewis, M. J. Hyperhomocysteinemia after an oral methionine load acutely impairs endothelial function in healthy adults. *Circulation* (1998); **98**(18): 1848-1852.

Beltran, B., Mathur, A., Duchen, M. R., Erusalimsky, J. D. and Moncada, S. The effect of nitric oxide on cell respiration: A key to understanding its role in cell survival or death. *Proc Natl Acad Sci U S A* (2000); **97**(26): 14602-14607.

Bendall, J. K., Cave, A. C., Heymes, C., Gall, N. and Shah, A. M. Pivotal role of a gp91(phox)-containing NADPH oxidase in angiotensin II-induced cardiac hypertrophy in mice. *Circulation* (2002); **105**(3): 293-296.

Bengel, F. M., Permanetter, B., Ungerer, M., Nekolla, S. and Schwaiger, M. Non-invasive estimation of myocardial efficiency using positron emission tomography and carbon-11 acetate--comparison between the normal and failing human heart. *Eur J Nucl Med* (2000); **27**(3): 319-326.

Berry, C., Anderson, N., Kirk, A. J., Dominiczak, A. F. and McMurray, J. J. Renin angiotensin system inhibition is associated with reduced free radical concentrations in arteries of patients with coronary heart disease. *Heart* (2001); **86**(2): 217-220.

Biasucci, L. M., Liuzzo, G., Fantuzzi, G., Caligiuri, G., Rebuzzi, A. G., Ginnetti, F., Dinarello, C. A. and Maseri, A. Increasing levels of interleukin (IL)-1Ra and IL-6 during the first 2 days of hospitalization in unstable angina are associated with increased risk of in-hospital coronary events. *Circulation* (1999); **99**(16): 2079-2084.

Bing, C., Brown, M., King, P., Collins, P., Tisdale, M. J. and Williams, G. Increased gene expression of brown fat uncoupling protein (UCP)1 and skeletal muscle UCP2 and UCP3 in MAC16-induced cancer cachexia. *Cancer Res* (2000); **60**(9): 2405-2410.

Blake, G. J., Dada, N., Fox, J. C., Manson, J. E. and Ridker, P. M. A prospective evaluation of lipoprotein-associated phospholipase A(2) levels and the risk of future cardiovascular events in women. *J Am Coll Cardiol* (2001); **38**(5): 1302-1306.

Blanc, J., Alves-Guerra, M. C., Esposito, B., Rousset, S., Gourdy, P., Ricquier, D., Tedgui, A., Miroux, B. and Mallat, Z. Protective role of uncoupling protein 2 in atherosclerosis. *Circulation* (2003); **107**(3): 388-390.

Blau, H. M., Pavlath, G. K., Hardeman, E. C., Chiu, C. P., Silberstein, L., Webster, S. G., Miller, S. C. and Webster, C. Plasticity of the differentiated state. *Science* (1985); **230**(4727): 758-766.

Boehm, E. A., Jones, B. E., Radda, G. K., Veech, R. L. and Clarke, K. Increased uncoupling proteins and decreased efficiency in palmitate-perfused hyperthyroid rat heart. *Am J Physiol Heart Circ Physiol* (2001); **280**(3): H977-H983.

Boivin, M., Camirand, A., Carli, F., Hoffer, L. J. and Silva, J. E. Uncoupling protein-2 and -3 messenger ribonucleic acids in adipose tissue and skeletal muscle of healthy males: variability, factors affecting expression, and relation to measures of metabolic rate. *J Clin Endocrinol Metab* (2000); **85**(5): 1975-1983.

Borecky, J., Maia, I. G. and Arruda, P. Mitochondrial uncoupling proteins in mammals and plants. *Biosci Rep* (2001); **21**(2): 201-212.

Borst, P., Loos, J. A., Christ, E. J. and Slater, E. C. Uncoupling activity of long-chain fatty acids. *Biochim Biophys Acta* (1962); **62**: 509-518.

Borutaite, V., Matthias, A., Harris, H., Moncada, S. and Brown, G. C. Reversible inhibition of cellular respiration by nitric oxide in vascular inflammation. *Am J Physiol Heart Circ Physiol* (2001); **281**(6): H2256-H2260.

Boss, O., Hagen, T. and Lowell, B. B. Uncoupling proteins 2 and 3: potential regulators of mitochondrial energy metabolism. *Diabetes* (2000); **49**(2): 143-156.

Boss, O., Samec, S., Desplanches, D., Mayet, M.-H., Seydoux, J., Muzzin, P. and Giacobino, J.-P. Effect of endurance training on mRNA expression of the uncoupling proteins 1, 2, and 3 in the rat. *The FASEB Journal* (1998); **12**: 335-339.

Boss, O., Samec, S., Dulloo, A., Seydoux, J., Muzzin, P. and Giacobino, J. P. Tissue-dependent upregulation of rat uncoupling protein-2 expression in response to fasting or cold. *FEBS Lett* (1997); **412**(1): 111-114.

Boss, O., Samec, S., Kuhne, F., Bijlenga, P., Assimacopoulos-Jeannet, F., Seydoux, J., Giacobino, J. P. and Muzzin, P. Uncoupling protein-3 expression in rodent skeletal muscle is modulated by food intake but not by changes in environmental temperature. *J Biol Chem* (1998); **273**(1): 5-8.

Boss, O., Samec, S., Paoloni-Giacobino, A., Rossier, C., Dulloo, A., Seydoux, J., Muzzin, P. and Giacobino, J. P. Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression. *FEBS Lett* (1997); **408**(1): 39-42.

Bouchard, C., Perusse, L., Chagnon, Y. C., Warden, C. and Ricquier, D. Linkage between markers in the vicinity of the uncoupling protein 2 gene and resting metabolic rate in humans. *Hum Mol Genet* (1997); **6**(11): 1887-1889.

Brand, K., Page, S., Rogler, G., Bartsch, A., Brandl, R., Knuechel, R., Page, M., Kaltschmidt, C., Baeuerle, P. A. and Neumeier, D. Activated transcription factor nuclear factor-kappa B is present in the atherosclerotic lesion. *J Clin Invest* (1996); **97**(7): 1715-1722.

Brand, M. D., Chien, L. F., Ainscow, E. K., Rolfe, D. F. and Porter, R. K. The causes and functions of mitochondrial proton leak. *Biochim Biophys Acta* (1994); **1187**(2): 132-139.

Braun, A., Kammerer, S., Maier, E., Bohme, E. and Roscher, A. A. Polymorphisms in the gene for the human B2-bradykinin receptor. New tools in assessing a genetic risk for bradykinin-associated diseases. *Immunopharmacology* (1996); **33**(1-3): 32-35.

Braun, A., Kammerer, S., Maier, E., Bohme, E. and Roscher, A. A. Polymorphisms in the gene for the human B2-bradykinin receptor. New tools in assessing a genetic risk for bradykinin-associated diseases. *Immunopharmacology* (1996); **33**(1-3): 32-5.

Braun, A., Maier, E., Kammerer, S., Muller, B. and Roscher, A. A. A novel sequence polymorphism in the promoter region of the human B2-bradykinin receptor gene. *Hum Genet* (1996); **97**(5): 688-9.

Brink, M., Price, S. R., Chrast, J., Bailey, J. L., Anwar, A., Mitch, W. E. and Delafontaine, P. Angiotensin II induces skeletal muscle wasting through enhanced protein degradation and down-regulates autocrine insulin-like growth factor I. *Endocrinology* (2001); **142**(4): 1489-1496.

Brink, M., Wellen, J. and Delafontaine, P. Angiotensin II causes weight loss and decreases circulating insulin-like growth factor I in rats through a pressor-independent mechanism. *J Clin Invest* (1996); **97**(11): 2509-2516.

Bristow, M. R. The adrenergic nervous system in heart failure. *N Engl J Med* (1984); **311**(13): 850-851.

Bristow, M. R. Changes in myocardial and vascular receptors in heart failure. *J Am Coll Cardiol* (1993); **22**(4 Suppl A): 61A-71A.

British Heart Foundation (2006). Coronary heart disease statistics. London, British Heart Foundation.

Brookes, P. S., Bolanos, J. P. and Heales, S. J. The assumption that nitric oxide inhibits mitochondrial ATP synthesis is correct. *FEBS Lett* (1999); **446**(2-3): 261-263.

Brown, C. C., Jr., Fry, D. L. and Ebert, R. V. The mechanics of pulmonary ventilation in patients with heart diseases. *Am J Med* (1954); **17**(4): 438-446.

Brown, G. C. and Cooper, C. E. Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. *FEBS Lett* (1994); **356**(2-3): 295-298.

Brownlee, M. Negative consequences of glycation. *Metabolism* (2000); **49**(2 Suppl 1): 9-13.

Brownlee, M. Biochemistry and molecular cell biology of diabetic complications. *Nature* (2001); **414**(6865): 813-820.

Brull, D., Dhamrait, S., Myerson, S., Erdmann, J., Woods, D., World, M., Pennell, D., Humphries, S., Regitz-Zagrosek, V. and Montgomery, H. Bradykinin B2BKR receptor polymorphism and left-ventricular growth response. *Lancet* (2001); **358**(9288): 1155-1156.

Brull, D., Dhamrait, S., Myerson, S., Erdmann, J., Woods, D., World, M., Pennell, D., Humphries, S., Regitz-Zagrosek, V. and Montgomery, H. Bradykinin B2BKR receptor polymorphism and left-ventricular growth response. *Lancet* (2001); **358**(9288): 1155-6.

Brull, D. J., Dhamrait, S., Moulding, R., Rumley, A., Lowe, G. D., World, M. J., Humphries, S. E. and Montgomery, H. E. The effect of fibrinogen genotype on fibrinogen levels after strenuous physical exercise. *Thromb Haemost* (2002); **87**(1): 37-41.

Brull, D. J., Sanders, J., Rumley, A., Lowe, G. D., Humphries, S. E. and Montgomery, H. E. Impact of angiotensin converting enzyme inhibition on post-coronary artery bypass interleukin 6 release. *Heart* (2002); **87**(3): 252-255.

Brull, D. J., Serrano, N., Zito, F., Jones, L., Montgomery, H. E., Rumley, A., Sharma, P., Lowe, G. D., World, M. J., Humphries, S. E. and Hingorani, A. D. Human CRP Gene Polymorphism Influences CRP Levels. Implications for the Prediction and Pathogenesis of Coronary Heart Disease. *Arterioscler Thromb Vasc Biol* (2003).

Brunotte, F., Thompson, C. H., Adamopoulos, S., Coats, A., Unitt, J., Lindsay, D., Kaklamanis, L., Radda, G. K. and Rajagopalan, B. Rat skeletal muscle metabolism in experimental heart failure: effects of physical training. *Acta Physiol Scand* (1995); **154**(4): 439-447.

Brunton, L. Therapeutics of the Circulation. London (1908), Murray.

Burke, A. P., Farb, A., Malcom, G. T., Liang, Y. H., Smialek, J. and Virmani, R. Coronary risk factors and plaque morphology in men with coronary disease who died suddenly. *N Engl J Med* (1997); **336**(18): 1276-1282.

Burke, A. P., Kolodgie, F. D., Farb, A., Weber, D. K., Malcom, G. T., Smialek, J. and Virmani, R. Healed plaque ruptures and sudden coronary death: evidence that subclinical rupture has a role in plaque progression. *Circulation* (2001); **103**(7): 934-940.

Buser, P. T., Camacho, S. A., Wu, S. T., Higgins, C. B., Jasmin, G., Parmley, W. W. and Wikman-Coffelt, J. The effect of dobutamine on myocardial performance and high-energy phosphate metabolism at different stages of heart failure in cardiomyopathic hamsters: a 31P MRS study. *Am Heart J* (1989); **118**(1): 86-91.

Cabrero, A., Alegret, M., Sanchez, R., Adzet, T., Laguna, J. C. and Vazquez, M. Peroxisome proliferator-activated receptor alpha (PPARalpha) activators, bezafibrate and Wy-14,643, increase uncoupling protein-3 mRNA levels without modifying the mitochondrial membrane potential in primary culture of rat preadipocytes. *Arch Biochem Biophys* (2000); **380**(2): 353-359.

Cabrero, A., Alegret, M., Sanchez, R. M., Adzet, T., Laguna, J. C. and Vazquez, M. Down-regulation of uncoupling protein-3 and -2 by thiazolidinediones in C2C12 myotubes. *FEBS Lett* (2000); **484**(1): 37-42.

Cadenas, S., Echtay, K. S., Harper, J. A., Jekabsons, M. B., Buckingham, J. A., Grau, E., Abuin, A., Chapman, H., Clapham, J. C. and Brand, M. D. The basal proton conductance of skeletal muscle mitochondria from transgenic mice overexpressing or lacking uncoupling protein-3. *J Biol Chem* (2002); **277**(4): 2773-2778.

Cai, H. and Harrison, D. G. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* (2000); **87**(10): 840-844.

Calver, A., Collier, J., Moncada, S. and Vallance, P. Effect of local intra-arterial NG-monomethyl-L-arginine in patients with hypertension: the nitric oxide dilator mechanism appears abnormal. *J Hypertens* (1992); **10**(9): 1025-1031.

Cambien, F., Alhenc-Gelas, F., Herbeth, B., Andre, J. L., Rakotovao, R., Gonzales, M. F., Allegrini, J. and Bloch, C. Familial resemblance of plasma angiotensin-converting enzyme level: the Nancy Study. *Am J Hum Genet* (1988); **43**(5): 774-780.

Cambien, F., Poirier, O., Lecerf, L., Evans, A., Cambou, J. P., Arveiler, D., Luc, G., Bard, J. M., Bara, L., Ricard, S. and et al. Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction. *Nature* (1992); **359**(6396): 641-644.

Cameron-Smith, D., Burke, L. M., Angus, D. J., Tunstall, R. J., Cox, G. R., Bonen, A., Hawley, J. A. and Hargreaves, M. A short-term, high-fat diet up-regulates lipid metabolism and gene expression in human skeletal muscle. *Am J Clin Nutr* (2003); **77**(2): 313-318.

Camirand, A., Marie, V., Rabelo, R. and Silva, J. E. Thiazolidinediones stimulate uncoupling protein-2 expression in cell lines representing white and brown adipose tissues and skeletal muscle. *Endocrinology* (1998); **139**(1): 428-431.

Campbell, D. J., Kladis, A. and Duncan, A. M. Bradykinin peptides in kidney, blood, and other tissues of the rat. *Hypertension* (1993); **21**(2): 155-165.

Cappuccio, F. P. Ethnicity and cardiovascular risk: variations in people of African ancestry and South Asian origin. *J Hum Hypertens* (1997); **11**(9): 571-576.

Cappuccio, F. P., Oakeshott, P., Strazzullo, P. and Kerry, S. M. Application of Framingham risk estimates to ethnic minorities in United Kingdom and implications for primary prevention of heart disease in general practice: cross sectional population based study. *Bmj* (2002); **325**(7375): 1271.

Cargill, M., Altshuler, D., Ireland, J., Sklar, P., Ardlie, K., Patil, N., Shaw, N., Lane, C. R., Lim, E. P., Kalyanaraman, N., Nemesh, J., Ziaugra, L., Friedland, L., Rolfe, A., Warrington, J., Lipshutz, R., Daley, G. Q. and Lander, E. S. Characterization of single-nucleotide polymorphisms in coding regions of human genes. *Nat Genet* (1999); **22**(3): 231-238.

Casademont, J. and Miro, O. Electron transport chain defects in heart failure. *Heart Failure Reviews* (2002); **7**: 131-139.

Cassell, P. G., Neverova, M., Janmohamed, S., Uwakwe, N., Qureshi, A., McCarthy, M. I., Saker, P. J., Albon, L., Kopelman, P., Noonan, K., Easlick, J., Ramachandran, A., Snehalatha, C., Pecqueur, C., Ricquier, D., Warden, C. and Hitman, G. A. An uncoupling protein 2 gene variant is associated with a raised body mass index but not Type II diabetes. *Diabetologia* (1999); **42**(6): 688-692.

Cassell, P. G., Saker, P. J., Huxtable, S. J., Kousta, E., Jackson, A. E., Hattersley, A. T., Frayling, T. M., Walker, M., Kopelman, P. G., Ramachandran, A., Snehelatha, C., Hitman, G. A. and McCarthy, M. I. Evidence that single nucleotide polymorphism in the uncoupling protein 3 (UCP3) gene influences fat distribution in women of European and Asian origin. *Diabetologia* (2000); **43**(12): 1558-1564.

Cassis, L., Helton, M., English, V. and Burke, G. Angiotensin II regulates oxygen consumption. *Am J Physiol Regul Integr Comp Physiol* (2002); **282**(2): R445-453.

Casteilla, L., Rigoulet, M. and Penicaud, L. Mitochondrial ROS metabolism: modulation by uncoupling proteins. *IUBMB Life* (2001); **52**(3-5): 181-188.

Castell, L. M., Poortmans, J. R., Leclercq, R., Brasseur, M., Duchateau, J. and Newsholme, E. A. Some aspects of the acute phase response after a marathon race, and the effects of glutamine supplementation. *Eur J Appl Physiol Occup Physiol* (1997); **75**(1): 47-53.

Castelli, W. P., Garrison, R. J., Wilson, P. W., Abbott, R. D., Kalousdian, S. and Kannel, W. B. Incidence of coronary heart disease and lipoprotein cholesterol levels. The Framingham Study. *JAMA* (1986); **256**(20): 2835-2838.

Castro, L., Rodriguez, M. and Radi, R. Aconitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide. *J Biol Chem* (1994); **269**(47): 29409-29415.

Celermajer, D. S., Sorensen, K. E., Georgakopoulos, D., Bull, C., Thomas, O., Robinson, J. and Deanfield, J. E. Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. *Circulation* (1993); **88**(5 Pt 1): 2149-2155.

Chakravarti, A., Buetow, K. H., Antonarakis, S. E., Waber, P. G., Boehm, C. D. and Kazazian, H. H. Nonuniform recombination within the human beta-globin gene cluster. *Am J Hum Genet* (1984); **36**(6): 1239-1258.

Challoner, D. R. and Steinberg, D. Effect of free fatty acid on the oxygen consumption of perfused rat heart. *Am J Physiol* (1966); **210**(2): 280-286.

Chan, C. B., MacDonald, P. E., Saleh, M. C., Johns, D. C., Marban, E. and Wheeler, M. B. Overexpression of uncoupling protein 2 inhibits glucose-stimulated insulin secretion from rat islets. *Diabetes* (1999); **48**(7): 1482-1486.

Chance, B., Sies, H. and Boveris, A. Hydroperoxide metabolism in mammalian organs. *Physiol Rev* (1979); **59**(3): 527-605.

Chandler, B. M., Sonnenblick, E. H., Spann, J. F., Jr. and Pool, P. E. Association of depressed myofibrillar adenosine triphosphatase and reduced contractility in experimental heart failure. *Circ Res* (1967); **21**(5): 717-725.

Chen, X. and Catravas, J. D. Neutrophil-mediated endothelial angiotensin-converting enzyme dysfunction: role of oxygen-derived free radicals. *Am J Physiol* (1993); **265**(3 Pt 1): L243-249.

Cheung, V. G., Conlin, L. K., Weber, T. M., Arcaro, M., Jen, K. Y., Morley, M. and Spielman, R. S. Natural variation in human gene expression assessed in lymphoblastoid cells. *Nat Genet* (2003); **33**(3): 422-425.

Chidsey, C. A., Harrison, D. C. and Braunwald, E. Augmentation of the plasma nor-epinephrine response to exercise in patients with congestive heart failure. *N Engl J Med* (1962); **267**: 650-654.

Ching, S. F., Hayes, L. W. and Slakey, L. L. Angiotensin-converting enzyme in cultured endothelial cells. Synthesis, degradation, and transfer to culture medium. *Arteriosclerosis* (1983); **3**(6): 581-588.

Chisolm, G. M. and Steinberg, D. The oxidative modification hypothesis of atherogenesis: an overview. *Free Radic Biol Med* (2000); **28**(12): 1815-1826.

Chiu, H. C., Kovacs, A., Ford, D. A., Hsu, F. F., Garcia, R., Herrero, P., Saffitz, J. E. and Schaffer, J. E. A novel mouse model of lipotoxic cardiomyopathy. *J Clin Invest* (2001); **107**(7): 813-822.

Chowienczyk, P. J., Watts, G. F., Cockcroft, J. R. and Ritter, J. M. Impaired endothelium-dependent vasodilation of forearm resistance vessels in hypercholesterolaemia. *Lancet* (1992); **340**(8833): 1430-1432.

Chua, T. P., Ponikowski, P. P., Harrington, D., Chambers, J. and Coats, A. J. Contribution of peripheral chemoreceptors to ventilation and the effects of their suppression on exercise tolerance in chronic heart failure. *Heart* (1996); **76**(6): 483-489.

CIBIS-II. The Cardiac Insufficiency Bisoprolol Study II (CIBIS-II): a randomised trial. *Lancet* (1999); **353**(9146): 9-13.

Clapham, J. C., Arch, J. R., Chapman, H., Haynes, A., Lister, C., Moore, G. B., Piercy, V., Carter, S. A., Lehner, I., Smith, S. A., Beeley, L. J., Godden, R. J., Herrity, N., Skehel, M., Changani, K. K., Hockings, P. D., Reid, D. G., Squires, S. M., Hatcher, J., Trail, B., Latcham, J., Rastan, S., Harper, A. J., Cadenas, S., Buckingham, J. A., Brand, M. D. and Abuin, A. Mice overexpressing human uncoupling protein-3 in skeletal muscle are hyperphagic and lean. *Nature* (2000); **406**(6794): 415-418.

Clark, A., Volterrani, M., Swan, J. W., Hue, D., Hooper, J. and Coats, A. J. Leg blood flow, metabolism and exercise capacity in chronic stable heart failure. *Int J Cardiol* (1996); **55**(2): 127-135.

Clark, A. L. and Coats, A. J. Usefulness of arterial blood gas estimations during exercise in patients with chronic heart failure. *Br Heart J* (1994); **71**(6): 528-530.

Clark, A. L., Poole-Wilson, P. A. and Coats, A. J. Relation between ventilation and carbon dioxide production in patients with chronic heart failure. *J Am Coll Cardiol* (1992); **20**(6): 1326-1332.

Clark, A. L., Poole-Wilson, P. A. and Coats, A. J. Exercise limitation in chronic heart failure: central role of the periphery. *J Am Coll Cardiol* (1996); **28**(5): 1092-1102.

Clausmeyer, S., Sturzebecher, R. and Peters, J. An alternative transcript of the rat renin gene can result in a truncated prorenin that is transported into adrenal mitochondria. *Circ Res* (1999); **84**(3): 337-344.

Cleeter, M. W., Cooper, J. M., Darley-Usmar, V. M., Moncada, S. and Schapira, A. H. Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. *FEBS Lett* (1994); **345**(1): 50-54.

Clementi, E., Brown, G. C., Foxwell, N. and Moncada, S. On the mechanism by which vascular endothelial cells regulate their oxygen consumption. *Proc Natl Acad Sci U S A* (1999); **96**(4): 1559-1562.

Cline, G. W., Vidal-Puig, A. J., Dufour, S., Cadman, K. S., Lowell, B. B. and Shulman, G. I. In vivo effects of uncoupling protein-3 gene disruption on mitochondrial energy metabolism. *J Biol Chem* (2001); **276**(23): 20240-20244.

Cohen, L. B. and Salzberg, B. M. Optical measurement of membrane potential. *Rev Physiol Biochem Pharmacol* (1978); **83**: 35-88.

Cohn, J. N. and Tognoni, G. A randomized trial of the angiotensin-receptor blocker valsartan in chronic heart failure. *N Engl J Med* (2001); **345**(23): 1667-1675.

Colhoun, H. M., McKeigue, P. M. and Davey Smith, G. Problems of reporting genetic associations with complex outcomes. *Lancet* (2003); **361**(9360): 865-872.

Collins, F. S., Guyer, M. S. and Charkravarti, A. Variations on a theme: cataloging human DNA sequence variation. *Science* (1997); **278**(5343): 1580-1581.

Collins, T. J., Lipp, P., Berridge, M. J., Li, W. and Bootman, M. D. Inositol 1,4,5-trisphosphate-induced Ca2+ release is inhibited by mitochondrial depolarization. *Biochem J* (2000); **347**(Pt 2): 593-600.

Colman, E. Dinitrophenol and obesity: an early twentieth-century regulatory dilemma. *Regul Toxicol Pharmacol* (2007); **48**(2): 115-117.

Colman, R. W., White, J. V., Scovell, S., Stadnicki, A. and Sartor, R. B. Kininogens are antithrombotic proteins In vivo. *Arterioscler Thromb Vasc Biol* (1999); **19**(9): 2245-2250.

Communal, C., Singh, K., Pimentel, D. R. and Colucci, W. S. Norepinephrine stimulates apoptosis in adult rat ventricular myocytes by activation of the beta-adrenergic pathway. *Circulation* (1998); **98**(13): 1329-1334.

Conrad, M., Jakupoglu, C., Moreno, S. G., Lippl, S., Banjac, A., Schneider, M., Beck, H., Hatzopoulos, A. K., Just, U., Sinowatz, F., Schmahl, W., Chien, K. R., Wurst, W., Bornkamm, G. W. and Brielmeier, M. Essential role for mitochondrial thioredoxin reductase in hematopoiesis, heart development, and heart function. *Mol Cell Biol* (2004); **24**(21): 9414-9423.

CONSENSUS Trial Study Group. Effects of enalapril on mortality in severe congestive heart failure. Results of the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS). *N Engl J Med* (1987); **316**(23): 1429-1435.

Conway, M. A., Allis, J., Ouwerkerk, R., Niioka, T., Rajagopalan, B. and Radda, G. K. Detection of low phosphocreatine to ATP ratio in failing hypertrophied human myocardium by 31P magnetic resonance spectroscopy. *Lancet* (1991); **338**(8773): 973-976.

