AN EXAMINATION OF GENETIC POLYMORPHISMS IN THE ENZYME HEME OXYGENASE-1 AND THEIR RELATIONSHIP TO CARDIOVASCULAR DISEASE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Jeanette M. Ferguson, B.S.

* * * * *

The Ohio State University
2005

Dissertation Committee:

Dr. Philip F. Binkley, Adviser

Dr. Glen E. Cooke

Dr. W. James Waldman

Approved by

________________________
Adviser
Pathology Graduate Program
ABSTRACT

Heme oxygenase-1 (HO-1) plays a critical role in protecting the cardiovascular system from the damaging effects of oxidative stress. The anti-inflammatory, antioxidant, antiproliferative and vasodilatory effects of its reaction products (carbon monoxide, free ferrous iron, and bilirubin) mediate cardiovascular protection. Two promoter polymorphisms found in HMOX1, the gene encoding HO-1, are reported to have contrasting effects on gene transcription. The A allele of the single nucleotide polymorphism (SNP) located at base –413 has been shown to increase transcriptional activity, while a (GT)n microsatellite promoter polymorphism has been shown to decrease HMOX1 transcription when the GT repeat number exceeds 25. These polymorphisms have been associated with cardiovascular disease, and have different frequency distributions based upon ethnicity.

The main goal of the experiments in this dissertation was to examine the relationship between the HMOX1 promoter polymorphisms and cardiovascular disease, while placing special emphasis on the African American ethnic group. Genotyping of the polymorphisms was performed by sequence analysis of the HMOX1 promoter. The microsatellite allele was classified as large (L) if the GT repeat was ≥ 26 and small (S) if the GT repeat was < 26. The HMOX1 SNP-413 was genotyped according to the bases present at position –413 of the sequences. In general, the African American subjects
were significantly associated with the large repeat microsatellite polymorphism and the T allele of the SNP-413. The Caucasian subjects were significantly associated with the small repeat microsatellite polymorphism and the A allele of the SNP-413. The L microsatellite allele and A SNP-413 allele was the most frequent allele combination seen in all subjects. Coronary artery disease was significantly associated with the LL microsatellite genotype in African Americans and the AA SNP-413 genotype in Caucasians. Congestive heart failure was significantly associated with the LL microsatellite genotype and TT SNP-413 genotype in African Americans. The author concludes that the large microsatellite polymorphism of HMOX1 is associated with cardiovascular disease in the African American population, and these subjects are possibly more susceptible to diseases resulting from oxidative stress due to deficient HO-1 activity.
Dedicated to my loving and patient family.
ACKNOWLEDGMENTS

I wish to thank my adviser, Philip F. Binkley, MD, MPH, for always believing in my abilities as a scientist and for expanding my knowledge of the world of statistics. I would also like to thank W. James Waldman, PhD, and Glen E. Cooke, MD, the members of my Graduate Committee. Your support and guidance throughout my graduate school career was a tremendous help to me, and I am forever grateful to you for sharing it with me.

The work in this dissertation would never have been possible without the knowledge and assistance provided by the following researchers: Yiwen Liu-Stratton, PhD, Mackenzie Taylor, Amanda Lesinski, and Patty Hatton. Our times together were great, and I will always treasure our friendship. Thank you for helping me to achieve my dream. I could never have gotten through this without you.

I would like to acknowledge Dennis Mathias for assisting me with my figures, Gregory Lesinski, PhD, for his advice on dissertation writing, and The Ohio State University Davis Heart and Lung Institute Microarray Genetics Core and Plant-Microbe Genomics Facility for running my sequencing reactions.

I would also like to thank all of the family and friends who provided me with love and support throughout my time in graduate school. To Janice Pohorence, Katy
Pohorence, Rick and Pam Pohorence, and Ashley Q. Hejmanowski, PhD: thank you for helping me to keep my goals in focus and to tough out the hard times

Finally, I would like to thank my husband, Bryan Ferguson, for putting up with all of the stressed-out temper tantrums, for editing chapters that seemed to be in a foreign language, and for just being a truly magnificent person. I will never be able to repay you for all of the gifts you have given me. Thank you for helping me to achieve all of my dreams. I hope someday to help you reach yours.
VITA

December 17, 1975.......................Born – Cleveland, Ohio

May 1998...............................B.S. Biology, Ohio Northern University

1998-Present..........................Graduate Research Associate, The Ohio State University

PUBLICATIONS


FIELDS OF STUDY

Major Field: Pathology
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract ...........................................................................................................</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication .......................................................................................................</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgements ...........................................................................................</td>
<td>v</td>
</tr>
<tr>
<td>Vita ..................................................................................................................</td>
<td>vi</td>
</tr>
<tr>
<td>List of Tables ..................................................................................................</td>
<td>xiv</td>
</tr>
<tr>
<td>List of Figures ...............................................................................................</td>
<td>xvi</td>
</tr>
<tr>
<td>List of Abbreviations .....................................................................................</td>
<td>xvii</td>
</tr>
<tr>
<td><strong>Chapters:</strong></td>
<td></td>
</tr>
<tr>
<td>1. Introduction ...............................................................................................</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Background ...............................................................................................</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Hypothesis and Research Objectives ......................................................</td>
<td>3</td>
</tr>
<tr>
<td>1.3 Organization .............................................................................................</td>
<td>3</td>
</tr>
<tr>
<td>2. Heme and the Heme Oxygenase Enzyme System ...............................................</td>
<td>5</td>
</tr>
<tr>
<td>2.1 Heme and the Heme Oxygenase Enzyme Reaction ..........................................</td>
<td>5</td>
</tr>
<tr>
<td>2.2 The Isoforms of Heme Oxygenase ..................................................................</td>
<td>8</td>
</tr>
<tr>
<td>2.3 The Induction of Heme Oxygenase-1 Expression ..........................................</td>
<td>13</td>
</tr>
<tr>
<td>2.4 The Reaction Products of Heme Catabolism ...............................................</td>
<td>15</td>
</tr>
<tr>
<td>2.5 Heme Oxygenase-1 Deficiency ......................................................................</td>
<td>16</td>
</tr>
<tr>
<td>2.5.1 Human HO-1 Deficiency ...........................................................................</td>
<td>17</td>
</tr>
<tr>
<td>2.5.2 Heme Oxygenase Knock Out Mice ................................................................</td>
<td>21</td>
</tr>
<tr>
<td>2.5.3 A Comparison of Human HO-1 Deficiency and HO-1 Knock Out Mice ................</td>
<td>23</td>
</tr>
<tr>
<td>2.6 The Polymorphisms of the HMOX1 Gene ....................................................</td>
<td>24</td>
</tr>
<tr>
<td>2.6.1 The HMOX1 T(-413)A Single Nucleotide Polymorphism ................................</td>
<td>25</td>
</tr>
<tr>
<td>2.6.2 The HMOX1 (GT)n Microsatellite Polymorphism ........................................</td>
<td>26</td>
</tr>
<tr>
<td>3. The Role of Heme Oxygenase-1 in the Cardiovascular System ............................</td>
<td>28</td>
</tr>
<tr>
<td>3.1 Introduction ...............................................................................................</td>
<td>28</td>
</tr>
<tr>
<td>3.2 Coronary Artery Disease and Atherosclerosis .............................................</td>
<td>29</td>
</tr>
<tr>
<td>3.2.1 Background ............................................................................................</td>
<td>29</td>
</tr>
<tr>
<td>3.2.2 The Role of Oxidant Stress in Coronary Artery Disease and Atherosclerosis</td>
<td>30</td>
</tr>
</tbody>
</table>
3.2.3 The Association of HO-1 with Coronary Artery Disease and Atherosclerosis ................................................................. 31

3.3 Cardiomyopathy and Congestive Heart Failure ................................................... 33
  3.3.1 Background ...................................................................................... 33
  3.3.2 The Role of Oxidant Stress in Cardiomyopathy and Congestive Heart Failure ............................................................................. 35
  3.3.3 The Association of HO-1 with Cardiomyopathy and Congestive Heart Failure ............................................................................... 36

3.4 Transplant Rejection ....................................................................................... 38
  3.4.1 Background ...................................................................................... 38
  3.4.2 The Role of Oxidant Stress in Transplant Rejection ....................... 40
  3.4.3 The Association of HO-1 with Transplant Rejection ....................... 41

3.5 The Relationship Between the Heme Oxygenase and Nitric Oxide Synthase Enzyme Systems .............................................................................. 42
  3.5.1 The Nitric Oxide Synthase Pathway and the Cardiovascular System .............................................................................................. 42
  3.5.2 The Interaction between Heme Oxygenase and Nitric Oxide Synthase ........................................................................................... 44
  3.5.3 The Combined Actions of Heme Oxygenase and Nitric Oxide Synthase in the Cardiovascular System ........................................... 46

3.6 The Protective Role of HO-1 in the Cardiovascular System .......................... 48
  3.6.1 The Actions of Carbon Monoxide.................................................... 49
  3.6.2 The Actions of Biliverdin and Bilirubin .......................................... 51
  3.6.3 The Actions of Ferrous Iron............................................................. 52
  3.6.4 The Overexpression of HO-1 ........................................................... 54

