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

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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.
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Dedicated to my wife, Nitasha and daughters, Meghana and Ashana.
Table of Contents

Personal Declaration ............................................................................................................ 2
Foreword .............................................................................................................................. 3
Abstract .............................................................................................................................. 4
Acknowledgements ............................................................................................................. 6
Table of Contents ................................................................................................................ 9
List of Figures ...................................................................................................................... 15
List of Tables ....................................................................................................................... 24
List of Abbreviations .......................................................................................................... 27

1. INTRODUCTION ............................................................................................................ 32

1.1. Coronary Heart Disease ............................................................................................. 33
  1.1.1. Atherosclerosis ...................................................................................................... 34
  1.1.2. The spectrum of disease in CHD ............................................................................. 37
  1.1.3. Risk factors for CHD ............................................................................................. 39
    1.1.3.1. Conventional risk factors ............................................................................... 39
    1.1.3.2. Genetic factors ................................................................................................ 42
    1.1.3.3. Novel risk factors ........................................................................................... 43

1.2. Heart Failure ................................................................................................................ 45
  1.2.1. Definition ............................................................................................................... 45
  1.2.2. Epidemiology and causes ...................................................................................... 45
  1.2.3. Pathophysiology .................................................................................................... 47
  1.2.4. Central vs. peripheral abnormalities in chronic heart failure ............................. 51
    1.2.4.1. Normal cardiac metabolism ......................................................................... 52
    1.2.4.2. Cardiac muscle metabolic abnormalities in heart failure ......................... 55
    1.2.4.3. Skeletal muscle metabolic abnormalities in heart failure ......................... 57
  1.2.5. Treatments ............................................................................................................. 62

1.3. Genetic association studies ......................................................................................... 63

1.4. Renin-Angiotensin-Aldosterone System (RAAS) ........................................................ 67
  1.4.1. Tissue ACE .......................................................................................................... 68
  1.4.2. Receptors for Angiotensin II and kinins ................................................................ 71
  1.4.3. Nitric oxide (NO-) ................................................................................................ 73

1.5. Mitochondria ............................................................................................................... 77
  1.5.1. Overview ............................................................................................................... 77
  1.5.2. Mitochondrial metabolism .................................................................................... 80
  1.5.3. Measuring mitochondrial function in living intact cells ....................................... 82
    1.5.3.1. TMRM ........................................................................................................... 85
    1.5.3.2. JC-1 ............................................................................................................... 86
  1.5.4. Defects in mitochondrial metabolism .................................................................... 87
  1.5.5. ACE and mitochondria .......................................................................................... 88

1.6. Oxidative Stress .......................................................................................................... 89
  1.6.1. Mitochondrial ROS generation ............................................................................. 92
  1.6.2. Other sources of oxidative stress ........................................................................ 94
2.3.4.5.  UCP2Del/Ins variant ................................................................. 151

2.4  Cell culture ........................................................................................................ 152
  2.4.1.  General cell culture methods for immortalised cell lines ...................... 154
    2.4.1.1.  Thawing and seeding cells for culture ........................................... 154
    2.4.1.2.  Cryopreservation of cells .............................................................. 155
  2.4.2.  Growing characteristics of C2C12 cell line ........................................ 155
  2.4.3.  Growing characteristics of H9C2 ........................................................... 155
  2.4.4.  Culture of primary cells ......................................................................... 156
    2.4.4.1.  Adult ventricular cardiomyocytes from the Sprague Dawley rat ... 156
    2.4.4.2.  Isolated human ventricular cardiomyocytes .................................. 158
    2.4.4.3.  Culture of human umbilical vein endothelial cells (HUVECs) ....... 159

2.5.  Measuring mitochondrial function in live cells ........................................... 159
  2.5.1.  Flow cytometric measurement of \( \Delta \psi_m \) ...................................... 160
    2.5.1.1.  Principles of flow cytometry ............................................................ 160
    2.5.1.2.  Flow cytometric analysis of \( \Delta \psi_m \) ........................................ 161
  2.5.2.  Confocal scanning laser microscopic measurement of \( \Delta \psi_m \) ......... 162
  2.5.3.  Measurement of cellular ROS generation ........................................... 164
  2.5.4.  Measurement of \textit{in vitro} ACE activity ........................................... 164
  2.5.5.  Measurement of cellular oxygen consumption .................................... 167

2.6.  Measurement of mRNA ................................................................................. 168
  2.6.1.  RNA isolation ......................................................................................... 168
  2.6.2.  cDNA synthesis ...................................................................................... 169
  2.6.3.  Design of primers .................................................................................... 169
  2.6.4.  Reverse-transcription (RT)-PCR ............................................................ 170
  2.6.5.  Quantitative real-time RT-PCR using the LightCycler® ......................... 171

2.7.  Statistical analyses .......................................................................................... 172

3.  RESULTS: IS THERE AN ASSOCIATION BETWEEN GENETIC
    VARIATION IN THE BRADYKININ B2 RECEPTOR AND PROSPECTIVE
    CARDIOVASCULAR PHENOTYPES? ................................................................. 174
  3.1.  Bassingbourn (Big Heart) 2 Study and \textit{BDKB2} +9/-9 gene variant ....... 175
  3.2.  NPHSII and the \textit{BDKB}2 gene variant ..................................................... 181
  3.3.  Discussion ..................................................................................................... 186

4.  RESULTS: THE BRADYKININ B2 RECEPTOR GENE AND HUMAN
    PERFORMANCE .................................................................................................... 192
  4.1.  Human physical performance .................................................................... 194
  4.2.  British Olympic athletes ............................................................................. 198
  4.3.  Discussion ..................................................................................................... 199
5. RESULTS: THE EFFECT OF ACE-INHIBITION ON MYOCYTE MITOCHONDRIAL FUNCTION ................................................. 203

5.1. Flow cytometry .................................................................................................................................................. 204
  5.1.1. Baseline characteristics ................................................................................................................................. 204
  5.1.2. TMRM mitochondrial probe ......................................................................................................................... 205
    5.1.2.1. Dose titration ............................................................................................................................................. 205
    5.1.2.2. The effect of mCCCP on cellular TMRM fluorescence .............................................................................. 208
  5.1.3. JC-1 mitochondrial probe ............................................................................................................................. 210
    5.1.3.1. Dose titration ........................................................................................................................................... 210
    5.1.3.2. JC-1 and manipulation of Δψₘ ................................................................................................................. 213
  5.1.4. ACE inhibitor effects on Δψₘ assessed in whole cells by flow cytometry ..................................................... 216
    5.1.4.1. TMRM .................................................................................................................................................... 216
    5.1.4.2. JC-1 ...................................................................................................................................................... 218

5.2. Confocal analysis ............................................................................................................................................... 220
  5.2.1. The effect of ACE inhibition on Δψₘ assessed by CLSM .............................................................................. 224
    5.2.1.1. C2C12 skeletal myotubes ....................................................................................................................... 224
    5.2.1.2. Cardiac cells .............................................................................................................................................. 224
  5.2.2. CLSM measurement of ROS in cardiomyocytes treated with ACEi ......................................................... 225

5.3. Cellular oxygen consumption ........................................................................................................................... 233
  5.3.1. C₂₁₂ cells treated with LPS ........................................................................................................................ 233
  5.3.2. ACE inhibitor treatment ............................................................................................................................... 235
  5.3.3. C₂₁₂ treatment with Angiotensin II ........................................................................................................... 235

5.4. UCP3 mRNA expression in C₂₁₂ myocytes ....................................................................................................... 236

5.5. Discussion .......................................................................................................................................................... 239

6. RESULTS: IS THERE AN ASSOCIATION BETWEEN VARIATION IN THE UCP3/UCP2 GENETIC LOCUS AND CARDIOVASCULAR OR PERFORMANCE PHENOTYPES? ................................................. 245

6.1. Genetic variation in UCP2 and UCP3 and LV mass ....................................................................................... 247
  6.1.1. UCP2-866G>A genotype ............................................................................................................................. 247
  6.1.2. UCP3-55C>T genotype ............................................................................................................................... 248

6.2. Prospective cardiovascular risk and genetic variation of UCP2 / 3 ................................................................ 252
  6.2.1. UCP2-866G>A .............................................................................................................................................. 252
  6.2.2. UCP3-55C>T ............................................................................................................................................... 258
  6.2.3. UCP3/2 haplotypes ...................................................................................................................................... 261

6.3. Skeletal muscle performance and UCP genotypes ......................................................................................... 263
  6.3.1. Skeletal muscle efficiency .......................................................................................................................... 263
  6.3.2. British Olympic Athletes ........................................................................................................................... 267

6.4. Diabetic study, oxidative stress and UCP genotypes (UDACS) ................................................................. 271
6.5. Acute inflammatory response and changes in serum ACE activity during intense physical exercise ................................................................. 274
6.5.2. ACE genotype and serum ACE activity during training ............... 274
6.5.2. Serum markers and UCP3-55C>T gene variant ............................ 277
6.5.3. Serum markers and UCP2-866G>A gene variant ..................... 278

6.6. Discussion .................................................................................. 281
6.6.1. LV mass .................................................................................. 281
6.6.2. Prospective CHD risk & oxidative stress .................................. 283
6.6.3. Skeletal muscle efficiency & endurance phenotypes ............... 288
6.6.4. The effects of endurance training on serum ACE activity ....... 292

7. RESULTS: IS THERE AN ASSOCIATION BETWEEN THE UCP3/UCP2 GENETIC LOCUS AND CIRCULATING ACE ACTIVITY? ............ 294

7.1. Healthy Caucasian and native South Africans .............................. 296
7.2. Diabetic patients (UDACS) .......................................................... 304
7.3. Type 1 Danish diabetics ............................................................... 311
7.4. Discussion .................................................................................. 317

8. RESULTS: DO UNCOUPLING PROTEINS 2 OR 3 ALTER ACE EXPRESSION IN HUMAN ENDOTHELIAL CELLS? ........................................ 322

8.1. Uncoupling proteins are expressed at the mRNA level in HUVECs .... 322
8.2. Manipulation of \( \Delta \psi_m \) in HUVECs .................................................. 322
8.3. ROS generation in HUVECs ....................................................... 325
8.4. The effects of prolonged hypoxia, uncoupling and high glucose on \( \Delta \psi_m \) and superoxide generation in cultured HUVECs ...................... 326
8.5. The effects of UCP antisense on HUVEC mitochondrial function ..... 327
8.6. The effect of UCP antisense on ACE expression in endothelial cells .... 330
8.7. Discussion .................................................................................. 332

9. CONCLUSIONS ............................................................................. 336

9.1. Genetic variation in the bradykinin \( \beta_2 \) receptor (BDKRB2) gene .......................... 336
9.2. ACE & mitochondria ................................................................. 341
9.3. UCP2 and UCP3 and the cardiovascular system ......................... 345
9.3.1. UCPs and prospective cardiac growth ..................................... 345
9.3.2. UCPs and CHD risk ............................................................... 349
9.3.3. UCPs and oxidative stress ......................................................... 351
9.3.4. UCPs and skeletal muscle efficiency ........................................ 352

9.4. UCP & ACE ............................................................................. 356
  9.4.1. Genetic studies ................................................................. 356
  9.4.2. In vitro work ...................................................................... 358

9.5. Study limitations and future directions ........................................ 362

10. APPENDICES ............................................................................ 363
    Appendix 1: Published manuscripts arising from this thesis ............ 363
    Appendix 2: Published letters arising from this thesis .................... 367
    Appendix 3: Abstracts of other papers arising during this thesis ...... 376
    Appendix 4: Assessment of circulating oxidative stress in UDACS ... 379

11. BIBLIOGRAPHY ........................................................................ 384
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>The structure of the normal artery (left) and the genesis of the atherosclerotic plaque (right)</td>
<td>36</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Diagram showing the interrelationship between ventricular end-diastolic volume (EDV) and stroke volume (SV)</td>
<td>47</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>Maladaptive mechanisms in heart failure include sympathetic (SNS) activation and RAAS activation</td>
<td>49</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>ATP synthesising and utilising reactions in the cardiomyocyte</td>
<td>51</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>Cartoon depiction of myocardial metabolism</td>
<td>53</td>
</tr>
<tr>
<td>Figure 1.6</td>
<td>Muscle hypothesis of chronic heart failure</td>
<td>59</td>
</tr>
<tr>
<td>Figure 1.7</td>
<td>The Renin-Angiotensin and Kallikrein-Kinin Systems</td>
<td>69</td>
</tr>
<tr>
<td>Figure 1.8</td>
<td>Cartoon depiction of classical nitric oxide (NO•) generation within endothelial cells by nitric oxide synthase (NOS)</td>
<td>73</td>
</tr>
<tr>
<td>Figure 1.9</td>
<td>Cross section through a mitochondrion, showing the inner and outer membranes</td>
<td>79</td>
</tr>
<tr>
<td>Figure 1.10</td>
<td>The mitochondrial proton circuit is analogous to an electrical circuit</td>
<td>81</td>
</tr>
<tr>
<td>Figure 1.11</td>
<td>States of respiration in isolated mitochondria</td>
<td>83</td>
</tr>
<tr>
<td>Figure 1.12</td>
<td>Chemical structure and excitation/emission spectra for TMRM</td>
<td>85</td>
</tr>
<tr>
<td>Figure 1.13</td>
<td>Chemical structure and excitation/emission spectra for JC-1</td>
<td>86</td>
</tr>
<tr>
<td>Figure 1.14</td>
<td>Reactive oxygen species (ROS) are generated at complexes I and III of the electron transport chain</td>
<td>94</td>
</tr>
<tr>
<td>Figure 1.15</td>
<td>Angiotensin II (Ang II) activation of membrane bound NAD(P)H oxidase system</td>
<td>96</td>
</tr>
</tbody>
</table>
Figure 1.16. Phylogenetic tree of the mitochondrial anion-carrier superfamily........107

Figure 1.17. Postulated tripartite structure of uncoupling proteins.........................108

Figure 1.18. Possible mechanisms of uncoupling protein proton translocation.........109

Figure 1.19. Human UCP2/UCP3 gene locus (chromosome 11q13) and common
variants........................................................................................................124

Figure 2.1. Example of a MADGE gel......................................................................148

Figure 2.2. Overview of a flow cytometer................................................................160

Figure 2.3. ‘Optics’ folder within the Laser Sharp confocal microscopy software..165

Figure 2.4. Laser Sharp imaging software during live confocal image acquisition...166

Figure 2.5. Cartoon depiction of oxyspot system to measure cellular oxygen
consumption....................................................................................................167

Figure 3.1. Proportional change in LV mass as assessed by CMR according to
BDKRB2 +9/-9 genotype in the BH2 study.....................................................180

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..180

Figure 3.3. Association between systolic blood pressure at recruitment in NPHS II and
CHD..................................................................................................................185