Cooke, J. P. and Dzau, V. J. Derangements of the nitric oxide synthase pathway, L-arginine, and cardiovascular diseases. *Circulation* (1997); **96**(2): 379-382.

Cortright, R. N., Zheng, D., Jones, J. P., Fluckey, J. D., DiCarlo, S. E., Grujic, D., Lowell, B. B. and Dohm, G. L. Regulation of skeletal muscle UCP-2 and UCP-3 gene expression by exercise and denervation. *Am J Physiol* (1999); **276**(1 Pt 1): E217-221.

Cossarizza, A., Baccarani-Contri, M., Kalashnikova, G. and Franceschi, C. A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1). *Biochem Biophys Res Commun* (1993); **197**(1): 40-45.

Cossarizza, A., Ceccarelli, D. and Masini, A. Functional heterogeneity of an isolated mitochondrial population revealed by cytofluorometric analysis at the single organelle level. *Exp Cell Res* (1996); **222**(1): 84-94.

Costello, A., Gray, S. and Donnelly, R. Effects of rosiglitazone and oleic acid on UCP-3 expression in L6 myotubes. *Diabetes Obes Metab* (2003); **5**(2): 136-138.

Costerousse, O., Allegrini, J., Lopez, M. and Alhenc-Gelas, F. Angiotensin I-converting enzyme in human circulating mononuclear cells: genetic polymorphism of expression in T-lymphocytes. *Biochem J* (1993); **290**(Pt 1): 33-40.

Cotton, J. M., Kearney, M. T., MacCarthy, P. A., Grocott-Mason, R. M., McClean, D. R., Heymes, C., Richardson, P. J. and Shah, A. M. Effects of nitric oxide synthase inhibition on Basal function and the force-frequency relationship in the normal and failing human heart in vivo. *Circulation* (2001); **104**(19): 2318-2323.

Couplan, E., del Mar Gonzalez-Barroso, M., Alves-Guerra, M. C., Ricquier, D., Goubern, M. and Bouillaud, F. No evidence for a basal, retinoic, or superoxide-induced uncoupling activity of the uncoupling protein 2 present in spleen or lung mitochondria. *J Biol Chem* (2002); **277**(29): 26268-26275.

Cowie, M. R., Wood, D. A., Coats, A. J., Thompson, S. G., Suresh, V., Poole-Wilson, P. A. and Sutton, G. C. Survival of patients with a new diagnosis of heart failure: a population based study. *Heart* (2000); **83**(5): 505-510.

Cox, R., Bouzekri, N., Martin, S., Southam, L., Hugill, A., Golamaully, M., Cooper, R., Adeyemo, A., Soubrier, F., Ward, R., Lathrop, G. M., Matsuda, F. and Farrall, M. Angiotensin-1-converting enzyme (ACE) plasma concentration is influenced by multiple ACE-linked quantitative trait nucleotides. *Hum Mol Genet* (2002); **11**(23): 2969-2977.

Coyle, E. F., Sidossis, L. S., Horowitz, J. F. and Beltz, J. D. Cycling efficiency is related to the percentage of type I muscle fibers. *Med Sci Sports Exerc* (1992); **24**(7): 782-788.

Crackower, M. A., Sarao, R., Oudit, G. Y., Yagil, C., Kozieradzki, I., Scanga, S. E., Oliveira-dos-Santos, A. J., da Costa, J., Zhang, L., Pei, Y., Scholey, J., Ferrario, C. M., Manoukian, A. S., Chappell, M. C., Backx, P. H., Yagil, Y. and Penninger, J. M. Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature* (2002); **417**(6891): 822-828.

Curtin, N. A., Clapham, J. C. and Barclay, C. J. Excess recovery heat production by isolated muscles from mice overexpressing uncoupling protein-3. *J Physiol* (2002); **542**(Pt 1): 231-235.

Cushman, D. W. and Cheung, H. S. Concentrations of angiotensin-converting enzyme in tissues of the rat. *Biochim Biophys Acta* (1971); **250**(1): 261-265.

Cusin, I., Rouru, J., Visser, T., Burger, A. G. and Rohner-Jeanrenaud, F. Involvement of thyroid hormones in the effect of intracerebroventricular leptin infusion on uncoupling protein-3 expression in rat muscle. *Diabetes* (2000); **49**(7): 1101-1105.

Cutting, W. C. and Tainter, M. L. Actions of dinitrophenol. *Proc Soc Exper Biol Med* (1932); **29**: 1268-1269.

Dahlof, B., Devereux, R. B., Kjeldsen, S. E., Julius, S., Beevers, G., Faire, U., Fyhrquist, F., Ibsen, H., Kristiansson, K., Lederballe-Pedersen, O., Lindholm, L. H., Nieminen, M. S., Omvik, P., Oparil, S. and Wedel, H. Cardiovascular morbidity and mortality in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. *Lancet* (2002); **359**(9311): 995-1003.

Dalgaard, L. T., Sorensen, T. I., Andersen, T., Hansen, T. and Pedersen, O. An untranslated insertion variant in the uncoupling protein 2 gene is not related to body mass index and changes in body weight during a 26- year follow-up in Danish Caucasian men. *Diabetologia* (1999); **42**(12): 1413-1416.

Dalgaard, L. T., Sorensen, T. I., Drivsholm, T., Borch-Johnsen, K., Andersen, T., Hansen, T. and Pedersen, O. A prevalent polymorphism in the promoter of the UCP3 gene and its relationship to body mass index and long term body weight change in the Danish population. *J Clin Endocrinol Metab* (2001); **86**(3): 1398-1402.

Danesh, J., Collins, R. and Peto, R. Lipoprotein(a) and coronary heart disease. Meta-analysis of prospective studies. *Circulation* (2000); **102**(10): 1082-1085.

Danesh, J., Lewington, S., Thompson, S. G., Lowe, G. D., Collins, R., Kostis, J. B., Wilson, A. C., Folsom, A. R., Wu, K., Benderly, M., Goldbourt, U., Willeit, J., Kiechl, S., Yarnell, J. W., Sweetnam, P. M., Elwood, P. C., Cushman, M., Psaty, B. M., Tracy, R. P., Tybjaerg-Hansen, A., Haverkate, F., de Maat, M. P., Fowkes, F. G., Lee, A. J., Smith, F. B., Salomaa, V., Harald, K., Rasi, R., Vahtera, E., Jousilahti, P., Pekkanen, J., D'Agostino, R., Kannel, W. B., Wilson, P. W., Tofler, G., Arocha-Pinango, C. L., Rodriguez-Larralde, A., Nagy, E., Mijares, M., Espinosa, R., Rodriquez-Roa, E., Ryder, E., Diez-Ewald, M. P., Campos, G., Fernandez, V., Torres, E., Coll, E., Marchioli, R., Valagussa, F., Rosengren, A., Wilhelmsen, L., Lappas, G., Eriksson, H., Cremer, P., Nagel, D., Curb, J. D., Rodriguez, B., Yano, K., Salonen, J. T., Nyyssonen, K., Tuomainen, T. P., Hedblad, B., Lind, P., Loewel, H., Koenig, W., Meade, T. W., Cooper, J. A., De Stavola, B., Knottenbelt, C., Miller, G. J., Bauer, K. A., Rosenberg, R. D., Sato, S., Kitamura, A., Naito, Y., Iso, H., Rasi, V., Palosuo, T., Ducimetiere, P., Amouyel, P., Arveiler, D., Evans, A. E., Ferrieres, J., Juhan-Vague, I., Bingham, A., Schulte, H., Assmann, G., Cantin, B., Lamarche, B., Despres, J. P., Dagenais, G. R., Tunstall-Pedoe, H., Woodward, M., Ben-Shlomo, Y., Davey Smith, G., Palmieri, V., Yeh, J. L., Rudnicka, A., Ridker, P., Rodeghiero, F., Tosetto, A., Shepherd, J., Ford, I., Robertson, M., Brunner, E., Shipley, M., Feskens, E. J. and Kromhout, D. Plasma fibrinogen level and the risk of major cardiovascular diseases and nonvascular mortality: an individual participant meta-analysis. *Jama* (2005); **294**(14): 1799-1809.

Danser, A. H. Local renin-angiotensin systems. *Mol Cell Biochem* (1996); **157**(1-2): 211-216.

Danser, A. H., Saris, J. J., Schuijt, M. P. and van Kats, J. P. Is there a local renin-angiotensin system in the heart? *Cardiovasc Res* (1999); **44**(2): 252-265.

Danser, A. H., Schalekamp, M. A., Bax, W. A., van den Brink, A. M., Saxena, P. R., Riegger, G. A. and Schunkert, H. Angiotensin-converting enzyme in the human heart. Effect of the deletion/insertion polymorphism. *Circulation* (1995); **92**(6): 1387-1388.

Dasarathy, Y., Lanzillo, J. J. and Fanburg, B. L. Stimulation of bovine pulmonary artery endothelial cell ACE by dexamethasone: involvement of steroid receptors. *Am J Physiol* (1992); **263**(6 Pt 1): L645-649.

Davies, C. H., Davia, K., Bennett, J. G., Pepper, J. R., Poole-Wilson, P. A. and Harding, S. E. Reduced contraction and altered frequency response of isolated ventricular myocytes from patients with heart failure. *Circulation* (1995); **92**(9): 2540-2549.

Davies, M. J., Bland, J. M., Hangartner, J. R., Angelini, A. and Thomas, A. C. Factors influencing the presence or absence of acute coronary artery thrombi in sudden ischaemic death. *Eur Heart J* (1989); **10**(3): 203-208.

Day, I. N., Humphries, S. E., Richards, S., Norton, D. and Reid, M. High-throughput genotyping using horizontal polyacrylamide gels with wells arranged for microplate array diagonal gel electrophoresis (MADGE). *Biotechniques* (1995); **19**(5): 830-835.

de Beer, F. C., Soutar, A. K., Baltz, M. L., Trayner, I. M., Feinstein, A. and Pepys, M. B. Low density lipoprotein and very low density lipoprotein are selectively bound by aggregated C-reactive protein. *J Exp Med* (1982); **156**(1): 230-242.

de Belder, A. J., Radomski, M. W., Why, H. J., Richardson, P. J., Bucknall, C. A., Salas, E., Martin, J. F. and Moncada, S. Nitric oxide synthase activities in human myocardium. *Lancet* (1993); **341**(8837): 84-85.

De Sousa, E., Veksler, V., Bigard, X., Mateo, P. and Ventura-Clapier, R. Heart failure affects mitochondrial but not myofibrillar intrinsic properties of skeletal muscle. *Circulation* (2000); **102**(15): 1847-1853.

Decking, U. K., Williams, J. P., Dahmann, R., Stumpe, T., Kelm, M. and Schrader, J. The nitric oxide-induced reduction in cardiac energy supply is not due to inhibition of creatine kinase. *Cardiovasc Res* (2001); **51**(2): 313-321.

Delles, C., Zimmerli, L. U., McGrane, D. J., Koh-Tan, C. H., Pathi, V. L., McKay, A. J., Steedman, T., Dargie, H. J., Hamilton, C. A. and Dominiczak, A. F. Vascular stiffness is related to superoxide generation in the vessel wall. *J Hypertens* (2008); **26**(5): 946-955.

Depierre, D. and Roth, M. Fluorimetric determination of dipeptidyl carboxypeptidase. (angiotensin-I-converting enzyme). *Enzyme* (1975); **19**(2): 65-70.

Depre, C., Young, M. E., Ying, J., Ahuja, H. S., Han, Q., Garza, N., Davies, P. J. and Taegtmeyer, H. Streptozotocin-induced changes in cardiac gene expression in the absence of severe contractile dysfunction. *J Mol Cell Cardiol* (2000); **32**(6): 985-996.

Dhamrait, S. S., Payne, J. R., Li, P., Jones, A., Toor, I. S., Cooper, J. A., Hawe, E., Palmen, J. M., Wootton, P. T., Miller, G. J., Humphries, S. E. and Montgomery, H. E. Variation in bradykinin receptor genes increases the cardiovascular risk associated with hypertension. *Eur Heart J* (2003); **24**(18): 1672-1680.

Dhamrait, S. S., Stephens, J. W., Cooper, J. A., Acharya, J., Mani, A. R., Moore, K., Miller, G. J., Humphries, S. E., Hurel, S. J. and Montgomery, H. E. Cardiovascular risk in healthy men and

markers of oxidative stress in diabetic men are associated with common variation in the gene for uncoupling protein 2. *Eur Heart J* (2004); **25**(6): 468-475.

Di Lisa, F., Blank, P. S., Colonna, R., Gambassi, G., Silverman, H. S., Stern, M. D. and Hansford, R. G. Mitochondrial membrane potential in single living adult rat cardiac myocytes exposed to anoxia or metabolic inhibition. *J Physiol (Lond)* (1995); **486**(Pt 1): 1-13.

Diaz, G., Falchi, A. M., Gremo, F., Isola, R. and Diana, A. Homogeneous longitudinal profiles and synchronous fluctuations of mitochondrial transmembrane potential. *FEBS Lett* (2000); **475**(3): 218-224.

Divisova, J., Vavrinkova, H., Tutterova, M., Kazdova, L. and Meschisvili, E. Effect of ACE inhibitor captopril and L-arginine on the metabolism and on ischemia-reperfusion injury of the isolated rat heart. *Physiol Rev* (2001); **50**(2): 143-152.

Djouadi, F., Weinheimer, C. J., Saffitz, J. E., Pitchford, C., Bastin, J., Gonzalez, F. J. and Kelly, D. P. A gender-related defect in lipid metabolism and glucose homeostasis in peroxisome proliferator-activated receptor alpha- deficient mice. *J Clin Invest* (1998); **102**(6): 1083-1091.

Do, M. S., Kim, J. B., Yoon, T. J., Park, C. H., Rayner, D. V. and Trayhurn, P. Induction of uncoupling protein-2 (UCP2) gene expression on the differentiation of rat preadipocytes to adipocytes in primary culture. *Mol Cells* (1999); **9**(1): 20-24.

Donoghue, M., Hsieh, F., Baronas, E., Godbout, K., Gosselin, M., Stagliano, N., Donovan, M., Woolf, B., Robison, K., Jeyaseelan, R., Breitbart, R. E. and Acton, S. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res* (2000); **87**(5): E1-9.

Doris, P. A. Hypertension genetics, single nucleotide polymorphisms, and the common disease:common variant hypothesis. *Hypertension* (2002); **39**(2 Pt 2): 323-331.

Downs, J. R., Clearfield, M., Weis, S., Whitney, E., Shapiro, D. R., Beere, P. A., Langendorfer, A., Stein, E. A., Kruyer, W. and Gotto, A. M., Jr. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study. *Jama* (1998); **279**(20): 1615-1622.

Doyle, J. T., Dawber, T. R., Kannel, W. B., Heslin, A. S. and Kahn, H. A. Cigarette smoking and coronary heart disease. Combined experience of the Albany and Framingham studies. *N Engl J Med* (1962); **26**: 796-801.

Drexler, H. Nitric oxide synthases in the failing human heart: a doubled-edged sword? *Circulation* (1999); **99**(23): 2972-2975.

Drexler, H., Banhardt, U., Meinertz, T., Wollschlager, H., Lehmann, M. and Just, H. Contrasting peripheral short-term and long-term effects of converting enzyme inhibition in patients with congestive heart failure. A double-blind, placebo-controlled trial. *Circulation* (1989); **79**(3): 491-502.

Drexler, H., Hiroi, M., Riede, U., Banhardt, U., Meinertz, T. and Just, H. Skeletal muscle blood flow, metabolism and morphology in chronic congestive heart failure and effects of short- and long-term angiotensin-converting enzyme inhibition. *Am J Cardiol* (1988); **62**(8): 82E-85E.

Drexler, H., Kastner, S., Strobel, A., Studer, R., Brodde, O. E. and Hasenfuss, G. Expression, activity and functional significance of inducible nitric oxide synthase in the failing human heart. *J Am Coll Cardiol* (1998); **32**(4): 955-963.

Drexler, H., Riede, U., Munzel, T., Konig, H., Funke, E. and Just, H. Alterations of skeletal muscle in chronic heart failure. *Circulation* (1992); **85**(5): 1751-1759.

Duchen, M. R., Leyssens, A. and Crompton, M. Transient mitochondrial depolarizations reflect focal sarcoplasmic reticular calcium release in single rat cardiomyocytes. *J Cell Biol* (1998); **142**(4): 975-988.

Duchen, M. R., Surin, A. and Jacobson, J. Imaging mitochondrial function in intact cells. *Methods Enzymol* (2003); **361**: 353-389.

Duranteau, J., Chandel, N. S., Kulisz, A., Shao, Z. and Schumacker, P. T. Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes. *J Biol Chem* (1998); **273**(19): 11619-11624.

Duval, C., Negre-Salvayre, A., Dogilo, A., Salvayre, R., Penicaud, L. and Casteilla, L. Increased reactive oxygen species production with antisense oligonucleotides directed against uncoupling protein 2 in murine endothelial cells. *Biochem Cell Biol* (2002); **80**(6): 757-764.

Dzau, V. J. Local expression and pathophysiological role of renin-angiotensin in the blood vessels and heart. *Basic Res Cardiol* (1993); **88 Suppl 1**: 1-14.

Dzau, V. J., Bernstein, K., Celermajer, D., Cohen, J., Dahlof, B., Deanfield, J., Diez, J., Drexler, H., Ferrari, R., van Gilst, W., Hansson, L., Hornig, B., Husain, A., Johnston, C., Lazar, H., Lonn, E., Luscher, T., Mancini, J., Mimran, A., Pepine, C., Rabelink, T., Remme, W., Ruilope, L., Ruzicka, M., Schunkert, H., Swedberg, K., Unger, T., Vaughan, D. and Weber, M. The relevance of tissue angiotensin-converting enzyme: manifestations in mechanistic and endpoint data. *Am J Cardiol* (2001); **88**(9 Suppl): 1L-20L.

Echtay, K. S., Murphy, M. P., Smith, R. A., Talbot, D. A. and Brand, M. D. Superoxide activates mitochondrial uncoupling protein 2 from the matrix side. Studies using targeted antioxidants. *J Biol Chem* (2002); **277**(49): 47129-47135.

Echtay, K. S., Roussel, D., St-Pierre, J., Jekabsons, M. B., Cadenas, S., Stuart, J. A., Harper, J. A., Roebuck, S. J., Morrison, A., Pickering, S., Clapham, J. C. and Brand, M. D. Superoxide activates mitochondrial uncoupling proteins. *Nature* (2002); **415**(6867): 96-99.

Echtay, K. S., Winkler, E., Frischmuth, K. and Klingenberg, M. Uncoupling proteins 2 and 3 are highly active H(+) transporters and highly nucleotide sensitive when activated by coenzyme Q (ubiquinone). *Proc Natl Acad Sci U S A* (2001); **98**(4): 1416-1421.

Ehara, S., Ueda, M., Naruko, T., Haze, K., Itoh, A., Otsuka, M., Komatsu, R., Matsuo, T., Itabe, H., Takano, T., Tsukamoto, Y., Yoshiyama, M., Takeuchi, K., Yoshikawa, J. and Becker, A. E. Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. *Circulation* (2001); **103**(15): 1955-60.

Ehrenberg, B., Montana, V., Wei, M. D., Wuskell, J. P. and Loew, L. M. Membrane potential can be determined in individual cells from the nernstian distribution of cationic dyes. *Biophys J* (1988); **53**(5): 785-794.

Eklund, A., Blaschke, E. and Danielsson, B. Subcellular localization of angiotensin-converting enzyme in the human alveolar macrophage. *Scand J Clin Lab Invest* (1987); **47**(1): 47-54.

Emanueli, C., Maestri, R., Corradi, D., Marchione, R., Minasi, A., Tozzi, M. G., Salis, M. B., Straino, S., Capogrossi, M. C., Olivetti, G. and Madeddu, P. Dilated and failing cardiomyopathy in bradykinin B(2) receptor knockout mice. *Circulation* (1999); **100**(23): 2359-2365.

Emaus, R. K., Grunwald, R. and Lemasters, J. J. Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectral and metabolic properties. *Biochim Biophys Acta* (1986); **850**(3): 436-448.

Enerback, S., Jacobsson, A., Simpson, E. M., Guerra, C., Yamashita, H., Harper, M. E. and Kozak, L. P. Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. *Nature* (1997); **387**(6628): 90-94.

Erdmann, B., Fuxe, K. and Ganten, D. Subcellular localization of angiotensin II immunoreactivity in the rat cerebellar cortex. *Hypertension* (1996); **28**(5): 818-824.

Essop, M. F., Razeghi, P., McLeod, C., Young, M. E., Taegtmeyer, H. and Sack, M. N. Hypoxia-induced decrease of UCP3 gene expression in rat heart parallels metabolic gene switching but fails to affect mitochondrial respiratory coupling. *Biochem Biophys Res Commun* (2004); **314**(2): 561-564.

Esterbauer, H., Oberkofler, H., Krempler, F., Strosberg, A. D. and Patsch, W. The Uncoupling Protein-3 (UCP3) Gene is Transcribed from Tissue- Specific Promoters in Humans, but not in Rodents. *J Biol Chem* (2000).

Esterbauer, H., Schneitler, C., Oberkofler, H., Ebenbichler, C., Paulweber, B., Sandhofer, F., Ladurner, G., Hell, E., Strosberg, A. D., Patsch, J. R., Krempler, F. and Patsch, W. A common polymorphism in the promoter of UCP2 is associated with decreased risk of obesity in middle-aged humans. *Nat Genet* (2001); **28**(2): 178-183.

Evans, A. E., Poirier, O., Kee, F., Lecerf, L., McCrum, E., Falconer, T., Crane, J., O'Rourke, D. F. and Cambien, F. Polymorphisms of the angiotensin-converting-enzyme gene in subjects who die from coronary heart disease. *Q J Med* (1994); **87**(4): 211-4.

Evans, J. L., Goldfine, I. D., Maddux, B. A. and Grodsky, G. M. Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. *Endocr Rev* (2002); **23**(5): 599-622.

Falk, E. Plaque rupture with severe pre-existing stenosis precipitating coronary thrombosis. Characteristics of coronary atherosclerotic plaques underlying fatal occlusive thrombi. *Br Heart J* (1983); **50**(2): 127-134.

Farb, A., Burke, A. P., Tang, A. L., Liang, T. Y., Mannan, P., Smialek, J. and Virmani, R. Coronary plaque erosion without rupture into a lipid core. A frequent cause of coronary thrombosis in sudden coronary death. *Circulation* (1996); **93**(7): 1354-1363.

Farkas, D. L., Wei, M. D., Febbroriello, P., Carson, J. H. and Loew, L. M. Simultaneous imaging of cell and mitochondrial membrane potentials. *Biophys J* (1989); **56**(6): 1053-1069.

Farmer, J. A. and Torre-Amione, G. Atherosclerosis and inflammation. *Curr Atheroscler Rep* (2002); **4**(2): 92-98.

Faussner, A., Bathon, J. M. and Proud, D. Comparison of the responses of B1 and B2 kinin receptors to agonist stimulation. *Immunopharmacology* (1999); **45**(1-3): 13-20.

Feinleib, M., Garrison, R. J., Fabsitz, R., Christian, J. C., Hrubec, Z., Borhani, N. O., Kannel, W. B., Rosenman, R., Schwartz, J. T. and Wagner, J. O. The NHLBI twin study of cardiovascular disease risk factors: methodology and summary of results. *Am J Epidemiol* (1977); **106**(4): 284-285.

Feitosa, M. F., Gaskill, S. E., Rice, T., Rankinen, T., Bouchard, C., Rao, D. C., Wilmore, J. H., Skinner, J. S. and Leon, A. S. Major gene effects on exercise ventilatory threshold: the HERITAGE Family Study. *J Appl Physiol* (2002); **93**(3): 1000-1006.

Ferder, L., Inserra, F., Romano, L., Ercole, L. and Pszenny, V. Effects of angiotensin-converting enzyme inhibition on mitochondrial number in the aging mouse. *Am J Physiol* (1993); **265**(1 Pt 1): C15-18.

Ferder, L., Romano, L. A., Ercole, L. B., Stella, I. and Inserra, F. Biomolecular changes in the aging myocardium: the effect of enalapril. *Am J Hypertens* (1998); **11**(11 Pt 1): 1297-1304.

Fernstrom, M., Tonkonogi, M. and Sahlin, K. Effects of acute and chronic endurance exercise on mitochondrial uncoupling in human skeletal muscle. *J Physiol* (2004); **554**(Pt 3): 755-763.

Ferraro, B., Galli, F., Frei, B., Kingdon, E., Canestrari, F., Rice-Evans, C., Buoncristiani, U., Davenport, A. and Moore, K. P. Peroxynitrite-induced oxidation of plasma lipids is enhanced in stable hemodialysis patients. *Kidney Int* (2003); **63**(6): 2207-13.

Figueroa, C. D., Dietze, G. and Muller-Esterl, W. Immunolocalization of bradykinin B2 receptors on skeletal muscle cells. *Diabetes* (1996); **45 Suppl 1**: S24-28.

Fink, L. I., Wilson, J. R. and Ferraro, N. Exercise ventilation and pulmonary artery wedge pressure in chronic stable congestive heart failure. *Am J Cardiol* (1986); **57**(4): 249-253.

Finkel, T. Signal transduction by reactive oxygen species in non-phagocytic cells. *J Leukoc Biol* (1999); **65**(3): 337-340.

Flather, M. D., Yusuf, S., Kober, L., Pfeffer, M., Hall, A., Murray, G., Torp-Pedersen, C., Ball, S., Pogue, J., Moye, L. and Braunwald, E. Long-term ACE-inhibitor therapy in patients with heart failure or left-ventricular dysfunction: a systematic overview of data from individual patients. ACE-Inhibitor Myocardial Infarction Collaborative Group. *Lancet* (2000); **355**(9215): 1575-1581.