3.7 The Effect of HO-1 Inhibition or Deficiency on the Cardiovascular System. 58

3.8 The Pharmacological Interactions of HO-1 .................................................... 60

3.9 The HMOX1 Polymorphisms and their Relationship to Cardiovascular Disease ............................................................................................... 63
  3.9.1 The Single Nucleotide Polymorphism at Base -413 of HMOX1..... 63
  3.9.2 The HMOX1 Promoter Microsatellite Polymorphism.................. 63

4. General Methods and Materials ........................................................................ 66
  4.1 Introduction ............................................................................................ 66
  4.2 Extraction of Genomic DNA ................................................................. 66
  4.3 Polymerase Chain Reaction (PCR) ......................................................... 67
  4.4 Gel Electrophoresis ............................................................................... 69
  4.5 Sequence Analysis ................................................................................ 69
5. Identification of Limitations with the Methodology of the Protocol to Examine the HMOX1 Exon 3 Dinucleotide Deletion

5.1 Introduction
5.2 Methods and Materials
5.2.1 Subjects
5.2.2 Extraction of Genomic DNA
5.2.3 Polymerase Chain Reaction
5.2.4 Gel Electrophoresis
5.2.5 Sequence Analysis
5.3 Results and Discussion

6. An Examination of the Association Between the HMOX1 Promoter Polymorphisms and the African American Population

6.1 Introduction
6.2 Methods and Materials
6.2.1 Subjects
6.2.2 Extraction of Genomic DNA
6.2.3 Polymerase Chain Reaction
6.2.4 Gel Electrophoresis
6.2.5 Sequencing Analysis
6.2.6 Genotyping and Statistical Analysis
6.3 Results
6.3.1 The Frequency Distribution of the HMOX1 Microsatellite Polymorphism
6.3.2 The Frequency Distribution of the HMOX1 Single Nucleotide Polymorphism at -413
6.3.3 The Frequency of both HMOX1 Promoter Polymorphisms in the Caucasian and African American Populations
6.4 Discussion
6.4.1 The Importance of Using Racial and Ethnic Classification in Genetic Research
6.4.2 A Discussion of the HMOX1 Microsatellite Polymorphism Results
6.4.3 A Discussion of the HMOX1 SNP-413 Results
6.4.4 A Discussion of the Combination of HMOX1 Promoter Polymorphisms in the African American and Caucasian Populations
6.4.5 A Discussion of Experimental Limitations and Future Research
7. An Examination of the HMOX1 Promoter Polymorphisms in Caucasian and African American Patients with Coronary Artery Disease ........................................ 104

7.1 Introduction ................................................................................................... 104
7.2 Methods and Materials ................................................................................ 105
  7.2.1 Subjects .......................................................................................... 105
  7.2.2 Extraction of Genomic DNA .......................................................... 105
  7.2.3 Polymerase Chain Reaction ........................................................... 105
  7.2.4 Gel Electrophoresis ........................................................................ 106
  7.2.5 Sequencing Analysis ...................................................................... 106
  7.2.6 Genotyping and Statistical Analysis .............................................. 106
7.3 Results ........................................................................................................... 106
  7.3.1 Demographic Information for the Patient and Control Populations .................................................................................................................. 106
  7.3.2 The Frequency Distribution of the HMOX1 Microsatellite Polymorphism ........................................................................................................ 109
  7.3.3 The Frequency Distribution of the HMOX1 Single Nucleotide Polymorphism at -413 .......................................................... 115
  7.3.4 The Frequency of both HMOX1 Promoter Polymorphisms in the Control and Patient Populations .......................................................... 118
7.4 Discussion ..................................................................................................... 121
  7.4.1 A Discussion of the HMOX1 Microsatellite Polymorphism Results ........................................................................................................ 121
  7.4.2 A Discussion of the HMOX1 SNP-413 Results............................. 123
  7.4.3 A Discussion of the Relationship Between the HMOX1 Promoter Polymorphisms .......................................................... 125
  7.4.4 A Discussion of Experimental Limitations and Future Research .. 126

8. An Examination of the HMOX1 Promoter Polymorphisms in Caucasian and African American Patients with Congestive Heart Failure ........................................ 128

8.1 Introduction .......................................................................................... 128
8.2 Methods and Materials ................................................................................ 129
  8.2.1 Subjects .......................................................................................... 129
  8.2.2 Extraction of Genomic DNA .......................................................... 129
  8.2.3 Polymerase Chain Reaction ........................................................... 129
  8.2.4 Gel Electrophoresis ........................................................................ 130
  8.2.5 Genotyping the eNOS Polymorphisms .......................................... 130
  8.2.6 Sequencing Analysis ...................................................................... 131
8.2.7 HMOX1 Genotyping ................................................................. 131
8.2.8 Statistical Analysis ................................................................. 131
8.3 Results ......................................................................................... 132
  8.3.1 Demographic Information for the Patient and Control
    Populations .................................................................................. 132
  8.3.2 The Frequency Distribution of the HMOX1 Microsatellite
    Polymorphism .............................................................................. 135
  8.3.3 The Frequency Distribution of the HMOX1 Single Nucleotide
    Polymorphism at -413 ................................................................ 141
  8.3.4 The Frequency of both HMOX1 Promoter Polymorphisms in
    the Control and Patient Populations ........................................... 143
  8.3.5 The Frequency of the eNOS Glu298Asp and Intron 4
    Polymorphisms ........................................................................... 146
    8.3.5.1 The eNOS Glu298Asp Polymorphism .............................. 146
    8.3.5.2 The eNOS Intron 4 Polymorphism ................................. 147
8.4 Discussion .................................................................................... 149
  8.4.1 A Discussion of Ethnicity and Congestive Heart Failure ............ 149
  8.4.2 A Discussion of the HMOX1 Microsatellite Polymorphism
    Results ......................................................................................... 150
  8.4.3 A Discussion of the HMOX1 SNP-413 Results ......................... 151
  8.4.4 A Discussion of the Combination of the HMOX1 Promoter
    Polymorphisms ........................................................................... 152
  8.4.5 A Discussion of the Presence of the eNOS Polymorphisms and
    HMOX1 Polymorphisms in the CHF Population ....................... 152
    8.4.5.1 The eNOS Glu298Asp Polymorphism ......................... 152
    8.4.5.2 The eNOS Intron 4 Polymorphism .............................. 154
  8.4.6 A Discussion about the HMOX1 Microsatellite Polymorphism
    and the Results of the A-HeFT Trial ........................................ 155
9. An Examination of Heme Oxygenase-1 Expression in Human Left Ventricle
   Tissue Samples ........................................................................... 157
  9.1 Introduction .................................................................................. 157
  9.2 Methods and Materials ............................................................. 158
    9.2.1 Subjects .............................................................................. 158
    9.2.2 Extraction of Genomic DNA .............................................. 158
    9.2.3 Polymerase Chain Reaction .............................................. 158
    9.2.4 Gel Electrophoresis ......................................................... 159
    9.2.5 Sequencing Analysis ......................................................... 159
    9.2.6 Genotyping ...................................................................... 159

xii
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>The Isoforms of the Heme Oxygenase Enzyme</td>
</tr>
<tr>
<td>2.2</td>
<td>Positive and Negative Effects of the Reaction Products from Heme Oxygenase Activity</td>
</tr>
<tr>
<td>3.1</td>
<td>Summary of Ischemic and Nonischemic Cardiomyopathy</td>
</tr>
<tr>
<td>3.2</td>
<td>The Pharmacological Interactions of HO-1</td>
</tr>
<tr>
<td>4.1</td>
<td>PCR Primers Used for Experiments</td>
</tr>
<tr>
<td>6.1</td>
<td>Demographic Information</td>
</tr>
<tr>
<td>6.2</td>
<td>Allele and Genotype Frequencies for the HMOX1 Microsatellite Polymorphism</td>
</tr>
<tr>
<td>6.3</td>
<td>Allele and Genotype Frequencies for the HMOX1 SNP-413</td>
</tr>
<tr>
<td>6.4</td>
<td>Genotype Frequencies of HMOX1 Microsatellite Polymorphism vs. SNP-413</td>
</tr>
<tr>
<td>6.5</td>
<td>GT Repeat Pairs with greater than 1% Frequency in the Caucasian Population and their Relationship to the HMOX1 SNP-413 Genotypes</td>
</tr>
<tr>
<td>6.6</td>
<td>GT Repeat Pairs with greater than 1% Frequency in the African American Population and their Relationship to the HMOX1 SNP-413 Genotypes</td>
</tr>
<tr>
<td>7.1</td>
<td>Demographic Information</td>
</tr>
<tr>
<td>7.2</td>
<td>Allele and Genotype Frequencies for the HMOX1 Microsatellite Polymorphism</td>
</tr>
<tr>
<td>7.3</td>
<td>Allele and Genotype Frequencies for the HMOX1 SNP-413</td>
</tr>
<tr>
<td>7.4</td>
<td>Genotype Frequencies of the HMOX1 Microsatellite Polymorphism vs. SNP-413</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>The Structure of the Heme Molecule</td>
<td>6</td>
</tr>
<tr>
<td>2.2</td>
<td>The Heme Oxygenase Reaction</td>
<td>8</td>
</tr>
<tr>
<td>2.3</td>
<td>Origins of Pathology Seen in Human HO-1 Deficiency</td>
<td>20</td>
</tr>
<tr>
<td>2.4</td>
<td>The Sequence of the HMOX1 Promoter Region</td>
<td>25</td>
</tr>
<tr>
<td>3.1</td>
<td>The Interactions Between NOS and HO-1 Enzyme Systems</td>
<td>46</td>
</tr>
<tr>
<td>3.2</td>
<td>The Role of NOS and HO-1 in Regulating VEGF</td>
<td>48</td>
</tr>
<tr>
<td>5.1</td>
<td>HMOX1 Exon 3 with the Dinucleotide Deletion Highlighted in Yellow</td>
<td>71</td>
</tr>
<tr>
<td>5.2</td>
<td>Gel for HMOX1 Exon 3 Dinucleotide Deletion Specific PCR</td>
<td>75</td>
</tr>
<tr>
<td>5.3</td>
<td>HMOX1 Exon 3 with Target Sequences for PCR Primers Highlighted</td>
<td>77</td>
</tr>
<tr>
<td>5.4</td>
<td>HMOX1 Exon 3 with Target for Sequencing Primers Highlighted</td>
<td>77</td>
</tr>
<tr>
<td>6.1</td>
<td>Frequency Distribution for Dinucleotide Repeats of the HMOX1 Microsatellite Polymorphism</td>
<td>84</td>
</tr>
<tr>
<td>7.1</td>
<td>Frequency Distribution for Dinucleotide Repeats of the HMOX1 Microsatellite Polymorphism</td>
<td>110</td>
</tr>
<tr>
<td>7.2</td>
<td>Frequency Distribution for Dinucleotide Repeats of the HMOX1 Microsatellite Polymorphism Divided by Ethnicity</td>
<td>111</td>
</tr>
<tr>
<td>8.1</td>
<td>Frequency Distribution for Dinucleotide Repeats of the HMOX1 Microsatellite Polymorphism</td>
<td>136</td>
</tr>
</tbody>
</table>
8.2 Frequency Distribution for Dinucleotide Repeats of the HMOX1 Microsatellite Polymorphism Divided by Ethnicity......................................................... 137