Figure 4.1. Distribution of baseline delta efficiency and training related changes in
delta efficiency.................................................................................................195

Figure 4.2. Baseline delta efficiency according to BDKRB2 (+9/-9) genotype in study
subjects homozygous for the ACE I/D polymorphism.................................197

Figure 4.3. ACE/BDKRB2 combined genotypes by running distance in BOA study 199

Figure 5.1. Dot plot representation of murine C2C12 myoblasts cells analysed by flow
cytometer ........................................................................................................204
Figure 5.2. Flow cytometric contour map of the same C_2C_{12} blasts .................205

Figure 5.3. A 3D density plot of TMRM staining characteristics of C_2C_{12} blasts.....206

Figure 5.4. TMRM fluorescence of C_2C_{12} blasts expressed on a histogram plot ......206

Figure 5.5. Contour plots of C_2C_{12} blasts incubated with increasing concentrations of TMRM for 30 minutes. .................................................................207

Figure 5.6. Flow cytometric analysis of TMRM stained C_2C_{12} cells..................208

Figure 5.7. Flow cytometric measurement of TMRM fluorescence in untreated (coupled) and mClCCP-treated (uncoupled) C_2C_{12} myoblasts...............209

Figure 5.8. Relative change in TMRM fluorescence in C_2C_{12} myoblasts after treatment with 20nM mClCCP (10000 cells) .........................................................209

Figure 5.9. Proportion of pre-adipocytes demonstrating green and orange fluorescence when incubated with increasing concentrations of JC-1 for 15 minutes .211

Figure 5.10. Proportion of pre-adipocytes demonstrating green and orange fluorescence when incubated with increasing concentrations of JC-1 for 60 minutes 211

Figure 5.11. Proportion of preadipocytes (total N=10000) exhibiting green monomer fluorescence incubated with increasing dose of JC-1 (varying time and concentrations).............................................................................212

Figure 5.12. Proportion of preadipocytes (total N=10000) exhibiting orange aggregate fluorescence incubated with increasing dose of JC-1.........................212

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. Lower panel: pre-treatment with 20μM 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.............................214
Figure 5.14. Dot plot series showing (upper panels) rat H9C2 cardiac blasts incubated for 30 mins at 37°C with increasing concentrations of the mitochondrial probe JC-1 followed by 2 channel flow cytometric analysis. 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.

Figure 5.15. JC-1 aggregate/monomer (A:G) fluorescence ratio in C2C12 myotubes incubated with vehicle, mClCCP or nigericin.

Figure 5.16. Flow cytometric histogram plot demonstrating TMRM stained C2C12 myotubes which have been untreated or ramiprilat (10⁻⁵M) treated for 24 hours.

Figure 5.17. Mean change in TMRM dye fluorescence following incubation of C2C12 myotubes with different ACE inhibitors at 10⁻⁵M for 24 - 48 hours.

Figure 5.18. Ratio of JC-1 aggregate to monomer fluorescence of C2C12 myotubes incubated with increasing dose of ACEi Ramiprilat for 24 hours.

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.

Figure 5.20. Confocal analysis of mitochondrial TMRM fluorescence in C2C12 myotubes.

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 Δψm.

Figure 5.22. C2C12 myotubes stained with TMRM and analysed by confocal microscopy.

Figure 5.23. Fields of C2C12 cells stained with TMRM and imaged with CLSM.

Figure 5.24. H9C2 cardiac blasts stained with TMRM and analysed by confocal microscopy.
Figure 5.25. CLSM analysis of Δψ\textsubscript{m} in rat adult ventricular primaries .......................... 229

Figures 5.26-28. TMRM fluorescence in human adult ventricular cardiomyocytes ... 230

Figure 5.29. Changes in TMRM and DCF-DA fluorescence in vehicle and Ramiprilat treated cardiomyocytes after sequential laser scanning .......................... 231

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\textsuperscript{-5}M for 24hr .......................... 232

Figure 5.31. Oxygen consumption measured in C\textsubscript{2}C\textsubscript{12} myotubes pre-treated with vehicle or lipopolysaccharide (LPS; 1μg.ml\textsuperscript{-1}) for 24 hours. Oxygen consumption was lower in LPS treated cells. After addition of uncoupler (arrow) the oxygen tension curves run in parallel ................................... 234

Figure 5.32. Respiratory control ratio (RCR) in vehicle, LPS and angiotensin II treated C\textsubscript{2}C\textsubscript{12} cells .............................................................................. 234

Figure 5.33. Oxyspot graph depicting oxygen consumption in C\textsubscript{2}C\textsubscript{12} myocytes treated with vehicle or Ramiprilat (10\textsuperscript{-5}M) for 24 hours ............................................. 235

Figure 5.34. Graph depicting oxygen consumption in C\textsubscript{2}C\textsubscript{12} myocytes pre-treated with Angiotensin II (10\textsuperscript{-7}M) for 1 hour ................................................................. 236

Figure 5.35. Quantitative RT-PCR analysis of UCP3:GAPDH mRNA transcript ratio in C\textsubscript{2}C\textsubscript{12} myotubes treated with increasing concentration of the ACE inhibitor ramiprilat for 24 hours ........................................................................ 238

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\textsubscript{2}C\textsubscript{12} myotubes analysed by quantitative RT-PCR ........................................... 238

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 ........................................................................ 251
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.................................................................257

Figure 6.3. Relative CHD risk in NPHS II according to UCP3/UCP2 haplotypes....262

Figure 6.4. UCP3/UCP2 haplotype frequencies in NPHS II .............................................262

Figure 6.5. Training related changes in delta efficiency .....................................................266

Figure 6.6. UCP3-55C>T and UCP2-866G>A haplotype distribution according to running distance and ethnicity of British Olympic athletes.................269

Figure 6.7. Serum ACE activity by quartile of age and by ACE I/D genotype during basic army training in 61 recruits. .........................................................276

Figure 6.8. Absolute changes in serum ACE activity during basic army training according to ACE genotype. .................................................................277

Figure 6.9. Mean serum ACE activity during basic training stratified by UCP genotypes .................................................................280

Figure 7.1. Linkage disequilibrium between common variants in the UCP3/2 gene cluster in the RSA (D’ black) and BH3 (D’red) studies. .........................297

Figure 7.2. Scatter plots of serum ACE activity vs. age in BH3 and RSA studies ...301

Figure 7.3. Serum ACE activity by UCP3 and UCP2 genotypes in the RSA and BH3 studies. .........................................................................................302

Figure 7.4. Predicted UCP3-55C>T, UCP2-866G>A and UCP2D/I haplotype effects on serum ACE activity in the BH3 and RSA study samples ...............303

Figure 7.5. Distribution of serum ACE activity in male Caucasians drawn from UDACS and negative correlation with age.........................................304
Figure 7.6. Mean serum ACE activity in male Caucasian subjects from UDACS according to drug treatment with an ACE inhibitor or angiotensin type 1 receptor blocker .................................................................306

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 .................................................306

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. 307

Figure 7.9. Linkage disequilibrium between common variants in the UCP3/2 gene cluster in UDACS type 1 (Black) and type 2 (red) diabetics .........................309

Figure 7.10. Mean serum ACE activity by UCP3 and UCP2 genotypes in ACEi- and ARB-naïve subjects from UDACS according to type of diabetes ..............310

Figure 7.11. The distribution of serum ACE activity amongst a sample of Danish type 1 diabetics. .........................................................................................311

Figure 7.12. Mean serum ACE activity in Danish type 1 diabetics according to drug treatment with an ACE inhibitor or angiotensin type 1 receptor blocker 313

Figure 7.13. Linkage disequilibrium between common variants in the UCP3/2 gene cluster in the Danish type 1 diabetics ..........................................................314

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 UCP2D/I genotypes ........................................................................................315

Figure 7.15. The statistical interaction between UCP3-55C>T, UCP2-866G>A and UCP2D/I genotypes in determining serum ACE activity (adjusted for age) in Danish type 1 diabetic patients ..................................................316

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 ......................................................323
Figure 8.2. Relative JC-1 aggregate to monomer (A:M) fluorescence in HUVECs pretreated with mClCCP, rotenone and nigericin..................................................324

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. ........................................................................................................325

Figure 8.4. Effects of 24 hours of high glucose, protonphore mClCCP and hypoxia (5% O2) on HUVEC Δψ_m and superoxide production (measured by JC-1 aggregate to monomer fluorescence ratio and DHE fluorescence, respectively)........................................................................................................327

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.................................................................328

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. ........................................................................................................329

Figure 8.7. Dihydroethidium fluorescence in HUVECs incubated with antisense to uncoupling proteins for 48 hours. ................................................................................................................329

Figure 8.8. ACE mRNA expression in cultured HUVECs following treatment for 48hr with 10mM UCP antisense. ........................................................................................................331

Figure 8.9. ACE activity measured in the culture media of HUVECs following treatment with UCP antisense for 48 hours ........................................................................331

Figure 9.1. Schematic summary of BDKRB2 gene-environment studies conducting in this thesis.................................................................338

Figure 9.2. Cartoon depiction of the putative mitochondrial actions of ACE and its downstream effectors, bradykinin (BK) and angiotensin II (Ang II). .....343

Figure 9.3. Schematic summary of UCP gene-environment studies conducting in this thesis and in appendix 4. .................................................................347
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. …380

Figure 10.2. Plasma TAOS and F₂-isoprostane in UDACS substudy of 20 diabetic closely matched men selected for homozygosity for UCP2-866G>A genotype and stratified by CHD status. ………………………………………………383
List of Tables

Table 1.1 The common causes of heart failure ................................................................. 46

Table 1.2. Published studies of common variants in the human UCP2 gene and association with various phenotypes .......................................................... 126

Table 1.3. Published studies of common variants in the human UCP3 gene and association with various phenotypes ......................................................... 128

Table 2.1 PCR mix for 100 reactions .................................................................................. 146

Table 2.2. Forward and reverse primers for genotyping common gene variants. ....... 153

Table 2.3. Typical flow cytometer detector settings for analysis of Δψm in live cells using JC-1 fluorescent dye ................................................................. 162

Table 2.4. Source of DNA sequences for primer design .................................................. 170

Table 3.1. Baseline characteristics of Big Heart 2 Study subjects .................................. 176

Table 3.2. Baseline characteristics of Big Heart 2 Study sample by BDKRB2 +9/-9 gene variant .................................................................................................. 178

Table 3.3. Training related changes in cardiac parameters by BDKRB2 +9/-9 genotype and ACE/BDKRB2 haplotypes ......................................................... 179

Table 3.4. Baseline characteristics of men from NPHS II according to BDKRB2(+9, -9) genotype. ....................................................................................... 183

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. ........................................ 184

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 ................................................................. 195
Table 5.1. Typical settings for confocal imaging ................................................................. 221

Table 5.2. Forward and reverse primers for RT-PCR and amplicon size ...................... 237

Table 6.1. Baseline characteristics of Big Heart 2 Study sample by UCP2-866G>A and UCP3-55C>T variants .............................................................. 249

Table 6.2 Training related changes in cardiac parameters by UCP2-866G>A and UCP3-55C>T genotypes and haplotypes ............................................ 250

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............. 253

Table 6.4. Baseline characteristics in NPHS II by UCP2-866G>A genotype. Data are mean (SD) unless otherwise stated ............................................. 254

Table 6.5. CHD risk in NPHS II in subjects stratified by UCP2-866G>A genotype. 255

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 ................................................................. 256

Table 6.7. Baseline characteristics of study subjects in NPHS II by UCP3-55C>T genotype. ........................................................................................................ 259

Table 6.8. CHD risk in NPHS II by UCP3-55C>T genotype ........................................... 260

Table 6.9. UCP3/2 haplotype distribution in NPHSII ..................................................... 261

Table 6.10. UCP3/2 haplotype frequencies in cases and controls from NPHSII ....... 262

Table 6.11. Baseline characteristics of the 131 subjects in the delta efficiency study .264

Table 6.12. Training related changes in delta efficiency according to UCP2-866G>A and UCP3-55C>T genotypes ............................................................. 265

Table 6.13. UCP3-55C>T genotype distribution amongst 81 British Olympic athletes according to competitive distance event .......................................... 267
Table 6.14. *UCP2*-866G>A genotype distribution amongst 81 British Olympic athletes according to competitive distance event. .................................................................268

Table 6.15. *UCP3/2* haplotype distribution in the BOA study, both overall and amongst the greater number of Caucasian subjects. .................................................................269

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.........................270

Table 6.17. Baseline characteristics of diabetic Caucasian men recruited in UDACS. 272

Table 6.18. Baseline characteristics of subjects from the Bassingbourn 3 Study........274

Table 6.19. *UCP* haplotype distribution and frequency in the Bassingbourn studies...279

Table 7.1. *UCP3*-55C>T, *UCP2*-866G>A and *UCP2* Del/Ins genotype distributions for the RSA and BH3 studies. .................................................................297

Table 7.2. Distribution and linkage disequilibrium (D’) between *UCP* genotypes in the RSA and Bassingbourn (Big Heart) 3 studies .................................................298

Table 7.3. Comparison of *UCP* haplotype distribution in the RSA and BH3 studies (graphic depiction of same on right) .........................................................300

Table 7.4. *UCP* haplotype distribution and linkage disequilibrium (D’) in UDACS. 309

Table 7.5. *UCP* haplotype distribution and linkage disequilibrium (D’) in the Danish type 1 diabetic patients..............................................................314

Table 8.1. Forward and reverse primers for RT-PCR and amplicon size. ..............323

Table 10.1. Plasma TAOS and esterified F2-isoprostanes in relation to *UCP2*-866G>A genotype and CHD status in the UDACS substudy. ............................382
List of Abbreviations

\(\Delta \psi_m\) Mitochondrial membrane potential
\(\Delta \mu_{\text{H}^+}\) Electrochemical gradient
\(\Delta \rho, \text{PMF}\) Proton motive force
\(\Delta \text{pH}\) Proton gradient
\(\Delta G\) 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\(_1\)R Angiotensin II type 1 receptor
AT\(_2\)R Angiotensin II type 2 receptor
ATP Adenosine triphosphate