Fleury, C., Neverova, M., Collins, S., Raimbault, S., Champigny, O., Levi-Meyrueis, C., Bouillaud, F., Seldin, M. F., Surwit, R. S., Ricquier, D. and Warden, C. H. Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. *Nat Genet* (1997); **15**(3): 269-272.

Floryk, D. and Houstek, J. Tetramethyl rhodamine methyl ester (TMRM) is suitable for cytofluorometric measurements of mitochondrial membrane potential in cells treated with digitonin. *Biosci Rep* (1999); **19**(1): 27-34.

Folland, J., Leach, B., Little, T., Hawker, K., Myerson, S., Montgomery, H. and Jones, D. Angiotensin-converting enzyme genotype affects the response of human skeletal muscle to functional overload. *Exp Physiol* (2000); **85**(5): 575-579.

Fonarow, G. C., Chelimsky-Fallick, C., Stevenson, L. W., Luu, M., Hamilton, M. A., Moriguchi, J. D., Tillisch, J. H., Walden, J. A. and Albanese, E. Effect of direct vasodilation with hydralazine versus angiotensin-converting enzyme inhibition with captopril on mortality in advanced heart failure: the Hy-C trial. *J Am Coll Cardiol* (1992); **19**(4): 842-850.

Foster, P. S. The role of phosphoinositide metabolism in Ca2+ signalling of skeletal muscle cells. *Int J Biochem* (1994); **26**(4): 449-468.

Fox, K. M. and Investigators, E. Efficacy of perindopril in reduction of cardiovascular events among patients with stable coronary artery disease: randomised, double-blind, placebo-controlled, multicentre trial (the EUROPA study). *Lancet* (2003); **362**(9386): 782-788.

Franciosa, J. A., Leddy, C. L., Wilen, M. and Schwartz, D. E. Relation between hemodynamic and ventilatory responses in determining exercise capacity in severe congestive heart failure. *Am J Cardiol* (1984); **53**(1): 127-134.

Francis, G. S. Pathophysiology of chronic heart failure. *Am J Med* (2001); **110 Suppl 7A**: 37S-46S.

Freyssenet, D., Berthon, P. and Denis, C. Mitochondrial biogenesis in skeletal muscle in response to endurance exercises. *Arch Physiol Biochem* (1996); **104**(2): 129-141.

Fukunaga, Y., Itoh, H., Hosoda, K., Doi, K., Matsuda, J., Son, C., Yamashita, J., Chun, T. H., Tanaka, T., Inoue, M., Masatsugu, K., Saito, T., Sawada, N. and Nakao, K. Altered gene expression of uncoupling protein-2 and -3 in stroke-prone spontaneously hypertensive rats. *J Hypertens* (2000); **18**(9): 1233-1238.

Furchgott, R. F. and Zawadski, J. V. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle ny acetylcholine. *Nature* (1980); **288**: 373-376.

Fusegawa, Y., Goto, S., Handa, S., Kawada, T. and Ando, Y. Platelet spontaneous aggregation in platelet-rich plasma is increased in habitual smokers. *Thromb Res* (1999); **93**(6): 271-278.

Gabriel, S. B., Schaffner, S. F., Nguyen, H., Moore, J. M., Roy, J., Blumenstiel, B., Higgins, J., DeFelice, M., Lochner, A., Faggart, M., Liu-Cordero, S. N., Rotimi, C., Adeyemo, A., Cooper, R., Ward, R., Lander, E. S., Daly, M. J. and Altshuler, D. The structure of haplotype blocks in the human genome. *Science* (2002); **296**(5576): 2225-2229.

Gainer, J. V., Brown, N. J., Bachvarova, M., Bastien, L., Maltais, I., Marceau, F. and Bachvarov, D. R. Altered frequency of a promoter polymorphism of the kinin B2 receptor gene in hypertensive African-Americans. *Am J Hypertens* (2000); **13**(12): 1268-1273.

Gambassi, G., Lapane, K. L., Sgadari, A., Carbonin, P., Gatsonis, C., Lipsitz, L. A., Mor, V. and Bernabei, R. Effects of angiotensin-converting enzyme inhibitors and digoxin on health outcomes of very old patients with heart failure. SAGE Study Group. Systematic Assessment of Geriatric drug use via Epidemiology. *Arch Intern Med* (2000); **160**(1): 53-60.

Garcia, M. J., McNamara, P. M., Gordon, T. and Kannel, W. B. Morbidity and mortality in diabetics in the Framingham population. Sixteen year follow-up study. *Diabetes* (1974); **23**(2): 105-111.

Garrison, R. J., Wilson, P. W., Castelli, W. P., Feinleib, M., Kannel, W. B. and McNamara, P. M. Obesity and lipoprotein cholesterol in the Framingham offspring study. *Metabolism* (1980); **29**(11): 1053-1060.

Gaussin, V., Tomlinson, J. E., Depre, C., Engelhardt, S., Antos, C. L., Takagi, G., Hein, L., Topper, J. N., Liggett, S. B., Olson, E. N., Lohse, M. J., Vatner, S. F. and Vatner, D. E. Common genomic response in different mouse models of beta-adrenergic-induced cardiomyopathy. *Circulation* (2003); **108**(23): 2926-2933.

Gayagay, G., Yu, B., Hambly, B., Boston, T., Hahn, A., Celermajer, D. S. and Trent, R. J. Elite endurance athletes and the ACE I allele--the role of genes in athletic performance. *Hum Genet* (1998); **103**(1): 48-50.

Geng, Y., Hansson, G. K. and Holme, E. Interferon-gamma and tumor necrosis factor synergize to induce nitric oxide production and inhibit mitochondrial respiration in vascular smooth muscle cells. *Circ Res* (1992); **71**(5): 1268-1276.

Gertz, E. W., Wisneski, J. A., Stanley, W. C. and Neese, R. A. Myocardial substrate utilization during exercise in humans. Dual carbon-labeled carbohydrate isotope experiments. *J Clin Invest* (1988); **82**(6): 2017-2025.

Gimeno, R. E., Dembski, M., Weng, X., Deng, N., Shyjan, A. W., Gimeno, C. J., Iris, F., Ellis, S. J., Woolf, E. A. and Tartaglia, L. A. Cloning and characterization of an uncoupling protein homolog: a potential molecular mediator of human thermogenesis. *Diabetes* (1997); **46**(5): 900-906.

Gohlke, P., Linz, W., Scholkens, B. A., Kuwer, I., Bartenbach, S., Schnell, A. and Unger, T. Angiotensin-converting enzyme inhibition improves cardiac function. Role of bradykinin. *Hypertension* (1994); **23**(4): 411-418.

Gohlke, P., Pees, C. and Unger, T. AT2 receptor stimulation increases aortic cyclic GMP in SHRSP by a kinin-dependent mechanism. *Hypertension* (1998); **31**(1 Pt 2): 349-355.

Goldman, J., Pfister, D. and Vulmirovich, R. Potentiation of insulin stimulation of hexose transport by kallikrein and bradykinin in isolated rat adipocytes. *Mol Cell Endocrinol* (1987); **50**: 183-191.

Gomez-Ambrosi, J., Fruhbeck, G. and Martinez, J. A. Leptin, but not a beta 3-adrenergic agonist, upregulates muscle uncoupling protein-3 messenger RNA expression: short-term thermogenic interactions. *Cell Mol Life Sci* (1999); **55**(6-7): 992-997.

Gomez-Sanchez, C. E., Zhou, M. Y., Cozza, E. N., Morita, H., Foecking, M. F. and Gomez-Sanchez, E. P. Aldosterone biosynthesis in the rat brain. *Endocrinology* (1997); **138**(8): 3369-3373.

Gong, D. W., He, Y., Karas, M. and Reitman, M. Uncoupling protein-3 is a mediator of thermogenesis regulated by thyroid hormone, beta3-adrenergic agonists, and leptin. *J Biol Chem* (1997); **272**(39): 24129-24132.

Gong, D. W., He, Y. and Reitman, M. L. Genomic organization and regulation by dietary fat of the uncoupling protein 3 and 2 genes. *Biochem Biophys Res Commun* (1999); **256**(1): 27-32.

Gong, D. W., Monemdjou, S., Gavrilova, O., Leon, L. R., Marcus-Samuels, B., Chou, C. J., Everett, C., Kozak, L. P., Li, C., Deng, C., Harper, M. E. and Reitman, M. L. Lack of obesity and normal response to fasting and thyroid hormone in mice lacking uncoupling protein-3. *J Biol Chem* (2000); **275**(21): 16251-16257.

Gordon, S. E., Davis, B. S., Carlson, C. J. and Booth, F. W. ANG II is required for optimal overload-induced skeletal muscle hypertrophy. *Am J Physiol Endocrinol Metab* (2001); **280**(1): E150-9.

Gosgnach, W., Challah, M., Coulet, F., Michel, J. B. and Battle, T. Shear stress induces angiotensin converting enzyme expression in cultured smooth muscle cells: possible involvement of bFGF. *Cardiovasc Res* (2000); **45**(2): 486-492.

Graff, C., Clayton, D. A. and Larsson, N. G. Mitochondrial medicine--recent advances. *J Intern Med* (1999); **246**(1): 11-23.

Granger, C. B., McMurray, J. J., Yusuf, S., Held, P., Michelson, E. L., Olofsson, B., Ostergren, J., Pfeffer, M. A. and Swedberg, K. Effects of candesartan in patients with chronic heart failure and reduced left-ventricular systolic function intolerant to angiotensin-converting-enzyme inhibitors: the CHARM-Alternative trial. *Lancet* (2003); **362**(9386): 772-776.

Grassi, G., Seravalle, G., Cattaneo, B. M., Lanfranchi, A., Vailati, S., Giannattasio, C., Del Bo, A., Sala, C., Bolla, G. B. and Pozzi, M. Sympathetic activation and loss of reflex sympathetic control in mild congestive heart failure. *Circulation* (1995); **92**(11): 3206-3211.

Griendling, K. K. and Ushio-Fukai, M. Reactive oxygen species as mediators of angiotensin II signaling. *Regul Pept* (2000); **91**(1-3): 21-27.

Griffiths, E. J. Mitochondria--potential role in cell life and death. *Cardiovasc Res* (2000); **46**(1): 24-27.
Guzik, T. J., West, N. E., Black, E., McDonald, D., Ratnatunga, C., Pillai, R. and Channon, K. M. Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors. *Circ Res* (2000); **86**(9): E85-90.
Haffner, S. M., Lehto, S., Ronnemaa, T., Pyorala, K. and Laakso, M. Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *N Engl J Med* (1998); **339**(4): 229-234.
Halsall, D., Luan, J., Saker, P., Huxtable, S., Farooqi, I., Keogh, J., Wareham, N. and O'Rahilly, S. Uncoupling protein 3 genetic variants in human obesity: the c-55t promoter polymorphism is negatively correlated with body mass index in a UK Caucasian population. *Int J Obes Relat Metab Disord* (2001); **25**(4): 472-477.
Hambrecht, R., Adams, V., Gielen, S., Linke, A., Mobius-Winkler, S., Yu, J., Niebauer, J., Jiang, H., Fiehn, E. and Schuler, G. Exercise intolerance in patients with chronic heart failure and increased expression of inducible nitric oxide synthase in the skeletal muscle. *J Am Coll Cardiol* (1999); **33**(1): 174-179.
Hambrecht, R., Fiehn, E., Yu, J., Niebauer, J., Weigl, C., Hilbrich, L., Adams, V., Riede, U. and Schuler, G. Effects of endurance training on mitochondrial ultrastructure and fiber type distribution in skeletal muscle of patients with stable chronic heart failure. *J Am Coll Cardiol* (1997); **29**(5): 1067-1073.
Hammond, R. L., Augustyniak, R. A., Rossi, N. F., Churchill, P. C., Lapanowski, K. and O'Leary, D. S. Heart failure alters the strength and mechanisms of the muscle metaboreflex. *Am J Physiol Heart Circ Physiol* (2000); **278**(3): H818-828.
Hansson, L. The benefits of lowering elevated blood pressure: a critical review of studies of cardiovascular morbidity and mortality in hypertension. *J Hypertens* (1996); **14**(5): 537-544.
Hansson, L. and Zanchetti, A. The Hypertension Optimal Treatment Study (the HOT Study). *Blood Press* (1993); **2**(1): 62-68.
Hardie, D. G. and Carling, D. The AMP-activated protein kinase--fuel gauge of the mammalian cell? *Eur J Biochem* (1997); **246**(2): 259-273.
Harding, S. E., Jones, S. M., O'Gara, P., del Monte, F., Vescovo, G. and Poole-Wilson, P. A. Isolated ventricular myocytes from failing and non-failing human heart; the relation of age and clinical status of patients to isoproterenol response. *J Mol Cell Cardiol* (1992); **24**(5): 549-564.
Hare, J. M., Givertz, M. M., Creager, M. A. and Colucci, W. S. Increased sensitivity to nitric oxide synthase inhibition in patients with heart failure: potentiation of beta-adrenergic inotropic responsiveness. *Circulation* (1998); **97**(2): 161-166.
Harper, J. A., Stuart, J. A., Jekabsons, M. B., Roussel, D., Brindle, K. M., Dickinson, K., Jones, R. B. and Brand, M. D. Artifactual uncoupling by uncoupling protein 3 in yeast mitochondria at the concentrations found in mouse and rat skeletal-muscle mitochondria. *Biochem J* (2002); **361**(Pt 1): 49-56.
Harridge, S. D., Magnusson, G. and Gordon, A. Skeletal muscle contractile characteristics and fatigue resistance in patients with chronic heart failure. *Eur Heart J* (1996); **17**(6): 896-901.
Harris, R. C. and Cheng, H. F. The intrarenal renin-angiotensin system: a paracrine system for the local control of renal function separate from the systemic axis. *Exp Nephrol* (1996); **4 Suppl 1**: 2-7.
Harrison, D., Griendling, K. K., Landmesser, U., Hornig, B. and Drexler, H. Role of oxidative stress in atherosclerosis. *Am J Cardiol* (2003); **91**(3A): 7A-11A.
Hashimoto, K., Hirose, M., Furukawa, S., Hayakawa, H. and Kimura, E. Changes in hemodynamics and bradykinin concentration in coronary sinus blood in experimental coronary artery occlusion. *Jpn Heart J* (1977); **18**(5): 679-689.
Hatakeyama, H., Miyamori, I., Fujita, T., Takeda, Y., Takeda, R. and Yamamoto, H. Vascular aldosterone. Biosynthesis and a link to angiotensin II-induced hypertrophy of vascular smooth muscle cells. *J Biol Chem* (1994); **269**(39): 24316-24320.
Hawe, E., Talmud, P. J., Miller, G. J. and Humphries, S. E. Family history is a coronary heart disease risk factor in the Second Northwick Park Heart Study. *Ann Hum Genet* (2003); **67**(Pt 2): 97-106.
Heart Protection Study Collaborative Group. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. *Lancet* (2002); **360**(9326): 7-22.
Heaton, G. M., Wagenvoord, R. J., Kemp, A., Jr. and Nicholls, D. G. Brown-adipose-tissue mitochondria: photoaffinity labelling of the regulatory site of energy dissipation. *Eur J Biochem* (1978); **82**(2): 515-521.
Hecht, D. and Zick, Y. Selective inhibition of protein tyrosine phosphatase activities by H2O2 and vanadate in vitro. *Biochem Biophys Res Commun* (1992); **188**(2): 773-779.
Heidkaemper, D., Winkler, E., Muller, V., Frischmuth, K., Liu, Q., Caskey, T. and Klingenberg, M. The bulk of UCP3 expressed in yeast cells is incompetent for a nucleotide regulated H+ transport. *FEBS Lett* (2000); **480**(2-3): 265-270.

Hennekens, C. H., Buring, J. E., Manson, J. E., Stampfer, M., Rosner, B., Cook, N. R., Belanger, C., LaMotte, F., Gaziano, J. M., Ridker, P. M., Willett, W. and Peto, R. Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. *N Engl J Med* (1996); **334**(18): 1145-1149.

Hennes, M. M., O'Shaughnessy, I. M., Kelly, T. M., LaBelle, P., Egan, B. M. and Kissebah, A. H. Insulin-resistant lipolysis in abdominally obese hypertensive individuals. Role of the renin-angiotensin system. *Hypertension* (1996); **28**(1): 120-126.

Henriksen, E. J. and Jacob, S. Effects of captopril on glucose transport activity in skeletal muscle of obese Zucker rats. *Metabolism* (1995); **44**(2): 267-272.

Henriksen, E. J., Jacob, S., Kinnick, T. R., Youngblood, E. B., Schmit, M. B. and Dietze, G. J. ACE inhibition and glucose transport in insulinresistant muscle: roles of bradykinin and nitric oxide. *Am J Physiol* (1999); **277**(1 Pt 2): R332-336.

Herbst, U., Toborek, M., Kaiser, S., Mattson, M. P. and Hennig, B. 4-Hydroxynonenal induces dysfunction and apoptosis of cultured endothelial cells. *J Cell Physiol* (1999); **181**(2): 295-303.

Herings, R. M., de Boer, A., Stricker, B. H., Leufkens, H. G. and Porsius, A. Hypoglycaemia associated with use of inhibitors of angiotensin converting enzyme. *Lancet* (1995); **345**(8959): 1195-1198.

Hesselink, M. K., Greenhaff, P. L., Constantin-Teodosiu, D., Hultman, E., Saris, W. H., Nieuwlaat, R., Schaart, G., Kornips, E. and Schrauwen, P. Increased uncoupling protein 3 content does not affect mitochondrial function in human skeletal muscle in vivo. *J Clin Invest* (2003); **111**(4): 479-486.

Hesselink, M. K., Keizer, H. A., Borghouts, L. B., Schaart, G., Kornips, C. F., Slieker, L. J., Sloop, K. W., Saris, W. H. and Schrauwen, P. Protein expression of UCP3 differs between human type 1, type 2a, and type 2b fibers. *Faseb J* (2001); **15**(6): 1071-1073.

Heymes, C., Vanderheyden, M., Bronzwaer, J. G., Shah, A. M. and Paulus, W. J. Endomyocardial nitric oxide synthase and left ventricular preload reserve in dilated cardiomyopathy. *Circulation* (1999); **99**(23): 3009-3016.

Hidalgo, C. and Jaimovich, E. Inositol trisphosphate and excitation-contraction coupling in skeletal muscle. *J Bioenerg Biomembr* (1989); **21**(2): 267-281.

Higginbotham, M. B., Morris, K. G., Conn, E. H., Coleman, R. E. and Cobb, F. R. Determinants of variable exercise performance among patients with severe left ventricular dysfunction. *Am J Cardiol* (1983); **51**(1): 52-60.

Hilbers, U., Peters, J., Bornstein, S. R., Correa, F. M., Johren, O., Saavedra, J. M. and Ehrhart-Bornstein, M. Local renin-angiotensin system is involved in K+-induced aldosterone secretion from human adrenocortical NCI-H295 cells. *Hypertension* (1999); **33**(4): 1025-1030.

Hill, M. F. and Singal, P. K. Antioxidant and oxidative stress changes during heart failure subsequent to myocardial infarction in rats. *Am J Pathol* (1996); **148**(1): 291-300.

Himms-Hagen, J. Brown adipose tissue thermogenesis: interdisciplinary studies. *Faseb J* (1990); **4**(11): 2890-2898.

Hingorani, A. D. Polymorphisms in endothelial nitric oxide synthase and atherogenesis: John French Lecture 2000. *Atherosclerosis* (2001); **154**(3): 521-527.

Hirotani, S., Otsu, K., Nishida, K., Higuchi, Y., Morita, T., Nakayama, H., Yamaguchi, O., Mano, T., Matsumura, Y., Ueno, H., Tada, M. and Hori, M. Involvement of nuclear factor-kappaB and apoptosis signal-regulating kinase 1 in G-protein-coupled receptor agonist-induced cardiomyocyte hypertrophy. *Circulation* (2002); **105**(4): 509-515.

Hoeks, J., van Baak, M. A., Hesselink, M. K., Hul, G. B., Vidal, H., Saris, W. H. and Schrauwen, P. Effect of beta1- and beta2-adrenergic stimulation on energy expenditure, substrate oxidation, and UCP3 expression in humans. *Am J Physiol Endocrinol Metab* (2003); **285**(4): E775-E782.

Horak, A. R. and Opie, L. H. Energy metabolism of the heart in catecholamine-induced myocardial injury. Concentration-dependent effects of epinephrine on enzyme release, mechanical function, and "oxygen wastage". *Adv Myocardiol* (1983); **4**: 23-43.

Horvath, T. L., Diano, S., Miyamoto, S., Barry, S., Gatti, S., Alberati, D., Livak, F., Lombardi, A., Moreno, M., Goglia, F., Mor, G., Hamilton, J., Kachinskas, D., Horwitz, B. and Warden, C. H. Uncoupling proteins-2 and 3 influence obesity and inflammation in transgenic mice. *Int J Obes Relat Metab Disord* (2003); **27**(4): 433-442.

Houle, S., Landry, M., Audet, R., Bouthillier, J., Bachvarov, D. R. and Marceau, F. Effect of allelic polymorphism of the B(1) and B(2) receptor genes on the contractile responses of the human umbilical vein to kinins. *J Pharmacol Exp Ther* (2000); **294**(1): 45-51.

Houser, S. R. and Margulies, K. B. Is depressed myocyte contractility centrally involved in heart failure? *Circ Res* (2003); **92**(4): 350-358.

Houser, S. R., Piacentino, V., 3rd, Mattiello, J., Weisser, J. and Gaughan, J. P. Functional properties of failing human ventricular myocytes. *Trends Cardiovasc Med* (2000); **10**(3): 101-107.

Houser, S. R., Piacentino, V., 3rd and Weisser, J. Abnormalities of calcium cycling in the hypertrophied and failing heart. *J Mol Cell Cardiol* (2000); **32**(9): 1595-1607.

Huang, S., Chen, X. H., Payne, J. R., Pennell, D. J., Gohlke, P., Smith, M. J., Day, I. N., Montgomery, H. E. and Gaunt, T. R. Haplotype of growth hormone and angiotensin I-converting enzyme genes, serum angiotensin I-converting enzyme and ventricular growth: pathway inference in pharmacogenetics. *Pharmacogenet Genomics* (2007); **17**(4): 291-294.

Hugel, S., Horn, M., de Groot, M., Remkes, H., Dienesch, C., Hu, K., Ertl, G. and Neubauer, S. Effects of ACE inhibition and beta-receptor blockade on energy metabolism in rats postmyocardial infarction. *Am J Physiol* (1999); **277**(6 Pt 2): H2167-2175.

Humphries, S. E., Hawe, E., Dhamrait, S., Miller, G. J. and Talmud, P. J. In search of genetic precision. *Lancet* (2003); **361**(9372): 1908-1909; author reply 1909-10.

Humphries, S. E., Ridker, P. M. and Talmud, P. J. Genetic testing for cardiovascular disease susceptibility: a useful clinical management tool or possible misinformation? *Arterioscler Thromb Vasc Biol* (2004); **24**(4): 628-636.

Hwang, C. S. and Lane, M. D. Up-regulation of uncoupling protein-3 by fatty acid in C2C12 myotubes. *Biochem Biophys Res Commun* (1999); **258**(2): 464-469.

Ide, T., Tsutsui, H., Hayashidani, S., Kang, D., Suematsu, N., Nakamura, K., Utsumi, H., Hamasaki, N. and Takeshita, A. Mitochondrial DNA damage and dysfunction associated with oxidative stress in failing hearts after myocardial infarction. *Circ Res* (2001); **88**(5): 529-535.

Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E. and Chaudhuri, G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A* (1987); **84**(24): 9265-9269.

Imaizumi, T., Takeshita, A., Nakamura, N., Sakai, K., Hirooka, Y., Suzuki, S., Yoshida, M. and Nakamura, M. Effects of captopril on forearm oxygen consumption during dynamic handgrip exercise in patients with congestive heart failure. *Jpn Heart J* (1990); **31**(6): 817-828.

Isami, S., Kishikawa, H., Araki, E., Uehara, M., Kaneko, K., Shirotani, T., Todaka, M., Ura, S., Motoyoshi, S., Matsumoto, K., Miyamura, N. and Shichiri, M. Bradykinin enhances GLUT4 translocation through the increase of insulin receptor tyrosine kinase in primary adipocytes: evidence that bradykinin stimulates the insulin signalling pathway. *Diabetologia* (1996); **39**(4): 412-420.

Ishigai, Y., Mori, T., Ikeda, T., Fukuzawa, A. and Shibano, T. Role of bradykinin-NO pathway in prevention of cardiac hypertrophy by ACE inhibitor in rat cardiomyocytes. *Am J Physiol* (1997); **273**(6 Pt 2): H2659-H2663.

Iwai, N., Matsunaga, M., Kita, T., Tei, M. and Kawai, C. Regulation of angiotensin converting enzyme activity in cultured human vascular endothelial cells. *Biochem Biophys Res Commun* (1987); **149**(3): 1179-1185.

Jaburek, M., Varecha, M., Gimeno, R. E., Dembski, M., Jezek, P., Zhang, M., Burn, P., Tartaglia, L. A. and Garlid, K. D. Transport function and regulation of mitochondrial uncoupling proteins 2 and 3. *J Biol Chem* (1999); **274**(37): 26003-26007.

Jacob, R. and Gulch, R. W. The functional significance of ventricular geometry for the transition from hypertrophy to cardiac failure. Does a critical degree of structural dilatation exist? *Basic Res Cardiol* (1998); **93**(6): 423-429.

Jacob, S., Henriksen, E. J., Fogt, D. L. and Dietze, G. J. Effects of trandolapril and verapamil on glucose transport in insulin-resistant rat skeletal muscle. *Metabolism* (1996); **45**(5): 535-541.

Jacobson, J. and Duchen, M. R. 'What nourishes me, destroys me': towards a new mitochondrial biology. *Cell Death Differ* (2001); **8**(10): 963-966.