9.1 Relative HMOX1 Expression vs. Genotypes of Promoter Polymorphisms ...... 170

9.2 Average HO-1 Protein Concentration vs. Genotypes of Promoter Polymorphisms........................................................................................................... 173
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
<tr>
<td>Ala</td>
<td>alanine</td>
</tr>
<tr>
<td>ANG II</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>apoE</td>
<td>apolipoprotein E</td>
</tr>
<tr>
<td>ARE</td>
<td>antioxidant response element</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>CAD</td>
<td>coronary artery disease</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>cGMP</td>
<td>guanosine 3’,5’ cyclic monophosphate</td>
</tr>
<tr>
<td>CHF</td>
<td>congestive heart failure</td>
</tr>
<tr>
<td>CM</td>
<td>cardiomyopathy</td>
</tr>
<tr>
<td>CO</td>
<td>carbon monoxide</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>DCM</td>
<td>idiopathic dilated cardiomyopathy</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>ferrous iron</td>
</tr>
<tr>
<td>g</td>
<td>gravity</td>
</tr>
<tr>
<td>G</td>
<td>guanine or guanosine</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamic acid</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5’ trisphosphate</td>
</tr>
<tr>
<td>HIF-1</td>
<td>hypoxia inducible transcription factor 1</td>
</tr>
<tr>
<td>His</td>
<td>histidine</td>
</tr>
<tr>
<td>HMOX1</td>
<td>gene encoding heme oxygenase-1</td>
</tr>
<tr>
<td>HMOX2</td>
<td>gene encoding heme oxygenase-2</td>
</tr>
<tr>
<td>HO-1</td>
<td>heme oxygenase-1</td>
</tr>
</tbody>
</table>
HO-2  heme oxygenase-2
HO-3  heme oxygenase-3
HSF 1  heat shock factor 1
ICM  ischemic cardiomyopathy
IL-10  interleukin 10
iNOS  inducible nitric oxide synthase
kb  kilobase
kDa  kilodalton
LDL  low density lipoprotein
Leu  leucine
LVAD  left ventricular assist device
M  molar
MAPK  mitogen-activated protein kinase
MCP-1  monocyte chemoattractant protein 1
MCS-F  macrophage colony stimulating factor
mg  microgram
MI  myocardial infarction
ml  milliliter
mM  millimolar
MMP  matrix metalloproteinase
mRNA  messenger ribonucleic acid
NAC  N-acetylcysteine
NADPH  nicotinamide adenine dinucleotide phosphate (reduced)
NFκB  nuclear factor Kappa B
ng  nanograms
nm  nanomolar
nNOS  neuronal nitric oxide synthase
NO  nitric oxide
NOS  nitric oxide synthase
Nrf2  nuclear factor-E2-related factor 2
NSAID  nonsteroidal anti-inflammatory drugs
PBS  phosphate buffer solution
PCR  polymerase chain reaction
PDGF  platelet derived growth factor
PTCA  percutaneous transluminal coronary angioplasty
RNA  ribonucleic acid
ROS  reactive oxygen species
RT-PCR  reverse transcriptase polymerase chain reaction
sGC  soluble guanylate cyclase
SNP  single nucleotide polymorphism
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>thymine or thymidine</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate-EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>Tris/EDTA</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor – alpha</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>ug</td>
<td>micrograms</td>
</tr>
<tr>
<td>ul</td>
<td>microliter</td>
</tr>
<tr>
<td>uM</td>
<td>micromolar</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated regions</td>
</tr>
<tr>
<td>Val</td>
<td>valine</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
</tr>
<tr>
<td>Φ</td>
<td>Phi</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

1.1 Background

Heme oxygenase (HO) plays a central role in heme detoxification and iron recycling by degrading heme into carbon monoxide (CO), free iron, and biliverdin. HO-1, encoded by the gene HMOX1, is the inducible form of the enzyme. It is induced by stimuli that increase the production of reactive oxygen species or decrease cellular glutathione stores.[Camhi, 1998; Imenschuh, 2000; Lavrovsky, 1993; Poss, 1997b] The transcription of HO-1 is also stimulated by cGMP upregulated by nitric oxide.[Polte, 2000] Studies have shown that HO-1 plays a protective role in congestive heart failure, atherosclerosis, and ischemia/reperfusion injury.[Ishikawa, 1997; Kimpara, 1997; Yachie, 1999; Yamada, 2000]

HO provides protection in the cardiovascular system through the actions of its reaction products, biliverdin, free iron, and CO. After its production, biliverdin is immediately converted into bilirubin through the actions of biliverdin reductase. Bilirubin possesses oxyradical scavenging properties and is consumed upon the overproduction of oxyradicals as a suicide antioxidant. Research has shown that this compound has the ability to inhibit low density lipoprotein oxidation, to limit excessive
recruitment of inflammatory cells to sites of endothelial injury, to supplement the antioxidant activities of vitamins C and E, and to act as a protective agent in instances of reperfusion injury.[Clark, 2000; Galbraith, 1999; Poss, 1997b; Wang, 1998] This antioxidant is a valuable player in maintaining the integrity of the cardiovascular system.

The free iron released from heme degradation is in the ferrous (Fe$^{2+}$) state. It upregulates an iron-transporter pump that facilitates the removal of excess Fe$^{2+}$ from the cell and induces ferritin expression. Ferritin limits the generation of free radicals by binding free Fe$^{2+}$ that would normally participate in the Fenton reaction and promote the formation of reactive oxygen species. [Cotran, 1999]

CO has been shown to play a role in the cardiovascular system similar to that of nitric oxide. It can activate guanylyl cyclase to cause an increase in cGMP resulting in vascular smooth muscle cell relaxation and the inhibition of platelet aggregation. [Immenusch, 2000] CO has also been shown to inhibit smooth muscle cell proliferation, and to modulate the activity of cytochrome P450-dependent monooxygenase, an intracellular source of oxidant generation. [Poss, 1997b; Zaret, 1992] The actions of CO help to maintain steady blood flow throughout the cardiovascular system, as well as contributing to the regulation of systemic blood pressure. [NHLBI, 2005]

Examination of the HO-1 gene has lead to the identification of three distinct genetic abnormalities: a two-nucleotide deletion in exon 3, a single nucleotide polymorphism at position –413, and a promoter microsatellite polymorphism. The goal of the experiments in this dissertation is to examine the relationship between these polymorphisms and cardiovascular disease, placing special emphasis on the frequency distribution of these polymorphisms in the African American population.
1.2 Hypothesis and Research Objectives

The hypothesis for this dissertation is that the HMOX1 promoter polymorphisms are significantly associated with the African American population, making them more susceptible to cardiovascular diseases resulting from oxidative stress. This hypothesis led to the following goals:

1. Determine the frequency of the HMOX1 promoter polymorphisms in the general African American population.
2. Determine the frequency of the HMOX1 promoter polymorphisms in a population consisting of Caucasians and African Americans with coronary artery disease.
3. Determine the frequency of the HMOX1 promoter polymorphisms in a population of Caucasian and African American congestive heart failure patients.
4. Examine the combined effects of the HMOX1 promoter polymorphisms on gene expression.