BAT Brown adipose tissue
BDKRB1 Bradykinin \(\beta_1\) receptor
BDKRB2 Bradykinin \(\beta_2\) 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 monophosphate
cGMP Cyclic guanosine-3’,5-monophosphate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CHF</td>
<td>Chronic heart failure</td>
</tr>
<tr>
<td>CMR</td>
<td>Cardiac magnetic resonance imaging</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CuZn-SOD</td>
<td>Copper-Zinc superoxide dismutase</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DBP</td>
<td>Diastolic blood pressure</td>
</tr>
<tr>
<td>DDAH</td>
<td>Dimethylarginine dimethylaminohydrolase</td>
</tr>
<tr>
<td>DE</td>
<td>Delta efficiency</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelium derived relaxing factor</td>
</tr>
<tr>
<td>EDV</td>
<td>End diastolic volume</td>
</tr>
<tr>
<td>EGFR</td>
<td>Endothelial growth factor receptor</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial isoform of nitric oxide synthase</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FAD⁺</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FADH</td>
<td>Reduced form of flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FME</td>
<td>Final military exercise</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GTN</td>
<td>Glyceryl trinitrate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>H⁺</td>
<td>Hydrogen ion (proton)</td>
</tr>
<tr>
<td>HCM</td>
<td>Hypertrophic cardiomyopathy</td>
</tr>
<tr>
<td>hsCRP</td>
<td>high sensitivity CRP</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>JC-1</td>
<td>5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide</td>
</tr>
<tr>
<td>JC-1 A:M</td>
<td>JC-1 aggregate to monomer fluorescence</td>
</tr>
<tr>
<td>JGA</td>
<td>Juxtaglomerular apparatus</td>
</tr>
<tr>
<td>KKS</td>
<td>Kallikrein-kinin system</td>
</tr>
<tr>
<td>LD</td>
<td>Linkage disequilibrium</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle/ventricular</td>
</tr>
<tr>
<td>LVH</td>
<td>Left ventricular hypertrophy</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>mi-CK</td>
<td>Mitochondrial isoform of creatine kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>Manganese-dependent isoform of superoxide dismutase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Reduced form of nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced form of nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal isoform of nitric oxide synthase</td>
</tr>
<tr>
<td>NO⁺</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NPHSII</td>
<td>Second Northwick Park Heart Study</td>
</tr>
<tr>
<td>NSTEMI</td>
<td>Non-ST elevation myocardial infarction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>O₂⁻•</td>
<td>Superoxide</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>OS</td>
<td>Oxidative stress</td>
</tr>
<tr>
<td>Ox-LDL</td>
<td>Oxidised low density lipoprotein</td>
</tr>
<tr>
<td>PMF, Δₚ</td>
<td>Proton motive force</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCr</td>
<td>Phosphocreatine</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
</tr>
<tr>
<td>PPRE</td>
<td>PPAR response element</td>
</tr>
<tr>
<td>QTL</td>
<td>Quantitative trait locus</td>
</tr>
<tr>
<td>RAAS</td>
<td>Renin angiotensin aldosterone system</td>
</tr>
<tr>
<td>RER</td>
<td>Respiratory exchange ratio</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RSA Study</td>
<td>Republic of South Africa Study</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>RV</td>
<td>Right ventricle/ventricular</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic blood pressure</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic nervous system</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>SV</td>
<td>Stroke volume</td>
</tr>
<tr>
<td>TAOS</td>
<td>Total antioxidant status</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>TMRE</td>
<td>Tetramethylrhodamine ethyl ester</td>
</tr>
<tr>
<td>TMRM</td>
<td>Tetramethylrhodamine methyl ester</td>
</tr>
<tr>
<td>TRE</td>
<td>Thyroid response element</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling protein</td>
</tr>
<tr>
<td>UDACS</td>
<td>University College Diabetes and Cardiovascular disease Study</td>
</tr>
<tr>
<td>VO₂</td>
<td>Rate of oxygen uptake</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
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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 β2 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 105,000 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 (PGI2). 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 95\textsuperscript{th} percentile (due predominantly to one of a number of mutations in the LDL receptor gene) and early-onset CHD in the 3\textsuperscript{rd} or 4\textsuperscript{th} 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 \textit{et al.} 2004).

1.1.3.3 Novel risk factors

Almost half of all MIs occur in patients without significant hyperlipidaemia (Ridker \textit{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 \textit{et al.} 1996; Ridker \textit{et al.} 1997; Tracy \textit{et al.} 1997; Ridker \textit{et al.} 1998; Koenig \textit{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 \textit{et al.} 1998) where it may have several pro-atherosclerotic actions including binding oxidised low density lipoprotein (oxLDL) (de Beer \textit{et al.} 1982), inducing adhesion molecule expression on endothelium (Pasceri \textit{et al.} 2000) and reducing NO\textsuperscript{•} bioavailability (Venugopal \textit{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 A₂ (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
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

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

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 output.

![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).](image-url)
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 juxtaglomerular 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

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**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:

\[
\Delta G = \Delta H - T\Delta S
\]

\(\Delta G\) = Gibbs energy change, \(\Delta H\) = enthalpy change, \(T\) = temperature
\(\Delta S\) = entropy change of the system

\(\Delta G\) 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 \(\Delta G\). The \(\Delta G\) required for ATP synthesis (also known as the phosphorylation potential) is obtained from the \(\Delta G\) 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:

\[ 2\text{ADP} \rightarrow \text{ATP} + \text{AMP}. \]

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 acylcarnitin 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 (PPARα)-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 31P 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 ΔG<sub>ΔG</sub>≈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 PPARγ 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 (VO₂) 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 VO₂, 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 VO₂, 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 (Vₑ/VₐCO₂ 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 CO₂-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).
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\textsubscript{2}-sensitivity has been reported in CHF (Chua \textit{et al.} 1996).

1.2.5 \textbf{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\textsubscript{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 \textit{et al.} 1992; AIRE Study Investigators 1993; Kober \textit{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 \textit{et al.} 1997; Pitt \textit{et al.} 2000; Cohn \textit{et al.} 2001; Granger \textit{et al.} 2003), and with aldosterone antagonism also providing additional mortality benefit (Pitt \textit{et al.} 1999; Pitt \textit{et al.} 2003). Selective beta-blockade has been shown in randomised placebo-controlled trials to reduce mortality (CIBIS-II 1999; MERIT-HF 1999; Packer \textit{et al.} 2002) and hospitalisation from heart failure (Packer \textit{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 \textit{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 $\alpha_2$ 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 β2 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 (AT1R) and type 2 (AT2R) receptors. Ang II is the effector peptide of the RAAS cascade, acting via the AT1R 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 AT2R is thought to oppose the actions of the AT1R (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 (Arg1-Pro2-Pro3-Gly4-Phe5-Ser6-Pro7-Phe8-Arg9) 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 glycercyl 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).

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**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 (O₂•⁻) 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 O₂•⁻ 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. Endogenous 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 (F₁,F₀-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 (F₀ subunit) and an ATP synthase (F₁ subunit). If the secondary pump were in isolation, it would hydrolyse ATP to ADP and Pᵢ.

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 $F_0F_1$-ATPase down this gradient, providing energy for generation of ATP. ATP exits the matrix through the ADP/ATP translocator.
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, $\Delta \rho$) 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). $\Delta \rho$ consists of two components: a H$^+$ gradient (pH gradient or $\Delta p$H) and a gradient due to the electrical potential generated between the matrix and intermembranous space ($\Delta \psi_m$). In the mitochondrion, the $\Delta p$H component is small, only 0.5pH units, and $\Delta \psi_m$ approximates $\Delta \rho$.

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, $V = I \times R$, 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.

Protonophores, 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 Δψᵢ. The rate of proton flow can be estimated from the respiration rate of mitochondria measured, for example, with an oxygen electrode.

Figure 1.10. The mitochondrial proton circuit is analogous to an electrical circuit (after Nicholls and Ferguson 2002). Voltage (V) is equivalent to proton motive force (Δp); 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 O₂, presented in Figure 1.11. Maximal respiration occurs (state 3) under optimal conditions when substrate, ADP and O₂ 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). Protonophore uncouplers can initialise rapid respiration, similar to state 3, by collapsing Δψₘ, 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 Δψₘ, intramitochondrial calcium, redox state, ROS generation, apoptosis and mitochondrial distribution and movement within cells.
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):

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

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.

**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
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 Δψ_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 equilibrate rapidly according to the magnitude of Δψ_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. Chemical structure and excitation/emission spectra for TMRM
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 Δψ_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 Δψ_m (Reers et al. 1991; Michelakis et al. 2002) and has been found to be more reliable than several other compounds, such as DiOC₆, 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$_2^•$), hydrogen peroxide (H$_2$O$_2$), 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$_2^•$, H$_2$O$_2$, 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-KB 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, \( \text{H}_2\text{O}_2 \), 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-KB nuclear translocation, involve ROS as a common step (Schreck et al. 1991). However, the ROS that activate NF-KB are more restricted to hydroperoxidases, such as \( \text{H}_2\text{O}_2 \). NF-KB has been directly implicated in atherogenesis, and activated NF-KB 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, TNF\( \alpha \) 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). \( \text{O}_2^{•−} \) can rapidly and irreversibly inhibit calcium-induced force generation in isolated rat cardiomyocytes (Miller et al. 1995).

\( \text{NO}^{•−} \) 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 \( \text{O}_2 \) consumption is due to \( \text{O}_2^{•−} \) production. \( \text{O}_2 \) appears to have access to the electron donating sites in complexes I and III, to allow formation of \( \text{O}_2^{•−} \). 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$_2$O$_2$ 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$_2$O$_2$ 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 $\Delta_p$-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 $\beta$-carotene, ubiquinone, lipoic acid, and urate (Nordberg et al. 2001).
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 AT1R, 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 AT₁R antagonism, and is enhanced by AT₂R 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 O₂⁻ and H₂O₂. 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.
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

1.7 RAAS AND METABOLISM

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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 24 hours 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 CO₂, 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 (VO₂) (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 B₂ 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 AT₁R, mediates cardiac fibrosis and hypertrophy (Kato et al. 1991). However, the protective effect of AT₁R blockade on the myocardium may be due to unopposed stimulation of AT₂R, which is kinin and NO• dependent (Gohlke et al. 1998; Jalowy et al. 1998). Perfusion with a combination of an
ACEi and an AT\textsubscript{1}R 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 O₂ (Brown et al. 1994; Cleeter et al. 1994). It thus reduces O₂ 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 O₂ allows cytochrome c oxidase to act as an O₂ sensor within cells, with the affinity of NO• for cytochrome c oxidase dependent on the O₂ 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 H2O2 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 thermogenesis 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. Postulated tripartite structure of uncoupling proteins. The conserved arginine residues shown are necessary for nucleotide binding.
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. Possible mechanisms of uncoupling protein proton translocation

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
PPARα agonists appear to have limited effects \textit{in vitro}, increasing liver UCP2 mRNA expression only (Kelly \textit{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 \textit{et al.} 1997; Yoshitomi \textit{et al.} 1998). As to be expected, tri-iodothyronine (T$_3$) increased UCP2 mRNA expression in WAT, skeletal and cardiac muscle in rodents (Lanni \textit{et al.} 1997; Masaki \textit{et al.} 1997; Lanni \textit{et al.} 1999) and in WAT and skeletal muscle in humans (Barbe \textit{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 \textit{et al.} 1998). Denervation increased UCP2 mRNA expression in mixed muscle (gastrocnemius) in both the rat and mouse (Cortright \textit{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 \textit{et al.} 1999). The differences may have been produced by differences in concomitant feeding in the experimental protocols.

\textbf{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 Δψₘ 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 O₂•⁻ (Echtay et al. 2002; Echtay et al.
The $\text{O}_2^\ast$ 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 $\beta$-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% O₂) 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 Δψₘ and decreased cell death, calcium overload and loss of Δψₘ 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).
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

<table>
<thead>
<tr>
<th>Author (Year)</th>
<th><em>UCP2</em> variant</th>
<th>Study group (n)</th>
<th>Phenotype</th>
<th>Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klannermark (1998)</td>
<td>Ala55Val</td>
<td>Caucasian (55 with metabolic syndrome, 51 controls)</td>
<td>BMI, resting energy expenditure</td>
<td>No association</td>
</tr>
<tr>
<td>Urhammer (1998)</td>
<td>Ala55Val</td>
<td>Caucasian (144 juvenile onset obesity, up to 369 healthy controls)</td>
<td>Obesity, insulin resistance</td>
<td>No association</td>
</tr>
<tr>
<td></td>
<td>3’ UTR 45bp D/I</td>
<td>Obese (44 – 966)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rare:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5’UTR Exon 1 C^{19}&gt;T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5’UTR Exon 1 C^{27}&gt;G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5’UTR Exon 2 C^{97}&gt;T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 4 Gly85Ser</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ala232Thr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walder (1998)</td>
<td>Ala55Val</td>
<td>Pima Indians (82)</td>
<td>Sleeping and daily metabolic rate (SMR, DMR)</td>
<td>Ala/Val higher SMR (P=0.007)</td>
</tr>
<tr>
<td></td>
<td>3’ UTR 45bp D/I</td>
<td></td>
<td></td>
<td>D/I higher SMR (P=0.02), DMR (P=0.04)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cassell (1999)</td>
<td>3’ UTR 45bp D/I</td>
<td>South Indian (708)</td>
<td>Obesity, type 2 diabetes, leptin</td>
<td>II raised BMI in South Indian Women (P=0.018)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UK Caucasian (247)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dalgaard (1999)</td>
<td>3’ UTR 45bp D/I</td>
<td>Danish Caucasian men (791 obese; 915 lean)</td>
<td>Body mass index</td>
<td>No association</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Variant</td>
<td>Population</td>
<td>Phenotype</td>
<td>Results</td>
</tr>
<tr>
<td>---------------------</td>
<td>---------</td>
<td>-----------------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Nordfors (2000)</td>
<td>3’ UTR 45bp D/I</td>
<td>Swedish Caucasian (41)</td>
<td>Body fat accumulation during peritoneal dialysis</td>
<td>DD increase in body fat and body weight ($P&lt;0.05$)</td>
</tr>
<tr>
<td>Evans (2000)</td>
<td>3’ UTR 45bp D/I</td>
<td>German Caucasians (579)</td>
<td>Morbid obesity, BMI</td>
<td>I allele increased in obesity and higher BMI ($P=0.002, 0.005$)</td>
</tr>
<tr>
<td>Yanovski (2000)</td>
<td>3’ UTR 45bp D/I</td>
<td>Children age 6-10 years (105) African American, Caucasian, Asian</td>
<td>BMI, body fat, resting energy expenditure</td>
<td>DD lower BMI, lower body fat.</td>
</tr>
<tr>
<td>Esterbauer (2001)</td>
<td>-866G&gt;A</td>
<td>Danish Caucasian (340 obese, 256 lean)</td>
<td>Obesity / BMI</td>
<td>AA reduced risk of obesity (RR 0.61 $P = 0.007$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>German Caucasian (791)</td>
<td></td>
<td>GG increases population risk of obesity by ~ 15%</td>
</tr>
<tr>
<td>Buemann (2001)</td>
<td>Ala55Val</td>
<td>Caucasian (8 Ala/Ala, 8 Val/Val)</td>
<td>Incremental exercise efficiency (energy expenditure at increasing workload)</td>
<td>Efficiency higher in Val/Val ($P&lt;0.05$)</td>
</tr>
</tbody>
</table>
Table 1.3. Published studies of common variants in the human UCP3 gene and association with various phenotypes

<table>
<thead>
<tr>
<th>Author (Year)</th>
<th>UCP3 variant</th>
<th>Study group (n)</th>
<th>Phenotype</th>
<th>Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meirhaeghe (2000)</td>
<td>-55C&gt;T</td>
<td>French Caucasian (116 obese, up to 718 controls, 171 type 2 diabetes)</td>
<td>Type 2 diabetes, obesity, lipids</td>
<td>CC increased risk of diabetes ($P&lt;0.05$), TT higher LDL ($P=0.001$)</td>
</tr>
<tr>
<td>Urhammer (1998)</td>
<td>Tyr99Tyr</td>
<td>Caucasian (156 juvenile onset obesity, up to 380 healthy controls)</td>
<td>Obesity, insulin resistance</td>
<td>No association</td>
</tr>
<tr>
<td>Otabe (2000)</td>
<td>-55C&gt;T</td>
<td>Caucasians: Controls (231) Obese (401)</td>
<td>Obesity</td>
<td>-55TT higher BMI in lean ($P=0.03$) and obese ($P=0.003$) No other associations</td>
</tr>
<tr>
<td>Walder (1998)</td>
<td>Tyr99Tyr</td>
<td>Pima Indians (82)</td>
<td>Sleeping and daily metabolic rate (SMR, DMR)</td>
<td>No associations</td>
</tr>
</tbody>
</table>
Table 1.3 (contd) Published studies of common variants in the human *UCP3* gene and association with various phenotypes.