Jacobson, J. and Duchen, M. R. Mitochondrial oxidative stress and cell death in astrocytes--requirement for stored Ca2+ and sustained opening of the permeability transition pore. *J Cell Sci* (2002); **115**(Pt 6): 1175-1188.

Jacobson, J., Duchen, M. R. and Heales, S. J. Intracellular distribution of the fluorescent dye nonyl acridine orange responds to the mitochondrial membrane potential: implications for assays of cardiolipin and mitochondrial mass. *J Neurochem* (2002); **82**(2): 224-233.

Jalowy, A., Schulz, R., Dorge, H., Behrends, M. and Heusch, G. Infarct size reduction by AT1-receptor blockade through a signal cascade of AT2-receptor activation, bradykinin and prostaglandins in pigs. *J Am Coll Cardiol* (1998); **32**(6): 1787-1796.

James, P. E., Jackson, S. K., Grinberg, O. Y. and Swartz, H. M. The effects of endotoxin on oxygen consumption of various cell types in vitro: an EPR oximetry study. *Free Radic Biol Med* (1995); **18**(4): 641-647.

Jamshidi, Y., Montgomery, H. E., Hense, H. W., Myerson, S. G., Torra, I. P., Staels, B., World, M. J., Doering, A., Erdmann, J., Hengstenberg, C., Humphries, S. E., Schunkert, H. and Flavell, D. M. Peroxisome proliferator--activated receptor alpha gene regulates left ventricular growth in response to exercise and hypertension. *Circulation* (2002); **105**(8): 950-955.

Jaspard, E., Wei, L. and Alhenc-Gelas, F. Differences in the properties and enzymatic specificities of the two active sites of angiotensin I-converting enzyme (kininase II). Studies with bradykinin and other natural peptides. *J Biol Chem* (1993); **268**(13): 9496-9503.

Jin, Z. Q. and Chen, X. Ramipril-induced delayed myocardial protection against free radical injury involves bradykinin B2 receptor-NO pathway and protein synthesis. *Br J Pharmacol* (1998); **125**(3): 556-562.

Jondeau, G., Katz, S. D., Zohman, L., Goldberger, M., McCarthy, M., Bourdarias, J. P. and LeJemtel, T. H. Active skeletal muscle mass and cardiopulmonary reserve. Failure to attain peak aerobic capacity during maximal bicycle exercise in patients with severe congestive heart failure. *Circulation* (1992); **86**(5): 1351-1356.

Jones, B. H., Standridge, M. K., Taylor, J. W. and Moustaid, N. Angiotensinogen gene expression in adipose tissue: analysis of obese models and hormonal and nutritional control. *Am J Physiol* (1997); **273**(1 Pt 2): R236-242.

Jonsson, J. R., Game, P. A., Head, R. J. and Frewin, D. B. The expression and localisation of the angiotensin-converting enzyme mRNA in human adipose tissue. *Blood Press* (1994); **3**(1-2): 72-75.

Kalsi, K. K., Smolenski, R. T., Pritchard, R. D., Khaghani, A., Seymour, A. M. and Yacoub, M. H. Energetics and function of the failing human heart with dilated or hypertrophic cardiomyopathy. *Eur J Clin Invest* (1999); **29**(6): 469-477.

Kanazawa, H., Otsuka, T., Hirata, K. and Yoshikawa, J. Association between the angiotensin-converting enzyme gene polymorphisms and tissue oxygenation during exercise in patients with COPD. *Chest* (2002); **121**(3): 697-701.

Kannel, W. B., Dawber, T. R., Friedman, G. D., Glennon, W. E. and McNamara, P. M. Risk Factors in Coronary Heart Disease. An Evaluation of Several Serum Lipids as Predictors of Coronary Heart Disease; the Framingham Study. *Ann Intern Med* (1964); **61**: 888-899.

Kannel, W. B., Neaton, J. D., Wentworth, D., Thomas, H. E., Stamler, J., Hulley, S. B. and Kjelsberg, M. O. Overall and coronary heart disease mortality rates in relation to major risk factors in 325,348 men screened for the MRFIT. Multiple Risk Factor Intervention Trial. *Am Heart J* (1986); **112**(4): 825-836.

Kannel, W. B., Schwartz, M. J. and McNamara, P. M. Blood pressure and risk of coronary heart disease: the Framingham study. *Dis Chest* (1969); **56**(1): 43-52.

Karjalainen, J., Kujala, U. M., Stolt, A., Mantysaari, M., Viitasalo, M., Kainulainen, K. and Kontula, K. Angiotensinogen gene M235T polymorphism predicts left ventricular hypertrophy in endurance athletes. *J Am Coll Cardiol* (1999); **34**(2): 494-499.

Karmazyn, M., Liu, Q., Gan, X. T., Brix, B. J. and Fliegel, L. Aldosterone increases NHE-1 expression and induces NHE-1-dependent hypertrophy in neonatal rat ventricular myocytes. *Hypertension* (2003); **42**(6): 1171-1176.

Kato, H., Suzuki, H., Tajima, S., Ogata, Y., Tominaga, T., Sato, A. and Saruta, T. Angiotensin II stimulates collagen synthesis in cultured vascular smooth muscle cells. *J Hypertens* (1991); **9**(1): 17-22.

Katsuki, T., Yasu, T., Ohmura, N., Nakada, I., Fujii, M. and Saito, M. Role of skeletal muscle metabolism in exercise capacity of patients with myocardial infarction studied by phosphorus-31 nuclear magnetic resonance. *Jpn Circ J* (1995); **59**(6): 315-322.

Katsuya, T., Horiuchi, M., Chen, Y. D., Koike, G., Pratt, R. E., Dzau, V. J. and Reaven, G. M. Relations between deletion polymorphism of the angiotensin-converting enzyme gene and insulin resistance, glucose intolerance, hyperinsulinemia, and dyslipidemia. *Arterioscler Thromb Vasc Biol* (1995); **15**(6): 779-782.

Kawabata, H., Ryomoto, T. and Ishikawa, K. Effect of angiotensin converting enzyme inhibitor and angiotensin II type 1 receptor antagonist on metabolism and contraction in ischemia-reperfused rabbit heart. *Jpn Circ J* (2000); **64**(4): 276-282.

Keavney, B., McKenzie, C. A., Connell, J. M., Julier, C., Ratcliffe, P. J., Sobel, E., Lathrop, M. and Farrall, M. Measured haplotype analysis of the angiotensin-I converting enzyme gene. *Hum Mol Genet* (1998); **7**(11): 1745-1751.

Keidar, S., Heinrich, R., Kaplan, M., Hayek, T. and Aviram, M. Angiotensin II administration to atherosclerotic mice increases macrophage uptake of oxidized ldl: a possible role for interleukin-6. *Arterioscler Thromb Vasc Biol* (2001); **21**(9): 1464-1469.

Keij, J. F., Bell-Prince, C. and Steinkamp, J. A. Staining of mitochondrial membranes with 10-nonyl acridine orange, MitoFluor Green, and MitoTracker Green is affected by mitochondrial membrane potential altering drugs. *Cytometry* (2000); **39**(3): 203-210.

Keith, M., Geranmayegan, A., Sole, M. J., Kurian, R., Robinson, A., Omran, A. S. and Jeejeebhoy, K. N. Increased oxidative stress in patients with congestive heart failure. *J Am Coll Cardiol* (1998); **31**(6): 1352-1356.

Kelly, D. P. and Strauss, A. W. Inherited cardiomyopathies. *N Engl J Med* (1994); **330**(13): 913-919.

Kelly, L. J., Vicario, P. P., Thompson, G. M., Candelore, M. R., Doebber, T. W., Ventre, J., Wu, M. S., Meurer, R., Forrest, M. J., Conner, M. W., Cascieri, M. A. and Moller, D. E. Peroxisome

proliferator-activated receptors gamma and alpha mediate in vivo regulation of uncoupling protein (UCP-1, UCP-2, UCP-3) gene expression. *Endocrinology* (1998); **139**(12): 4920-4927.

Kemp, G. J., Thompson, C. H., Stratton, J. R., Brunotte, F., Conway, M., Adamopoulos, S., Arnolda, L., Radda, G. K. and Rajagopalan, B. Abnormalities in exercising skeletal muscle in congestive heart failure can be explained in terms of decreased mitochondrial ATP synthesis, reduced metabolic efficiency, and increased glycogenolysis. *Heart* (1996); **76**: 35-41.

Kennon, B., Petrie, J. R., Small, M. and Connell, J. M. Angiotensin-converting enzyme gene and diabetes mellitus. *Diabet Med* (1999); **16**(6): 448-458.

Kichuk, M. R., Seyedi, N., Zhang, X., Marboe, C. C., Michler, R. E., Addonizio, L. J., Kaley, G., Nasjletti, A. and Hintze, T. H. Regulation of nitric oxide production in human coronary microvessels and the contribution of local kinin formation. *Circulation* (1996); **94**(1): 44-51.

Kim, Y. M., Bombeck, C. A. and Billiar, T. R. Nitric oxide as a bifunctional regulator of apoptosis. *Circ Res* (1999); **84**(3): 253-256.

Kimes, B. W. and Brandt, B. L. Properties of a clonal muscle cell line derived from rat heart. *Experimental Cell Research* (1976); **98**: 367-381.

Kimm, S. Y., Glynn, N. W., Aston, C. E., Damcott, C. M., Poehlman, E. T., Daniels, S. R. and Ferrell, R. E. Racial differences in the relation between uncoupling protein genes and resting energy expenditure. *Am J Clin Nutr* (2002); **75**(4): 714-719.

King, H., Aubert, R. E. and Herman, W. H. Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. *Diabetes Care* (1998); **21**(9): 1414-1431.

Kingma, J. H., van Gilst, W. H., Peels, C. H., Dambrink, J. H., Verheugt, F. W. and Wielenga, R. P. Acute intervention with captopril during thrombolysis in patients with first anterior myocardial infarction. Results from the Captopril and Thrombolysis Study (CATS). *Eur Heart J* (1994); **15**(7): 898-907.

Kitakaze, M., Node, K., Minamino, T., Asanuma, H., Ueda, Y., Kosaka, H., Kuzuya, T. and Hori, M. Inhibition of angiotensin-converting enzyme increases the nitric oxide levels in canine ischemic myocardium. *J Mol Cell Cardiol* (1998); **30**(11): 2461-2466.

Kitaoka, H., Takata, J., Hitomi, N., Furuno, T., Seo, H., Chikamori, T. and Doi, Y. L. Effect of angiotensin-converting enzyme inhibitor (enalapril or imidapril) on ventilation during exercise in patients with chronic heart failure secondary to idiopathic dilated cardiomyopathy. *Am J Cardiol* (2000); **85**(5): 658-660, A10.

Kizaki, T., Suzuki, K., Hitomi, Y., Taniguchi, N., Saitoh, D., Watanabe, K., Onoe, K., Day, N. K., Good, R. A. and Ohno, H. Uncoupling protein 2 plays an important role in nitric oxide production of lipopolysaccharide-stimulated macrophages. *Proc Natl Acad Sci U S A* (2002); **99**(14): 9392-9397.

Knopp, A., Thierfelder, S., Koopmann, R., Biskup, C., Bohle, T. and Benndorf, K. Anoxia generates rapid and massive opening of KATP channels in ventricular cardiac myocytes. *Cardiovasc Res* (1999); **41**(3): 629-640.

Kober, L., Torp-Pedersen, C., Carlsen, J. E., Bagger, H., Eliasen, P., Lyngborg, K., Videbaek, J., Cole, D. S., Auclert, L. and Pauly, N. C. A clinical trial of the angiotensin-converting-enzyme inhibitor trandolapril in patients with left ventricular dysfunction after myocardial infarction. Trandolapril Cardiac Evaluation (TRACE) Study Group. *N Engl J Med* (1995); **333**(25): 1670-1676.

Koenig, W., Sund, M., Frohlich, M., Fischer, H. G., Lowel, H., Doring, A., Hutchinson, W. L. and Pepys, M. B. C-Reactive protein, a sensitive marker of inflammation, predicts future risk of coronary heart disease in initially healthy middle-aged men: results from the MONICA (Monitoring Trends and Determinants in Cardiovascular Disease) Augsburg Cohort Study, 1984 to 1992. *Circulation* (1999); **99**(2): 237-242.

Kojda, G. and Kottenberg, K. Regulation of basal myocardial function by NO. *Cardiovasc Res* (1999); **41**(3): 514-523.

Konat, G. W., Kofke, W. A. and Miric, S. Toxicity of compound A to C6 rat glioma cells. *Metab Brain Dis* (2003); **18**(1): 11-15.

Kong, C. W., Hsu, T. G., Lu, F. J., Chan, W. L. and Tsai, K. Leukocyte mitochondria depolarization and apoptosis in advanced heart failure: clinical correlations and effect of therapy. *J Am Coll Cardiol* (2001); **38**(6): 1693-1700.

Kowluru, R., Yamazaki, T., McNamara, B. C. and Jefcoate, C. R. Metabolism of exogenous cholesterol by rat adrenal mitochondria is stimulated equally by physiological levels of free Ca2+ and by GTP. *Mol Cell Endocrinol* (1995); **107**(2): 181-188.

Koya, D. and King, G. L. Protein kinase C activation and the development of diabetic complications. *Diabetes* (1998); **47**(6): 859-866.

Kramer, R. E. Evidence that angiotensin II decreases mitochondrial calcium in the glomerulosa cell. *Mol Cell Endocrinol* (1990); **74**(2): 87-100.

Krauss, S., Zhang, C. Y. and Lowell, B. B. A significant portion of mitochondrial proton leak in intact thymocytes depends on expression of UCP2. *Proc Natl Acad Sci U S A* (2002); **99**(1): 118-122.

Krauss, S., Zhang, C. Y. and Lowell, B. B. The mitochondrial uncoupling-protein homologues. *Nat Rev Mol Cell Biol* (2005); **6**(3): 248-261.

Krempler, F., Esterbauer, H., Weitgasser, R., Ebenbichler, C., Patsch, J. R., Miller, K., Xie, M., Linnemayr, V., Oberkofler, H. and Patsch, W. A functional polymorphism in the promoter of UCP2 enhances obesity risk but reduces type 2 diabetes risk in obese middle-aged humans. *Diabetes* (2002); **51**(11): 3331-3335.

Krieger, C. and Duchen, M. R. Mitochondria, Ca(2+) and neurodegenerative disease. *Eur J Pharmacol* (2002); **447**(2-3): 177-188.

Kubo, S. H., Rector, T. S., Bank, A. J., Williams, R. E. and Heifetz, S. M. Endothelium-dependent vasodilation is attenuated in patients with heart failure. *Circulation* (1991); **84**(4): 1589-1596.

Kubota, T., Mori, H., Tamori, Y., Okazawa, H., Fukuda, T., Miki, M., Ito, C., Fleury, C., Bouillaud, F. and Kasuga, M. Molecular screening of uncoupling protein 2 gene in patients with noninsulin-dependent diabetes mellitus or obesity. *J Clin Endocrinol Metab* (1998); **83**(8): 2800-2804.

Kudoh, A., Dietze, G. J. and Rabito, S. F. Insulin enhances the bradykinin response in L8 rat skeletal myoblasts. *Diabetes* (2000); **49**(2): 190-194.

Kudoh, A. and Matsuki, A. Effects of angiotensin-converting enzyme inhibitors on glucose uptake. *Hypertension* (2000); **36**(2): 239-244.

Kuller, L. H., Tracy, R. P., Shaten, J. and Meilahn, E. N. Relation of C-reactive protein and coronary heart disease in the MRFIT nested case-control study. Multiple Risk Factor Intervention Trial. *Am J Epidemiol* (1996); **144**(6): 537-547.

Kunsch, C. and Medford, R. M. Oxidative stress as a regulator of gene expression in the vasculature. *Circ Res* (1999); **85**(8): 753-766.

Lakatta, E. G. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part III: cellular and molecular clues to heart and arterial aging. *Circulation* (2003); **107**(3): 490-497.

Lakka, H. M., Laaksonen, D. E., Lakka, T. A., Niskanen, L. K., Kumpusalo, E., Tuomilehto, J. and Salonen, J. T. The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men. *Jama* (2002); **288**(21): 2709-2716.

Lam, K. Y. and Leung, P. S. Regulation and expression of a renin-angiotensin system in human pancreas and pancreatic endocrine tumours. *Eur J Endocrinol* (2002); **146**(4): 567-572.

Lander, E. S. The new genomics: global views of biology. *Science* (1996); **274**(5287): 536-539.

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., McKernan, K., Meldrim, J., Mesirov, J. P., Miranda, C., Morris, W., Naylor, J., Raymond, C., Rosetti, M., Santos, R., Sheridan, A., Sougnez, C., Stange-Thomann, N., Stojanovic, N., Subramanian, A., Wyman, D., Rogers, J., Sulston, J., Ainscough, R., Beck, S., Bentley, D., Burton, J., Clee, C., Carter, N., Coulson, A., Deadman, R., Deloukas, P., Dunham, A., Dunham, I., Durbin, R., French, L., Grafham, D., Gregory, S., Hubbard, T., Humphray, S., Hunt, A., Jones, M., Lloyd, C., McMurray, A., Matthews, L., Mercer, S., Milne, S., Mullikin, J. C., Mungall, A., Plumb, R., Ross, M., Shownkeen, R., Sims, S., Waterston, R. H., Wilson, R. K., Hillier, L. W., McPherson, J. D., Marra, M. A., Mardis, E. R., Fulton, L. A., Chinwalla, A. T., Pepin, K. H., Gish, W. R., Chissoe, S. L., Wendl, M. C., Delehaunty, K. D., Miner, T. L., Delehaunty, A., Kramer, J. B., Cook, L. L., Fulton, R. S., Johnson, D. L., Minx, P. J., Clifton, S. W., Hawkins, T., Branscomb, E., Predki, P., Richardson, P., Wenning, S., Slezak, T., Doggett, N., Cheng, J. F., Olsen, A., Lucas, S., Elkin, C., Uberbacher, E., Frazier, M., Gibbs, R. A., Muzny, D. M., Scherer, S. E., Bouck, J. B., Sodergren, E. J., Worley, K. C., Rives, C. M., Gorrell, J. H., Metzker, M. L., Naylor, S. L., Kucherlapati, R. S., Nelson, D. L., Weinstock, G. M., Sakaki, Y., Fujiyama, A., Hattori, M., Yada, T., Toyoda, A., Itoh, T., Kawagoe, C., Watanabe, H., Totoki, Y., Taylor, T., Weissenbach, J., Heilig, R., Saurin, W., Artiguenave, F., Brottier, P., Bruls, T., Pelletier, E., Robert, C., Wincker, P., Smith, D. R., Doucette-Stamm, L., Rubenfield, M., Weinstock, K., Lee, H. M., Dubois, J., Rosenthal, A., Platzer, M., Nyakatura, G., Taudien, S., Rump, A., Yang, H., Yu, J., Wang, J., Huang, G., Gu, J., Hood, L., Rowen, L., Madan, A., Qin, S., Davis, R. W., Federspiel, N. A., Abola, A. P., Proctor, M. J., Myers, R. M., Schmutz, J., Dickson, M., Grimwood, J., Cox, D. R., Olson, M. V., Kaul, R., Shimizu, N., Kawasaki, K., Minoshima, S., Evans, G. A., Athanasiou, M., Schultz, R., Roe, B. A., Chen, F., Pan, H., Ramser, J., Lehrach, H., Reinhardt, R., McCombie, W. R., de la Bastide, M., Dedhia, N., Blocker, H., Hornischer, K., Nordsiek, G., Agarwala, R., Aravind, L., Bailey, J. A., Bateman, A., Batzoglou, S., Birney, E., Bork, P., Brown, D. G., Burge, C. B., Cerutti, L., Chen, H. C., Church, D., Clamp, M., Copley, R. R., Doerks, T., Eddy, S. R., Eichler, E. E., Furey, T. S., Galagan, J., Gilbert, J. G., Harmon, C., Hayashizaki, Y., Haussler, D., Hermjakob, H., Hokamp, K., Jang, W., Johnson, L. S., Jones, T. A., Kasif, S., Kaspryzk, A., Kennedy, S., Kent, W. J., Kitts, P., Koonin, E. V., Korf, I., Kulp, D., Lancet, D., Lowe, T. M., McLysaght, A., Mikkelsen, T., Moran, J. V., Mulder, N., Pollara, V. J., Ponting, C. P., Schuler, G., Schultz, J., Slater, G., Smit, A. F., Stupka, E., Szustakowski, J., Thierry-Mieg, D., Thierry-Mieg, J., Wagner, L.,

Wallis, J., Wheeler, R., Williams, A., Wolf, Y. I., Wolfe, K. H., Yang, S. P., Yeh, R. F., Collins, F., Guyer, M. S., Peterson, J., Felsenfeld, A., Wetterstrand, K. A., Patrinos, A., Morgan, M. J., de Jong, P., Catanese, J. J., Osoegawa, K., Shizuya, H., Choi, S. and Chen, Y. J. Initial sequencing and analysis of the human genome. *Nature* (2001); **409**(6822): 860-921.

Lander, E. S. and Schork, N. J. Genetic dissection of complex traits. *Science* (1994); **265**(5181): 2037-2048.

Lang, B. F., Burger, G., O'Kelly, C. J., Cedergren, R., Golding, G. B., Lemieux, C., Sankoff, D., Turmel, M. and Gray, M. W. An ancestral mitochondrial DNA resembling a eubacterial genome in miniature. *Nature* (1997); **387**(6632): 493-497.

Langberg, H., Bjorn, C., Boushel, R., Hellsten, Y. and Kjaer, M. Exercise-induced increase in interstitial bradykinin and adenosine concentrations in skeletal muscle and peritendinous tissue in humans. *J Physiol* (2002); **542**(Pt 3): 977-983.

Langdown, M. L., Smith, N. D., Sugden, M. C. and Holness, M. J. Excessive glucocorticoid exposure during late intrauterine development modulates the expression of cardiac uncoupling proteins in adult hypertensive male offspring. *Pflugers Arch* (2001); **442**(2): 248-255.

Lanni, A., Beneduce, L., Lombardi, A., Moreno, M., Boss, O., Muzzin, P., Giacobino, J. P. and Goglia, F. Expression of uncoupling protein-3 and mitochondrial activity in the transition from hypothyroid to hyperthyroid state in rat skeletal muscle. *FEBS Lett* (1999); **444**(2-3): 250-254.

Lanni, A., De Felice, M., Lombardi, A., Moreno, M., Fleury, C., Ricquier, D. and Goglia, F. Induction of UCP2 mRNA by thyroid hormones in rat heart. *FEBS Lett* (1997); **418**(1-2): 171-174.

LaNoue, K. F., Strzelecki, T., Strzelecka, D. and Koch, C. Regulation of the uncoupling protein in brown adipose tissue. *J Biol Chem* (1986); **261**(1): 298-305.

Larkin, S., Mull, E., Miao, W., Pittner, R., Albrandt, K., Moore, C., Young, A., Denaro, M. and Beaumont, K. Regulation of the third member of the uncoupling protein family, UCP3, by cold and thyroid hormone. *Biochem Biophys Res Commun* (1997); **240**(1): 222-227.

Lebovitz, R. M., Zhang, H., Vogel, H., Cartwright, J., Jr., Dionne, L., Lu, N., Huang, S. and Matzuk, M. M. Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. *Proc Natl Acad Sci U S A* (1996); **93**(18): 9782-9787.

Lee, F. Y., Li, Y., Zhu, H., Yang, S., Lin, H. Z., Trush, M. and Diehl, A. M. Tumor necrosis factor increases mitochondrial oxidant production and induces expression of uncoupling protein-2 in the regenerating mice [correction of rat] liver. *Hepatology* (1999); **29**(3): 677-687.

Leri, A., Claudio, P. P., Li, Q., Wang, X., Reiss, K., Wang, S., Malhotra, A., Kajstura, J. and Anversa, P. Stretch-mediated release of angiotensin II induces myocyte apoptosis by activating p53 that enhances the local renin-angiotensin system and decreases the Bcl-2-to-Bax protein ratio in the cell. *J Clin Invest* (1998); **101**(7): 1326-1342.

Lesnefsky, E. J., Moghaddas, S., Tandler, B., Kerner, J. and Hoppel, C. L. Mitochondrial dysfunction in cardiac disease: ischemia--reperfusion, aging, and heart failure. *J Mol Cell Cardiol* (2001); **33**(6): 1065-1089.

Levy, D., Garrison, R. J., Savage, D. D., Kannel, W. B. and Castelli, W. P. Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. *N Engl J Med* (1990); **322**(22): 1561-6.

Levy, D., Garrison, R. J., Savage, D. D., Kannel, W. B. and Castelli, W. P. Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. *N Engl J Med* (1990); **322**(22): 1561-1566.

Levy, D., Kenchaiah, S., Larson, M. G., Benjamin, E. J., Kupka, M. J., Ho, K. K., Murabito, J. M. and Vasan, R. S. Long-term trends in the incidence of and survival with heart failure. *N Engl J Med* (2002); **347**(18): 1397-1402.

Lewontin, R. C. The Interaction of Selection and Linkage. I. General considerations; heterotic models. *Genetics* (1964); **50**: 49-67.

Li, L., Desantiago, J., Chu, G., Kranias, E. G. and Bers, D. M. Phosphorylation of phospholamban and troponin I in beta-adrenergic-induced acceleration of cardiac relaxation. *Am J Physiol Heart Circ Physiol* (2000); **278**(3): H769-779.

Li, Y., Huang, T. T., Carlson, E. J., Melov, S., Ursell, P. C., Olson, J. L., Noble, L. J., Yoshimura, M. P., Berger, C., Chan, P. H., Wallace, D. C. and Epstein, C. J. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* (1995); **11**(4): 376-381.

Libby, P. Inflammation in atherosclerosis. *Nature* (2002); **420**(6917): 868-874.