1.3 Organization

This dissertation is organized into nine chapters:

1. Introduction
2. Heme and the Heme Oxygenase Enzyme System
3. The Role of Heme Oxygenase-1 in the Cardiovascular System
4. General Methods and Materials
5. Identification of Limitations within the Methodology of the Protocol to Examine the HMOX1 Exon 3 Dinucleotide Deletion
6. An Examination of the Association Between the HMOX1 Promoter Polymorphisms and the African American Population
7. An Examination of the HMOX1 Promoter Polymorphisms in Caucasian and African American Patients with Coronary Artery Disease

8. An Examination of the HMOX1 Promoter Polymorphisms in Caucasian and African American Patients with Congestive Heart Failure

9. An Examination of Heme Oxygenase-1 Expression in Human Left Ventricle Tissue Samples
CHAPTER 2

HEME AND THE HEME OXYGENASE ENZYME SYSTEM

2.1 Heme and the Heme Oxygenase Enzyme Reaction

The heme oxygenase enzyme system is active in almost all species and tissues. This enzyme is crucial because it catalyzes the degradation of heme and acts as a regulator of heme homeostasis and hemoprotein levels. Heme, also known as ferroprotoporphyrin IX, is a complex of an iron atom linked to the four ligand groups of porphyrin (Figure 2.1). It is highly conserved across species, as it is required for most forms of life and is a vital part of many useful redox reactions. [Galbraith, 1999; Maines, 1997; Wagener, 2003] Heme is synthesized in all nucleated cells of the body, with the most rapid rates of synthesis occurring in bone marrow erythroid cells and liver hepatocytes. These cells are responsible for the production of great quantities of hemoproteins. [Wagener, 2003] Heme is necessary for the formation and catalytic activity of numerous hemoproteins, including nitric oxide synthase (NOS), hemoglobin, cytochrome P450, and soluble guanylate cyclase (sGC). [Immenschuh, 2000; Maines,
When serving as the prosthetic moiety for such hemoproteins, heme facilitates vital cellular functions such as oxygen delivery, signal transduction, electron transport, gene transcription/translation, cell differentiation, and cell proliferation. [Dong, 2000; Immenschuh, 2000; Maines, 1997; Ryter, 2000a; Wagener, 2003]

**Figure 2.1: The Structure of the Heme Molecule**

When not bound to proteins, heme plays a detrimental role in organisms by reacting with molecular oxygen and catalyzing the production of cytotoxic reactive oxygen species (ROS). These ROS are destructive to cells and tissues causing DNA damage, lipid peroxidation, and protein denaturation. [Dong, 2000; Immenschuh, 2000;
Maines, 1997] Free heme is also lipophilic and therefore intercalates into cell
membranes and impairs lipid bilayers and organelles, causing tissues to undergo
cytoskeleton destabilization and mitochondrial dysfunction. [Platt, 1998; Wagener, 2003]
Free heme is found only under pathological conditions because under normal conditions,
y any excess heme produced by tissues for incorporation into hemoproteins is degraded by
heme oxygenase. Pathological situations involving oxidative stress allow free heme to be
released through the actions of free radicals. The free radicals denature hemoproteins and
damage cellular membranes allowing the newly released free heme to enter into and
damage cells. Oxidative stress also decreases the demand for hemoprotein production,
leading to increased amounts of newly synthesized free heme. Excessive amounts of free
heme may overwhelm the heme oxygenase system resulting in decreased heme
degradation and exacerbating cellular and tissue damage. [Maines, 1997; Ryter, 2000b;
Wagener, 2003]

Heme oxygenase is the only physiological method of heme degradation, and
therefore plays a critical role in the regulation of the cellular heme pool. [Dong, 2000;
Immenschuh, 2000] It also controls the distribution of intracellular iron by transferring
heme iron to the cell’s ferritin-iron pool. [Ryter, 2000a; Suematsu, 1999] Figure 2.2
illustrates the heme oxygenase enzyme reaction. This enzyme degrades the
protoporphyrin ring of heme, which consists of four pyrrole rings linked by methene
bridges, via oxidation resulting in the production of equimolar quantities of biliverdin,
free ferrous iron (Fe^{2+}), and carbon monoxide (CO). Biliverdin reductase then rapidly
transforms biliverdin into bilirubin, which is excreted into bile. [Dong, 2000; Galbraith,
1999; Perrella, 2003; Suematsu, 2001]
Heme catabolism requires the synchronous activity of NADPH-cytochrome P450 reductase, the reducing agent NADPH, and molecular oxygen. Three molecules of both oxygen and NADPH are used in the oxidation of a single heme molecule. The degradation of heme by heme oxygenase is the only colorimetric reaction in humans, and is readily observable when bruised skin fades from black (the color of heme released from injured erythrocytes) to green (the color of biliverdin) to yellow (the color of bilirubin). [Galbraith, 1999; Maines, 1997]

![Figure 2.2: The Heme Oxygenase Enzyme Reaction](image)

**Figure 2.2 : The Heme Oxygenase Enzyme Reaction**

### 2.2 The Isoforms of Heme Oxygenase

Two known isoforms of the heme oxygenase enzyme are expressed in humans, and a summary of each is found in Table 2.1. Both isoforms are anchored to the endoplasmic reticulum by a hydrophobic sequence of amino acids located at the enzymes’ carboxyl terminus. [Yoshida, 1988; Wagener, 2003] Heme oxygenase-1 (HO-
1) is a highly inducible 32 kDA protein expressed ubiquitously in various organs and cells in response to numerous stress related stimuli. [Suematsu, 2001] An in depth discussion of HO-1 will follow in Section 2.3. Heme oxygenase-2 (HO-2) is a constitutively synthesized 36 kDa protein expressed in many organs throughout the body. [Otterbein, 2000b; Perrella, 2003] There is little change in HO-2 protein expression, except with glucocorticoid treatment. The response of HO-2 to adrenal glucocorticoids is due to a single glucocorticoid response element present in its promoter. [Cornelussen, 2002; Maines, 1997] HMOX2, the gene for the HO-2 isoform, maps to chromosome 16p13.3. [Kutty, 1994]

A third isoform, heme oxygenase-3 (HO-3), was first described in rats as a 33 kDA protein resulting from a single transcript of approximately 2.4 kb. The HO-3 mRNA had similar tissue expression patterns as HO-2, but HO-3 protein was unable to be detected. [McCoubrey, 1997] Recently, Hayashi et al performed experiments to further characterize the gene structure of this isoform and found it was actually two HO-3-related genes, HO-3a and HO-3b. These genes lacked introns and contained sequences similar to exons 2-5 of the HMOX2 gene. RT-PCR analysis and Western blot analysis revealed these genes did not form mRNA or protein. The lack of introns, inability of related mRNA’s to be amplified by RT-PCR, and lack of related proteins led the investigators to hypothesize that HO-3a and HO-3b were pseudogenes. [Abraham, 2005; Hayashi, 2004] A pseudogene is a DNA sequence showing a high degree of sequence homology to a nonallelic functional gene but which is nonfunctional itself. It often consists of the coding DNA from another gene. [Strachan, 1996] The results suggest that
there is no functional HO-3 gene, and that the proposed isoform described by McCoubrey
*et al* is really a processed pseudogene derived from HO-2 transcripts. [Abraham, 2005;
Hayashi, 2004]
<table>
<thead>
<tr>
<th></th>
<th>HO-1</th>
<th>HO-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene and map locus</td>
<td>HMOX1 22q12</td>
<td>HMOX2 16p13.3</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>32 kDa</td>
<td>36 kDa</td>
</tr>
<tr>
<td>Examples of Physiological Roles</td>
<td>Heme degradation, Antioxidant defense, Regulation of hemoprotein activity</td>
<td>Heme degradation, Heme binding</td>
</tr>
<tr>
<td>Inducers of Expression</td>
<td>Anything producing oxidative stress</td>
<td>Adrenal glucocorticoids</td>
</tr>
<tr>
<td>Constitutive Tissue Expression</td>
<td>Spleen, liver</td>
<td>Most tissues, especially the brain, testes, spleen and liver</td>
</tr>
<tr>
<td>Code for Transcription Product</td>
<td>Single message of approximately 1.8 kb</td>
<td>Two or more transcripts resulting from the combination of three different 5’ UTRs and two different poly A signals</td>
</tr>
</tbody>
</table>

Table 2.1: The Isoforms of the Heme Oxygenase Enzyme
In mammals, HO-1 and HO-2 are primarily responsible for the degradation of heme, and they are evolutionarily conserved across species. HO-2 acts as the main catalyst of heme degradation under normal unstressed conditions, while HO-1 plays a major role during tissue responses to pathophysiological states. [Maines, 1997; Suematsu, 2001; Wagener, 2003] HO-2 possesses two heme-binding sites, and therefore has the capacity to bind free heme. Because of this, HO-2 is felt to act as the first line of defense against oxidative stress. [Ryter, 2000b; Wagener, 2003] During a sudden accumulation of free heme, HO-2 binds as much heme as possible. It then acts along with newly expressed HO-1 to catalyze heme degradation, therefore preventing cellular and tissue damage.