<table>
<thead>
<tr>
<th>Study</th>
<th>Variants Described</th>
<th>Populations Studied</th>
<th>Phenotypes Assessed</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argyropoulos 1998</td>
<td>Val102Ile</td>
<td>Several ethnic populations</td>
<td>Obesity, type 2 diabetes</td>
<td>Rare mutations associated with morbid obesity and diabetes</td>
</tr>
<tr>
<td></td>
<td>Rare: R143X</td>
<td>Mende tribe of Sierra Leone</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exon 6 splice-donor mutation</td>
<td>African-Americans</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Caucasians</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tyr99Tyr</td>
<td>Caucasian (75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Val102Ile</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tyr210Tyr</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
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 AT1R 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:

\[
\frac{\Delta \text{ work performed per min}}{\Delta \text{ energy expended per min}} \times 100 \%
\]

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), UCP2-866G>A and UCP3-55C>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-Bjørgaard and colleagues (Pedersen-Bjørgaard 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 >12mmol.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 MgCl₂**: 20.33 MgCl₂ dissolved in 100ml dH₂O;
- **1M Tris pH 7.5**: 12.11g Tris made up to 100ml in dH₂O, 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 dH₂O and stored at 4°C;
- **0.5M Na₂EDTA**: 37.22g EDTA, up to 200ml in dH₂O, adjust with NaOH to pH 8.0;
- **10% SDS**: 10g Sodium dodecyl sulphate, made up to 100ml dH₂O;
- **Nuclear lysis mix**: 1ml Tris-HCl pH 8.2, 2.34g NaCl, 0.4ml 0.5M Na₂EDTA pH 8.0, 10ml 10% SDS, made up to 90ml in dH₂O;
- **5M sodium perchlorate**: 70.24g sodium perchlorate, up to 100ml in dH₂O;
- **TE buffer pH 7.6**: 1.21g Tris, 0.37g EDTA, made up to 100ml dH₂O.
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 1300g (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 1300g 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 1300g 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 -20°C overnight. The mixture was subsequently centrifuged at 1300g 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 -20°C.

2.2.3. **DNA extraction protocol from saline mouthwash (buccal cells)**

Lysis Buffer consisted of 1000ml dH\textsubscript{2}O 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 eppendorf.

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 eppendorf.

6. 1000μl of 100% ethanol was added and the eppendorf 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 37°C for 24 hours and stored at 4°C (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 95°C 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 95°C). 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.

\[2.3.2. \textbf{PCR experimental protocol}\]

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 200g for 1 minute to ensure the DNA was at the bottom of the wells to prevent cross-contamination. A Finnipipette 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 200g 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 80°C for 10 minutes.

Table 2.1 PCR mix for 100 reactions

<table>
<thead>
<tr>
<th>Reagent (μl)</th>
<th>ACE I/D</th>
<th>BDKRB2 (+9/-9)</th>
<th>UCP2-866G&gt;A</th>
<th>UCP3-55C&gt;T</th>
<th>UCP2 D/I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polmix</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Magnesium 50mmol.l⁻¹</td>
<td>60</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Forward primer</td>
<td>2.75</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1.5</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Third primer</td>
<td>7.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dH₂O</td>
<td>1620</td>
<td>1617</td>
<td>1617</td>
<td>1617</td>
<td>1617</td>
</tr>
<tr>
<td>Taq polymerase 5u/μl</td>
<td>4</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>BSA</td>
<td>-</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>W1 (1%)</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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 71.2° 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-methacryloylopropyltrimethoxysilane (‘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 dH2O 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 ethylenediaminetetraacetic 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. Example of a MADGE gel.** 96 genomic DNA samples (including 4 blanks) have been amplified by PCR for the UCP2D/I variant. The gel was then loaded with each individual PCR product. The wells are arranged in a 12x9 grid with lanes arranged at 71.2° 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:

\[
\begin{align*}
95\degree C & \quad 5 \text{ min} \\
95\degree C & \quad 45 \text{ sec} \\
54\degree C & \quad 45 \text{ sec} \\
72\degree C & \quad 30 \text{ sec} \\
72\degree C & \quad 5 \text{ min} \\
\end{align*}
\]
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. **UCP3-55C>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

\[
\begin{align*}
95^\circ C & \text{ for 40s} \\
55^\circ C & \text{ for 30s} \\
72^\circ C & \text{ for 1m}
\end{align*}
\]

30 cycles

72º for 5 mins

The 194bp product was digested overnight at 37ºC with the restriction enzyme *BsuR1* 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 dH₂O.
2.3.4.4. UCP2-866G>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

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

\[
5'\text{-A CGCGT-3'}
\]

\[
3'\text{-TGCGC A-5'}
\]

On digestion, the 360bp PCR product would therefore yield 290+70bp fragments in G allele carriers only. For 100 digestion reactions, 20µl *MluI* was mixed with 150µl buffer R⁺ and this diluted with 320µl dH₂O. 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:
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 CO₂ incubators (Wolf Laboratories, UK), humidified and set to 5% CO₂, 37°C unless otherwise stated.
Table 2.2. Forward and reverse primers for genotyping common gene variants.

<table>
<thead>
<tr>
<th>Gene variant</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Restriction enzyme or 3(^{rd}) primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BDKRB2 (+9,-9)</strong></td>
<td>5'-TCTGCTTTCTGGCTCCGAG-3'</td>
<td>5'-AGCGGCATGGGCACTTCAGT-3'</td>
<td>-</td>
</tr>
<tr>
<td><strong>UCP3-55C&gt;T</strong></td>
<td>5'-GGATAAGGTTCAGTCAGGC-3'</td>
<td>5'-AAGGGATGAGGGAGGAGAAAA-3'</td>
<td>BsuR1</td>
</tr>
<tr>
<td><strong>UCP2-866G&gt;A</strong></td>
<td>5'-CACGCTTCTCTGCAAGGAC-3'</td>
<td>5'-AGGCCGTACGGAGATGGACCG-3'</td>
<td>MluI</td>
</tr>
<tr>
<td><strong>UCP2 Del/Ins</strong></td>
<td>5'-CAGTGAGGAAGTGAGGAGG-3'</td>
<td>5'-GGGGCAGGAAGATTTCC-3'</td>
<td>-</td>
</tr>
<tr>
<td><strong>ACE I/D</strong></td>
<td>5'-CATCCTTTCTCCATTTCTC-3'</td>
<td>5'-TCGGATTACAGCCCTGATACAG-3'</td>
<td>5'-ATTCAGAGCTGGAAATAAAATT-3'</td>
</tr>
</tbody>
</table>
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 equilibrate 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 $10^6$ cells.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$_2$C$_{12}$ 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$_2$C$_{12}$ 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$_2$C$_{12}$ 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 C₂C₁₂ 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)**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>mM</th>
<th>g.L⁻¹</th>
<th>MWt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile distilled water 1000ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium chloride (BDH)</td>
<td>116.3</td>
<td>6.8</td>
<td>58.44</td>
</tr>
<tr>
<td>Potassium chloride (BDH)</td>
<td>5.4</td>
<td>0.4</td>
<td>74.55</td>
</tr>
<tr>
<td>Magnesium sulphate (BDH)</td>
<td>0.4</td>
<td>0.1</td>
<td>246.48</td>
</tr>
<tr>
<td>HEPES (BDH)</td>
<td>20.0</td>
<td>4.76</td>
<td>238.3</td>
</tr>
<tr>
<td>Sodium dihydrogen orthophosphate (BDH)</td>
<td>0.9</td>
<td>0.12</td>
<td>137.99</td>
</tr>
<tr>
<td>D-Glucose (BDH)*</td>
<td>5.6</td>
<td>1</td>
<td>180.16</td>
</tr>
</tbody>
</table>

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) CaCl₂

Buffer 4 (200ml)

Buffer 1 +  50µM (100µl of 100mM) CaCl₂

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 CaCl₂ 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\textsubscript{2} and 5% CO\textsubscript{2}. The culture medium was endothelial growth medium (EGM™ BulletKits\textsuperscript{®} 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\textsuperscript{-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\textsubscript{2} and 5%CO\textsubscript{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 (ΔΨ\textsubscript{m}) and ROS generation. The fluorescent probes TMRM and JC-1 were obtained from Molecular Probes (Eugene, Oregon).
2.5.1. Flow cytometric measurement of Δψₘ

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 dichroic 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 dichroic 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 Δψ_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% CO₂). 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 Δψₘ in live cells using JC-1 fluorescent dye

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Detector</th>
<th>Voltage</th>
<th>Amp Gain</th>
<th>Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>FSC</td>
<td>E-1</td>
<td>4.87</td>
<td>Lin</td>
</tr>
<tr>
<td>P2</td>
<td>SSC</td>
<td>280</td>
<td>5.41</td>
<td>Lin</td>
</tr>
<tr>
<td>P3</td>
<td>FL-1</td>
<td>420</td>
<td>1</td>
<td>Log</td>
</tr>
<tr>
<td>P4</td>
<td>FL-2</td>
<td>385</td>
<td>1</td>
<td>Log</td>
</tr>
<tr>
<td>P5</td>
<td>FL-3</td>
<td>150</td>
<td>1</td>
<td>Log</td>
</tr>
</tbody>
</table>

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 Δψₘ

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 dichroic 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 -20°C. 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 dichroic 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 dichroic mirror (reflects light below 560nm) through a green light filter (515±30nm) to PMT1. Red-orange fluorescence is reflected at the second dichroic 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 Eppendorf's 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 eppendorf. 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 spectrophotometer. 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 20mer 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 $\geq 3.0$ mmol.L$^{-1}$ for use with the LightCycler®.

Table 2.4. Source of DNA sequences for primer design

<table>
<thead>
<tr>
<th>cDNA sequence</th>
<th>Accession number</th>
<th>cDNA sequence</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human GAPDH</td>
<td>J04038</td>
<td>Mouse UCP3</td>
<td>AF032902</td>
</tr>
<tr>
<td>Human UCP2</td>
<td>NM_003355 / AF019409</td>
<td>Mouse ACE</td>
<td>M55333</td>
</tr>
<tr>
<td>Human UCP3</td>
<td>U84763 / AF050113</td>
<td>Rat GAPDH</td>
<td>AF106860</td>
</tr>
<tr>
<td>Human ACE</td>
<td>J04144</td>
<td>Rat UCP2</td>
<td>AB017043</td>
</tr>
<tr>
<td>Mouse GAPDH</td>
<td>NM_008084</td>
<td>Rat UCP3</td>
<td>U92069</td>
</tr>
<tr>
<td>Mouse UCP2</td>
<td>AB012159</td>
<td>Rat ACE</td>
<td>U03734</td>
</tr>
</tbody>
</table>

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, $\geq 3.0$mM 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 $\chi^2$ 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.
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**

<table>
<thead>
<tr>
<th>Trait</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>19.6 (2.4)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>117.6 (11.7)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>66.1 (10.5)</td>
</tr>
<tr>
<td>Body mass index (kg.m$^{-2}$)</td>
<td>23.1 (2.2)</td>
</tr>
<tr>
<td>Lean mass (kg)</td>
<td>58.8 (6.0)</td>
</tr>
<tr>
<td>Adipose tissue (kg)</td>
<td>12.2 (3.9)</td>
</tr>
<tr>
<td>LV mass (g)</td>
<td>183.9 (25.1)</td>
</tr>
<tr>
<td>$BDKRB2$ +9/+9/9-9/-9 (n)</td>
<td>33 / 60 / 16</td>
</tr>
<tr>
<td>-9 allele frequency</td>
<td>0.422 [0.356-0.488]</td>
</tr>
</tbody>
</table>
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 ($\chi^2=1.8; P=0.18$) 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

<table>
<thead>
<tr>
<th>Trait</th>
<th>BDKRB2 +9/-9 Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+9/+9  n = 33</td>
</tr>
<tr>
<td>Age (years)</td>
<td>19.3 (2.1)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>117.0 (11.9)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>63.2 (8.9)</td>
</tr>
<tr>
<td>Body mass index (kg.m⁻²)</td>
<td>22.9 (1.9)</td>
</tr>
<tr>
<td>Lean mass (kg)</td>
<td>58.4 (4.1)</td>
</tr>
<tr>
<td>Adipose tissue mass (kg)</td>
<td>11.7 (4.1)</td>
</tr>
</tbody>
</table>
Table 3.3. Training related changes in cardiac parameters by BDKRB2 +9/-9 genotype and ACE/BDKRB2 haplotypes

<table>
<thead>
<tr>
<th>Cardiac traits</th>
<th>BDKRB2 +9/-9 Genotype</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-9/-9</td>
<td>-9/+9</td>
<td>+9/+9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV mass pre-training (g)</td>
<td>180.0 (19.8)</td>
<td>184.2 (27.3)</td>
<td>181.9 (24.6)</td>
<td>0.82</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>LVMI&lt;sup&gt;LM&lt;/sup&gt; pre-training (x10&lt;sup&gt;-3&lt;/sup&gt;)</td>
<td>3.25 (0.26)</td>
<td>3.13 (0.35)</td>
<td>3.11 (0.39)</td>
<td>0.43</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>LV mass post training (g)</td>
<td>184.6 (22.0)</td>
<td>192.5 (28.2)</td>
<td>195.6 (22.8)</td>
<td>0.38</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>LVMI&lt;sup&gt;LM&lt;/sup&gt; post training (x10&lt;sup&gt;-3&lt;/sup&gt;)</td>
<td>3.26 (0.35)</td>
<td>3.16 (0.36)</td>
<td>3.30 (0.34)</td>
<td>0.24</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>Change in LV mass (g)</td>
<td>4.6 (11.3)</td>
<td>8.3 (13.1)</td>
<td>13.7 (13.9)</td>
<td>0.06</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Change in LV mass (%)</td>
<td>2.7 (6.4)</td>
<td>4.8 (7.2)</td>
<td>8.1 (8.5)</td>
<td>0.04</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Change in LVMI&lt;sup&gt;LM&lt;/sup&gt; (%)</td>
<td>0.1 (5.3)</td>
<td>2.3 (7.7)</td>
<td>5.8 (9.1)</td>
<td>0.06</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Change in LV stroke volume (%)</td>
<td>2.36 (27.0)</td>
<td>10.8 (23.5)</td>
<td>13.5 (19.1)</td>
<td>0.28</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>Change in RV stroke volume (%)</td>
<td>3.1 (23.1)</td>
<td>8.8 (21.2)</td>
<td>16.5 (18.0)</td>
<td>0.08</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

| BDKRB2 / ACE haplotypes *              |     |     |     |     |     |     |     |
|----------------------------------------|-----|-----|-----|-----|-----|-----|
|                                        | -9-9 II | -9-9 DD | +9+9 II | +9+9 DD | P ANOVA | P Linear |
| 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<sup>LM</sup> pre-training (x10<sup>-3</sup>) | 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<sup>LM</sup> post training (x10<sup>-3</sup>) | 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<sup>LM</sup> 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 \textit{BDKRB2} +9/-9 genotype in the BH2 study.