Lin, C. S., Hackenberg, H. and Klingenberg, E. M. The uncoupling protein from brown adipose tissue mitochondria is a dimer. A hydrodynamic study. *FEBS Lett* (1980); **113**(2): 304-306.

Lindenmayer, G. E., Sordahl, L. A. and Schwartz, A. Reevaluation of oxidative phosphorylation in cardiac mitochondria from normal animals and animals in heart failure. *Circ Res* (1968); **23**(3): 439-450.

Lindmark, E., Diderholm, E., Wallentin, L. and Siegbahn, A. Relationship between interleukin 6 and mortality in patients with unstable coronary artery disease: effects of an early invasive or noninvasive strategy. *Jama* (2001); **286**(17): 2107-2113.

Lindsay, D. C., Holdright, D. R., Clarke, D., Anand, I. S., Poole-Wilson, P. A. and Collins, P. Endothelial control of lower limb blood flow in chronic heart failure. *Heart* (1996); **75**(5): 469-476.

Linz, W. and Scholkens, B. A. Influence of local converting enzyme inhibition on angiotensin and bradykinin effects in ischemic rat hearts. *J Cardiovasc Pharmacol* (1987); **10**(Suppl 7): S75-82.

Linz, W. and Scholkens, B. A. Role of bradykinin in the cardiac effects of angiotensin-converting enzyme inhibitors. *J Cardiovasc Pharmacol* (1992); **20 Suppl 9**: S83-90.

Linz, W. and Scholkens, B. A. A specific B2-bradykinin receptor antagonist HOE 140 abolishes the antihypertrophic effect of ramipril. *Br J Pharmacol* (1992); **105**(4): 771-772.

Linz, W., Scholkens, B. A. and Han, Y. F. Beneficial effects of the converting enzyme inhibitor, ramipril, in ischemic rat hearts. *J Cardiovasc Pharmacol* (1986); **8**(Suppl 10): S91-99.

Linz, W., Wiemer, G., Gohlke, P., Unger, T. and Scholkens, B. A. Contribution of kinins to the cardiovascular actions of angiotensin-converting enzyme inhibitors. *Pharmacol Rev* (1995); **47**(1): 25-49.

Linz, W., Wiemer, G. and Scholkens, B. A. Bradykinin prevents left ventricular hypertrophy in rats. *J Hypertens Suppl* (1993); **11 Suppl 5**: S96-S97.

Linz, W., Wiemer, G. and Scholkens, B. A. Role of kinins in the pathophysiology of myocardial ischaemia. In vitro and in vivo studies. *Diabetes* (1996); **45**(Suppl 1): S51-58.

Linz, W., Wiemer, G. and Scholkens, B. A. Role of kinins in the pathophysiology of myocardial ischemia. In vitro and in vivo studies. *Diabetes* (1996); **45 Suppl 1**: S51-58.

Lipkin, D. P., Jones, D. A., Round, J. M. and Poole-Wilson, P. A. Abnormalities of skeletal muscle in patients with chronic heart failure. *Int J Cardiol* (1988); **18**(2): 187-195.

Liu, J., Wang, C., Murakami, Y., Gong, G., Ishibashi, Y., Prody, C., Ochiai, K., Bache, R. J., Godinot, C. and Zhang, J. Mitochondrial ATPase and high-energy phosphates in failing hearts. *Am J Physiol Heart Circ Physiol* (2001); **281**(3): H1319-1326.

Liu, Y. H., Yang, X. P., Sharov, V. G., Nass, O., Sabbah, H. N., Peterson, E. and Carretero, O. A. Effects of angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor antagonists in rats with heart failure. Role of kinins and angiotensin II type 2 receptors. *J Clin Invest* (1997); **99**(8): 1926-1935.

Lo, L. W., Koch, C. J. and Wilson, D. F. Calibration of oxygen-dependent quenching of the phosphorescence of Pd-meso-tetra (4-carboxyphenyl) porphine: a phosphor with general application for measuring oxygen concentration in biological systems. *Anal Biochem* (1996); **236**(1): 153-160.

Loew, L. M., Tuft, R. A., Carrington, W. and Fay, F. S. Imaging in five dimensions: time-dependent membrane potentials in individual mitochondria. *Biophys J* (1993); **65**(6): 2396-2407.

Loke, K. E., Laycock, S. K., Mital, S., Wolin, M. S., Bernstein, R., Oz, M., Addonizio, L., Kaley, G. and Hintze, T. H. Nitric oxide modulates mitochondrial respiration in failing human heart. *Circulation* (1999); **100**(12): 1291-1297.

Lopez, J. R. and Parra, L. Inositol 1,4,5-trisphosphate increases myoplasmic [Ca2+] in isolated muscle fibers. Depolarization enhances its effects. *Cell Calcium* (1991); **12**(8): 543-557.

Lung, C. C., Chan, E. K. and Zuraw, B. L. Analysis of an exon 1 polymorphism of the B2 bradykinin receptor gene and its transcript in normal subjects and patients with C1 inhibitor deficiency. *J Allergy Clin Immunol* (1997); **99**(1 Pt 1): 134-146.

Lung, C. C., Chan, E. K. and Zuraw, B. L. Analysis of an exon 1 polymorphism of the B2 bradykinin receptor gene and its transcript in normal subjects and patients with C1 inhibitor deficiency. *J Allergy Clin Immunol* (1997); **99**(1 Pt 1): 134-46.

MacCarthy, P. A., Grieve, D. J., Li, J. M., Dunster, C., Kelly, F. J. and Shah, A. M. Impaired endothelial regulation of ventricular relaxation in cardiac hypertrophy: role of reactive oxygen species and NADPH oxidase. *Circulation* (2001); **104**(24): 2967-2974.

MacCarthy, P. A. and Shah, A. M. Impaired endothelium-dependent regulation of ventricular relaxation in pressure-overload cardiac hypertrophy. *Circulation* (2000); **101**(15): 1854-1860.

Madeddu, P., Varoni, M. V., Palomba, D., Emanueli, C., Demontis, M. P., Glorioso, N., Dessi-Fulgheri, P., Sarzani, R. and Anania, V. Cardiovascular phenotype of a mouse strain with disruption of bradykinin B2-receptor gene. *Circulation* (1997); **96**(10): 3570-3578.

Malik, I., Danesh, J., Whincup, P., Bhatia, V., Papacosta, O., Walker, M., Lennon, L., Thomson, A. and Haskard, D. Soluble adhesion molecules and prediction of coronary heart disease: a prospective study and meta-analysis. *Lancet* (2001); **358**(9286): 971-976.

Mancini, D. M., Coyle, E., Coggan, A., Beltz, J., Ferraro, N., Montain, S. and Wilson, J. R. Contribution of intrinsic skeletal muscle changes to 31P NMR skeletal muscle metabolic abnormalities in patients with chronic heart failure. *Circulation* (1989); **80**(5): 1338-1346.

Mancini, D. M., Henson, D., LaManca, J. and Levine, S. Evidence of reduced respiratory muscle endurance in patients with heart failure. *J Am Coll Cardiol* (1994); **24**(4): 972-81.

Mancini, G. B. and Stewart, D. J. Why were the results of the Heart Outcomes Prevention Evaluation (HOPE) trial so astounding? *Can J Cardiol* (2001); **17 Suppl A**: 15A-7A.

Mano, A., Tatsumi, T., Shiraishi, J., Keira, N., Nomura, T., Takeda, M., Nishikawa, S., Yamanaka, S., Matoba, S., Kobara, M., Tanaka, H., Shirayama, T., Takamatsu, T., Nozawa, Y. and Matsubara, H. Aldosterone directly induces myocyte apoptosis through calcineurin-dependent pathways. *Circulation* (2004); **110**(3): 317-23.

Mao, W., Yu, X. X., Zhong, A., Li, W., Brush, J., Sherwood, S. W., Adams, S. H. and Pan, G. UCP4, a novel brain-specific mitochondrial protein that reduces membrane potential in mammalian cells. *FEBS Lett* (1999); **443**(3): 326-330.

Marenberg, M. E., Risch, N., Berkman, L. F., Floderus, B. and de Faire, U. Genetic susceptibility to death from coronary heart disease in a study of twins. *N Engl J Med* (1994); **330**(15): 1041-6.

Marin-Garcia, J., Goldenthal, M. J. and Moe, G. W. Abnormal cardiac and skeletal muscle mitochondrial function in pacing-induced cardiac failure. *Cardiovasc Res* (2001); **52**(1): 103-110.

Marshall, R. P., McAnulty, R. J. and Laurent, G. J. Angiotensin II is mitogenic for human lung fibroblasts via activation of the type 1 receptor. *Am J Respir Crit Care Med* (2000); **161**(6): 1999-2004.

Marui, N., Offermann, M. K., Swerlick, R., Kunsch, C., Rosen, C. A., Ahmad, M., Alexander, R. W. and Medford, R. M. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *J Clin Invest* (1993); **92**(4): 1866-1874.

Masaki, T., Yoshimatsu, H., Kakuma, T., Hidaka, S., Kurokawa, M. and Sakata, T. Enhanced expression of uncoupling protein 2 gene in rat white adipose tissue and skeletal muscle following chronic treatment with thyroid hormone. *FEBS Lett* (1997); **418**(3): 323-326.

Masaki, T., Yoshimatsu, H. and Sakata, T. Expression of rat uncoupling protein family mRNA levels by chronic treatment with thyroid hormone. *Int J Obes Relat Metab Disord* (2000); **24 Suppl 2**: S162-S164.

Maskin, C. S., Forman, R., Sonnenblick, E. H., Frishman, W. H. and LeJemtel, T. H. Failure of dobutamine to increase exercise capacity despite hemodynamic improvement in severe chronic heart failure. *Am J Cardiol* (1983); **51**(1): 177-182.

Massie, B., Conway, M., Yonge, R., Frostick, S., Ledingham, J., Sleight, P., Radda, G. and Rajagopalan, B. Skeletal muscle metabolism in patients with congestive heart failure: relation to clinical severity and blood flow. *Circulation* (1987); **76**(5): 1009-1019.

Massie, B. M., Conway, M., Rajagopalan, B., Yonge, R., Frostick, S., Ledingham, J., Sleight, P. and Radda, G. Skeletal muscle metabolism during exercise under ischemic conditions in congestive heart failure. Evidence for abnormalities unrelated to blood flow. *Circulation* (1988); **78**(2): 320-326.

Massie, B. M., Conway, M., Yonge, R., Frostick, S., Sleight, P., Ledingham, J., Radda, G. and Rajagopalan, B. 31P nuclear magnetic resonance evidence of abnormal skeletal muscle metabolism in patients with congestive heart failure. *Am J Cardiol* (1987); **60**(4): 309-315.

Mathew, J., Sleight, P., Lonn, E., Johnstone, D., Pogue, J., Yi, Q., Bosch, J., Sussex, B., Probstfield, J. and Yusuf, S. Reduction of cardiovascular risk by regression of electrocardiographic markers of left ventricular hypertrophy by the angiotensin-converting enzyme inhibitor ramipril. *Circulation* (2001); **104**(14): 1615-1621.

Mathur, A., Hong, Y., Kemp, B. K., Barrientos, A. A. and Erusalimsky, J. D. Evaluation of fluorescent dyes for the detection of mitochondrial membrane potential changes in cultured cardiomyocytes. *Cardiovascular Research* (2000); **46**: 126-138.

Matsubara, H. Pathophysiological role of angiotensin II type 2 receptor in cardiovascular and renal diseases. *Circ Res* (1998); **83**(12): 1182-1191.

Matsuda, J., Hosoda, K., Itoh, H., Son, C., Doi, K., Tanaka, T., Fukunaga, Y., Inoue, G., Nishimura, H., Yoshimasa, Y., Yamori, Y. and Nakao, K. Cloning of rat uncoupling protein-3 and uncoupling protein-2 cDNAs: their gene expression in rats fed high-fat diet. *FEBS Lett* (1997); **418**(1-2): 200-204.

Mayer, B. and Hemmens, B. Biosynthesis and action of nitric oxide in mammalian cells. *Trends Biochem Sci* (1997); **22**(12): 477-481.

Mayfield, R. K., Shimojo, N. and Jaffa, A. A. Skeletal muscle kallikrein. Potential role in metabolic regulation. *Diabetes* (1996); **45 Suppl 1**: S20-23.

Mazzocchi, G., Malendowicz, L. K., Markowska, A., Albertin, G. and Nussdorfer, G. G. Role of adrenal renin-angiotensin system in the control of aldosterone secretion in sodium-restricted rats. *Am J Physiol Endocrinol Metab* (2000); **278**(6): E1027-1030.

McKelvie, R. S., Teo, K. K., McCartney, N., Humen, D., Montague, T. and Yusuf, S. Effects of exercise training in patients with congestive heart failure: a critical review. *J Am Coll Cardiol* (1995); **25**(3): 789-796.

McMurray, J., Chopra, M., Abdullah, I., Smith, W. E. and Dargie, H. J. Evidence of oxidative stress in chronic heart failure in humans. *Eur Heart J* (1993); **14**(11): 1493-1498.

Meade, T. W., Mellows, S., Brozovic, M., Miller, G. J., Chakrabarti, R. R., North, W. R., Haines, A. P., Stirling, Y., Imeson, J. D. and Thompson, S. G. Haemostatic function and ischaemic heart disease: principal results of the Northwick Park Heart Study. *Lancet* (1986); **2**(8506): 533-537.

Meirhaeghe, A., Amouyel, P., Helbecque, N., Cottel, D., Otabe, S., Froguel, P. and Vasseur, F. An uncoupling protein 3 gene polymorphism associated with a lower risk of developing Type II diabetes and with atherogenic lipid profile in a French cohort. *Diabetologia* (2000); **43**(11): 1424-1428.

MERIT-HF. Effect of metoprolol CR/XL in chronic heart failure: Metoprolol CR/XL Randomised Intervention Trial in Congestive Heart Failure (MERIT-HF). *Lancet* (1999); **353**(9169): 2001-2007.

Mettauer, B., Zoll, J., Sanchez, H., Lampert, E., Ribera, F., Veksler, V., Bigard, X., Mateo, P., Epailly, E., Lonsdorfer, J. and Ventura-Clapier, R. Oxidative capacity of skeletal muscle in heart failure patients versus sedentary or active control subjects. *J Am Coll Cardiol* (2001); **38**(4): 947-954.

Mezzano, S. A., Ruiz-Ortega, M. and Egido, J. Angiotensin II and renal fibrosis. *Hypertension* (2001); **38**(3 Pt 2): 635-638.

Michel, B., Grima, M., Nirina, L. B., Ingert, C., Coquard, C., Barthelmebs, M. and Imbs, J. L. Inhibitory effect of reactive oxygen species on angiotensin I-converting enzyme (kininase II). *Clin Exp Pharmacol Physiol* (2001); **28**(3): 212-218.

Michelakis, E. D., Hampl, V., Nsair, A., Wu, X., Harry, G., Haromy, A., Gurtu, R. and Archer, S. L. Diversity in mitochondrial function explains differences in vascular oxygen sensing. *Circ Res* (2002); **90**(12): 1307-1315.

Michelakis, E. D., Rebeyka, I., Wu, X., Nsair, A., Thebaud, B., Hashimoto, K., Dyck, J. R., Haromy, A., Harry, G., Barr, A. and Archer, S. L. O2 sensing in the human ductus arteriosus: regulation of voltage-gated K+ channels in smooth muscle cells by a mitochondrial redox sensor. *Circ Res* (2002); **91**(6): 478-486.

Milano, C. A., Dolber, P. C., Rockman, H. A., Bond, R. A., Venable, M. E., Allen, L. F. and Lefkowitz, R. J. Myocardial expression of a constitutively active alpha 1B-adrenergic receptor in transgenic mice induces cardiac hypertrophy. *Proc Natl Acad Sci U S A* (1994); **91**(21): 10109-10113.

Miles, L. A., Fless, G. M., Levin, E. G., Scanu, A. M. and Plow, E. F. A potential basis for the thrombotic risks associated with lipoprotein(a). *Nature* (1989); **339**(6222): 301-303.

Miller, D. J. and MacFarlane, N. G. Intracellular effects of free radicals and reactive oxygen species in cardiac muscle. *J Hum Hypertens* (1995); **9**(6): 465-473.

Miller, G. J., Bauer, K. A., Barzegar, S., Foley, A. J., Mitchell, J. P., Cooper, J. A. and Rosenberg, R. D. The effects of quality and timing of venepuncture on markers of blood coagulation in healthy middle-aged men. *Thromb Haemost* (1995); **73**(1): 82-6.

Miller, G. J., Bauer, K. A., Barzegar, S., Foley, A. J., Mitchell, J. P., Cooper, J. A. and Rosenberg, R. D. The effects of quality and timing of venepuncture on markers of blood coagulation in healthy middle-aged men. *Thromb Haemost* (1995); **73**(1): 82-86.

Miller, S. A., Dykes, D. D. and Polesky, H. F. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* (1988); **16**(3): 1215.

Millet, L., Vidal, H., Andreelli, F., Larrouy, D., Riou, J. P., Ricquier, D., Laville, M. and Langin, D. Increased uncoupling protein-2 and -3 mRNA expression during fasting in obese and lean humans. *J Clin Invest* (1997); **100**(11): 2665-2670.

Minners, J., Lacerda, L., McCarthy, J., Meiring, J. J., Yellon, D. M. and Sack, M. N. Ischemic and pharmacological preconditioning in Girardi cells and C2C12 myotubes induce mitochondrial uncoupling. *Circ Res* (2001); **89**(9): 787-792.

Minotti, J. R., Christoph, I., Oka, R., Weiner, M. W., Wells, L. and Massie, B. M. Impaired skeletal muscle function in patients with congestive heart failure. Relationship to systemic exercise performance. *J Clin Invest* (1991); **88**(6): 2077-2082.

Minshall, R. D., Tan, F., Nakamura, F., Rabito, S. F., Becker, R. P., Marcic, B. and Erdos, E. G. Potentiation of the actions of bradykinin by angiotensin I-converting enzyme inhibitors. The role of expressed human bradykinin B2 receptors and angiotensin I-converting enzyme in CHO cells [published erratum appears in Circ Res 1998 Jan 9-23;82(1):137]. *Circ Res* (1997); **81**(5): 848-856.

Mitchell, P. and Moyle, J. Chemiosmotic hypothesis of oxidative phosphorylation. *Nature* (1967); **213**(72): 137-139.

Mjos, O. D. Effect of free fatty acids on myocardial function and oxygen consumption in intact dogs. *J Clin Invest* (1971); **50**(7): 1386-1389.

Modriansky, M., Murdza-Inglis, D. L., Patel, H. V., Freeman, K. B. and Garlid, K. D. Identification by site-directed mutagenesis of three arginines in uncoupling protein that are essential for nucleotide binding and inhibition. *J Biol Chem* (1997); **272**(40): 24759-24762.

Mohan, P., Brutsaert, D. L., Paulus, W. J. and Sys, S. U. Myocardial contractile response to nitric oxide and cGMP. *Circulation* (1996); **93**(6): 1223-1229.

Moncada, S. and Erusalimsky, J. D. Does nitric oxide modulate mitochondrial energy generation and apoptosis? *Nat Rev Mol Cell Biol* (2002); **3**(3): 214-220.

Moncada, S. and Higgs, A. The L-arginine-nitric oxide pathway. *N Engl J Med* (1993); **329**(27): 2002-2012.

Montgomery, H., Clarkson, P., Barnard, M., Bell, J., Brynes, A., Dollery, C., Hajnal, J., Hemingway, H., Mercer, D., Jarman, P., Marshall, R., Prasad, K., Rayson, M., Saeed, N., Talmud, P., Thomas, L., Jubb, M., World, M. and Humphries, S. Angiotensin-converting-enzyme gene insertion/deletion polymorphism and response to physical training. *Lancet* (1999); **353**(9152): 541-545.

Montgomery, H., Clarkson, P., Barnard, M., Bell, J., Brynes, A., Dollery, C., Hajnal, J., Hemingway, H., Mercer, D., Jarman, P., Marshall, R., Prasad, K., Rayson, M., Saeed, N., Talmud, P., Thomas, L., Jubb, M., World, M. and Humphries, S. Angiotensin-converting-enzyme gene insertion/deletion polymorphism and response to physical training [see comments]. *Lancet* (1999); **353**(9152): 541-545.

Montgomery, H. and Dansek, A. H. In search of genetic precision. *Lancet* (2003); **361**(9372): 1909; author reply 1909-10.

Montgomery, H. and Dhamrait, S. ACE genotype and performance. *J Appl Physiol* (2002); **92**(4): 1774-1775; author reply 1776-7.

Montgomery, H. E., Clarkson, P., Dollery, C. M., Prasad, K., Losi, M. A., Hemingway, H., Statters, D., Jubb, M., Girvain, M., Varnava, A., World, M., Deanfield, J., Talmud, P., McEwan, J. R., McKenna, W. J. and Humphries, S. Association of angiotensin-converting enzyme gene I/D polymorphism with change in left ventricular mass in response to physical training. *Circulation* (1997); **96**(3): 741-747.

Montgomery, H. E., Clarkson, P., Nwose, O. M., Mikailidis, D. P., Jagroop, I. A., Dollery, C., Moult, J., Benhizia, F., Deanfield, J., Jubb, M., World, M., McEwan, J. R., Winder, A. and Humphries, S. The acute rise in plasma fibrinogen concentration with exercise is influenced by the G-453-A polymorphism of the beta-fibrinogen gene. *Arterioscler Thromb Vasc Biol* (1996); **16**(3): 386-391.

Montgomery, H. E., Marshall, R., Hemingway, H., Myerson, S., Clarkson, P., Dollery, C., Hayward, M., Holliman, D. E., Jubb, M., World, M., Thomas, E. L., Brynes, A. E., Saeed, N., Barnard, M., Bell, J. D., Prasad, K., Rayson, M., Talmud, P. J. and Humphries, S. E. Human gene for physical performance. *Nature* (1998); **393**(6682): 221-222.

Morrow, J. D. and Roberts, L. J., 2nd. Mass spectrometry of prostanoids: F2-isoprostanes produced by non-cyclooxygenase free radical-catalyzed mechanism. *Methods Enzymol* (1994); **233**: 163-74.

Motoshima, H., Araki, E., Nishiyama, T., Taguchi, T., Kaneko, K., Hirashima, Y., Yoshizato, K., Shirakami, A., Sakai, K., Kawashima, J., Shirotani, T., Kishikawa, H. and Shichiri, M. Bradykinin enhances insulin receptor tyrosine kinase in 32D cells reconstituted with bradykinin and insulin signaling pathways. *Diabetes Res Clin Pract* (2000); **48**(3): 155-170.

MRFIT Research Group. Relationship between baseline risk factors and coronary heart disease and total mortality in the Multiple Risk Factor Intervention Trial. *Prev Med* (1986); **15**(3): 254-273.

Murakami, H., Yayama, K., Miao, R. Q., Wang, C., Chao, L. and Chao, J. Kallikrein gene delivery inhibits vascular smooth muscle cell growth and neointima formation in the rat artery after balloon angioplasty. *Hypertension* (1999); **34**(2): 164-170.

Murakami, K., Mizushige, K., Noma, T., Kimura, S., Abe, Y. and Matsuo, H. Effects of perindopril on left ventricular remodeling and aortic regurgitation in rats assessed by echocardiography. *Angiology* (2000); **51**(11): 943-952.

Murakami, K., Mizushige, K., Noma, T., Tsuji, T., Kimura, S. and Kohno, M. Perindopril effect on uncoupling protein and energy metabolism in failing rat hearts. *Hypertension* (2002); **40**(3): 251-255.

Murphey, L. J., Gainer, J. V., Vaughan, D. E. and Brown, N. J. Angiotensin-converting enzyme insertion/deletion polymorphism modulates the human in vivo metabolism of bradykinin. *Circulation* (2000); **102**(8): 829-832.

Murphy, M. P., Echtay, K. S., Blaikie, F. H., Asin-Cayuela, J., Cocheme, H. M., Green, K., Buckingham, J. A., Taylor, E. R., Hurrell, F., Hughes, G., Miwa, S., Cooper, C. E., Svistunenko, D. A., Smith, R. A. and Brand, M. D. Superoxide activates uncoupling proteins by generating carbon-centered radicals and initiating lipid peroxidation: studies using a mitochondria-targeted spin trap derived from alpha-phenyl-N-tert-butylnitrone. *J Biol Chem* (2003); **278**(49): 48534-48545.

Murray, A. J., Anderson, R. E., Watson, G. C., Radda, G. K. and Clarke, K. Uncoupling proteins in human heart. *Lancet* (2004); **364**(9447): 1786-1788.

Myerson, S., Hemingway, H., Budget, R., Martin, J., Humphries, S. and Montgomery, H. Human angiotensin I-converting enzyme gene and endurance performance. *J Appl Physiol* (1999); **87**(4): 1313-1316.

Myerson, S. G., Montgomery, H. E., Whittingham, M., Jubb, M., World, M. J., Humphries, S. E. and Pennell, D. J. Left Ventricular Hypertrophy With Exercise and ACE Gene Insertion/Deletion Polymorphism : A Randomized Controlled Trial With Losartan. *Circulation* (2001); **103**(2): 226-230.

Nagase, I., Yoshida, T. and Saito, M. Up-regulation of uncoupling proteins by beta-adrenergic stimulation in L6 myotubes. *FEBS Lett* (2001); **494**(3): 175-180.

Nakae, I., Mitsunami, K., Omura, T., Yabe, T., Tsutamoto, T., Matsuo, S., Takahashi, M., Morikawa, S., Inubushi, T., Nakamura, Y., Kinoshita, M. and Horie, M. Proton magnetic resonance spectroscopy can detect creatine depletion associated with the progression of heart failure in cardiomyopathy. *J Am Coll Cardiol* (2003); **42**(9): 1587-1593.