Under normal conditions, four major organs abundantly express heme oxygenase activity: the brain, liver, spleen, and testes. Monoclonal antibodies against rat HO-1 and HO-2 were used to examine isoform distribution under normal conditions, and showed HO-1 and HO-2 have very unique distribution patterns. [Maines, 1997; Suematsu, 2001] Under unstimulated conditions, HO-1 shows marked expression in spleen macrophages and liver Kupffer cells. Since the spleen and liver are two locations for the reticuloendothelial system, which removes senescent erythrocytes from circulation, upregulation of HO-1 expression results from the continuous exposure of these cells to hemoglobin-derived free heme molecules released as the erythrocytes are destroyed. [Suematsu, 2001; Wagener, 2003] HO-2 is highly expressed in the brain and testes. [Perrella, 2003] In these locations, it is believed to play the role of a physiological regulator of cellular function. [Wagener, 2003]
Even though they are located on different genes and have different gene expression patterns, HO-1 and HO-2 have similar mechanisms of heme catalysis, substrate specificity, and cofactor/coenzyme requirements. [Maines, 1997] They also have approximately 43% nucleotide and amino acid sequence similarity. [Immenschuh, 2000]

A 24-amino acid long segment is perfectly conserved among all forms of HO-1 and HO-2, with the only difference being the substitution of a leucine residue in HO-1 for a methionine residue in HO-2. This segment is hydrophobic and forms a pocket that binds heme pyrrole rings 1 and 2 through electrostatic interactions. The following portion of this conserved segment is known as the HO signature: Leu-Leu-Val-Ala-His-Ala-Tyr-Thr-Arg. The histidine residue found in the conserved segment is vital for both isoforms. In HO-1, the histidine facilitates heme catalysis, and in HO-2 it is essential for enzyme activity. The heme pocket does not recognize the metal moiety of metalloporphyrins and only has specificity for the porphyrin ring side chains. This means that non-physiological metalloproteins, such as tin protoporphyrin or zinc protoporphyrin, can compete against the natural substrate of heme oxygenase and inhibit enzyme activity. [Maines, 1997]

2.3 The Induction of Heme Oxygenase-1 Expression

The experiments in this dissertation focus on the HO-1 isoform due to its strong participation in cellular defense mechanisms against oxidative stress. [Ryter, 2000a] Oxidative stress occurs when the rate of ROS generation exceeds the ability of a cell’s natural defenses to protect itself. This could arise from an increase in the cellular production of ROS or from a depletion of cellular glutathione, an important intracellular antioxidant. [Immenschuh, 2000; Poss, 1997a; Ryter, 2000b; Ryter, 2002] Oxidative
stress is associated with many diseases, including atherosclerosis, acute renal failure, hypertension, ischemia/reperfusion, cancer, and Alzheimer’s disease. These same disease states involve increased expression of HO-1, leading many investigators to rely on HO-1 expression as an indicator of cellular stress and injury. [Otterbein, 2000b]

Expression of HMOX1, the gene encoding HO-1, occurs ubiquitously in mammalian cells and is considered an adaptive cellular response to prevent damage from oxidative stress. [Immenschuh, 2000; Ryter, 2000a] It is induced by multiple stimuli, all possessing the ability to generate ROS. These include heme, heat shock, ischemia/reperfusion, radiation, hypoxia, hyperoxia, inflammation, heavy metals, lipopolysaccharides, nitric oxide, and hydrogen peroxide. [Benjamin, 1998; Cornelussen, 2002; Immenschuh, 2000; Maines, 1997; Otterbein, 2000b] Many inducers of HO-1 promote the destabilization of intracellular hemoproteins, resulting in the release of the heme moiety. This creates a pro-oxidant situation readily diffused by the actions of HO-1. [Platt, 1998] The magnitude of HO-1 induction by oxidative stress can be greatly reduced or inhibited by the addition of antioxidants, such as N-acetyl cysteine (NAC), or iron chelators. [Immenschuh, 2000; Ryter, 2000a]

HMOX1 is located at chromosome 22q12 and is approximately 14 kb long, consisting of five exons and four introns. [Maines, 1997; Shibahara, 1989] Transcriptional control is mediated by multiple regulatory elements localized in the promoter 5’-flanking region of the gene. [Immenschuh, 2000] These include sites for oxidative stress-responsive transcription factors such as NFκB, activator protein-1 (AP-1), and Nrf2. The promoter also contains a heat shock element, antioxidant response elements, and hypoxia response elements. [Cornelussen, 2002; Dong, 2000; Ishikawa,
2003; Lavrovsky, 1993; Maines, 1997; Wagener, 2003] The NFκB site links the HMOX1 gene to inflammatory responses and is activated by free radicals. [Cornelussen, 2002; Dong, 2000] During oxidative stress, heat shock factor 1 (HSF1) is hyperphosphorylated in a ras-dependent manner by members of the MAPK subfamilies. This induces the HSF1 monomers to oligomerize as homotrimers, which then bind to an upstream sequence-specific heat shock element causing gene transcription. HMOX1 transcription is also upregulated after hypoxia-inducible transcription factor 1 (HIF-1) binds to the promoter hypoxia response elements. [Benjamin, 1998]

2.4 The Reaction Products of Heme Catabolism

The induction of HO-1 provides protection against the damaging effects of oxidative stress. The specific mechanisms by which HO-1 exerts cytoprotection are not totally clear, but the bilirubin, ferrous iron, and CO released during heme catabolism are active participants. [Nath, 2000; Otterbein, 2000b] The reaction products also help to regulate biological responses involving inflammation, cell survival, and cell proliferation. [Cornelussen, 2002; Perrella, 2003] HO-1 also contributes to cytoprotection by down-regulating hemoproteins, such as NOS, COX, and cytochrome P450 monooxygenase, whose reactions can contribute to oxidative stress. [Haider, 2002; Suematsu, 2001] Depending on cellular conditions and HO-1 expression levels, the reaction products could also cause deleterious effects. [Dong, 2000; Galbraith, 1999] A delicate balance between heme and the HO-1 system must be maintained in the tissue microenvironment to prevent oxidative damage. Examples of known activities for the reaction products are
summarized in Table 2.2. Further details about their protective actions in the cardiovascular system will be presented in Section 3.6.

<table>
<thead>
<tr>
<th></th>
<th>Positive Effects</th>
<th>Negative Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Carbon Monoxide</strong></td>
<td>Vasodilation, Anti-inflammatory actions, Anti-proliferative actions, Signal transduction</td>
<td>Poisonous to hemoproteins</td>
</tr>
<tr>
<td><strong>Biliverdin/Bilirubin</strong></td>
<td>Antioxidant</td>
<td>Damaging to biomembranes and cytotoxic</td>
</tr>
<tr>
<td><strong>Ferrous Iron</strong></td>
<td>Cytoprotection through ferritin upregulation</td>
<td>Pro-oxidant causing cellular injury</td>
</tr>
</tbody>
</table>

Table 2.2: Positive and Negative Effects of the Reaction Products from Heme Oxygenase Activity

2.5 Heme Oxygenase-1 Deficiency

HO-1 acts to rid cells of pro-oxidants released by destabilized hemoproteins and replaces them with the antioxidants biliverdin and bilirubin. It recruits ferritin, which sequesters iron and prevents iron-dependent oxidative stress. HO-1 also suppresses the production of cytokines and induces vasodilation through the release of CO. [Platt, 1998] Even though the reaction products have the potential for causing cellular and tissue damage, a lack of HO-1 function causes devastating results as illustrated by human HO-1
deficiency and HO-1 knock out mice. The following sections describe the only known case of human HO-1 deficiency, and the first studies utilizing HO-1 knock out mice. A discussion of the differences in the pathological manifestation of the HO-1 deficiency in humans versus mice also appears. Additional experiments dealing with knock out mice and the cardiovascular system are detailed in Section 3.7 of this dissertation.

2.5.1 Human HO-1 Deficiency

Yachie et al described the only known case of human HO-1 deficiency in 1999. It occurred in a Japanese male who at 26 months old first presented with recurrent fever and a generalized erythematous rash. By age 6, the patient displayed severe growth retardation, developmental delay, marked hepatomegaly, asplenia, and constant hematuria and proteinuria. Microscopic analysis provided evidence of iron deposition in both renal and hepatic tissue. The patient’s renal glomeruli showed detachment of the endothelium, with subendothelial deposition of an unidentified material. Blood analysis revealed increased leukocytes, platelets, and ferritin, as well as microcytic hypochromic anemia. The patient had marked abnormalities of the coagulation/fibinolysis system associated with elevated thrombomodulin and von Willebrand factor indicating the presence of severe, persistent endothelial damage. Hyperlipidemia, with extremely high total cholesterol and triglyceride values, was also present. The patient’s total bilirubin levels were constantly low and serum heme concentration extremely high, which signaled an abnormality in the hemoglobin metabolic pathway. This led Yachie et al to examine the patient’s heme oxygenase activity. [Yachie, 1999]

Immunohistochemistry of hepatic tissue samples from the patient revealed a complete absence of HO-1. Peripheral blood mononuclear cells cultured from the patient
did not produce any detectable HO-1 after stimulation with cadmium, a known inducer of HO-1. Lymphoblastoid cell lines from the patient produced only HO-2 and no detectable HO-1 after cadmium stimulation. The cell lines from the patient were also extremely sensitive to hemin-induced cell injury. Cell lines created from control samples and the patient’s parents produced comparable levels of HO-1 and HO-2 with cadmium stimulation. [Yachie, 1999]