![Figure 3.1](image1)

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

![Figure 3.2](image2)
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 ($\chi^2=0.003; P=0.96$; 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 \( \text{BDKRB2} \) 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 \( \text{BDKRB2}(+9,+9) \) 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 \( \text{BDKRB2}(-9,-9) \) 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 \( \text{BDKRB2} \) 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.**

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

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.

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

* 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. Association between systolic blood pressure at recruitment in NPHS II and CHD amongst

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, β₁ (and, to a lesser extent β₂) 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-Arg⁹-bradykinin and Lys-des-Arg⁹-bradykinin. Intact kinins and their fragments act upon the β₁ and β₂ 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 \textit{BDKRB2}(-9) allele is associated with symptomatic hereditary angioedema in cases of C1 inhibitor deficiency (Lung \textit{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 \textit{higher} \textit{BDKRB2} expression/activity seemingly associated with \textit{reduced} cardiovascular risk in the hypertensive state. KKS protection may be mediated through a number of potential mechanisms including \textit{BDKRB2}-mediated inhibition of vascular smooth muscle cell growth (Murakami \textit{et al.} 1999), coronary vasodilatation (Su \textit{et al.} 2000), and local nitric oxide synthesis (Kichuk \textit{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 \textit{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 \textit{in vivo} (Swartz \textit{et al.} 1980), but also mediates cross-talk between membrane-bound ACE and the \textit{BDKRB2}, leading to a reduction in \textit{BDKRB2} desensitisation, reduced receptor endocytosis and an increase in bradykinin receptor affinity (Minshall \textit{et al.} 1997). Moreover, selective AT$_1$R 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.
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 ±3.1kg.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.

![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.

<table>
<thead>
<tr>
<th>BDKRB2 Genotype</th>
<th>Delta efficiency% (sd; n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACE DD, n=45</td>
</tr>
<tr>
<td>+9+9</td>
<td>23.45 (2.81; 8)</td>
</tr>
<tr>
<td>+9-9</td>
<td>24.06 (3.15; 12)</td>
</tr>
<tr>
<td>-9-9</td>
<td>25.30 (1.65; 15)</td>
</tr>
<tr>
<td>+9 allele</td>
<td>23.90 (3.03; 20)</td>
</tr>
</tbody>
</table>

ANOVA

<table>
<thead>
<tr>
<th></th>
<th>0.233</th>
<th>0.005</th>
<th>0.003</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear trend</td>
<td>0.097</td>
<td>0.013</td>
<td>0.002</td>
</tr>
<tr>
<td>+9 allele vs. -9-9</td>
<td>0.104</td>
<td>0.0008</td>
<td>0.001</td>
</tr>
</tbody>
</table>
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 BDKRB2 -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).
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 B₂ 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.
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 C2C12 undifferentiated myoblasts and preadipocytes because of their ease of culture. Cytometer PMT settings (voltage, gain) were adjusted until C2C12 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 C2C12 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).
5.1.2. TMRM mitochondrial probe

5.1.2.1. Dose titration

C₂C₁₂ 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 C2C12 blasts.** Size (FSC) and granularity (SSC) are on linear x- and y-axes, respectively, and TMRM red fluorescence on the logarithmic z-axis.

![3D density plot of TMRM staining characteristics of C2C12 blasts.](image)

Figure 5.4. **TMRM fluorescence of C2C12 blasts expressed on a histogram plot, with number of events (cells; y-axis) against TMRM fluorescence (FL-2; x-axis; logarithmic scale, arbitrary units).**

![Histogram of TMRM fluorescence of C2C12 blasts.](image)
C₂C₁₂ 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 C₂C₁₂ 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 mCICCP on cellular TMRM fluorescence

C₂C₁₂ blasts were incubated with increasing doses of TMRM, followed by trypsinisation and resuspension in Kreb’s Buffer containing the mitochondrial respiratory uncoupler mCICCP 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 C₂C₁₂ cells. Control (coupled) cells are shown in the red histogram. Treatment with the mitochondrial respiratory uncoupler mCICCP (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) C2C12 myoblasts. (N=3 at each point)

Figure 5.8. Relative change in TMRM fluorescence in C2C12 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 C2C12 myotubes incubated with increasing concentrations of JC-1 (37°C, 30 minutes), with the comparative plots below of C2C12 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 C2C12 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 C2C12 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 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). 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 C2C12 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 C2C12 myotubes incubated with vehicle, mClCCP or nigericin (10000 cells, $N=3$ repeats), * $P < 10^{-7}$; † $P = 0.03$

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>mClCCP</th>
<th>Nigericin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean JC-1 A:M fluorescence ratio (indexed to 100)</td>
<td>100</td>
<td>72 ± 6</td>
<td>117 ± 11</td>
</tr>
</tbody>
</table>

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 C2C12 skeletal myotubes using a low dose (0.1 μM) of TMRM for 30 minutes. Treatment of C2C12 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$_2$C$_{12}$ cells were also incubated with 5 $\mu$M 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$_2$C$_{12}$ myotubes which have been untreated or ramiprilat ($10^{-5}$M) treated for 24 hours.
5.1.4.2. JC-1

Membrane potential assessed by JC-1 in C2C12 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 C2C12 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.

C₂C₁₂ myoblasts were seeded at low density (10³ 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 C2C12 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 mCICCP 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.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Laser</th>
<th>Power</th>
<th>Iris</th>
<th>Gain</th>
<th>Detection filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMRM</td>
<td>Argon</td>
<td>5%</td>
<td>1</td>
<td>29%</td>
<td>515/30</td>
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<tr>
<td>DCF</td>
<td>HeNe</td>
<td>30%</td>
<td>1</td>
<td>50%</td>
<td>590/70</td>
</tr>
<tr>
<td>JC-1</td>
<td>Argon</td>
<td>15%</td>
<td>1</td>
<td>13%</td>
<td>515/30</td>
</tr>
<tr>
<td></td>
<td>HeNe</td>
<td>13%</td>
<td>1</td>
<td>50%</td>
<td>590/70</td>
</tr>
</tbody>
</table>

( using 560 dichroic mirror)
Figure 5.20. Confocal analysis of mitochondrial TMRM fluorescence in C2C12 myotubes (x20 magnification).

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 fluorescence 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 $1 \times 10^4$ 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 C2C12 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 Δψ_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 Δψ_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

![Vehicle 24 hours](image)

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

![Ramiprilat 24 hours](image)

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

```
<table>
<thead>
<tr>
<th></th>
<th>Relative Fluorescence (arb units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>200</td>
</tr>
<tr>
<td>Ramiprilat 10^{-5}M</td>
<td>1200</td>
</tr>
</tbody>
</table>
```

p=0.000001
Figure 5.23. Fields of C2C12 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 C2C12 cells treated with either vehicle or ramiprilat for 24 hours (n=3)

p=0.01
Figure 5.24. H9C2 cardiac blasts stained with TMRM and analysed by confocal microscopy

i. vehicle treated

ii. ramiprilat $10^{-5}$M

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

\[ p = 0.008 \]
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 (Duchén 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 (10⁻⁵M) 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 10⁻⁵M and vehicle treated cells (Figure 5.28).
Figure 5.25. CLSM analysis of Δψ_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

Vehicle

Ramiprilat 10^{-5}M

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

* P < 0.00001
Figures 5.28-28. TMRM fluorescence in human adult ventricular cardiomyocytes

Figure 5.26. Confocal images of myocytes stained with TMRM

A. Vehicle

![Vehicle](image1)

B. mClCCP (5μM)

![mClCCP](image2)

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

![Time Series](image3)

$P = 0.8$

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

![Fluorescence](image4)

Vehicle

Ramiprilat 10-5M
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 dichroic 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.](image-url)
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$_2$C$_{12}$ 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$_2$C$_{12}$ 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$_2$C$_{12}$ 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. **C₂C₁₂ 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 C₂C₁₂ myocytes treated with vehicle or Ramiprilat (10⁻⁵M) for 24 hours (PO₂ indexed to 100)**

5.3.3. **C₂C₁₂ treatment with Angiotensin II**

Pre-treatment of C₂C₁₂ myocytes with Ang II (10⁻⁷ M) for 1 hour resulted in a significant increase in basal oxygen consumption in C₂C₁₂ myotubes (-7 ±0.6 mTorr.s⁻¹ vs. -23 ±8.2 mTorr.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 (\( \text{PO}_2 \) indexed to 100).

![Graph depicting oxygen consumption](image)

5.4 **UCP3 mRNA EXPRESSION IN \( C_2C_{12} \) MYOCYTES.**

Primers were designed as previously described (Table 5.2) with a melting temperature \( (T_m) \) of 60°C. 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 C2C12 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**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Amplicon size (bp)</th>
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</thead>
<tbody>
<tr>
<td>mGAPDH</td>
<td>TGCATCCTGCAGCAACCACTG</td>
<td>CACAGCTTTCCAGGGGCCA</td>
<td>141</td>
</tr>
<tr>
<td>mUCP3</td>
<td>CCCGATACATGAAAGCCT</td>
<td>AGATCCCGCAGTACC</td>
<td>203</td>
</tr>
<tr>
<td>mUCP2</td>
<td>GGTCCCGCTCCAGGCTCAGG</td>
<td>GCATTACGGGCAACATTG</td>
<td>138</td>
</tr>
</tbody>
</table>

Incubation of C2C12 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, C2C12 cells were incubated within a hypoxic (37°C, 5%CO₂, 1% O₂, 94% N₂) environment for 6 hours, with or without pre-treatment with ramiprilat 10⁻⁵M 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 C2C12 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 C2C12 myotubes analysed by quantitative RT-PCR (mean ± SD of 3-4 experiments)

* $P < 0.05$ vs. normoxia
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 dichroic 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 C2C12 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 C2C12 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 C2C12 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 Δψ_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 Δψ_m (Beltran et al. 2000; Moncada et al. 2002). In keeping with this, treatment of C2C12 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 C2C12 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.
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 ($\chi^2=0.67; P=0.41$) 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 UCP2AA 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 ($204 \pm 29$ g vs. $190 \pm 25$ g vs. $192 \pm 23$ 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\textsubscript{LM}) was significantly higher in TT homozygotes after training.

There was no evidence of LD between UCP2-866G>A and UCP3-55C>T genotypes (Δ = 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

<table>
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<th>Trait</th>
<th>UCP2 -866G&gt;A Genotype</th>
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<th>UCP3 -55C&gt;T Genotype</th>
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<td></td>
<td>GG n = 49</td>
<td>GA n = 69</td>
<td>AA n = 18</td>
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<td>Age (years)</td>
<td>19.0 (1.7)</td>
<td>19.9 (2.7)</td>
<td>19.9 (3.1)</td>
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<td>Systolic blood pressure (mmHg)</td>
<td>118 (12)</td>
<td>116 (10)</td>
<td>125 (14)*</td>
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<td>Diastolic blood pressure (mmHg)</td>
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<td>65 (9)</td>
<td>67 (11)</td>
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<td>Body mass index (kg.m(^{-2}))</td>
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<td>Lean mass (kg)</td>
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<td>58.5 (6.2)</td>
<td>59.0 (6.0)</td>
</tr>
<tr>
<td>Adipose tissue mass (kg)</td>
<td>11.1 (2.9)</td>
<td>12.5 (4.2)</td>
<td>14.0 (4.1)†</td>
</tr>
</tbody>
</table>

*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

<table>
<thead>
<tr>
<th>Cardiac traits</th>
<th>UCP2-866G&gt;A Genotype</th>
<th>UCP3-55C&gt;T Genotype</th>
<th>UCP3-55C&lt;T haplotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GG</td>
<td>GA</td>
<td>AA</td>
</tr>
<tr>
<td>LV mass pre-training (g)</td>
<td>184.2 (22.7)</td>
<td>182.7 (27.0)</td>
<td>191.2 (25.4)</td>
</tr>
<tr>
<td>LVMI&lt;sup&gt;LM&lt;/sup&gt; pre-training (x10&lt;sup&gt;-3&lt;/sup&gt;)</td>
<td>3.11 (0.31)</td>
<td>3.17 (0.36)</td>
<td>3.29 (0.39)</td>
</tr>
<tr>
<td>LV mass post training (g)</td>
<td>192.3 (22.7)</td>
<td>190.4 (24.8)</td>
<td>203.6 (28.6)</td>
</tr>
<tr>
<td>LVMI&lt;sup&gt;LM&lt;/sup&gt; post training (x10&lt;sup&gt;-3&lt;/sup&gt;)</td>
<td>3.17 (0.32)</td>
<td>3.21 (0.34)</td>
<td>3.37 (0.39)</td>
</tr>
<tr>
<td>Change in LV mass (%)</td>
<td>4.8 (8.8)</td>
<td>4.9 (7.9)</td>
<td>6.6 (6.7)</td>
</tr>
<tr>
<td>Change in LV stroke volume (%)</td>
<td>10.4 (31.8)</td>
<td>6.8 (26.7)</td>
<td>18.0 (31.3)</td>
</tr>
<tr>
<td>Change in RV stroke volume (%)</td>
<td>8.4 (19.6)</td>
<td>8.0 (22.7)</td>
<td>22.2 (25.9)</td>
</tr>
</tbody>
</table>

- LVMI<sup>LM</sup> = Left ventricle mass indexed to body surface area.