Nakamura, K., Fushimi, K., Kouchi, H., Mihara, K., Miyazaki, M., Ohe, T. and Namba, M. Inhibitory effects of antioxidants on neonatal rat cardiac myocyte hypertrophy induced by tumor necrosis factor-alpha and angiotensin II. *Circulation* (1998); **98**(8): 794-799.

Nakamura, Y., Nagase, I., Asano, A., Sasaki, N., Yoshida, T., Umekawa, T., Sakane, N. and Saito, M. Beta 3-adrenergic agonist up-regulates uncoupling proteins 2 and 3 in skeletal muscle of the mouse. *J Vet Med Sci* (2001); **63**(3): 309-314.

Nascimben, L., Friedrich, J., Liao, R., Pauletto, P., Pessina, A. C. and Ingwall, J. S. Enalapril treatment increases cardiac performance and energy reserve via the creatine kinase reaction in myocardium of Syrian myopathic hamsters with advanced heart failure. *Circulation* (1995); **91**(6): 1824-1833.

Navab, M., Hama, S. Y., Ready, S. T., Ng, C. J., Van Lenten, B. J., Laks, H. and Fogelman, A. M. Oxidized lipids as mediators of coronary heart disease. *Curr Opin Lipidol* (2002); **13**(4): 363-372.

Negre-Salvayre, A., Hirtz, C., Carrera, G., Cazenave, R., Troly, M., Salvayre, R., Penicaud, L. and Casteilla, L. A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. *Faseb J* (1997); **11**(10): 809-815.

Neri Serneri, G. G., Boddi, M., Coppo, M., Chechi, T., Zarone, N., Moira, M., Poggesi, L., Margheri, M. and Simonetti, I. Evidence for the existence of a functional cardiac renin-angiotensin system in humans. *Circulation* (1996); **94**(8): 1886-1893.

Neubauer, S., Horn, M., Cramer, M., Harre, K., Newell, J. B., Peters, W., Pabst, T., Ertl, G., Hahn, D., Ingwall, J. S. and Kochsiek, K. Myocardial phosphocreatine-to-ATP ratio is a predictor of mortality in patients with dilated cardiomyopathy. *Circulation* (1997); **96**(7): 2190-2196.

Newsholme, E. A., Blomstrand, E. and Ekblom, B. Physical and mental fatigue: metabolic mechanisms and importance of plasma amino acids. *Br Med Bull* (1992); **48**(3): 477-495.

Ni, A., Chao, L. and Chao, J. Transcription factor nuclear factor kappaB regulates the inducible expression of the human B1 receptor gene in inflammation. *J Biol Chem* (1998); **273**(5): 2784-2791.

Nicholls, D. G. Hamster brown-adipose-tissue mitochondria. The control of respiration and the proton electrochemical potential gradient by possible physiological effectors of the proton conductance of the inner membrane. *Eur J Biochem* (1974); **49**(3): 573-83.

Nicholls, D. G. A history of UCP1. *Biochem Soc Trans* (2001); **29**(Pt 6): 751-755.

Nicholls, D. G. and Ferguson, S. J. Bioenergetics 3. London (2002), Academic Press.

Nicholls, D. G. and Lindberg, O. Brown-adipose-tissue mitochondria. The influence of albumin and nucleotides on passive ion permeabilities. *Eur J Biochem* (1973); **37**(3): 523-530.

Nishikawa, T., Edelstein, D., Du, X. L., Yamagishi, S., Matsumura, T., Kaneda, Y., Yorek, M. A., Beebe, D., Oates, P. J., Hammes, H. P., Giardino, I. and Brownlee, M. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* (2000); **404**(6779): 787-790.

Nishio, E. and Watanabe, Y. The involvement of reactive oxygen species and arachidonic acid in alpha 1-adrenoceptor-induced smooth muscle cell proliferation and migration. *Br J Pharmacol* (1997); **121**(4): 665-670.

Nisoli, E., Carruba, M. O., Tonello, C., Macor, C., Federspil, G. and Vettor, R. Induction of fatty acid translocase/CD36, peroxisome proliferator- activated receptor-gamma2, leptin, uncoupling proteins 2 and 3, and tumor necrosis factor-alpha gene expression in human subcutaneous fat by lipid infusion. *Diabetes* (2000); **49**(3): 319-324.

Noma, T., Nishiyama, A., Mizushige, K., Murakami, K., Tsuji, T., Kohno, M., Rahman, M., Fukui, T., Abe, Y. and Kimura, S. Possible role of uncoupling protein in regulation of myocardial energy metabolism in aortic regurgitation model rats. *Faseb J* (2001); **15**(7): 1206-1208.

Nordberg, J. and Arner, E. S. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic Biol Med* (2001); **31**(11): 1287-1312.

Nordfors, L., Heimburger, O., Lonnqvist, F., Lindholm, B., Helmrich, J., Schalling, M. and Stenvinkel, P. Fat tissue accumulation during peritoneal dialysis is associated with a polymorphism in uncoupling protein 2. *Kidney Int* (2000); **57**(4): 1713-1719.

Notarius, C. F., Atchison, D. J. and Floras, J. S. Impact of heart failure and exercise capacity on sympathetic response to handgrip exercise. *Am J Physiol Heart Circ Physiol* (2001); **280**(3): H969-976.

Nuydens, R., Novalbos, J., Dispersyn, G., Weber, C., Borgers, M. and Geerts, H. A rapid method for the evaluation of compounds with mitochondria-protective properties. *J Neurosci Methods* (1999); **92**(1-2): 153-159.

O'Rahilly, S. Uncoupling protein 2: Adiposity angel and diabetes devil? *Nat Med* (2001); **7**(7): 770-772.

Olivetti, G., Abbi, R., Quaini, F., Kajstura, J., Cheng, W., Nitahara, J. A., Quaini, E., Di Loreto, C., Beltrami, C. A., Krajewski, S., Reed, J. C. and Anversa, P. Apoptosis in the failing human heart. *N Engl J Med* (1997); **336**(16): 1131-141.

Olson, R. E. and Schwartz, W. B. Myocardial metabolism in congestive heart failure. *Medicine (Baltimore)* (1951); **30**(1): 21-41.

Onder, G., Penninx, B. W., Balkrishnan, R., Fried, L. P., Chaves, P. H., Williamson, J., Carter, C., Di Bari, M., Guralnik, J. M. and Pahor, M. Relation between use of angiotensin-converting enzyme inhibitors and muscle strength and physical function in older women: an observational study. *Lancet* (2002); **359**(9310): 926-930.

Opasich, C., Aquilani, R., Dossena, M., Foppa, P., Catapano, M., Pagani, S., Pasini, E., Ferrari, R., Tavazzi, L. and Pastoris, O. Biochemical analysis of muscle biopsy in overnight fasting patients with severe chronic heart failure. *Eur Heart J* (1996); **17**(11): 1686-1693.

Opasich, C., Pasini, E., Aquilani, R., Cobelli, F., Solfrini, R., Ferrari, R. and Tavazzi, L. Skeletal muscle function at low work level as a model for daily activities in patients with chronic heart failure. *Eur Heart J* (1997); **18**(10): 1626-1631.

Ostrowski, K., Hermann, C., Bangash, A., Schjerling, P., Nielsen, J. N. and Pedersen, B. K. A trauma-like elevation of plasma cytokines in humans in response to treadmill running. *J Physiol* (1998); **513 (Pt 3)**: 889-894.

Otabe, S., Clement, K., Dina, C., Pelloux, V., Guy-Grand, B., Froguel, P. and Vasseur, F. A genetic variation in the 5' flanking region of the UCP3 gene is associated with body mass index in humans in interaction with physical activity. *Diabetologia* (2000); **43**(2): 245-249.

Otabe, S., Clement, K., Rich, N., Warden, C., Pecqueur, C., Neverova, M., Raimbault, S., Guy-Grand, B., Basdevant, A., Ricquier, D., Froguel, P. and Vasseur, F. Mutation screening of the human UCP 2 gene in normoglycemic and NIDDM morbidly obese patients: lack of association between new UCP 2 polymorphisms and obesity in French Caucasians. *Diabetes* (1998); **47**(5): 840-842.

Packer, M., Fowler, M. B., Roecker, E. B., Coats, A. J., Katus, H. A., Krum, H., Mohacsi, P., Rouleau, J. L., Tendera, M., Staiger, C., Holcslaw, T. L., Amann-Zalan, I. and DeMets, D. L. Effect of carvedilol on the morbidity of patients with severe chronic heart failure: results of the carvedilol prospective randomized cumulative survival (COPERNICUS) study. *Circulation* (2002); **106**(17): 2194-2199.

Palmer, R. M. J., Ferrige, A. G. and Moncada, S. Nitric oxide release accounts for the biological activity of endothelial-derived relaxing factor. *Nature* (1987); **327**: 524-526.

Panahloo, A., Andres, C., Mohamed-Ali, V., Gould, M. M., Talmud, P., Humphries, S. E. and Yudkin, J. S. The insertion allele of the ACE gene I/D polymorphism. A candidate gene for insulin resistance? *Circulation* (1995); **92**(12): 3390-3393.

Paolisso, G., Gambardella, A., Galzerano, D., D'Amore, A., Rubino, P., Verza, M., Teasuro, P., Varricchio, M. and D'Onofrio, F. Total-body and myocardial substrate oxidation in congestive heart failure. *Metabolism* (1994); **43**(2): 174-179.

Parenti, A., Morbidelli, L., Ledda, F., Granger, H. J. and Ziche, M. The bradykinin/B1 receptor promotes angiogenesis by up-regulation of endogenous FGF-2 in endothelium via the nitric oxide synthase pathway. *Faseb J* (2001); **15**(8): 1487-1489.

Parissis, J. T., Venetsanou, K. F., Kalantzi, M. V., Mentzikof, D. D. and Karas, S. M. Serum profiles of granulocyte-macrophage colony-stimulating factor and C-C chemokines in hypertensive patients with or without significant hyperlipidemia. *Am J Cardiol* (2000); **85**(6): 777-9, A9.

Park, J. Y., Takahara, N., Gabriele, A., Chou, E., Naruse, K., Suzuma, K., Yamauchi, T., Ha, S. W., Meier, M., Rhodes, C. J. and King, G. L. Induction of endothelin-1 expression by glucose: an effect of protein kinase C activation. *Diabetes* (2000); **49**(7): 1239-1248.

Parthasarathi, K., Ichimura, H., Quadri, S., Issekutz, A. and Bhattacharya, J. Mitochondrial reactive oxygen species regulate spatial profile of proinflammatory responses in lung venular capillaries. *J Immunol* (2002); **169**(12): 7078-7086.

Pasceri, V., Willerson, J. T. and Yeh, E. T. Direct proinflammatory effect of C-reactive protein on human endothelial cells. *Circulation* (2000); **102**(18): 2165-2168.

Pearlstein, D. P., Ali, M. H., Mungai, P. T., Hynes, K. L., Gewertz, B. L. and Schumacker, P. T. Role of mitochondrial oxidant generation in endothelial cell responses to hypoxia. *Arterioscler Thromb Vasc Biol* (2002); **22**(4): 566-573.

Pecqueur, C., Alves-Guerra, M. C., Gelly, C., Levi-Meyrueis, C., Couplan, E., Collins, S., Ricquier, D., Bouillaud, F. and Miroux, B. Uncoupling protein 2, in vivo distribution, induction upon oxidative stress, and evidence for translational regulation. *J Biol Chem* (2001); **276**(12): 8705-8712.

Pecqueur, C., Cassard-Doulcier, A. M., Raimbault, S., Miroux, B., Fleury, C., Gelly, C., Bouillaud, F. and Ricquier, D. Functional organization of the human uncoupling protein-2 gene, and juxtaposition to the uncoupling protein-3 gene. *Biochem Biophys Res Commun* (1999); **255**(1): 40-46.

Pedersen-Bjergaard, U., Agerholm-Larsen, B., Pramming, S., Hougaard, P. and Thorsteinsson, B. Activity of angiotensin-converting enzyme and risk of severe hypoglycaemia in type 1 diabetes mellitus. *Lancet* (2001); **357**(9264): 1248-1253.

Pedersen, B. K. and Hoffman-Goetz, L. Exercise and the immune system: regulation, integration, and adaptation. *Physiol Rev* (2000); **80**(3): 1055-1081.

Pedersen, S. B., Borglum, J. D., Kristensen, K., Norrelund, H., Otto, J., Jorgensen, L. and Richelsen, B. Regulation of uncoupling protein (UCP) 2 and 3 in adipose and muscle tissue by fasting and growth hormone treatment in obese humans. *Int J Obes Relat Metab Disord* (2000); **24**(8): 968-975.

Peeters, G. A., Sanguinetti, M. C., Eki, Y., Konarzewska, H., Renlund, D. G., Karwande, S. V. and Barry, W. H. Method for isolation of human ventricular myocytes from single endocardial and epicardial biopsies. *Am J Physiol* (1995); **268**(4 Pt 2): H1757-1764.

Peters, J., Kranzlin, B., Schaeffer, S., Zimmer, J., Resch, S., Bachmann, S., Gretz, N. and Hackenthal, E. Presence of renin within intramitochondrial dense bodies of the rat adrenal cortex. *Am J Physiol* (1996); **271**(3 Pt 1): E439-450.

Petros, A., Bennett, D. and Vallance, P. Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. *Lancet* (1991); **338**(8782-8783): 1557-1558.

Pfeffer, M. A., Braunwald, E., Moye, L. A., Basta, L., Brown, E. J., Jr., Cuddy, T. E., Davis, B. R., Geltman, E. M., Goldman, S., Flaker, G. C. and et al. Effect of captopril on mortality and morbidity in patients with left ventricular dysfunction after myocardial infarction. Results of the survival and ventricular enlargement trial. The SAVE Investigators. *N Engl J Med* (1992); **327**(10): 669-677.

Piepoli, M., Clark, A. L., Volterrani, M., Adamopoulos, S., Sleight, P. and Coats, A. J. Contribution of muscle afferents to the hemodynamic, autonomic, and ventilatory responses to exercise in patients with chronic heart failure: effects of physical training. *Circulation* (1996); **93**(5): 940-952.

Pieruzzi, F., Abassi, Z. A. and Keiser, H. R. Expression of renin-angiotensin system components in the heart, kidneys, and lungs of rats with experimental heart failure. *Circulation* (1995); **92**(10): 3105-3112.

Pimentel, D. R., Amin, J. K., Xiao, L., Miller, T., Viereck, J., Oliver-Krasinski, J., Baliga, R., Wang, J., Siwik, D. A., Singh, K., Pagano, P., Colucci, W. S. and Sawyer, D. B. Reactive oxygen species mediate amplitude-dependent hypertrophic and apoptotic responses to mechanical stretch in cardiac myocytes. *Circ Res* (2001); **89**(5): 453-460.

Pitt, B., Poole-Wilson, P. A., Segal, R., Martinez, F. A., Dickstein, K., Camm, A. J., Konstam, M. A., Riegger, G., Klinger, G. H., Neaton, J., Sharma, D. and Thiyagarajan, B. Effect of losartan compared with captopril on mortality in patients with symptomatic heart failure: randomised trial--the Losartan Heart Failure Survival Study ELITE II. *Lancet* (2000); **355**(9215): 1582-1587.

Pitt, B., Remme, W., Zannad, F., Neaton, J., Martinez, F., Roniker, B., Bittman, R., Hurley, S., Kleiman, J. and Gatlin, M. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. *N Engl J Med* (2003); **348**(14): 1309-1321.

Pitt, B., Segal, R., Martinez, F. A., Meurers, G., Cowley, A. J., Thomas, I., Deedwania, P. C., Ney, D. E., Snavely, D. B. and Chang, P. I. Randomised trial of losartan versus captopril in patients over 65 with heart failure (Evaluation of Losartan in the Elderly Study, ELITE). *Lancet* (1997); **349**(9054): 747-752.

Pitt, B., Zannad, F., Remme, W. J., Cody, R., Castaigne, A., Perez, A., Palensky, J. and Wittes, J. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *N Engl J Med* (1999); **341**(10): 709-717.

Poderoso, J. J., Carreras, M. C., Lisdero, C., Riobo, N., Schopfer, F. and Boveris, A. Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. *Arch Biochem Biophys* (1996); **328**(1): 85-92.

Poderoso, J. J., Peralta, J. G., Lisdero, C. L., Carreras, M. C., Radisic, M., Schopfer, F., Cadenas, E. and Boveris, A. Nitric oxide regulates oxygen uptake and hydrogen peroxide release by the isolated beating rat heart. *Am J Physiol* (1998); **274**(1 Pt 1): C112-119.

Possel, H., Noack, H., Keilhoff, G. and Wolf, G. Life imaging of peroxynitrite in rat microglial and astroglial cells: Role of superoxide and antioxidants. *Glia* (2002); **38**(4): 339-350.

Potter, D. D., Sobey, C. G., Tompkins, P. K., Rossen, J. D. and Heistad, D. D. Evidence that macrophages in atherosclerotic lesions contain angiotensin II. *Circulation* (1998); **98**(8): 800-807.

Pralong, W. F., Spat, A. and Wollheim, C. B. Dynamic pacing of cell metabolism by intracellular Ca2+ transients. *J Biol Chem* (1994); **269**(44): 27310-27314.

Prineas, R., Crow, R. and Blackburn, H. The Minnesota Code Manual of Electrocardiographic Findings. Standards and Procedures for Measurements and Classification. *Boston: John Wright, PSC Inc* (1982).

Prunet-Marcassus, B., Moulin, K., Carmona, M. C., Villarroya, F., Penicaud, L. and Casteilla, L. Inverse distribution of uncoupling proteins expression and oxidative capacity in mature adipocytes and stromal-vascular fractions of rat white and brown adipose tissues. *FEBS Lett* (1999); **464**(3): 184-188.

Pyorala, K., Laakso, M. and Uusitupa, M. Diabetes and atherosclerosis: an epidemiologic view. *Diabetes Metab Rev* (1987); **3**(2): 463-524.

Quinn, S. J., Williams, G. H. and Tillotson, D. L. Calcium oscillations in single adrenal glomerulosa cells stimulated by angiotensin II. *Proc Natl Acad Sci U S A* (1988); **85**(15): 5754-5758.

Rabito, S. F., Minshall, R. D., Nakamura, F. and Wang, L.-X. Bradykinin B2 receptors on skeletal muscle are coupled to inositol 1,4,5-triphosphate formation. *Diabetes* (1996); **45**(Suppl 1): S29-33.

Rabkin, S. W. Cilazapril and captopril accelerate recovery from hypoxia in myocardial cell aggregates in culture. *J Cardiovasc Pharmacol* (1992); **19**(3): 394-401.

Radi, R., Rodriguez, M., Castro, L. and Telleri, R. Inhibition of mitochondrial electron transport by peroxynitrite. *Arch Biochem Biophys* (1994); **308**(1): 89-95.

Rahemtulla, T. and Bhopal, R. Pharmacogenetics and ethnically targeted therapies. *Bmj* (2005); **330**(7499): 1036-1037.

Raidoo, D. M., Ramsaroop, R., Naidoo, S., Muller-Esterl, W. and Bhoola, K. D. Kinin receptors in human vascular tissue: their role in atheromatous disease. *Immunopharmacology* (1997); **36**(2-3): 153-160.

Rajagopalan, S., Kurz, S., Munzel, T., Tarpey, M., Freeman, B. A., Griendling, K. K. and Harrison, D. G. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest* (1996); **97**(8): 1916-1923.

Rajagopalan, S., Meng, X. P., Ramasamy, S., Harrison, D. G. and Galis, Z. S. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. *J Clin Invest* (1996); **98**(11): 2572-2579.

Rajfer, S. I., Nemanich, J. W., Shurman, A. J. and Rossen, J. D. Metabolic responses to exercise in patients with heart failure. *Circulation* (1987); **76**(6 Pt 2): VI46-153.

Rakhit, R. D., Mojet, M. H., Marber, M. S. and Duchen, M. R. Mitochondria as targets for nitric oxide-induced protection during simulated ischemia and reoxygenation in isolated neonatal cardiomyocytes. *Circulation* (2001); **103**(21): 2617-2623.

Rankinen, T., Perusse, L., Rauramaa, R., Rivera, M. A., Wolfarth, B. and Bouchard, C. The human gene map for performance and health-related fitness phenotypes: the 2001 update. *Med Sci Sports Exerc* (2002); **34**(8): 1219-33.

Rankinen, T., Wolfarth, B., Simoneau, J. A., Maier-Lenz, D., Rauramaa, R., Rivera, M. A., Boulay, M. R., Chagnon, Y. C., Perusse, L., Keul, J. and Bouchard, C. No association between the angiotensin-converting enzyme ID polymorphism and elite endurance athlete status. *J Appl Physiol* (2000); **88**(5): 1571-1575.

Ravussin, E. and Bogardus, C. A brief overview of human energy metabolism and its relationship to essential obesity. *Am J Clin Nutr* (1992); **55**(1 Suppl): 242S-245S.

Razeghi, P., Young, M. E., Alcorn, J. L., Moravec, C. S., Frazier, O. H. and Taegtmeyer, H. Metabolic gene expression in fetal and failing human heart. *Circulation* (2001); **104**(24): 2923-2931.

Rector, T. S., Johnson, G., Dunkman, W. B., Daniels, G., Farrell, L., Henrick, A., Smith, B. and Cohn, J. N. Evaluation by patients with heart failure of the effects of enalapril compared with hydralazine plus isosorbide dinitrate on quality of life. V-HeFT II. The V-HeFT VA Cooperative Studies Group. *Circulation* (1993); **87**(6 Suppl): VI71-177.

Reers, M., Smith, T. W. and Chen, L. B. J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. *Biochemistry* (1991); **30**(18): 4480-4486.

Regoli, D., Rizzi, A., Calo, G., Nsa Allogho, S. and Gobeil, F. B1 and B2 kinin receptors in various species. *Immunopharmacology* (1997); **36**(2-3): 143-147.

Reich, D. E., Cargill, M., Bolk, S., Ireland, J., Sabeti, P. C., Richter, D. J., Lavery, T., Kouyoumjian, R., Farhadian, S. F., Ward, R. and Lander, E. S. Linkage disequilibrium in the human genome. *Nature* (2001); **411**(6834): 199-204.

Reneland, R. and Lithell, H. Angiotensin-converting enzyme in human skeletal muscle. A simple in vitro assay of activity in needle biopsy specimens. *Scand J Clin Lab Invest* (1994); **54**(2): 105-111.

Rett, K., Wicklmayr, M., Dietze, G. J. and Haring, H. U. Insulin-induced glucose transporter (GLUT1 and GLUT4) translocation in cardiac muscle tissue is mimicked by bradykinin. *Diabetes* (1996); **45 Suppl 1**: S66-69.

Rial, E., Gonzalez-Barroso, M., Fleury, C., Iturrizaga, S., Sanchis, D., Jimenez-Jimenez, J., Ricquier, D., Goubern, M. and Bouillaud, F. Retinoids activate proton transport by the uncoupling proteins UCP1 and UCP2. *Embo J* (1999); **18**(21): 5827-5833.

Ricquier, D. and Bouillaud, F. Mitochondrial uncoupling proteins: from mitochondria to the regulation of energy balance. *J Physiol* (2000); **529 Pt 1**: 3-10.

Ridker, P. M., Buring, J. E., Shih, J., Matias, M. and Hennekens, C. H. Prospective study of C-reactive protein and the risk of future cardiovascular events among apparently healthy women. *Circulation* (1998); **98**(8): 731-733.

Ridker, P. M., Cushman, M., Stampfer, M. J., Tracy, R. P. and Hennekens, C. H. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med* (1997); **336**(14): 973-979.

Ridker, P. M., Rifai, N., Rose, L., Buring, J. E. and Cook, N. R. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. *N Engl J Med* (2002); **347**(20): 1557-1565.

Ridker, P. M., Rifai, N., Stampfer, M. J. and Hennekens, C. H. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation* (2000); **101**(15): 1767-1772.

Rieder, M. J., Taylor, S. L., Clark, A. G. and Nickerson, D. A. Sequence variation in the human angiotensin converting enzyme. *Nat Genet* (1999); **22**(1): 59-62.

Rigat, B., Hubert, C., Alhenc-Gelas, F., Cambien, F., Corvol, P. and Soubrier, F. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest* (1990); **86**(4): 1343-1346.

Risch, N. and Merikangas, K. The future of genetic studies of complex human diseases. *Science* (1996); **273**(5281): 1516-1517.

Risch, N. J. Searching for genetic determinants in the new millennium. *Nature* (2000); **405**(6788): 847-856.

Rogers, W. J., Johnstone, D. E., Yusuf, S., Weiner, D. H., Gallagher, P., Bittner, V. A., Ahn, S., Schron, E., Shumaker, S. A. and Sheffield, L. T. Quality of life among 5,025 patients with left ventricular dysfunction randomized between placebo and enalapril: the Studies of Left Ventricular Dysfunction. The SOLVD Investigators. *J Am Coll Cardiol* (1994); **23**(2): 393-400.

Rohacs, T., Nagy, G. and Spat, A. Cytoplasmic Ca2+ signalling and reduction of mitochondrial pyridine nucleotides in adrenal glomerulosa cells in response to K+, angiotensin II and vasopressin. *Biochem J* (1997); **322 (Pt 3)**: 785-792.

Rolfe, D. F. and Brand, M. D. Contribution of mitochondrial proton leak to skeletal muscle respiration and to standard metabolic rate. *Am J Physiol* (1996); **271**(4 Pt 1): C1380-C1389.

Rose, E. A., Gelijns, A. C., Moskowitz, A. J., Heitjan, D. F., Stevenson, L. W., Dembitsky, W., Long, J. W., Ascheim, D. D., Tierney, A. R., Levitan, R. G., Watson, J. T., Meier, P., Ronan, N. S., Shapiro, P. A., Lazar, R. M., Miller, L. W., Gupta, L., Frazier, O. H., Desvigne-Nickens, P., Oz, M. C. and Poirier, V. L. Long-term mechanical left ventricular assistance for end-stage heart failure. *N Engl J Med* (2001); **345**(20): 1435-1443.