Reverse transcription-PCR for HO-1 mRNA was run on RNA extracted from the cadmium stimulated lymphoblastoid cell lines in order to perform mutational analysis of the HMOX1 gene. Control samples and a sample from patient’s father displayed a single band of mRNA, while samples from the patient and his mother expressed mRNA of two different sizes. The patient predominantly possessed the smaller sized mRNA, with only a minor amount being near the normal size. Direct sequencing of the mRNA bands revealed that when compared to the known HMOX1 sequence, the larger paternal band contained a two-nucleotide deletion within exon 3 and the smaller maternal band displayed a complete loss of exon 2. The patient was shown to have both of these deletions. Yachie et al felt that the lack of HO-1 could result in extreme vulnerability to common stressful stimuli resulting in a cascade of inflammatory reactions and vascular endothelial damage. The resulting sustained oxidative injury, and lack of protection from HO-1, could then cause many of the complications seen in the patient. [Yachie, 1999]

Several months after being diagnosed with the HO-1 deficiency, the patient’s condition deteriorated with the onset of hypertension, subdural hemorrhage, and mental status changes. The patient ultimately died and Kawashima et al reported the results of his autopsy. In spite of his young age, the patient’s liver and adrenal glands contained
amyloid deposits, and his aorta contained fatty streaks and fibrous plaques. His kidneys showed an irregular distribution of foamy macrophages with iron pigments, and mesangiolipoproliferative glomerular changes. Kawashima et al felt that the erythrocyte fragmentation and hemolysis, the disseminated intravascular coagulation, and mesangiolipoproliferative glomerular changes seen in the patient resulted from endothelial injury and reticuloendothelial dysfunction due to a lack of HO-1. [Kawashima, 2002] A summary of their results can be seen in Figure 2.3. Spectral analysis of plasma samples taken from the patient revealed substantial oxidation of plasma hemoglobin to methemoglobin, a situation that promotes the formation of oxidized low density lipoprotein (LDL). Further analysis of the patient’s sample revealed oxidized LDL was present, establishing another source for the endothelial damage present in the HO-1 deficient patient. [Jeney, 2002]
Figure 2.3: Origins of Pathology Seen in Human HO-1 Deficiency
2.5.2 Heme Oxygenase Knock Out Mice

In 1997, Poss and Tonegawa generated the first HO-1 knock out mice by removing a 3.7 kb fragment from the HMOX1 gene. The deleted segment contained an intron sequence and approximately 85% of the total coding sequence for the HO-1 protein. Due to high embryonic lethality in knock out mating pairs, the mice had to be generated through *in vitro* fertilization techniques utilizing gametes from one HO-1 homozygous knock out and one HO-1 heterozygous knock out mouse. The resulting HO-1 deficient mice were smaller and thinner than their littermates, bred poorly, and commonly experienced premature mortality. As adults, the knock out mice developed normochromic, microcytic anemia, associated with abnormally low serum iron levels. They also exhibited pathological iron loading, accumulating hepatic and renal iron that contributed to macromolecular oxidative damage, tissue injury, and chronic inflammation. Progressive chronic inflammatory disease was shown by an enlarged spleen and lymph nodes, hepatic inflammatory cell infiltrates, vasculitis, and glomerulonephritis. [Kawashima, 2002] The pathologies displayed by the HO-1 knock out mice illustrate the critical role of HO-1 in the release and recycling of iron from murine hepatic and renal tissue stores. [Poss, 1997a]

Poss and Tonegawa also examined the role of HO-1 in cellular antioxidant defense. Murine embryonic fibroblasts cultured from the HO-1 deficient animals were exposed to several oxidants, including hemin and hydrogen peroxide. The cells displayed an increase in free radical production and a hypersensitivity to oxidant-induced cytotoxicity. Young adult HO-1 knock out mice challenged with endotoxin, were markedly sensitive to hepatic injury and mortality caused by oxidative stress. The results
of these experiments provided genetic evidence that HO-1 serves as an important enzymatic antioxidant system. [Poss, 1997b]

Yet et al exposed HO-1 knock out mice to hypoxia (10% oxygen) for five to seven weeks in order to examine the effect of HO-1 on cellular adaptation. After seven weeks of chronic hypoxia, the right ventricles from the HO-1 deficient mice were severely dilated and contained right ventricle infarcts with mural thrombi. Cardiomyocytes from these animals showed lipid peroxidation and oxidative damage. Many apoptotic cardiomyocytes could be detected in the areas surrounding the infarcted myocardium. This study revealed that an absence of HO-1 causes cardiomyocytes to have a maladaptive response to hypoxia and hypoxia induced pulmonary hypertension. [Yet, 1999]

Nath et al used HO-1 knock out mice to demonstrate the critical role of HO-1 in protection against acute hemoprotein induced toxicity. The investigators utilized a glycerol model of hemoprotein induced tissue injury consisting of intramuscular injections of hypertonic glycerol that would induce myolysis and hemolysis, subsequently exposing the tissues to large amounts of myoglobin and hemoglobin. Experiments involving the glycerol model and the direct infusion of hemoglobin resulted in the knock out mice exhibiting fulminant, irreversible kidney failure and mortality. This study showed that genetic deletion of HO-1 renders tissues vulnerable to the damaging effects of hemoproteins, and emphasized the importance of the enzyme’s antioxidant and heme catalyzing activities. [Nath, 2000]
2.5.3 A Comparison of Human HO-1 Deficiency and HO-1 Knock Out Mice

Mice lacking HO-1 tend to show predominant iron metabolic disorders and have long survival rates. In comparison, the case of human HO-1 deficiency resulted in severe injury of the endothelial cells and reticuloendothelial system, resulting in a greatly shortened survival rate. Both have similar clinical features, but differ greatly pathologically. Many foam cells were found in the liver and generalized reticuloendothelial tissue of the human patient, while none were found in the knock out mice. Despite his young age, the human deficient in HO-1 had marked amyloid deposition, while none is seen in senile HO-1 knock out mice. Kawashima et al conjectured that injury to the sinusoidal endothelium, together with possible macrophage dysfunction, might lead to the deposition of an amyloid precursor of unknown origins.

The human patient also had prominent atherosclerotic changes with fatty streaks and fibrous plaques not seen in the knock out mice. These fibrous plaques were characterized by the proliferation of smooth muscle cells, and did not contain foamy macrophages normally found in atherosclerotic plaques. [Kawashima, 2002] HO-1 expression has been shown to protect against excessive cell proliferation in the vascular system, and CO has been shown to inhibit the proliferation of smooth muscle cells. [Duckers, 2001] A lack of HO-1 may lead to the abnormal regulation of proliferation signals for smooth muscle cells. This could result the excess of smooth muscle cells seen in the fibrous plaques of the HO-1 deficient patient. In summary, it seems that the human case of HO-1 deficiency is more severely affected by oxidative stressors than HO-1 knock out mice. [Kawashima, 2002]
2.6 The Polymorphisms of the HMOX1 Gene

The devastating consequences of a lack of HO-1 activity are clearly illustrated by the case of human HO-1 deficiency and by studies utilizing HO-1 knock out mice. The complete deletion of exon 2 and the two-nucleotide deletion in exon 3 of the HMOX1 gene in the human case combined to prevent the production of HO-1. Aside from the exon 3 deletion study reported in Chapter 5 of this dissertation, no other research has been published dealing with the polymorphisms seen in the HO-1 deficient patient and their effect on disease. Three other polymorphisms have been identified in the 5’ flanking region, or promoter, of the HMOX1 gene: a single nucleotide polymorphism (SNP) at base –1135, a SNP at base –413, and a (GT)n microsatellite polymorphism at base –257. Only the SNP at –413 and the (GT)n microsatellite polymorphism have been found to influence promoter function and are associated with disease states. Figure 2.4 shows the HMOX1 promoter region and the locations of these important polymorphisms. The polymorphisms and their effects on HO-1 activity will be described in the following sections.
The SNP at –413 is highlighted in green, and the microsatellite polymorphism at –257 is highlighted in pink.

2.6.1 The HMOX1 T(-413)A Single Nucleotide Polymorphism

In 2003, Ono et al published the first accounts of the HMOX1 promoter T(-413)A SNP. Interest in the role of HO-1 in oxidative stress mediated pathologies prompted the investigators to perform complete sequence analysis on 96 Japanese individuals to screen for sequence variations in the HMOX1 promoter region. This resulted in the discovery of the SNP at -413. After identifying the SNP through sequencing, mutation-specific probes were then used for genetic analysis of an additional 1,902 Japanese subjects. The reported genotype frequency for 1,958 of the 1,998 subjects is as follows: AA = 22% (n = 425), AT = 46% (n = 902), and TT = 32% (n = 631). Cell culture experiments involving a HMOX1 promoter/luciferase fusion gene showed that the presence of the A allele
caused eight-fold greater promoter activity when compared to a fusion gene containing the T allele. [Ono, 2003] Studies dealing with the influence of the HMOX1 T(-413)A SNP on hypertension and ischemic heart disease are detailed in Section 3.9.1.