250
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 ($\chi^2$=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.

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

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

<table>
<thead>
<tr>
<th>Trait</th>
<th>GG (n = 1088)</th>
<th>GA (n = 1245)</th>
<th>AA (n = 362)</th>
<th>Probability 3 way</th>
<th>Probability G+ vs. AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>56.1 (3.4)</td>
<td>56.1 (3.4)</td>
<td>56.1 (3.5)</td>
<td>0.99</td>
<td>0.93</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>138.5 (19.2)</td>
<td>138.1 (18.9)</td>
<td>139.7 (20.3)</td>
<td>0.38</td>
<td>0.19</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>84.2 (11.2)</td>
<td>84.4 (11.4)</td>
<td>86.2 (11.1)</td>
<td><strong>0.01</strong></td>
<td><strong>0.003</strong></td>
</tr>
<tr>
<td>Body mass index (kg.m²⁻¹)</td>
<td>26.5 (3.5)</td>
<td>26.4 (3.5)</td>
<td>26.7 (3.4)</td>
<td>0.4</td>
<td>0.22</td>
</tr>
<tr>
<td>Current smoking % (n)</td>
<td>28.0% (305)</td>
<td>28.9% (360)</td>
<td>27.1% (98)</td>
<td>0.76</td>
<td>0.57</td>
</tr>
<tr>
<td>Diabetes n (%)</td>
<td>13 (0.012)</td>
<td>25 (0.020)</td>
<td>26 (0.024)</td>
<td>0.22</td>
<td>0.10</td>
</tr>
<tr>
<td>Cholesterol (mmol.l⁻¹)</td>
<td>5.71 (1.02)</td>
<td>5.72 (1.00)</td>
<td>5.78 (1.01)</td>
<td>0.59</td>
<td>0.34</td>
</tr>
<tr>
<td>Triglyceride (mmol.l⁻¹) *</td>
<td>1.78 (0.94)</td>
<td>1.78 (0.93)</td>
<td>1.88 (1.03)</td>
<td>0.2</td>
<td>0.07</td>
</tr>
<tr>
<td>CRP (mg.l⁻¹); n</td>
<td>1.25 (1.48); n=295</td>
<td>1.25 (1.37); n=321</td>
<td>1.53 (1.76); n=105</td>
<td>0.23</td>
<td>0.09</td>
</tr>
<tr>
<td>Fibrinogen (g.l⁻¹)</td>
<td>2.71 (0.52)</td>
<td>2.70 (0.51)</td>
<td>2.71 (0.51)</td>
<td>0.79</td>
<td>0.64</td>
</tr>
</tbody>
</table>

* geometric mean (approximate SD).
Table 6.5. CHD risk in NPHS II in subjects stratified by *UCP2*-866G>A genotype.

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

* 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.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>UCP2 AG+GG</th>
<th>UCP2 AA</th>
<th>Relative Risk* [95% CI]</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Events / Total No.</td>
<td>Events / Total No.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smoker</td>
<td>90 / 1668 (5.4%)</td>
<td>36 / 264 (13.6%)</td>
<td>2.71 [1.83 – 4.01]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoker</td>
<td>66 / 665 (9.9%)</td>
<td>12 / 98 (12.2%)</td>
<td>1.23 [0.66 -2.28]</td>
<td>0.51</td>
</tr>
<tr>
<td>BMI&lt;30kg.m²</td>
<td>133 / 2019 (6.6%)</td>
<td>32 / 297 (10.8%)</td>
<td>1.77 [1.20 -2.61]</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>23 / 311 (7.4%)</td>
<td>16 / 64 (25.0%)</td>
<td>3.17 [1.65 – 6.10]</td>
<td>0.001</td>
</tr>
<tr>
<td>SBP&lt;160mmHg</td>
<td>123 / 2021 (6.1%)</td>
<td>35 / 300 (11.7%)</td>
<td>1.94 [1.33 -2.84]</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>33 / 310 (10.7%)</td>
<td>13 / 62 (21.0%)</td>
<td>2.41 [1.26 – 4.60]</td>
<td>0.008</td>
</tr>
<tr>
<td>DBP&lt;95mmHg</td>
<td>110 / 1934 (5.7%)</td>
<td>35 / 283 (12.4%)</td>
<td>2.23 [1.52 -3.28]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>46 / 396 (11.6%)</td>
<td>13 / 79 (16.5%)</td>
<td>1.57 [0.85 – 2.92]</td>
<td>0.15</td>
</tr>
</tbody>
</table>

* Adjusted for age & practice; SBP Systolic blood pressure, DBP Diastolic blood pressure, BMI Body mass index
IS THERE AN ASSOCIATION BETWEEN GENETIC VARIATION IN THE BRADYKININ B2 RECEPTOR AND PROSPECTIVE CARDIOVASCULAR PHENOTYPES?

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

A. AA BMI>30 (15 / 64) 3.66
   G+ BMI>30 (23 / 311) 1.15
   AA BMI<30 (32 / 297) 1.77
   G+ BMI<30 (133 / 2019)

RERI 2.88 (0.41-8.41)

Relative Risk

B. AA SBP>160 (13 / 62) 4.20
   G+ SBP>160 (33 / 310) 1.74
   AA NT (35 / 300) 1.94
   G+ NT (123 / 2021)

RERI 1.22 (-1.22-4.41)

Relative Risk

C. AA DM (5 / 13) 7.90
   G+ DM (8 / 51) 2.86
   AA non-DM (43 / 349) 1.90
   G+ non-DM (148 / 2262)

RERI 5.30 (-2.26-25.15)

Relative Risk

BMI = Body mass index (kg.m²); 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 ($\chi^2 = 3.946$, $P = 0.05$) 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.

<table>
<thead>
<tr>
<th>Trait</th>
<th>UCP3 -55C&gt;T Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
</tr>
<tr>
<td></td>
<td>n = 1088</td>
</tr>
<tr>
<td>Age (years)</td>
<td>56.0 (3.5)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>137.1 (18.8)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>84.4 (10.9)</td>
</tr>
<tr>
<td>Body mass index (kg.m^-2)</td>
<td>26.3 (3.4)</td>
</tr>
<tr>
<td>Current smoking % (n)</td>
<td>29.0% (473)</td>
</tr>
<tr>
<td>Diabetes n (%)</td>
<td>2.5% (40)</td>
</tr>
<tr>
<td>Cholesterol (mmol.l^-1)</td>
<td>5.70 (1.01)</td>
</tr>
<tr>
<td>Triglyceride (mmol.l^-1) *</td>
<td>1.79 (0.95)</td>
</tr>
<tr>
<td>CRP (mg.l^-1)*; n</td>
<td>1.25 (1.48); n=295</td>
</tr>
<tr>
<td>Fibrinogen (g.l^-1)</td>
<td>2.73 (0.53)</td>
</tr>
</tbody>
</table>

Data are mean (SD) unless otherwise stated.  * Geometric means (approximate SD)
Table 6.8. CHD risk in NPHS II by *UCP3*-55C>T genotype

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

* 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 \( (\chi^2 \text{ test } P < 0.00005) \). 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**

<table>
<thead>
<tr>
<th>NPHS2</th>
<th><em>UCP2</em>-866G&gt;A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GG</td>
</tr>
<tr>
<td>CC</td>
<td>759</td>
</tr>
<tr>
<td><em>UCP3</em>&lt;br&gt;-55C&gt;T</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>273</td>
</tr>
<tr>
<td>TT</td>
<td>34</td>
</tr>
</tbody>
</table>
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.

![Graph showing relative risk for UCP3/UCP2 haplotypes]

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

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency</th>
<th>Controls</th>
<th>Cases</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCP3-55</td>
<td>UCP2-866</td>
<td>All</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>G</td>
<td>0.531</td>
<td>0.535</td>
<td>0.472</td>
</tr>
<tr>
<td>C</td>
<td>A</td>
<td>0.244</td>
<td>0.242</td>
<td>0.271</td>
</tr>
<tr>
<td>T</td>
<td>G</td>
<td>0.103</td>
<td>0.105</td>
<td>0.078</td>
</tr>
<tr>
<td>T</td>
<td>A</td>
<td>0.122</td>
<td>0.117</td>
<td>0.177</td>
</tr>
</tbody>
</table>

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

![Graph showing haplotype frequencies for UCP3/UCP2]

262
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 \( UCP2 \)-866G>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>
<thead>
<tr>
<th>Trait</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>20.7 (4.4)</td>
</tr>
<tr>
<td>Gender (proportion male)</td>
<td>67%</td>
</tr>
<tr>
<td>Mass (kg)</td>
<td>70.4 (9.4)</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.74 (0.08)</td>
</tr>
<tr>
<td>Delta efficiency (%)</td>
<td>24.6 (2.6)</td>
</tr>
<tr>
<td>( UCP3 )-55C&gt;T CC / CT / TT (n)</td>
<td>29 / 27 / 5</td>
</tr>
<tr>
<td>T allele frequency (95% C.I.)</td>
<td>0.303 (0.222 -0.385)</td>
</tr>
<tr>
<td>( UCP2 )-866G&gt;A GG / GA / AA (n)</td>
<td>21 / 22 / 15</td>
</tr>
<tr>
<td>A allele frequency (95% C.I.)</td>
<td>0.448 (0.358-0.539)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>UCP2-866G&gt;A genotype</th>
<th>Pre</th>
<th>Post</th>
<th>Absolute change (%)</th>
<th>Proportional change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG (21)</td>
<td>24.6 ± 2.6</td>
<td>24.4 ± 2.8</td>
<td>-0.2 ± 3.6</td>
<td>0.2 ± 14.6</td>
</tr>
<tr>
<td>GA (22)</td>
<td>24.3 ± 3.0</td>
<td>26.1 ± 3.1</td>
<td>1.7 ± 2.8</td>
<td>7.9 ± 12.6</td>
</tr>
<tr>
<td>AA (15)</td>
<td>24.0 ± 2.2</td>
<td>26.2 ± 3.3</td>
<td>2.3 ± 3.7</td>
<td>10.1 ± 15.1</td>
</tr>
<tr>
<td>A allele</td>
<td>24.2 ± 2.7</td>
<td>26.1 ± 3.1</td>
<td>2.0 ± 3.1</td>
<td>8.8 ± 13.5</td>
</tr>
</tbody>
</table>

*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

<table>
<thead>
<tr>
<th>UCP3-55</th>
<th>Pre</th>
<th>Post</th>
<th>Absolute change (%)</th>
<th>Proportional change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC (29)</td>
<td>24.2 ± 2.8</td>
<td>25.8 ± 2.9</td>
<td>1.5 ± 3.3</td>
<td>7.1 ± 13.4</td>
</tr>
<tr>
<td>CT (27)</td>
<td>24.6 ± 2.7</td>
<td>25.3 ± 3.3</td>
<td>0.6 ± 3.5</td>
<td>3.3 ± 14.7</td>
</tr>
<tr>
<td>TT (5)</td>
<td>26.1 ± 1.1</td>
<td>24.9 ± 1.2</td>
<td>-1.2 ± 1.8</td>
<td>-4.3 ± 6.6</td>
</tr>
</tbody>
</table>

*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

---

**Diagram A.**

- X-axis: Genotypes (CC, CT, TT, GG, GA, AA)
- Y-axis: Absolute change in delta efficiency (%)

**Diagram B.**

- X-axis: *UCP3*-55 genotypes (CC, CT, TT)
- Y-axis: Percentage change in delta efficiency (%)
- Z-axis: *UCP2*-866 genotypes (CC, CT, TT, GA, AA, GG)

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

<table>
<thead>
<tr>
<th>Running Distance</th>
<th>UCP3-55C&gt;T</th>
<th>Total</th>
<th>T allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CT</td>
<td>TT</td>
</tr>
<tr>
<td>≤200m</td>
<td>13</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>400-3000m</td>
<td>19</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>≥5000m</td>
<td>18</td>
<td>14</td>
<td>32</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>30</td>
<td>1</td>
</tr>
</tbody>
</table>

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 ($\chi^2 = 2.29; P = 0.13$) 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 ($\chi^2 = 0.195; P = 0.66$) 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.

<table>
<thead>
<tr>
<th>Running Distance</th>
<th>UCP2-866G&gt;A</th>
<th>Total</th>
<th>A allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GG</td>
<td>GA</td>
<td>AA</td>
</tr>
<tr>
<td>≤200m</td>
<td>12</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>400-3000m</td>
<td>14</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>≥5000m</td>
<td>14</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>35</td>
<td>6</td>
</tr>
</tbody>
</table>

*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.

<table>
<thead>
<tr>
<th>Overall</th>
<th>UCP2-866G&gt;A</th>
<th>Caucasian</th>
<th>UCP2-866G&gt;A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GG</td>
<td>GA</td>
<td>AA</td>
</tr>
<tr>
<td>UCP3</td>
<td>CC</td>
<td>27</td>
<td>20</td>
</tr>
<tr>
<td>-55C&gt;T</td>
<td>CT</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>UCP3</td>
<td>CC</td>
<td>22</td>
<td>17</td>
</tr>
<tr>
<td>-55C&gt;T</td>
<td>CT</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Haplotype frequency</th>
<th>≤ 200 m</th>
<th>≥ 400m</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overall</td>
<td>Cauc</td>
<td>Afro-Car</td>
</tr>
<tr>
<td>UCP3-55</td>
<td>UCP2-866</td>
<td>BH3</td>
<td>Overall</td>
</tr>
<tr>
<td>C</td>
<td>G</td>
<td>0.509</td>
<td>0.613</td>
</tr>
<tr>
<td>C</td>
<td>A</td>
<td>0.258</td>
<td>0.188</td>
</tr>
<tr>
<td>T</td>
<td>G</td>
<td>0.126</td>
<td>0.105</td>
</tr>
<tr>
<td>T</td>
<td>A</td>
<td>0.107</td>
<td>0.094</td>
</tr>
</tbody>
</table>

BH3, Bassingbourn 3 study; Cauc, Caucasian; Afro-Car, Afro-Caribbean
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, HbA1c 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.**