Rosenkranz, A. C., Dusting, G. J. and Ritchie, R. H. Hyperglycaemia abolishes the antihypertrophic efficacy of bradykinin in rat ventricular myocytes. *Clin Exp Pharmacol Physiol* (1999); **26**(7): 519-521.

Ross, R. Atherosclerosis: an inflammatory disease. *N Engl J Med* (1999); **340**: 115-126.

Rosser, D. M., Manji, M., Cooksley, H. and Bellingan, G. Endotoxin reduces maximal oxygen consumption in hepatocytes independent of any hypoxic insult. *Intensive Care Med* (1998); **24**(7): 725-729.

Rothman, K. J. No adjustments are needed for multiple comparisons. *Epidemiology* (1990); **1**(1): 43-6.

Rottenberg, H. Membrane potential and surface potential in mitochondria: uptake and binding of lipophilic cations. *J Membr Biol* (1984); **81**(2): 127-138.

Roychowdhury, S., Luthe, A., Keilhoff, G., Wolf, G. and Horn, T. F. Oxidative stress in glial cultures: detection by DAF-2 fluorescence used as a tool to measure peroxynitrite rather than nitric oxide. *Glia* (2002); **38**(2): 103-114.

Rubanyi, G. M. and Vanhoutte, P. M. Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. *Am J Physiol* (1986); **250**(5 Pt 2): H822-H827.

Rubin, S. A., Brown, H. V. and Swan, H. J. Arterial oxygenation and arterial oxygen transport in chronic myocardial failure at rest, during exercise and after hydralazine treatment. *Circulation* (1982); **66**(1): 143-148.

Russell, A. P., Somm, E., Praz, M., Crettenand, A., Hartley, O., Melotti, A., Giacobino, J. P., Muzzin, P., Gobelet, C. and Deriaz, O. UCP3 protein regulation in human skeletal muscle fibre types I, IIa and IIx is dependent on exercise intensity. *J Physiol* (2003); **550**(Pt 3): 855-861.

Saavedra, J. M. Brain and pituitary angiotensin. *Endocr Rev* (1992); **13**(2): 329-380.

Sabbah, H. N., Sharov, V., Riddle, J. M., Kono, T., Lesch, M. and Goldstein, S. Mitochondrial abnormalities in myocardium of dogs with chronic heart failure. *J Mol Cell Cardiol* (1992); **24**(11): 1333-1347.

Sabri, A., Hughie, H. H. and Lucchesi, P. A. Regulation of hypertrophic and apoptotic signaling pathways by reactive oxygen species in cardiac myocytes. *Antioxid Redox Signal* (2003); **5**(6): 731-740.

Sachidanandam, R., Weissman, D., Schmidt, S. C., Kakol, J. M., Stein, L. D., Marth, G., Sherry, S., Mullikin, J. C., Mortimore, B. J., Willey, D. L., Hunt, S. E., Cole, C. G., Coggill, P. C., Rice, C. M., Ning, Z., Rogers, J., Bentley, D. R., Kwok, P. Y., Mardis, E. R., Yeh, R. T., Schultz, B., Cook, L., Davenport, R., Dante, M., Fulton, L., Hillier, L., Waterston, R. H., McPherson, J. D., Gilman, B., Schaffner, S., Van Etten, W. J., Reich, D., Higgins, J., Daly, M. J., Blumenstiel, B., Baldwin, J., Stange-Thomann, N., Zody, M. C., Linton, L., Lander, E. S. and Altshuler, D. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature* (2001); **409**(6822): 928-933.

Sacks, F. M., Pfeffer, M. A., Moye, L. A., Rouleau, J. L., Rutherford, J. D., Cole, T. G., Brown, L., Warnica, J. W., Arnold, J. M., Wun, C. C., Davis, B. R. and Braunwald, E. The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators. *N Engl J Med* (1996); **335**(14): 1001-1009.

Sadoshima, J., Xu, Y., Slayter, H. S. and Izumo, S. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. *Cell* (1993); **75**(5): 977-984.

Safar, M. E., Toto-Moukouo, J. J., Bouthier, J. A., Asmar, R. E., Levenson, J. A., Simon, A. C. and London, G. M. Arterial dynamics, cardiac hypertrophy, and antihypertensive treatment. *Circulation* (1987); **75**(1 Pt 2): I156-61.

Saijonmaa, O. and Fyhrquist, F. Upregulation of angiotensin converting enzyme by atrial natriuretic peptide and cyclic GMP in human endothelial cells. *Cardiovasc Res* (1998); **40**(1): 206-210.

Saijonmaa, O., Nyman, T., Kosonen, R. and Fyhrquist, F. Upregulation of angiotensin-converting enzyme by vascular endothelial growth factor. *Am J Physiol Heart Circ Physiol* (2001); **280**(2): H885-891.

Salvioli, S., Ardizzoni, A., Franceschi, C. and Cossarizza, A. JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. *FEBS Lett* (1997); **411**(1): 77-82.

Samec, S., Seydoux, J. and Dulloo, A. G. Interorgan signaling between adipose tissue metabolism and skeletal muscle uncoupling protein homologs: is there a role for circulating free fatty acids? *Diabetes* (1998); **47**(11): 1693-1698.

Samec, S., Seydoux, J. and Dulloo, A. G. Skeletal muscle UCP3 and UCP2 gene expression in response to inhibition of free fatty acid flux through mitochondrial beta-oxidation. *Pflugers Arch* (1999); **438**(4): 452-457.

Sampson, M., Gopaul, N., Davies, I., Hughes, D. and Carrier, M. Plasma F2 Isoprostanes. Direct evidence of increased free radical damage during acute hyperglycaemia in type 2 diabetes. *Diabetes Care* (2002); **25**(3): 537-541.

Sanbe, A., Tanonaka, K., Kobayasi, R. and Takeo, S. Effects of long-term therapy with ACE inhibitors, captopril, enalapril and trandolapril, on myocardial energy metabolism in rats with heart failure following myocardial infarction. *J Mol Cell Cardiol* (1995); **27**(10): 2209-2222.

Sattar, N., Gaw, A., Scherbakova, O., Ford, I., O'Reilly, D. S., Haffner, S. M., Isles, C., Macfarlane, P. W., Packard, C. J., Cobbe, S. M. and Shepherd, J. Metabolic syndrome with and without C-reactive protein as a predictor of coronary heart disease and diabetes in the West of Scotland Coronary Prevention Study. *Circulation* (2003); **108**(4): 414-419.

Sawyer, D. B. and Colucci, W. S. Mitochondrial oxidative stress in heart failure: "oxygen wastage" revisited. *Circ Res* (2000); **86**(2): 119-120.

Scaduto, R. C., Jr. and Grotyohann, L. W. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophys J* (1999); **76**(1 Pt 1): 469-477.

Scandinavian Simvastatin Survival Study Group. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet* (1994); **344**(8934): 1383-1389.

Schieffer, B., Schieffer, E., Hilfiker-Kleiner, D., Hilfiker, A., Kovanen, P. T., Kaartinen, M., Nussberger, J., Harringer, W. and Drexler, H. Expression of angiotensin II and interleukin 6 in human coronary atherosclerotic plaques: potential implications for inflammation and plaque instability. *Circulation* (2000); **101**(12): 1372-1378.

Schleiff, E. and McBride, H. The central matrix loop drives import of uncoupling protein 1 into mitochondria. *J Cell Sci* (2000); **113 (Pt 12)**: 2267-2272.
Schling, P., Mallow, H., Trindl, A. and Loffler, G. Evidence for a local renin angiotensin system in primary cultured human preadipocytes. *Int J Obes Relat Metab Disord* (1999); **23**(4): 336-341.
Schmaier, A. H. Plasma kallikrein/kinin system: a revised hypothesis for its activation and its physiologic contributions. *Curr Opin Hematol* (2000); **7**(5): 261-265.
Schoelkens, B. A. and Linz, W. Bradykinin-mediated metabolic effects in isolated perfused rat hearts. *Agents Actions Suppl* (1992); **38 (Pt 2)**: 36-42.
Scholkens, B. A. Kinins in the cardiovascular system. *Immunopharmacology* (1996); **33**(1-3): 209-216.
Schonbeck, U., Varo, N., Libby, P., Buring, J. and Ridker, P. M. Soluble CD40L and cardiovascular risk in women. *Circulation* (2001); **104**(19): 2266-2268.
Schrauwen, P. and Hesselink, M. UCP2 and UCP3 in muscle controlling body metabolism. *J Exp Biol* (2002); **205**(Pt 15): 2275-2285.
Schrauwen, P., Hoppeler, H., Billeter, R., Bakker, A. H. and Pendergast, D. R. Fiber type dependent upregulation of human skeletal muscle UCP2 and UCP3 mRNA expression by high-fat diet. *Int J Obes Relat Metab Disord* (2001); **25**(4): 449-456.
Schrauwen, P., Xia, J., Walder, K., Snitker, S. and Ravussin, E. A novel polymorphism in the proximal UCP3 promoter region: effect on skeletal muscle UCP3 mRNA expression and obesity in male non-diabetic Pima Indians. *Int J Obes Relat Metab Disord* (1999); **23**(12): 1242-1245.
Schreck, R., Rieber, P. and Baeuerle, P. A. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *Embo J* (1991); **10**(8): 2247-2258.
Schulz, R., Dodge, K. L., Lopaschuk, G. D. and Clanachan, A. S. Peroxynitrite impairs cardiac contractile function by decreasing cardiac efficiency. *Am J Physiol* (1997); **272**(3 Pt 2): H1212-1219.
Schwartz, A. and Lee, K. Study of heart mitochondria and glycolytic metabolism in experimentally induced cardiac failure. *Circ Res* (1962); **10**: 321-332.
Scott, A. C., Wensel, R., Davos, C. H., Georgiadou, P., Ceri Davies, L., Coats, A. J., Francis, D. P. and Piepoli, M. F. Putative contribution of prostaglandin and bradykinin to muscle reflex hyperactivity in patients on Ace-inhibitor therapy for chronic heart failure. *Eur Heart J* (2004); **25**(20): 1806-1813.
Scott, A. C., Wensel, R., Davos, C. H., Kemp, M., Kaczmarek, A., Hooper, J., Coats, A. J. and Piepoli, M. F. Chemical mediators of the muscle ergoreflex in chronic heart failure: a putative role for prostaglandins in reflex ventilatory control. *Circulation* (2002); **106**(2): 214-220.
Sen, C. K. and Packer, L. Antioxidant and redox regulation of gene transcription. *Faseb J* (1996); **10**(7): 709-720.
Sernia, C. A critical appraisal of the intrinsic pancreatic angiotensin-generating system. *Jop* (2001); **2**(1): 50-55.
Sesti, G., Cardellini, M., Marini, M. A., Frontoni, S., D'Adamo, M., Del Guerra, S., Lauro, D., De Nicolais, P., Sbraccia, P., Del Prato, S., Gambardella, S., Federici, M., Marchetti, P. and Lauro, R. A common polymorphism in the promoter of UCP2 contributes to the variation in insulin secretion in glucose-tolerant subjects. *Diabetes* (2003); **52**(5): 1280-1283.
Shah, A. M. and MacCarthy, P. A. Paracrine and autocrine effects of nitric oxide on myocardial function. *Pharmacol Ther* (2000); **86**(1): 49-86.
Sharov, V. G., Todor, A. V., Silverman, N., Goldstein, S. and Sabbah, H. N. Abnormal mitochondrial respiration in failed human myocardium. *J Mol Cell Cardiol* (2000); **32**(12): 2361-2367.
Shen, W., Asai, K., Uechi, M., Mathier, M. A., Shannon, R. P., Vatner, S. F. and Ingwall, J. S. Progressive loss of myocardial ATP due to a loss of total purines during the development of heart failure in dogs: a compensatory role for the parallel loss of creatine. *Circulation* (1999); **100**(20): 2113-2118.
Shen, W., Hintze, T. H. and Wolin, M. S. Nitric oxide. An important signaling mechanism between vascular endothelium and parenchymal cells in the regulation of oxygen consumption. *Circulation* (1995); **92**(12): 3505-3512.
Shepherd, J., Cobbe, S. M., Ford, I., Isles, C. G., Lorimer, A. R., MacFarlane, P. W., McKillop, J. H. and Packard, C. J. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. *N Engl J Med* (1995); **333**(20): 1301-1307.
Shigenaga, M. K., Hagen, T. M. and Ames, B. N. Oxidative damage and mitochondrial decay in aging. *Proc Natl Acad Sci U S A* (1994); **91**(23): 10771-10778.
Shimokawa, T., Kato, M., Ezaki, O. and Hashimoto, S. Transcriptional regulation of muscle-specific genes during myoblast differentiation. *Biochem Biophys Res Commun* (1998); **246**(1): 287-292.
Sing, C. F., Stengard, J. H. and Kardia, S. L. Genes, environment, and cardiovascular disease. *Arterioscler Thromb Vasc Biol* (2003); **23**(7): 1190-6.

Siwik, D. A., Tzortzis, J. D., Pimental, D. R., Chang, D. L., Pagano, P. J., Singh, K., Sawyer, D. B. and Colucci, W. S. Inhibition of copper-zinc superoxide dismutase induces cell growth, hypertrophic phenotype, and apoptosis in neonatal rat cardiac myocytes in vitro. *Circ Res* (1999); **85**(2): 147-153.

Skarka, L., Bardova, K., Brauner, P., Flachs, P., Jarkovska, D., Kopecky, J. and Ostadal, B. Expression of mitochondrial uncoupling protein 3 and adenine nucleotide translocase 1 genes in developing rat heart: putative involvement in control of mitochondrial membrane potential. *J Mol Cell Cardiol* (2003); **35**(3): 321-330.

Sleight, P., Yusuf, S., Pogue, J., Tsuyuki, R., Diaz, R. and Probstfield, J. Blood-pressure reduction and cardiovascular risk in HOPE study. *Lancet* (2001); **358**(9299): 2130-2131.

Smiley, S. T., Reers, M., Mottola-Hartshorn, C., Lin, M., Chen, A., Smith, T. W., Steele, G. D., Jr. and Chen, L. B. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proc Natl Acad Sci U S A* (1991); **88**(9): 3671-3675.

Snowden, C. B., McNamara, P. M., Garrison, R. J., Feinleib, M., Kannel, W. B. and Epstein, F. H. Predicting coronary heart disease in siblings--a multivariate assessment: the Framingham Heart Study. *Am J Epidemiol* (1982); **115**(2): 217-222.

Solanes, G., Vidal-Puig, A., Grujic, D., Flier, J. S. and Lowell, B. B. The Human Uncoupling Protein-3 Gene: Genomic Structure, Chromosomal Localization, and Genetic Basis for Short and Long Form Transcripts. *The Journal of Biological Chemistry* (1997); **272**(41): 25433-25436.

SOLVD Investigators. Effect of enalapril on survival in patients with reduced left ventricular ejection fractions and congestive heart failure. *N Engl J Med* (1991); **325**(5): 293-302.

Sorensen, V. B., Wroblewski, H., Galatius, S., Haunso, S. and Kastrup, J. Exercise skeletal muscle blood flow is related to peripheral microvascular stiffness in idiopathic dilated cardiomyopathy. *Microvasc Res* (1999); **58**(3): 268-280.

Spinale, F. G. Matrix metalloproteinases: regulation and dysregulation in the failing heart. *Circ Res* (2002); **90**(5): 520-530.

St-Pierre, A. C., Ruel, I. L., Cantin, B., Dagenais, G. R., Bernard, P. M., Despres, J. P. and Lamarche, B. Comparison of various electrophoretic characteristics of LDL particles and their relationship to the risk of ischemic heart disease. *Circulation* (2001); **104**(19): 2295-2299.

Stadtman, E. R. and Levine, R. L. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids* (2003); **25**(3-4): 207-218.

Stamler, J., Vaccaro, O., Neaton, J. D. and Wentworth, D. Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. *Diabetes Care* (1993); **16**(2): 434-444.

Stanley, W. and Chandler, M. Energy metabolism in the normal and failing heart: potential for therapeutic interventions. *Heart Failure Reviews* (2002); **7**: 115-130.

Starling, R. C., Hammer, D. F. and Altschuld, R. A. Human myocardial ATP content and in vivo contractile function. *Mol Cell Biochem* (1998); **180**(1-2): 171-177.

Stary, H. C., Chandler, A. B., Dinsmore, R. E., Fuster, V., Glagov, S., Insull, W., Jr., Rosenfeld, M. E., Schwartz, C. J., Wagner, W. D. and Wissler, R. W. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* (1995); **92**(5): 1355-1374.

Stefanadis, C., Diamantopoulos, L., Vlachopoulos, C., Tsiamis, E., Dernellis, J., Toutouzas, K., Stefanadi, E. and Toutouzas, P. Thermal heterogeneity within human atherosclerotic coronary arteries detected in vivo: A new method of detection by application of a special thermography catheter. *Circulation* (1999); **99**(15): 1965-1971.

Steinberg, H. O., Chaker, H., Leaming, R., Johnson, A., Brechtel, G. and Baron, A. D. Obesity/insulin resistance is associated with endothelial dysfunction. Implications for the syndrome of insulin resistance. *J Clin Invest* (1996); **97**(11): 2601-2610.

Steinberg, H. O., Tarshoby, M., Monestel, R., Hook, G., Cronin, J., Johnson, A., Bayazeed, B. and Baron, A. D. Elevated circulating free fatty acid levels impair endothelium-dependent vasodilation. *J Clin Invest* (1997); **100**(5): 1230-1239.

Stemmer, P., Wisler, P. L. and Watanabe, A. M. (1991). Isolated myocytes in experimental cardiology. The Heart and Cardiovascular System. H. Fozzard, E. Haber, R. B. Jennings, A. Katz and H. E. Morgan. New York, Raven. **I:** 387-399.

Stephens, J. W., Gable, D. R., Hurel, S. J., Miller, G. J., Cooper, J. A. and Humphries, S. E. Increased plasma markers of oxidative stress are associated with coronary heart disease in males with diabetes mellitus and with 10-year risk in a prospective sample of males. *Clin Chem* (2006); **52**(3): 446-452.

Stephens, J. W. and Humphries, S. E. The molecular genetics of cardiovascular disease: clinical implications. *J Intern Med* (2003); **253**(2): 120-127.

Stitt, A. W., Bucala, R. and Vlassara, H. Atherogenesis and advanced glycation: promotion, progression, and prevention. *Ann N Y Acad Sci* (1997); **811**: 115-127; discussion 127-129.

Stoll, M., Meffert, S., Stroth, U. and Unger, T. Growth or antigrowth: angiotensin and the endothelium. *J Hypertens* (1995); **13**(12 Pt 2): 1529-1534.

Stranger, B. E., Nica, A. C., Forrest, M. S., Dimas, A., Bird, C. P., Beazley, C., Ingle, C. E., Dunning, M., Flicek, P., Koller, D., Montgomery, S., Tavare, S., Deloukas, P. and Dermitzakis, E. T. Population genomics of human gene expression. *Nat Genet* (2007); **39**(10): 1217-1224.

Stratton, J. R., Dunn, J. F., Adamopoulos, S., Kemp, G. J., Coats, A. J. and Rajagopalan, B. Training partially reverses skeletal muscle metabolic abnormalities during exercise in heart failure. *J Appl Physiol* (1994); **76**(4): 1575-1582.

Strong, J. P., Malcom, G. T., McMahan, C. A., Tracy, R. E., Newman, W. P., 3rd, Herderick, E. E. and Cornhill, J. F. Prevalence and extent of atherosclerosis in adolescents and young adults: implications for prevention from the Pathobiological Determinants of Atherosclerosis in Youth Study. *Jama* (1999); **281**(8): 727-735.

Stuart, J. A., Harper, J. A., Brindle, K. M., Jekabsons, M. B. and Brand, M. D. A mitochondrial uncoupling artifact can be caused by expression of uncoupling protein 1 in yeast. *Biochem J* (2001); **356**(Pt 3): 779-789.

Stuart, J. A., Harper, J. A., Brindle, K. M., Jekabsons, M. B. and Brand, M. D. Physiological levels of mammalian uncoupling protein 2 do not uncouple yeast mitochondria. *J Biol Chem* (2001); **276**(21): 18633-18639.

Su, J. B., Houel, R., Heloire, F., Barbe, F., Beverelli, F., Sambin, L., Castaigne, A., Berdeaux, A., Crozatier, B. and Hittinger, L. Stimulation of bradykinin B(1) receptors induces vasodilation in conductance and resistance coronary vessels in conscious dogs: comparison with B(2) receptor stimulation. *Circulation* (2000); **101**(15): 1848-1853.

Suematsu, N., Tsutsui, H., Wen, J., Kang, D., Ikeuchi, M., Ide, T., Hayashidani, S., Shiomi, T., Kubota, T., Hamasaki, N. and Takeshita, A. Oxidative stress mediates tumor necrosis factor-alpha-induced mitochondrial DNA damage and dysfunction in cardiac myocytes. *Circulation* (2003); **107**(10): 1418-1423.

Suga, H. Ventricular energetics. *Physiol Rev* (1990); **70**(2): 247-277.

Sullivan, M., Green, H. and Cobb, F. Skeletal muscle biochemistry and histology in ambulatory patients with long-term heart failure. *Circulation* (1990); **81**: 518-527.

Sullivan, M. J., Green, H. J. and Cobb, F. R. Altered skeletal muscle metabolic response to exercise in chronic heart failure. Relation to skeletal muscle aerobic enzyme activity. *Circulation* (1991); **84**(4): 1597-1607.

Sullivan, M. J. and Hawthorne, M. H. Exercise intolerance in patients with chronic heart failure. *Prog Cardiovasc Dis* (1995); **38**(1): 1-22.

Sullivan, M. J., Knight, J. D., Higginbotham, M. B. and Cobb, F. R. Relation between central and peripheral hemodynamics during exercise in patients with chronic heart failure. Muscle blood flow is reduced with maintenance of arterial perfusion pressure. *Circulation* (1989); **80**(4): 769-781.

Sun, X., Wray, C., Tian, X., Hasselgren, P. O. and Lu, J. Expression of uncoupling protein 3 is upregulated in skeletal muscle during sepsis. *Am J Physiol Endocrinol Metab* (2003); **285**(3): E512-520.

Sun, Y., Zhang, J., Zhang, J. Q. and Weber, K. T. Renin expression at sites of repair in the infarcted rat heart. *J Mol Cell Cardiol* (2001); **33**(5): 995-1003.

Suzuki, Y. J., Forman, H. J. and Sevanian, A. Oxidants as stimulators of signal transduction. *Free Radic Biol Med* (1997); **22**(1-2): 269-285.

Swan, L., Birnie, D. H., Padmanabhan, S., Inglis, G., Connell, J. M. and Hillis, W. S. The genetic determination of left ventricular mass in healthy adults. *Eur Heart J* (2003); **24**(6): 577-582.

Swartz, S. L., Williams, G. H., Hollenberg, N. K., Levine, L., Dluhy, R. G. and Moore, T. J. Captopril-induced changes in prostaglandin production: relationship to vascular responses in normal man. *J Clin Invest* (1980); **65**(6): 1257-1264.

Swedberg, K., Held, P., Kjekshus, J., Rasmussen, K., Ryden, L. and Wedel, H. Effects of the early administration of enalapril on mortality in patients with acute myocardial infarction. Results of the Cooperative New Scandinavian Enalapril Survival Study II (CONSENSUS II). *N Engl J Med* (1992); **327**(10): 678-684.

Taguchi, T., Kishikawa, H., Motoshima, H., Sakai, K., Nishiyama, T., Yoshizato, K., Shirakami, A., Toyonaga, T., Shirontani, T., Araki, E. and Shichiri, M. Involvement of bradykinin in acute exercise-induced increase of glucose uptake and GLUT-4 translocation in skeletal muscle: studies in normal and diabetic humans and rats. *Metabolism* (2000); **49**(7): 920-930.

Takezako, T., Saku, K., Zhang, B., Ou, J., Bai, H., Imai, K., Jimi, S., Shirai, K. and Arakawa, K. Angiotensin I converting enzyme gene polymorphism and insulin resistance in patients with angina pectoris. *Am J Hypertens* (1999); **12**(3): 291-297.

Tan, L. B., Jalil, J. E., Pick, R., Janicki, J. S. and Weber, K. T. Cardiac myocyte necrosis induced by angiotensin II. *Circ Res* (1991); **69**(5): 1185-1195.

Taniguchi, E., Harada, M., Kawaguchi, T., Koga, H., Kumemura, H., Hanada, S., Shishido, S., Baba, S., Kumashiro, R., Ueno, T., Sakisaka, S. and Sata, M. Expression of uncoupling protein-2 in biliary epithelial cells in primary biliary cirrhosis. *Liver* (2002); **22**(6): 451-458.

Taylor, A. L., Ziesche, S., Yancy, C., Carson, P., D'Agostino, R., Jr., Ferdinand, K., Taylor, M., Adams, K., Sabolinski, M., Worcel, M. and Cohn, J. N. Combination of isosorbide dinitrate and hydralazine in blacks with heart failure. *N Engl J Med* (2004); **351**(20): 2049-2057.

Taylor, M., Wallhaus, T. R., Degrado, T. R., Russell, D. C., Stanko, P., Nickles, R. J. and Stone, C. K. An evaluation of myocardial fatty acid and glucose uptake using PET with [18F]fluoro-6-thia-heptadecanoic acid and. *J Nucl Med* (2001); **42**(1): 55-62.

Taylor, R. R., Mamotte, C. D., Fallon, K. and van Bockxmeer, F. M. Elite athletes and the gene for angiotensin-converting enzyme. *J Appl Physiol* (1999); **87**(3): 1035-1037.