2.6.2 The HMOX1 (GT)n Microsatellite Polymorphism

Shibahara et al were the first to report the presence of an alternating (GT)n sequence located between base –257 and –198 of the HMOX1 promoter in their 1997 paper detailing the structural organization of the HMOX1 gene. [Shibahara, 1989] Such purine-pyrimidine alternating sequences are known to assume a Z-DNA conformation that negatively affects transcriptional activity. [Exner, 2004; Naylor, 1990; Shibahara, 1989] Chromosomal DNA is most commonly found in the B-DNA form consisting of a right-handed helix with 10 base pairs per turn. The Z-DNA form consists of a left-handed helix with 12 base pairs per turn. [Champe, 1994; Marks, 1996] This Z-DNA conformation has been cited as the basis for observed differences in the transcriptional activity of HMOX1 promoters containing varied (GT)n repeat lengths, and in vivo footprinting experiments have shown a complete lack of protein binding to the microsatellite region. [Exner, 2004] Taken together, this evidence suggests that the conformational changes resulting from the (GT)n microsatellite polymorphism interfere with the transcriptional regulation of HMOX1.

Numerous studies have examined the relationship between the length of the HMOX1 (GT)n microsatellite polymorphism and diseases involving oxidative stress. In
the different study populations, the GT repeat varies in size from 12 to 40 and has a bimodal distribution with the main alleles at 23 and 30 repeats. Several studies have examined the impact of the various repeat sizes on transcriptional activity through the use of HMOX1/luciferase fusion genes transfected into different cell lines. Cell lines containing GT repeats less than 25 tend to have increased transcriptional activity upon stimulation when compared to cells containing constructs with greater than 25 GT repeats. [Chen, 2002; Exner, 2004; Yamada, 2000] Hirai et al created lymphoblastoid cell lines from subjects with known GT repeat lengths and showed that oxidative stress induced HMOX1 gene expression and HO-1 enzyme activity were significantly higher in cell lines with repeats smaller than 27 than in cell lines with repeats greater than or equal to 33. [Exner, 2004; Hirai, 2003] The relationship between the (GT)n microsatellite polymorphism and the cardiovascular system is described in Section 3.9.2.
CHAPTER 3

THE ROLE OF HEME OXYGENASE-1 IN THE CARDIOVASCULAR SYSTEM

3.1 Introduction

The previous chapter described the important actions of HO-1 in preventing injury mediated by oxidative stress. This chapter focuses on the role of the HO-1 enzyme reaction in cardiovascular diseases associated with oxidative stress, such as coronary artery disease, cardiomyopathy, congestive heart failure, and transplant rejection. The relationship between the heme oxygenase and nitric oxide synthase (NOS) enzyme systems are examined. This chapter includes examples of how HO-1 inhibition exacerbates and HO-1 overexpression alleviates cardiovascular disease. Interactions between the enzyme and pharmacologic agents commonly used in the treatment of cardiovascular disease are described. Finally, a discussion of the HMOX1 promoter polymorphisms and their association with cardiovascular pathologies is presented.
3.2 Coronary Artery Disease and Atherosclerosis

3.2.1 Background

Coronary artery disease (CAD) is the leading cause of death in industrialized nations. It occurs when coronary arteries become hardened and narrowed due to a build up of atherosclerotic plaques on the inner artery walls. These plaques cause a reduction in blood flow to the heart, resulting in myocardial ischemia. The most common symptoms of CAD are chest pain/discomfort and shortness of breath. Clinically, it can manifest as a myocardial infarction (MI), angina pectoris, arrhythmia, heart failure, or sudden cardiac death. [Cotran, 1999; NHLBI, 2005] Risk factors for CAD include age, family history, hypercholesterolemia, hypertension, smoking, diabetes, obesity, and a sedentary lifestyle.

Atherosclerosis is an inflammatory process initiated as a consequence of endothelial injury. It is characterized by a progressive accumulation of lipids and fibrous elements resulting in plaque formation. [Ishikawa, 2003; Ishikawa, 2001a] Endothelial injury results from a variety of sources, including hyperlipidemia, hypertension, and smoking. Injury causes endothelial dysfunction, which is characterized by increased permeability, increased leukocyte adhesion, and monocyte adhesion/emigration to subendothelial locations. [Cotran, 1999]

Lipoproteins, especially low-density lipoprotein (LDL), accumulate in the extracellular subendothelial space of the artery. Monocytes and macrophages recruited to the subendothelial space create an environment rich in ROS, resulting in the formation of oxidized LDL. Macrophages ingest the oxidized LDL through scavenger receptors and become foam cells. These foam cells then accumulate in the core of the atherosclerotic
Plaque. [Hoekstra, 2004] Plaque development is then advanced by vascular smooth muscle cell (VSMC) proliferation and deposition of collagen and other extracellular matrix proteins. [Cotran, 1999]

The treatment strategy for CAD focuses on relieving symptoms, impeding atherosclerosis, preventing blood clot development, widening or bypassing blocked arteries, and reducing cardiac events. Treatment commonly involves lifestyle changes, cholesterol-lowering medications, anticoagulants, antiplatelet medications, ACE inhibitors, beta-blockers, calcium channel blockers, and nitroglycerin or long-acting nitrates. Percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass surgery are also used as treatment methods for CAD. [NHLBI, 2005]

3.2.2 The Role of Oxidant Stress in Coronary Artery Disease and Atherosclerosis

The development of atherosclerosis is greatly influenced by ROS. Common risk factors for atherosclerosis, such as diabetes, hypertension, and smoking, increase the amount of oxidative stress in the vascular system. [Ishikawa, 2003] The endothelial damage at the root of plaque formation can induce the formation of ROS via the actions of several enzymes, including xanthine oxidase, NADPH oxidase and uncoupled eNOS. [Ishikawa, 2003; Schwartz, 2001] Endothelial cells, VSMCs, macrophages, and foam cells found in atherosclerotic plaques also generate ROS. These ROS activate transcription factors, such as NFkB and AP-1, inducing genes that further the inflammatory process and plaque formation. [Ishikawa, 2003] ROS are also involved in the formation of oxidized LDL, a key step in the progression of atherosclerosis, and have
been implicated in vasospasm, loss of endothelial-dependent vasodilation, and other toxic effects linked to oxidized lipoproteins. [Cotran, 1999; Schwartz, 2001]

Commonly performed treatment procedures for CAD also promote oxidative stress. PTCA induces vascular injury, triggering an inflammatory reaction and VSMC proliferation. [Schillinger, 2004] Restenosis of the newly opened vessel can develop as a result of inflammation, constrictive vascular remodeling, and neointima formation by proliferating VSMCs. [Schillinger, 2004] PTCA and coronary artery bypass surgery reopen blocked blood vessels, resulting in the reperfusion of ischemic myocardial tissues. This process generates oxidative stress and accelerated inflammation leading to cardiomyocyte death, and is referred to as ischemia/reperfusion injury. [Yet, 2001]

3.2.3 The Association of HO-1 with Coronary Artery Disease and Atherosclerosis

Under normal physiological conditions, both HO-1 and HO-2 are expressed at low levels in VSMCs and cardiomyocytes. Under conditions of oxidative stress, HO-1 is highly induced in the heart and blood vessels. [Yet, 1999] Many stressors in the cardiovascular system induce HO-1, including proinflammatory cytokines, growth factors, hemodynamic changes, oxidized lipoproteins, ANG II, NO, hypertension, hypoxia, and heme. [Endler, 2004; Hoekstra, 2004; Ishikawa, 2003] These stressors create ROS that upregulate the transcription of HMOX1 by interacting with promoter NrF2 and antioxidant response element (ARE) sites. [Hosoya, 2005; Ishikawa, 2003] In the artery wall, HO-1 acts to scavenge ROS, resulting in the attenuation of monocyte adhesion and chemotaxis, and the reduction of lipid peroxidation. [Ishikawa, 2003] HO-1 also acts to protect cardiomyocytes from ischemia/reperfusion injury. [Yet, 2001] The
protective actions of HO-1 can be attributed to the enzyme’s reaction products and interactions with ANGII, NOS, and cyclooxygenase (COX). [Abraham, 2003; Haider, 2002; Li Volti, 2003; Mazza, 2003; Wagener, 2003]

In 1990, Wang et al first examined the expression of HO-1 in atherosclerotic lesions. Aortic sections from humans and apolipoprotein E (ApoE)-deficient mice were used for immunostaining and in situ hybridization experiments. HO-1 expression was displayed in the endothelium and foam cells of early lesions from both species. HO-1 expression was also detected in the medial VSMCs of advanced atherosclerotic lesions. Experiments exposing cultured murine peritoneal macrophages to either native LDL or oxidized LDL showed that the induction of HO-1 mRNA was limited to cultures treated with oxidized LDL. [Wang, 1998] Later research established that the expression of HO-1 in atherosclerotic lesions colocalized with oxidized LDL, strongly suggesting that HO-1 is induced in vivo by oxidized LDL. [Hoekstra, 2004; Ishikawa, 2001b]

Co-cultures of human aortic endothelial cells and VSMCs were used by Ishikawa et al to examine the role of HO-1 in early atherosclerosis. HO-1 expression did not occur under basal conditions and was not upregulated by native LDL. Minimally modified LDL highly induced HO-1, and this induction was augmented by hemin pretreatment. The increase of HO-1 resulted in a reduction of monocyte chemotaxis in response to oxidized LDL. Monocyte chemotaxis was also reduced if cell cultures were pretreated with biliverdin or bilirubin and enhanced with HO-1 inhibition. [Ishikawa, 1997] A later study established that the expression of HO-1 in aortic endothelial cells from an atherosclerosis susceptible mouse strain was upregulated by oxidized LDL. [Shi, 2000]
These results further solidified the link between HO-1 and the oxidative modification of LDL. [Ishikawa, 2003]