<table>
<thead>
<tr>
<th>Trait</th>
<th>No CHD (n=360)</th>
<th>CHD (n=105)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>59.2 (13.9)</td>
<td>67.5 (11.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)*</td>
<td>130 (128-149)</td>
<td>136.5 (123-151)</td>
<td>0.49</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)*</td>
<td>80 (75-87)</td>
<td>78 (70-84)</td>
<td>0.007</td>
</tr>
<tr>
<td>Body mass index (kg.m^{-2})*</td>
<td>30 (25.2-30.9)</td>
<td>29.1 (24.7-32.2)</td>
<td>0.03</td>
</tr>
<tr>
<td>Current smoking % (n)</td>
<td>11.4 (41)</td>
<td>21.9 (23)</td>
<td>0.02</td>
</tr>
<tr>
<td>Cholesterol (mmol.l^{-1})*</td>
<td>5.0 (1.0)</td>
<td>4.5 (1.0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TC:HDL*</td>
<td>3.7 (3.1-4.7)</td>
<td>3.8 (3.1-4.6)</td>
<td>0.77</td>
</tr>
<tr>
<td>Triglycerides (mmol.l^{-1})*</td>
<td>1.5 (1.0 -2.3)</td>
<td>1.8 (1.2-2.7)</td>
<td>0.03</td>
</tr>
<tr>
<td>CRP (mg.l^{-1})*</td>
<td>1.41 (0.83 – 2.38)</td>
<td>2.02 (1.04-3.30)</td>
<td>0.004</td>
</tr>
<tr>
<td>TAOS (%)</td>
<td>43.3 (13.2)</td>
<td>40.3 (13.7)</td>
<td>0.04</td>
</tr>
<tr>
<td>Type 2 diabetes % (n)</td>
<td>70.6 (254)</td>
<td>95.2 (100)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Duration of diabetes (years)†</td>
<td>11 (5-20)</td>
<td>12 (6-18)</td>
<td>0.67</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.7 (6.7 -7.8)</td>
<td>7.5 (6.5-8.8)</td>
<td>0.62</td>
</tr>
<tr>
<td>Creatinine (mmol.l^{-1})*</td>
<td>93 (84 -109)</td>
<td>105 (88-124)</td>
<td>0.001</td>
</tr>
<tr>
<td>Proteinuria (%)</td>
<td>10%</td>
<td>17%</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>UCP2-866G&gt;A Genotype frequency (GG/GA/AA)</strong></td>
<td>154/165/41</td>
<td>44/51/10</td>
<td>0.820</td>
</tr>
<tr>
<td><strong>UCP2-866A allele frequency (95%CI)</strong></td>
<td>0.34 (0.31-0.38)</td>
<td>0.34 (27-0.40)</td>
<td>0.99</td>
</tr>
</tbody>
</table>

**Drug Therapy**

<table>
<thead>
<tr>
<th>Trait</th>
<th>No CHD (%)</th>
<th>CHD (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (%)</td>
<td>34.6</td>
<td>47.3</td>
<td>0.05</td>
</tr>
<tr>
<td>Sulphonylureas (%)</td>
<td>29.6</td>
<td>40.3</td>
<td>0.04</td>
</tr>
<tr>
<td>Metformin (%)</td>
<td>42.4</td>
<td>63.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Aspirin (%)</td>
<td>39.6</td>
<td>77.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ACEI (%)</td>
<td>43.1</td>
<td>62.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Statins (%)</td>
<td>14.6</td>
<td>62.6</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Trait</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>19.4 (2.2)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>122.7 (11.2)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>71.4 (9.1)</td>
</tr>
<tr>
<td>Body mass index (kg.m^-2)</td>
<td>22.5 (2.6)</td>
</tr>
<tr>
<td>Current smokers (%)</td>
<td>48.9%</td>
</tr>
<tr>
<td>C-reactive protein (mg.l^-1)*</td>
<td>0.45 (0.20 - 1.26)</td>
</tr>
<tr>
<td>Interleukin-6 (pg.ml^-1)*</td>
<td>0.65 (0.40 – 1.04)</td>
</tr>
<tr>
<td>Fibrinogen (g.l^-1)</td>
<td>2.61 (0.56)</td>
</tr>
<tr>
<td>Serum ACE activity (nmol his-leu.ml^-1.min^-1)</td>
<td>29.7 (9.6)</td>
</tr>
</tbody>
</table>

**UCP3-55C>T CC / CT / TT (n)** 132 / 76 / 13
T allele frequency (95% C.I.) 0.23 (0.191 – 0.270)

**UCP2-866G>A GG / GA / AA (n)** 85 / 110 / 25
A allele frequency (95% C.I.) 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 ±10 nmol 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.
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 ($\chi^2 = 0.22; \ P=0.64$) 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 $22.5 \pm 2.7 \text{ kg.m}^{-2}$, CT $22.4 \pm 2.4 \text{ kg.m}^{-2}$, TT $20.9 \pm 2.1 \text{ kg.m}^{-2}$; $P=0.10$ 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 ($\chi^2 = 1.42; P = 0.23$) 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 ($\Delta = 0.11; P = 0.63$; Table 6.19). UCP3/UCP2 combined genotype
analysis demonstrated that \textit{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

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$P$=NS for difference
Figure 6.9. Mean serum ACE activity during basic training stratified by:

A.  \textit{UCP3-55C>T}
B.  \textit{UCP2-86G>A} genotypes
C.  \textit{UCP2/UCP3} haplotypes.

\begin{itemize}
  \item \textbf{A.} \textit{UCP3-55C>T} genotype
  \begin{itemize}
    \item CC
    \item CT
    \item TT
  \end{itemize}

  \begin{itemize}
    \item Mean serum ACE activity (nmol his-leu/ml/min)
  \end{itemize}

\begin{itemize}
  \item \textbf{B.} \textit{UCP2-86G>A} genotype
  \begin{itemize}
    \item GG
    \item GA
    \item AA
  \end{itemize}

  \begin{itemize}
    \item Mean serum ACE activity (nmol his-leu/ml/min)
  \end{itemize}

\begin{itemize}
  \item \textbf{C.} \textit{UCP2 / UCP3} haplotype
  \begin{itemize}
    \item A+ / C+
    \item A+ / TT
    \item GG / C+
    \item GG / TT
  \end{itemize}

  \begin{itemize}
    \item Mean serum ACE activity (nmol his-leu/ml/min)
  \end{itemize}
\end{itemize}

\begin{itemize}
  \item \textbf{Stage of training}
  \begin{itemize}
    \item Pre
    \item Mid
    \item Post
  \end{itemize}
\end{itemize}

A+ = \textit{UCP2-866A} allele carriers
C+ = \textit{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 PPARα-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), UCP2AA homozygosity was associated with 40% lower TAOS mirrored by a 100% increase in F\textsubscript{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 F₂-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(+) 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.
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.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>RSA</th>
<th>BH3</th>
<th>Comparison (X²)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotype</td>
<td>Rare allele frequency (95% CI)</td>
<td>Genotype</td>
<td>Rare allele frequency (95% CI)</td>
</tr>
<tr>
<td><em>UCP3</em>-55C&gt;T</td>
<td>146 / 40 / 4</td>
<td>0.126 (0.093 – 0.160)</td>
<td>132 / 76 / 13</td>
<td>0.231 (0.191 – 0.270)</td>
</tr>
<tr>
<td><em>UCP2</em>-866G&gt;A</td>
<td>97 / 81 / 13</td>
<td>0.280 (0.235 – 0.325)</td>
<td>85 / 110 / 25</td>
<td>0.364 (0.319 – 0.409)</td>
</tr>
<tr>
<td><em>UCP2</em> Del/Ins</td>
<td>140 / 45 / 2</td>
<td>0.131 (0.097 – 0.165)</td>
<td>105 / 93 / 16</td>
<td>0.292 (0.249 – 0.335)</td>
</tr>
</tbody>
</table>

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**

<table>
<thead>
<tr>
<th>RSA</th>
<th>UCP2-866G&gt;A</th>
<th>Bassingbourn (Big Heart) 3</th>
<th>UCP2-866G&gt;A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GG       GA    AA</td>
<td>CC      54    56    10</td>
<td>CC           54    56    10</td>
</tr>
<tr>
<td>UCP3</td>
<td>-55C&gt;T</td>
<td>CT     16    21    3</td>
<td>-55C&gt;T</td>
</tr>
<tr>
<td></td>
<td>TT       0     0     0</td>
<td>TT           3     9     1</td>
<td></td>
</tr>
<tr>
<td>D’ = 0; P=1.0</td>
<td>D’=0.13; P=0.49</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RSA</th>
<th>UCP2 Del/Ins</th>
<th>Bassingbourn (Big Heart) 3</th>
<th>UCP2 Del/Ins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DD   DI     I I</td>
<td>CC       107    30    2</td>
<td>CC           47    67    15</td>
</tr>
<tr>
<td>UCP3</td>
<td>-55C&gt;T</td>
<td>CT     24    14    0</td>
<td>-55C&gt;T</td>
</tr>
<tr>
<td></td>
<td>TT           4     0     0</td>
<td>TT           12    1     0</td>
<td></td>
</tr>
<tr>
<td>D’ = 0.06; P=0.95</td>
<td>D’=-0.31; P=0.0005</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RSA</th>
<th>UCP2 Del/Ins</th>
<th>Bassingbourn (Big Heart) 3</th>
<th>UCP2 Del/Ins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DD   DI     I I</td>
<td>GG       91     2     0</td>
<td>GG           68    15    0</td>
</tr>
<tr>
<td>UCP2</td>
<td>-866G&gt;A</td>
<td>GA     38    37    2</td>
<td>-866G&gt;A</td>
</tr>
<tr>
<td></td>
<td>AA            7     5     0</td>
<td>TT           3     10    9</td>
<td></td>
</tr>
<tr>
<td>D’ = 0.54; P&lt;0.0001</td>
<td>D’ = 0.58; P&lt;0.0001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
There was a highly significant difference in \textit{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 \textit{UCP3} promoter and \textit{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 ($47.2 \pm 12.1 \text{ nmol His-Leu.ml}^{-1}.\text{min}^{-1}$ and $27.4 \pm 9.0 \text{ nmol His-Leu.ml}^{-1}.\text{min}^{-1}$, respectively; $P = 2 \times 10^{-48}$).

Age-adjusted ACE activity amongst \textit{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 \textit{UCP2}-866A allele carriers in the BH3 study. However, there appeared to be differential regulation of serum ACE activity in both studies according to \textit{UCP2} genotypes (Figure 7.3), but only significant differences by \textit{UCP2D/I} genotypes in the BH3 study (linear trend $P=0.04$; I allele vs. DD homozygotes $P=0.02$). The statistical interaction between \textit{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)**

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Haplotype frequency</th>
<th>RSA</th>
<th>BH3</th>
<th>( P ) difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCP3-55</td>
<td>UCP2-866</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C G Del</td>
<td>0.612</td>
<td>0.463</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>C A Del</td>
<td>0.152</td>
<td>0.024</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>T G Del</td>
<td>0.094</td>
<td>0.120</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>C A Ins</td>
<td>0.092</td>
<td>0.225</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>T A Ins</td>
<td>0.023</td>
<td>0.009</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>C G Ins</td>
<td>0.015</td>
<td>0.058</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>T A Del</td>
<td>0.011</td>
<td>0.100</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>T G Ins</td>
<td>0</td>
<td>0</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

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/UCP2I* and the *UCP3-55T/UCP2-866G/UCP2I* 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/UCP2I* haplotype group was excluded. A SNP change from the *UCP3/2 CGD* haplotype to *CGI* 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 CAD* haplotype to *CAI* 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 UCP2D/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.
7. IS THERE AN ASSOCIATION BETWEEN THE UCP3/UCP2 GENETIC LOCUS AND CIRCULATING ACE ACTIVITY?

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 **UCP2I/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.

<table>
<thead>
<tr>
<th>Caucasian type 2 diabetics</th>
<th><em>UCP2</em>-866G&gt;A</th>
<th></th>
<th></th>
<th></th>
<th>Caucasian type 1 diabetics</th>
<th><em>UCP2</em>-866G&gt;A</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>UCP3</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>UCP3</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>164</td>
<td>156</td>
<td>27</td>
<td></td>
<td>CC</td>
<td>47</td>
<td>37</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>-55C&gt;T</td>
<td>57</td>
<td>92</td>
<td>35</td>
<td></td>
<td>-55C&gt;T</td>
<td>16</td>
<td>26</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>1</td>
<td>18</td>
<td>7</td>
<td></td>
<td>TT</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>D’ = 0.23; P &lt;0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D’ = 0.21; P = 0.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Caucasian type 2 diabetics</th>
<th><em>UCP2</em> Del/Ins</th>
<th></th>
<th></th>
<th></th>
<th>Caucasian type 1 diabetics</th>
<th><em>UCP2</em> Del/Ins</th>
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<tbody>
<tr>
<td><em>UCP3</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>UCP3</em></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>CC</td>
<td>159</td>
<td>158</td>
<td>30</td>
<td></td>
<td>CC</td>
<td>47</td>
<td>37</td>
<td>4</td>
<td></td>
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<tr>
<td>-55C&gt;T</td>
<td>102</td>
<td>75</td>
<td>6</td>
<td></td>
<td>-55C&gt;T</td>
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<tr>
<td>TT</td>
<td>23</td>
<td>2</td>
<td>1</td>
<td></td>
<td>TT</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>D’ = -0.19; P=0.0003</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D’ = -0.06; P = 0.97</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Caucasian type 2 diabetics</th>
<th><em>UCP2</em> Del/Ins</th>
<th></th>
<th></th>
<th></th>
<th>Caucasian type 1 diabetics</th>
<th><em>UCP2</em> Del/Ins</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td><em>UCP2</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>UCP2</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>198</td>
<td>33</td>
<td>3</td>
<td></td>
<td>GG</td>
<td>60</td>
<td>10</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>-866G&gt;A</td>
<td>84</td>
<td>178</td>
<td>10</td>
<td></td>
<td>-866G&gt;A</td>
<td>26</td>
<td>48</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>12</td>
<td>36</td>
<td>24</td>
<td></td>
<td>AA</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>D’ = 0.60; P&lt;0.0001</td>
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<td></td>
<td></td>
<td></td>
<td>D’ = 0.52; P&lt;0.0001</td>
<td></td>
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</tr>
</tbody>
</table>

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 UCP3 and UCP2 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 > 12 mmol.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; \( P < 1 \times 10^{-21} \) 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 UCP2D/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 UCP2D/I and significant negative LD between UCP3-55C>T and UCP2D/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 UCP2II 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 UCP2D/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.

<table>
<thead>
<tr>
<th>Danish type 1 UCP2-866G&gt;A</th>
<th>Danish type 1 UCP2 Del/Ins</th>
<th>Danish type 1 UCP2 Del/Ins</th>
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<tbody>
<tr>
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<td>UCP2</td>
</tr>
<tr>
<td></td>
<td>-866G&gt;A</td>
<td>-866G&gt;A</td>
</tr>
<tr>
<td></td>
<td>GG</td>
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</tr>
<tr>
<td></td>
<td>AA</td>
<td>12</td>
</tr>
<tr>
<td>-55C&gt;T</td>
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<td>CT</td>
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<td>3</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0</td>
</tr>
</tbody>
</table>

D’ = 0.23; P = 0.005

D’ = -0.26; P = 0.001

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 UCP2D/I genotypes.