Terry, C. M. and Callahan, K. S. Protein kinase C regulates cytokine-induced tissue factor transcription and procoagulant activity in human endothelial cells. *J Lab Clin Med* (1996); **127**(1): 81-93.

Teshima, Y., Akao, M., Jones, S. P. and Marban, E. Uncoupling protein-2 overexpression inhibits mitochondrial death pathway in cardiomyocytes. *Circ Res* (2003); **93**(3): 192-200.

Thomis, M. A., Beunen, G. P., Van Leemputte, M., Maes, H. H., Blimkie, C. J., Claessens, A. L., Marchal, G., Willems, E. and Vlietinck, R. F. Inheritance of static and dynamic arm strength and some of its determinants. *Acta Physiol Scand* (1998); **163**(1): 59-71.

Tigerstedt, R. and Bergman, P. G. Niere und Kreilauf. *Skand Arch Physiol* (1898); **8**: 223-271.

Tipnis, S. R., Hooper, N. M., Hyde, R., Karran, E., Christie, G. and Turner, A. J. A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. *J Biol Chem* (2000); **275**(43): 33238-33243.

Torzewski, J., Torzewski, M., Bowyer, D. E., Frohlich, M., Koenig, W., Waltenberger, J., Fitzsimmons, C. and Hombach, V. C-reactive protein frequently colocalizes with the terminal complement complex in the intima of early atherosclerotic lesions of human coronary arteries. *Arterioscler Thromb Vasc Biol* (1998); **18**(9): 1386-1392.

Toshima, S., Hasegawa, A., Kurabayashi, M., Itabe, H., Takano, T., Sugano, J., Shimamura, K., Kimura, J., Michishita, I., Suzuki, T. and Nagai, R. Circulating oxidized low density lipoprotein levels. A biochemical risk marker for coronary heart disease. *Arterioscler Thromb Vasc Biol* (2000); **20**(10): 2243-2247.

Tracy, R. P., Psaty, B. M., Macy, E., Bovill, E. G., Cushman, M., Cornell, E. S. and Kuller, L. H. Lifetime smoking exposure affects the association of C-reactive protein with cardiovascular disease risk factors and subclinical disease in healthy elderly subjects. *Arterioscler Thromb Vasc Biol* (1997); **17**(10): 2167-2176.

Tu, N., Chen, H., Winnikes, U., Reinert, I., Marmann, G., Pirke, K. M. and Lentes, K. U. Molecular cloning and functional characterization of the promoter region of the human uncoupling protein-2 gene. *Biochem Biophys Res Commun* (1999); **265**(2): 326-334.

Tu, N., Chen, H., Winnikes, U., Reinert, I., Marmann, G., Pirke, K. M. and Lentes, K. U. Structural organization and mutational analysis of the human uncoupling protein-2 (hUCP2) gene. *Life Sci* (1999); **64**(3): L41-50.

Tyni-Lenne, R., Gordon, A., Europe, E., Jansson, E. and Sylven, C. Exercise-based rehabilitation improves skeletal muscle capacity, exercise tolerance, and quality of life in both women and men with chronic heart failure. *J Card Fail* (1998); **4**(1): 9-17.

Urhammer, S. A., Dalgaard, L. T., Sorensen, T. I., Moller, A. M., Andersen, T., Tybjaerg-Hansen, A., Hansen, T., Clausen, J. O., Vestergaard, H. and Pedersen, O. Mutational analysis of the coding region of the uncoupling protein 2 gene in obese NIDDM patients: impact of a common amino acid polymorphism on juvenile and maturity onset forms of obesity and insulin resistance. *Diabetologia* (1997); **40**(10): 1227-1230.

Ushio-Fukai, M., Alexander, R. W., Akers, M. and Griendling, K. K. p38 Mitogen-activated protein kinase is a critical component of the redox-sensitive signaling pathways activated by angiotensin II. Role in vascular smooth muscle cell hypertrophy. *J Biol Chem* (1998); **273**(24): 15022-15029.

Vallance, P., Leone, A., Calver, A., Collier, J. and Moncada, S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet* (1992); **339**(8793): 572-575.

Van der Lee, K. A., Willemsen, P. H., Samec, S., Seydoux, J., Dulloo, A. G., Pelsers, M. M., Glatz, J. F., Van der Vusse, G. J. and Van Bilsen, M. Fasting-induced changes in the expression of genes controlling substrate metabolism in the rat heart. *J Lipid Res* (2001); **42**(11): 1752-1758.

Van Der Lee, K. A., Willemsen, P. H., Van Der Vusse, G. J. and Van Bilsen, M. Effects of fatty acids on uncoupling protein-2 expression in the rat heart. *Faseb J* (2000); **14**(3): 495-502.

Vanderkooi, J. M. and Wilson, D. F. A new method for measuring oxygen concentration in biological systems. *Adv Exp Med Biol* (1986); **200**: 189-193.

Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., Gocayne, J. D., Amanatides, P., Ballew, R. M., Huson, D. H., Wortman, J. R., Zhang, Q., Kodira, C. D., Zheng, X. H., Chen, L., Skupski, M., Subramanian, G., Thomas, P. D., Zhang, J., Gabor Miklos, G. L., Nelson, C., Broder, S., Clark, A. G., Nadeau, J., McKusick, V. A., Zinder, N., Levine, A. J., Roberts, R. J., Simon, M., Slayman, C., Hunkapiller, M., Bolanos, R., Delcher, A., Dew, I., Fasulo, D., Flanigan, M., Florea, L., Halpern, A., Hannenhalli, S., Kravitz, S., Levy, S., Mobarry, C., Reinert, K., Remington, K., Abu-Threideh, J., Beasley, E., Biddick, K., Bonazzi, V., Brandon, R., Cargill, M., Chandramouliswaran, I., Charlab, R., Chaturvedi, K., Deng, Z., Di Francesco, V., Dunn, P., Eilbeck, K., Evangelista, C., Gabrielian, A. E., Gan, W., Ge, W., Gong, F., Gu, Z., Guan, P., Heiman, T. J., Higgins, M. E., Ji, R. R., Ke, Z., Ketchum, K. A., Lai, Z., Lei, Y., Li, Z., Li, J., Liang, Y., Lin, X., Lu, F., Merkulov, G. V., Milshina, N., Moore, H. M., Naik, A. K., Narayan, V. A., Neelam, B., Nusskern, D., Rusch, D. B., Salzberg, S., Shao, W., Shue, B., Sun, J., Wang, Z., Wang, A., Wang, X., Wang, J., Wei, M., Wides, R., Xiao, C., Yan, C., Yao, A., Ye, J., Zhan, M., Zhang, W., Zhang, H., Zhao, Q., Zheng, L., Zhong, F., Zhong, W., Zhu, S., Zhao, S., Gilbert, D., Baumhueter, S., Spier, G., Carter, C., Cravchik, A., Woodage, T., Ali, F., An, H., Awe, A., Baldwin, D., Baden, H., Barnstead, M., Barrow, I., Beeson, K., Busam, D., Carver, A., Center, A., Cheng, M. L., Curry, L., Danaher, S., Davenport, L., Desilets, R., Dietz, S., Dodson, K., Doup, L., Ferriera, S., Garg, N., Gluecksmann, A., Hart, B., Haynes, J., Haynes, C., Heiner, C., Hladun, S., Hostin, D., Houck, J., Howland, T., Ibegwam, C., Johnson, J., Kalush, F., Kline, L., Koduru, S., Love, A., Mann, F., May, D., McCawley, S., McIntosh, T., McMullen, I., Moy, M., Moy, L., Murphy, B., Nelson, K., Pfannkoch, C., Pratts, E., Puri, V., Qureshi, H., Reardon, M., Rodriguez, R., Rogers, Y. H., Romblad, D., Ruhfel, B., Scott, R., Sitter, C., Smallwood, M., Stewart, E., Strong, R., Suh, E., Thomas, R., Tint, N. N., Tse, S., Vech, C., Wang, G., Wetter, J., Williams, S., Williams, M., Windsor, S., Winn-Deen, E., Wolfe, K., Zaveri, J., Zaveri, K., Abril, J. F., Guigo, R., Campbell, M. J., Sjolander, K. V., Karlak, B., Kejariwal, A., Mi, H., Lazareva, B., Hatton, T., Narechania, A., Diemer, K., Muruganujan, A., Guo, N., Sato, S., Bafna, V., Istrail, S., Lippert, R., Schwartz, R., Walenz, B., Yooseph, S., Allen, D., Basu, A., Baxendale, J., Blick, L., Caminha, M., Carnes-Stine, J., Caulk, P., Chiang, Y. H., Coyne, M., Dahlke, C., Mays, A., Dombroski, M., Donnelly, M., Ely, D., Esparham, S., Fosler, C., Gire, H., Glanowski, S., Glasser, K., Glodek, A., Gorokhov, M., Graham, K., Gropman, B., Harris, M., Heil, J., Henderson, S., Hoover, J., Jennings, D., Jordan, C., Jordan, J., Kasha, J., Kagan, L., Kraft, C., Levitsky, A., Lewis, M., Liu, X., Lopez, J., Ma, D., Majoros, W., McDaniel, J., Murphy, S., Newman, M., Nguyen, T., Nguyen, N., Nodell, M., Pan, S., Peck, J., Peterson, M., Rowe, W., Sanders, R., Scott, J., Simpson, M., Smith, T., Sprague, A., Stockwell, T., Turner, R., Venter, E., Wang, M., Wen, M., Wu, D., Wu, M., Xia, A., Zandieh, A. and Zhu, X. The sequence of the human genome. *Science* (2001); **291**(5507): 1304-1351.

Venugopal, S. K., Devaraj, S. and Yuhanna, I. Demonstration that C-reactive protein decreases eNOS expression and activity in human aortic endothelial cells. *Circulation* (2002); **106**: 1439-.

Vettor, R., Fabris, R., Serra, R., Lombardi, A. M., Tonello, C., Granzotto, M., Marzolo, M. O., Carruba, M. O., Ricquier, D., Federspil, G. and Nisoli, E. Changes in FAT/CD36, UCP2, UCP3 and GLUT4 gene expression during lipid infusion in rat skeletal and heart muscle. *Int J Obes Relat Metab Disord* (2002); **26**(6): 838-847.

Vickers, C., Hales, P., Kaushik, V., Dick, L., Gavin, J., Tang, J., Godbout, K., Parsons, T., Baronas, E., Hsieh, F., Acton, S., Patane, M., Nichols, A. and Tummino, P. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem* (2002); **277**(17): 14838-14843.

Vidal-Puig, A., Solanes, G., Grujic, D., Flier, J. S. and Lowell, B. B. UCP3: An Uncoupling Protein Homologue Expressed Preferentially and Abundantly in Skeletal Muscle and Brown Adipose Tissue. *Biochemical and Biophysical Research Communications* (1997); **235**: 79-82.

Vidal-Puig, A. J., Grujic, D., Zhang, C. Y., Hagen, T., Boss, O., Ido, Y., Szczepanik, A., Wade, J., Mootha, V., Cortright, R., Muoio, D. M. and Lowell, B. B. Energy metabolism in uncoupling protein 3 gene knockout mice. *J Biol Chem* (2000); **275**(21): 16258-16266.

Villard, E., Alonso, A., Agrapart, M., Challah, M. and Soubrier, F. Induction of angiotensin I-converting enzyme transcription by a protein kinase C-dependent mechanism in human endothelial cells. *J Biol Chem* (1998); **273**(39): 25191-25197.

Wald, D. S., Law, M. and Morris, J. K. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. *Bmj* (2002); **325**(7374): 1202-1206.

Walder, K., Norman, R. A., Hanson, R. L., Schrauwen, P., Neverova, M., Jenkinson, C. P., Easlick, J., Warden, C. H., Pecqueur, C., Raimbault, S., Ricquier, D., Silver, M. H., Shuldiner, A. R., Solanes, G., Lowell, B. B., Chung, W. K., Leibel, R. L., Pratley, R. and Ravussin, E. Association between uncoupling protein polymorphisms (UCP2-UCP3) and energy metabolism/obesity in Pima indians. *Hum Mol Genet* (1998); **7**(9): 1431-1435.

Wang, D. Z., Chao, L. and Chao, J. Hypotension in transgenic mice overexpressing human bradykinin B2 receptor. *Hypertension* (1997); **29**(1 Pt 2): 488-493.

Wang, X., Culotta, V. C. and Klee, C. B. Superoxide dismutase protects calcineurin from inactivation. *Nature* (1996); **383**(6599): 434-437.

Ward, M. W., Rego, A. C., Frenguelli, B. G. and Nicholls, D. G. Mitochondrial membrane potential and glutamate excitotoxicity in cultured cerebellar granule cells. *J Neurosci* (2000); **20**(19): 7208-7219.

Watanabe, T., Yanagishita, T., Konno, N., Geshi, E. and Katagiri, T. Reversal of early metabolic dysfunction in hypertensive rat left-ventricular myocytes by angiotensin-converting enzyme inhibition. *Jpn Heart J* (1997); **38**(4): 503-514.

Weber, K. T. and Brilla, C. G. Pathological hypertrophy and cardiac interstitium. Fibrosis and renin-angiotensin-aldosterone system. *Circulation* (1991); **83**(6): 1849-1865.

Weber, K. T. and Sun, Y. Recruitable ACE and tissue repair in the infarcted heart. *J Renin Angiotensin Aldosterone Syst* (2000); **1**(4): 295-303.

Wei, L., Alhenc-Gelas, F., Soubrier, F., Michaud, A., Corvol, P. and Clauser, E. Expression and characterization of recombinant human angiotensin I-converting enzyme. Evidence for a C-terminal transmembrane anchor and for a proteolytic processing of the secreted recombinant and plasma enzymes. *J Biol Chem* (1991); **266**(9): 5540-5546.

Weight, L. M., Alexander, D. and Jacobs, P. Strenuous exercise: analogous to the acute-phase response? *Clin Sci (Lond)* (1991); **81**(5): 677-683.

Weigle, D. S., Selfridge, L. E., Schwartz, M. W., Seeley, R. J., Cummings, D. E., Havel, P. J., Kuijper, J. L. and BeltrandelRio, H. Elevated free fatty acids induce uncoupling protein 3 expression in muscle: a potential explanation for the effect of fasting. *Diabetes* (1998); **47**(2): 298-302.

Weinbrenner, T., Cladellas, M., Isabel Covas, M., Fito, M., Tomas, M., Senti, M., Bruguera, J. and Marrugat, J. High oxidative stress in patients with stable coronary heart disease. *Atherosclerosis* (2003); **168**(1): 99-106.

Welch, G. N. and Loscalzo, J. Homocysteine and atherothrombosis. *N Engl J Med* (1998); **338**(15): 1042-1050.

Westin, W. and Mullane, K. Does captopril attenuate reperfusion-induced myocardial dysfunction by scavenging free radicals? *Circulation* (1988); **77**(Suppl 1): 30-39.

Wicklmayr, M., Dietze, G., Bottger, M. L. and Grunst, J. Evidence for an involvement of kinin liberation in the priming action of insulin on glucose uptake into skeletal muscle. *FEBS Lett* (1979); **98**: 61-65.

Wicklmayr, M., Dietze, G., Brunnbauer, H., Rett, K. and Mehnert, H. Dose-dependent effect of bradykinin on muscular blood flow and glucose uptake in man. *Hoppe Seylers Z Physiol Chem* (1983); **364**(7): 831-833.

Wilhelmsen, L., Svardsudd, K., Korsan-Bengtsen, K., Larsson, B., Welin, L. and Tibblin, G. Fibrinogen as a risk factor for stroke and myocardial infarction. *N Engl J Med* (2001); **311**: 501-505.

Williams, A. G., Rayson, M. P., Jubb, M., World, M., Woods, D. R., Hayward, M., Martin, J., Humphries, S. E. and Montgomery, H. E. The ACE gene and muscle performance. *Nature* (2000); **403**(6770): 614.

Williams, S. B., Goldfine, A. B., Timimi, F. K., Ting, H. H., Roddy, M. A., Simonson, D. C. and Creager, M. A. Acute hyperglycemia attenuates endothelium-dependent vasodilation in humans in vivo. *Circulation* (1998); **97**(17): 1695-1701.

Witztum, J. L. and Steinberg, D. The oxidative modification hypothesis of atherosclerosis: does it hold for humans? *Trends Cardiovasc Med* (2001); **11**(3-4): 93-102.

Wollenberger, A., Kletke, B. and Raabe, G. Some metabolic characteristics of mitochondria from chronically overloaded, hypertrophied hearts. *Exp Mol Pathol* (1965); **2**(251-260).

Woods, D., Hickman, M., Jamshidi, Y., Brull, D., Vassiliou, V., Jones, A., Humphries, S. and Montgomery, H. Elite swimmers and the D allele of the ACE I/D polymorphism. *Hum Genet* (2001); **108**(3): 230-2.

Woods, D., Sanders, J., Jones, A., Hawe, E., Gohlke, P., Humphries, S. E., Payne, J. and Montgomery, H. The serum angiotensin-converting enzyme and angiotensin II response to altered posture and acute exercise, and the influence of ACE genotype. *Eur J Appl Physiol* (2004); **91**(2-3): 342-348.

World Health Organisation. Arterial Hypertension and Ischaemic Heart Disease. Preventative aspects: Report of an expert committee. *WHO Technical Report Series* (1962); **231**: 3-28.

World Health Organisation. World Health Organisation Myocardial infarction community registers. *Public Health Eur* (1976); **5**: 157-164.

World Health Organisation (2002). World Health Report: Reducing risks, promoting healthy life. Geneva.

WoSCoPS Group. Baseline risk factors and their association with outcome in the West of Scotland Coronary Prevention Study. *Am J Cardiol* (1997); **79**(6): 756-762.

Xia, Y. and Zweier, J. L. Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. *Proc Natl Acad Sci U S A* (1997); **94**(13): 6954-6958.

Xu, T. S., Bowman, E. P., Glass, D. B. and Lambeth, J. D. Stimulation of adrenal mitochondrial cholesterol side-chain cleavage by GTP, steroidogenesis activator polypeptide (SAP), and sterol carrier protein2. GTP and SAP act synergistically. *J Biol Chem* (1991); **266**(11): 6801-6807.

Yaari, S., Goldbourt, Even-Zohar, S. and Neufeld, H. N. Associations of serum high density lipoprotein and total cholesterol with total, cardiovascular, and cancer mortality in a 7-year prospective study of 10 000 men. *Lancet* (1981); **1**(8228): 1011-105.

Yaffe, D. and Saxel, O. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* (1977); **270**(5639): 725-727.

Yamaguchi, F., Kawana, K., Tanonaka, K., Kamano, I., Igarashi, T., Gen, E., Fujimoto, Y., Maki, T., Sanbe, A., Nasa, Y. and Takeo, S. Improvement of exercise capacity of rats with chronic heart failure by long-term treatment with trandolapril. *Br J Pharmacol* (1999); **126**(7): 1585-1592.

Yang, N., MacArthur, D. G., Gulbin, J. P., Hahn, A. G., Beggs, A. H., Easteal, S. and North, K. ACTN3 Genotype Is Associated with Human Elite Athletic Performance. *Am J Hum Genet* (2003); **73**(3).

Yang, X. P., Liu, Y. H., Mehta, D., Cavasin, M. A., Shesely, E., Xu, J., Liu, F. and Carretero, O. A. Diminished cardioprotective response to inhibition of angiotensin-converting enzyme and angiotensin II type 1 receptor in B(2) kinin receptor gene knockout mice. *Circ Res* (2001); **88**(10): 1072-1079.

Yang, X. P., Liu, Y. H., Scicli, G. M., Webb, C. R. and Carretero, O. A. Role of kinins in the cardioprotective effect of preconditioning: study of myocardial ischemia/reperfusion injury in B2 kinin receptor knockout mice and kininogen-deficient rats. *Hypertension* (1997); **30**(3 Pt 2): 735-740.

Yanovski, J. A., Diament, A. L., Sovik, K. N., Nguyen, T. T., Li, H., Sebring, N. G. and Warden, C. H. Associations between uncoupling protein 2, body composition, and resting energy expenditure in lean and obese African American, white, and Asian children. *Am J Clin Nutr* (2000); **71**(6): 1405-1420.

Yayama, K., Yoshiya, M., Takahashi, K., Matsui, T., Takano, M. and Okamoto, H. Role of the kidney in the plasma clearance of angiotensinogen in the rat: plasma clearance and tissue distribution of 125I-angiotensinogen. *Life Sci* (1995); **57**(19): 1791-801.

Yoshitomi, H., Yamazaki, K., Abe, S. and Tanaka, I. Differential regulation of mouse uncoupling proteins among brown adipose tissue, white adipose tissue, and skeletal muscle in chronic beta 3 adrenergic receptor agonist treatment. *Biochem Biophys Res Commun* (1998); **253**(1): 85-91.

Yoshitomi, H., Yamazaki, K. and Tanaka, I. Mechanism of ubiquitous expression of mouse uncoupling protein 2 mRNA: control by cis-acting DNA element in 5'-flanking region. *Biochem J* (1999); **340**(Pt 2): 397-404.

Young, M. and Funder, J. W. Aldosterone and the heart. *Trends Endocrinol Metab* (2000); **11**(6): 224-226.

Young, M. E., Patil, S., Ying, J., Depre, C., Ahuja, H. S., Shipley, G. L., Stepkowski, S. M., Davies, P. J. and Taegtmeyer, H. Uncoupling protein 3 transcription is regulated by peroxisome proliferator-activated receptor (alpha) in the adult rodent heart. *Faseb J* (2001); **15**(3): 833-845.

Yu, X. X., Mao, W., Zhong, A., Schow, P., Brush, J., Sherwood, S. W., Adams, S. H. and Pan, G. Characterization of novel UCP5/BMCP1 isoforms and differential regulation of UCP4 and UCP5 expression through dietary or temperature manipulation. *Faseb J* (2000); **14**(11): 1611-1618.

Yucel, D., Aydogdu, S., Cehreli, S., Saydam, G., Canatan, H., Senes, M., Cigdem Topkaya, B. and Nebioglu, S. Increased oxidative stress in dilated cardiomyopathic heart failure. *Clin Chem* (1998); **44**(1): 148-154.

Yusuf, S., Dagenais, G., Pogue, J., Bosch, J. and Sleight, P. Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med* (2000); **342**(3): 154-160.

Yusuf, S., Sleight, P., Pogue, J., Bosch, J., Davies, R. and Dagenais, G. Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med* (2000); **342**(3): 145-153.

Zafari, A. M., Ushio-Fukai, M., Akers, M., Yin, Q., Shah, A., Harrison, D. G., Taylor, W. R. and Griendling, K. K. Role of NADH/NADPH oxidase-derived H2O2 in angiotensin II-induced vascular hypertrophy. *Hypertension* (1998); **32**(3): 488-495.

Zhang, B., Tanaka, H., Shono, N., Miura, S., Kiyonaga, A., Shindo, M. and Saku, K. The I allele of the angiotensin-converting enzyme gene is associated with an increased percentage of slow-twitch type I fibers in human skeletal muscle. *Clin Genet* (2003); **63**(2): 139-44.

Zhang, C. Y., Baffy, G., Perret, P., Krauss, S., Peroni, O., Grujic, D., Hagen, T., Vidal-Puig, A. J., Boss, O., Kim, Y. B., Zheng, X. X., Wheeler, M. B., Shulman, G. I., Chan, C. B. and Lowell, B. B. Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes. *Cell* (2001); **105**(6): 745-755.

Zhang, C. Y., Hagen, T., Mootha, V. K., Slieker, L. J. and Lowell, B. B. Assessment of uncoupling activity of uncoupling protein 3 using a yeast heterologous expression system. *FEBS Lett* (1999); **449**(2-3): 129-134.

Zhang, H., Huang, H. M., Carson, R. C., Mahmood, J., Thomas, H. M. and Gibson, G. E. Assessment of membrane potentials of mitochondrial populations in living cells. *Anal Biochem* (2001); **298**(2): 170-180.

Zhang, H., Schmeisser, A., Garlichs, C. D., Plotze, K., Damme, U., Mugge, A. and Daniel, W. G. Angiotensin II-induced superoxide anion generation in human vascular endothelial cells: role of membrane-bound NADH-/NADPH-oxidases. *Cardiovasc Res* (1999); **44**(1): 215-222.

Zhang, X., Xie, Y. W., Nasjletti, A., Xu, X., Wolin, M. S. and Hintze, T. H. ACE inhibitors promote nitric oxide accumulation to modulate myocardial oxygen consumption. *Circulation* (1997); **95**(1): 176-182.

Zhu, Y. C., Zhu, Y. Z., Spitznagel, H., Gohlke, P. and Unger, T. Substrate metabolism, hormone interaction, and angiotensin-converting enzyme inhibitors in left ventricular hypertrophy. *Diabetes* (1996); **45 Suppl 1**: S59-65.

Zhuo, J., Moeller, I., Jenkins, T., Chai, S. Y., Allen, A. M., Ohishi, M. and Mendelsohn, F. A. Mapping tissue angiotensin-converting enzyme and angiotensin AT1, AT2 and AT4 receptors. *J Hypertens* (1998); **16**(12 Pt 2): 2027-2037.

Zipes, D. P., Libby, P., Bonow, R. O. and Braunwald, E. Braunwald's Heart Disease: A Textbook of Cardiovascular Mediciine, Seventh Edition. Philadelphia (2005), Elsevier.

Zong, H., Ren, J. M., Young, L. H., Pypaert, M., Mu, J., Birnbaum, M. J. and Shulman, G. I. AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. *Proc Natl Acad Sci U S A* (2002); **99**(25): 15983-15987.

Zou, M. H., Shi, C. and Cohen, R. A. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J Clin Invest* (2002); **109**(6): 817-826.

Zurlo, F., Larson, K., Bogardus, C. and Ravussin, E. Skeletal muscle metabolism is a major determinant of resting energy expenditure. *J Clin Invest* (1990); **86**(5): 1423-1427.