Data are mean ±SEM;
LT= linear trend analysis, II vs. D+ = ANOVA of II vs. D allele carriers
Figure 7.15. 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. 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).
7.4 DISCUSSION

The *UCP3*-55C>T, *UCP2*-866G>A and *UCP2D/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 *UCP2D/I* in Caucasians and no LD between these 2 sites in South Africans (the negative degree of LD between *UCP3*-55C>T and *UCP2D/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 UCP3/2 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 UCP2D/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 *UCP2D/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.
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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>cDNA accession no.</th>
<th>Primers</th>
<th>Amplicon size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGAPDH</td>
<td>J04038</td>
<td>F: GGGGAAGGTGAAGGTCGGAGT</td>
<td>228</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CCTGGAAGATGGTGATGGGAT</td>
<td></td>
</tr>
<tr>
<td>hUCP3</td>
<td>U84763 / AF050113</td>
<td>F: CCTCACTACCGGGATT</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GTTGACGATAGCATCCCT</td>
<td></td>
</tr>
<tr>
<td>hUCP2</td>
<td>NM_003355 / AF019409</td>
<td>F: GCTTTGAAGAAGGGGAC</td>
<td>296</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTGTAACCGGACTTTAGCA</td>
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<tr>
<td>hACE</td>
<td>J04144</td>
<td>F: ACCAATGACACGGAAAG</td>
<td>207</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GTGGGTTCGTTCGG</td>
<td></td>
</tr>
</tbody>
</table>
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.** Relative JC-1 aggregate to monomer (A:M) fluorescence in HUVECs pretreated with mClCCP, rotenone and nigericin.

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.
8.4 THE EFFECTS OF PROLONGED HYPOXIA, UNCOUPLING AND HIGH GLUCOSE ON Δψₘ AND SUPEROXIDE GENERATION IN CULTURED HUVECS

HUVECs were incubated for 24 hours in different experimental conditions to induce cell stress. Superoxide generation and Δψₘ 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% O₂ and 5% CO₂.

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 Δψₘ 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 Δψₘ (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% O₂; N = 3) exhibited significant cell stress with a reduction in Δψₘ (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).
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 48 hours, 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 Δψₘ 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

<table>
<thead>
<tr>
<th>Strand</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hUCP1</td>
<td>5’- GAGTGAAGATGGGGGGGCCCTGACAGCCTCG -</td>
</tr>
<tr>
<td>hUCP1 antisense</td>
<td>3’- ACTTCTACCCCCGGACTGTC</td>
</tr>
<tr>
<td>hUCP2</td>
<td>5’- TCAAGCATCATGGGGTTCAAGGCCACA -</td>
</tr>
<tr>
<td>hUCP2 antisense</td>
<td>3’- CGTAGTACCAACCCAAAGTTCC</td>
</tr>
<tr>
<td>hUCP3</td>
<td>5’- CCAAGACTATGGGACTGAAGCCATTCA -</td>
</tr>
<tr>
<td>hUCP3 antisense</td>
<td>3’- CCTGATACCAACCTGACTTCG</td>
</tr>
<tr>
<td>Scrambled antisense</td>
<td>5’- AGCCATCCACGATTGATCA</td>
</tr>
</tbody>
</table>
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.

Figure 8.7. Dihydroethidium fluorescence in HUVECs incubated with antisense to uncoupling proteins for 48 hours.
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 ($7 \pm 3.5\%$ 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 $40 \pm 8.0\%$ 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).

\[ P<0.05 \text{ vs. vehicle; N=4 repeats} \]

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$\bullet$. It is possible that respiratory uncoupling by mClCCP or mitochondrial superoxide generation leads to cellular stress and a reduction in cellular NO$\bullet$ 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 O2•− and ONOO− production. Rotenone is a complex 1 inhibitor, binding to complex I downstream from its O2•− production site, therefore generating O2•− 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 O2•− 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 Δψm but generates O2•− from complexes I and III by reverse electron transport. Rotenone treatment during cellular hypoxia therefore binds complex I upstream from the site of O2•− production, thereby reducing O2•−. (Parhasarathi 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 Δψm and a dose dependant increase in superoxide generation. Similar patterns of change were demonstrated with chronic hypoxia (Pearlstein et al. 2002). However,
A 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 $O_2^{•-}$ 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 $O_2\cdot^-$ 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, $O_2\cdot^-$ 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) $O_2\cdot^-$ 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 β2 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 \textit{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 \textit{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 (\textit{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 (\textit{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 \textit{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 \textit{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, \textit{BDKRB2} gene knockout mice develop hypertension and LVH with exaggerated responses to salt and Ang II (Madeddu et al. 1997; Emanueli et al. 1999).
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.
Flow cytometric and confocal microscopic assays were developed using fluorescent potentiometric dyes to indirectly measure Δψₘ in cultured live cells. Treatment with the ACEi ramiprilat for 24 hours lead to a highly significant increase in Δψₘ 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 C₂C₁₂ 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 Δψₘ 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 C₂C₁₂ 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 O2 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•:O2 ratio, acts as an O2 sensor, and that constitutive low concentrations of NO• produced by eNOS or mtNOS is required by cells to exhibit control over O2 consumption, particularly as O2 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 O2 tension. It may be that the benefits in tissue contractile function and reductions in O2 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 O2 utilisation at complex IV and increase Δψm. 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 O2 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 O2 consumption, reduction in Δψm and compensatory activation of UCP (UCP*) to limit further ROS generation.
Pretreatment of C2C12 myotubes with LPS for 24 hours significantly suppressed cellular O2 consumption by 73%. Maximal respiratory cycling caused by treatment with the uncoupler mClCCP was unaffected by pre-treatment with LPS, suggesting an intact ETC. LPS has been shown to reduce cellular O2 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 O2 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 (F1,F0-ATPase) to maintain Δψm (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 (Beltrán 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 Δψm, and may lead to O2 utilisation at complexes I and III to generate superoxide (O2•−). After persistent inhibition of respiration by NO•, the depletion of glycolytic ATP and/or oxidative damage leads to a collapse in Δψm and cell death (Beltrán et al. 2000).

How does mClCCP drive cellular O2 consumption during maximal inhibition of mitochondrial respiration by NO•? This effect has not been previously reported. It is possible that sudden collapse of Δψm with a low dose of mClCCP, leads to futile electron cycling through complexes I to III and consumption of O2 at complexes I and III to produce O2•−, as demonstrated in HUVECs (vide infra). Alternatively, mClCCP
may decrease the bioavailability of NO•, possibly by ROS induced uncoupling of iNOS, and therefore allow O₂ to compete at complex IV.

An exciting novel finding was that Ang II directly increased the resting O₂ consumption of C₂C₁₂ 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 O₂ 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 O₂ consumption (Brown et al. 1994; Cleeter et al. 1994). The extra-mitochondrial NADPH oxidase pathway relies on activation of the AT₁R; 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).
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, \( UCP2AA \) homozygosity was associated with highly significant increases in markers of oxidative stress, with 40% lower TAOS mirrored by a 100% increase in \( F_2 \)-isoprostone 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). $O_2^•$ 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 $O_2^•$ 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 HbA1c 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 $O_2\cdot^{-}$ 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 O₂ 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 UCP2D/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 UCP2D/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 \textit{BDKRB2} gene and \textit{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 \textit{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 \textit{UCP2}-866G>A variant and then studied \textit{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, \textit{in vivo} assessment of culprit coronary plaque temperature could be made according to \textit{UCP2} genotype in patients presenting with unstable angina and acute MI using a thermography catheter (Stefanadis \textit{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 O₂ 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.


*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 B₁ receptor (B₁R) and constitutive B₂ receptor (B₂R) contain functional variants: the B₁R−699C (rather than G) and the B₂R(−9) (rather than +9) alleles are associated with greater mRNA expression and the B₂R(−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 B₁R−699GG homozygotes (HR 2.14 [1.42–3.22]; P<0.0001) and B₂R(+9,+9) individuals (HR 3.51 [1.69–7.28]; P=0.001) but not in B₁R−699C allele carriers (HR 0.82 [0.28–2.42]; P=0.76) or in B₂R(−9,−9) homozygotes (HR 1.25 [0.51-3.04]; P=0.63).

**Conclusions.** Common variation in the genes for the kinin B₁ and B₂ receptors influences prospective hypertensive coronary risk. These are the first reported human data to suggest a role for the B₁R in human coronary vascular disease, and the first prospective study to demonstrate a similar role for the B₂R.
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.


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 β2 receptor (B2R) 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 B2R 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 B2R -9/-9 having the highest DE at baseline. The ACE I/B2R -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 B2R 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

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. In their Research letter, Bernard Keavney and colleagues (Feb 1, p 396) dispute our finding, while your accompanying Editorial (p 357) 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 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, 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, 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


2. ACE Genotype and Performance

Montgomery HE, Dhamrait SS. 


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

3. ACE gene, physical activity, and physical fitness

Williams AG, Day SH, Dhamrait S.

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


APPENDIX 3: Other papers arising during this thesis


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 F₂-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-PGF₂α 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, HbA₁c 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.

ANOVA P=0.13, GG vs. A allele, P=0.04), which remained significant after adjustment for age, triglyceride, HDL-C, glucose, HbA1c 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

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.
of interaction between \textit{UCP2-866G>A} genotype and CHD status in determining plasma TAOS \((P=0.025\) for G allele \textit{vs. AA}).

To further corroborate these data, 20 men from UDACS were selected for homozygosity for the \textit{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\% \textit{vs. no-CHD} 48.4±12.9\%; \(P=0.22\)) and F\textsubscript{2}-isoprostanes higher (CHD 168.3±74.2pg.ml\textsuperscript{-1} \textit{vs. no-CHD} 111.7±79.1pg.ml\textsuperscript{-1}; \(P=0.05\)). However, there were highly significant differences in plasma TAOS between AA and GG homozygotes overall (AA 36.9±8.4\% \textit{vs. GG} 53.6±10.5\%; \(P<0.0001\)), in those without CHD (AA 42.1±6.1\% \textit{vs. GG} 54.7±7.0; \(P=0.016\)) and in those with CHD (AA 31.7±7.3\% \textit{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\textsubscript{2}-isoprostane concentrations were highly significantly elevated in AA homozygotes both overall (AA 175.4±64.5pg.ml\textsuperscript{-1} \textit{vs. GG} 104.6±72.8pg.ml\textsuperscript{-1}; \(P=0.011\)) and in those with CHD (AA 220.6±37.2pg.ml\textsuperscript{-1} \textit{vs. GG} 109.9±51.1pg.ml\textsuperscript{-1}; \(P=0.005\)), but not significantly in AA homozygotes without CHD (AA 119.1±40.2pg.ml\textsuperscript{-1} \textit{vs. GG} 105.9±27.5pg.ml\textsuperscript{-1}; \(P=0.58\); Figure 8.2b). There was evidence of significant interaction between genotype and CHD status in determining F\textsubscript{2}-isoprostanes \((P=0.014\) but not plasma TAOS \((P=0.19)\).
## Table 10.1. Plasma TAOS and esterified F₂-isoprostanes in relation to UCP2-866G>A genotype and CHD status in the UDACS substudy.

<table>
<thead>
<tr>
<th></th>
<th>No CHD</th>
<th></th>
<th>P</th>
<th>CHD</th>
<th></th>
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<tr>
<td></td>
<td>AA (n=5)</td>
<td>GG (n=5)</td>
<td></td>
<td>AA (n=5)</td>
<td>GG (n=5)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>64.6 (4.4)</td>
<td>65.4 (5.3)</td>
<td>0.80</td>
<td>64.0 (6.2)</td>
<td>65.1 (7.2)</td>
<td>0.79</td>
</tr>
<tr>
<td>BMI (kg.m⁻²)</td>
<td>32.7 (6.9)</td>
<td>29.6 (4.8)</td>
<td>0.43</td>
<td>30.34 (3.7)</td>
<td>36.2 (19.0)</td>
<td>0.52</td>
</tr>
<tr>
<td>Duration DM (years)</td>
<td>6 (0.5-20.5)</td>
<td>6 (4-12)</td>
<td>0.78</td>
<td>12 (7.5-21)</td>
<td>10 (8-17)</td>
<td>0.64</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.5 (0.7)</td>
<td>7.5 (1.3)</td>
<td>0.19</td>
<td>8.4 (2.1)</td>
<td>8.6 (1.5)</td>
<td>0.88</td>
</tr>
<tr>
<td>Glucose (mmol.l⁻¹)</td>
<td>9.2 (5.0-15.7)</td>
<td>8.1 (7.2-10.1)</td>
<td>0.47</td>
<td>9.6 (6.8-16.6)</td>
<td>9.2 (7.5-16.2)</td>
<td>0.99</td>
</tr>
<tr>
<td>Cholesterol (mmol.l⁻¹)</td>
<td>5.1 (0.8)</td>
<td>5.5 (1.0)</td>
<td>0.50</td>
<td>4.5 (0.3)</td>
<td>4.6 (0.9)</td>
<td>0.90</td>
</tr>
<tr>
<td>LDL (mmol.l⁻¹)</td>
<td>2.8 (0.6)</td>
<td>3.4 (1.0)</td>
<td>0.28</td>
<td>2.2 (0.6)</td>
<td>2.4 (0.5)</td>
<td>0.65</td>
</tr>
<tr>
<td>HDL (mmol.l⁻¹)</td>
<td>1.3 (0.4)</td>
<td>1.4 (0.3)</td>
<td>0.93</td>
<td>1.1 (0.2)</td>
<td>1.2 (0.1)</td>
<td>0.27</td>
</tr>
<tr>
<td>Tg (mmol.l⁻¹)</td>
<td>2.1 (0.6)</td>
<td>2.3 (1.5)</td>
<td>0.81</td>
<td>3.3 (1.4)</td>
<td>2.2 (1.7)</td>
<td>0.30</td>
</tr>
<tr>
<td>TAOS (%)</td>
<td>42.06 (6.12)*</td>
<td>54.68 (6.99)</td>
<td>0.02</td>
<td>31.70 (7.27)*</td>
<td>52.6 (6.25)</td>
<td>0.001</td>
</tr>
<tr>
<td>F₂ isoprostanes (pg. ml⁻¹)</td>
<td>119.1 (40.2)**</td>
<td>105.9 (27.48)</td>
<td>0.58</td>
<td>220.6 (37.2)**</td>
<td>109.9 (51.1)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

**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 F2-isoprostan 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


Casademont, J. and Miro, O. Electron transport chain defects in heart failure. *Heart Failure Reviews* (2002); 7: 131-139.


Drexler, H. Nitric oxide synthases in the failing human heart. *Circ Res* (1999); **85**(8): 82E-88E.


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 USA* (2001); **98**(4): 1416-1421.


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.


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.


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.


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.


Kramer, R. E. Evidence that angiotensin II decreases mitochondrial calcium in the glomerulosa cell. Mol Cell Endocrinol (1990); 74(2): 87-100.


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.


optotic signaling pathways in the study (4S).


Salvioli, S., Ardissoni, 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.


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-.