3.3 Cardiomyopathy and Congestive Heart Failure

3.3.1 Background

Cardiomyopathy (CM) is a heart disease caused by a primary abnormality in the myocardium. [Cotran, 1999] It is marked by cardiac hypertrophy, an adaptive remodeling response to increased cardiac wall stress caused by blood pressure or volume overload. [Hu, 2004] Direct myocardial damage causes a disruption in the heart’s pumping ability, resulting in inadequate blood flow. Pump dysfunction results from a loss of functional myocytes and/or a shift in myocyte contractility due in part to the reexpression of fetal isoforms involved in contraction and/or calcium hemostasis. [Sawyer, 2002] Congestive heart failure (CHF) is the inability of the heart to provide the body’s organs and tissues with an adequate blood supply and is the endpoint of CM. [Zaret, 1992] The following cellular changes occur in the ventricles of failing hearts: myocyte growth, loss of myocytes through apoptosis or necrosis, and myocyte slippage caused by the degradation of collagen due to activated matrix metalloproteinases (MMP). [Sawyer, 2002] There are two major classes of cardiomyopathies, nonischemic and ischemic, which are summarized in Table 3.1. Regardless of etiology, patients suffering from CHF present with the following symptoms: shortness of breath, fatigue, weakness, and edema. [NHLBI, 2005]
<table>
<thead>
<tr>
<th>Description of Condition</th>
<th>Ischemic Cardiomyopathy</th>
<th>Nonischemic or Idiopathic Dilated Cardiomyopathy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Congestive heart failure caused by coronary artery disease</td>
<td>Congestive heart failure characterized by progressive cardiac hypertrophy, dilation, and contractile dysfunction</td>
</tr>
<tr>
<td>Description of Diseased Heart</td>
<td>Enlarged, heavy heart with left ventricular hypertrophy and dilation; Scars from previous myocardial infarction often present</td>
<td>Weighs 2-3 times normal, is large and flabby, with dilation of all chambers</td>
</tr>
<tr>
<td>Description of Coronary Arteries</td>
<td>Moderate to severe atherosclerosis present</td>
<td>Usually free of significant atherosclerosis</td>
</tr>
<tr>
<td>Common Treatment Regimen</td>
<td>Medications to relieve both CHF and CAD; PTCA and coronary bypass surgery; Heart transplant</td>
<td>Medications to relieve symptoms of CHF and improve heart function, including cardiac glycosides, diuretics, and ACE inhibitors; Implantation of LVAD; Heart transplant</td>
</tr>
</tbody>
</table>

Sources: [Cotran, 1999; Zaret, 1992]

**Table 3.1: Summary of Ischemic and Nonischemic Cardiomyopathy**
3.3.2 The Role of Oxidant Stress in Cardiomyopathy and Congestive Heart Failure

Oxidative stress can induce myocyte hypertrophy, apoptosis, fetal gene expression and increased MMP activity, cellular changes that contribute to myocardial remodeling in cardiomyopathy. In order to slow or reverse the remodeling process, pharmacological agents such as vasodilators, ACE inhibitors, and beta-blockers, interrupt the intercellular pathways mediating the phenotype changes of cardiomyocytes. [Sawyer, 2002] CHF is a direct result of oxidative stress, as evidenced by elevated levels of malondialdehyde, an indicator of oxidative stress, in the blood of CHF patients. [Belch, 1991] ROS have been implicated in the development of myocyte apoptosis and fibrosis, two common characteristics in CHF. [Sharma, 2000] Oxidative stress also plays a unique role in ischemic cardiomyopathy through its involvement in atherosclerosis and the damaging effects of ischemia/reperfusion injury that can occur after PTCA or bypass surgery. [Clark, 2000b; Ishikawa, 1997; Sharma, 1999; Siow, 1999; Wang, 1998]

Large redox imbalances occur in failing hearts of both animal models of heart failure and CHF patients. [Feuerstein, 1998] After inducing hypertrophy and heart failure in young guinea pigs, Dhalla and Singal found higher antioxidant activity and lower levels of lipid peroxidation in hypertrophied hearts compared to failing hearts. The investigators proposed that a relative deficit in myocardial antioxidant capacity and redox state might play a role in the pathogenesis of heart failure. [Dhalla, 1994] The same experiments were repeated with the addition of long-term vitamin E therapy to examine the effects of this naturally occurring antioxidant on the progression from hypertrophy to heart failure. As previously seen, the hypertrophied hearts displayed decreased oxidative
stress compared to the failing hearts. Treatment with vitamin E improved the myocardial redox state and prevented the progression to heart failure. These results substantiated a role for increased oxidative stress in the pathogenesis of heart failure, and suggested a potential therapeutic value for antioxidant treatment in modulating the pathogenesis of heart failure. [Dhalla, 1996]

Tumor necrosis factor-α (TNF-α) and ANG II are known to provoke hypertrophic responses in the myocardium leading to heart failure. Nakamura et al hypothesized that ROS generated by the signal transduction pathways of TNF-α and ANG II may contribute to the myocyte hypertrophy. *In vitro* experiments involving neonatal rat cardiomyocytes revealed that ROS generation was induced in a dose-dependent manner by both TNF-α and ANG II, and that the presence of the ROS caused myocyte hypertrophy. Antioxidant treatment of cell cultures inhibited the hypertrophic response. [Nakamura, 1998] Tanaka et al furthered the results by examining the effects of ROS on hypertrophy in adult rat cardiomyocytes. Cell cultures were stimulated with endothelin-1 or phenylephrine resulting in an increase in ROS levels and subsequent hypertrophy. The stimulated cardiomyocytes also showed an increase in extracellular signal-regulated kinase (ERK) activity. Antioxidant treatment suppressed the increase in ROS and prevented ERK activation, therefore preventing myocyte hypertrophy. [Tanka, 2001]

### 3.3.3 The Association of HO-1 with Cardiomyopathy and Congestive Heart Failure

In 1993, Katayose et al examined HO-1 mRNA expression in rat hearts exposed to right ventricle pressure overload. An increase in HO-1 mRNA was seen in both ventricles, providing the first evidence that hemodynamic stress, a major contributor to
cardiac hypertrophy, contributes to the upregulation of HO-1. [Katayose, 1993] Later research involving an \textit{in vivo} murine pressure-overload model of left ventricle hypertrophy also found that increased HO-1 mRNA expression accompanied hypertrophy. In these experiments, hypertrophy was also associated with an increase in lipid peroxidation, further demonstrating the role of oxidative stress in the genesis of cardiac hypertrophy. [Date, 2002]

Raju et al examined the role of HO-1 in a canine model of chronic right-sided heart failure and found that right ventricles from failing hearts showed a significant increase in HO-1 mRNA and protein, accompanied by a significant increase in cGMP levels. The increase in HO-1 was also seen with norepinephrine infusion, leading the investigators to hypothesize that the increase in interstitial norepinephrine levels commonly seen in failing myocardium contributes to the induction of the HO-1 system. The induction of HO-1 may act as a defense mechanism to protect the myocardium against oxidative stress involved in heart failure. [Raju, 1999]

A volume overload model of heart failure used to examine HO-1 expression and activity in lung parenchyma during heart failure found that both HO-1 expression and activity were increased in the rats with heart failure. HO-1 was localized in pulmonary siderophages, pulmonary macrophages that phagocytize erythrocytes leaked from capillaries congested as a result of heart failure. Lam et al concluded that the increase in HO-1 activity and subsequent stimulation of downstream signaling by cGMP and p38 MAPK may play an important protective role during the pathogenesis of heart failure. [Lam, 2005]
Grabellus et al examined the distribution of HO-1 in vivo and in vitro to determine its relationship to improvements in cardiac function resulting from the use of left ventricular assist devices (LVAD). The improvement in cardiac function, known as reverse remodeling, is believed to be the result of reduced local hypoxia eliminating tissue stress. Left ventricle tissue sections were taken from patients with end-stage heart failure before and after LVAD support, with unused donor hearts serving as controls. When compared to the control samples, HO-1 protein levels were substantially increased in failing hearts before LVAD support. After LVAD support, HO-1 expression was significantly decreased. [Grabellus, 2002] The investigators also cultured neonatal rat cardiomyocytes under hypoxic conditions, resulting in a sixfold increase in HO-1 protein expression. When the cultures were exposed to a normoxic environment, HO-1 protein measurements returned to basal levels. The reversible upregulation of HO-1 seen in both the in vivo and in vitro studies supports the hypothesis that reverse remodeling is in part triggered by the restoration of cardiac normoxia and subsequent elimination of oxidative stress by mechanical unloading. [Grabellus, 2002]

3.4 Transplant Rejection

3.4.1 Background

Heart transplantation became a viable treatment method for patients in severe heart failure in the early 1970’s. [Zaret, 1992] It has become so successful that more than 85% of recipients survive the first year and more than 70% survive five years. [Heartcenteronline, 2005] The requirements for the ideal transplant recipient vary between hospitals, but generally include the following: (1.) the patient is in end-stage
heart disease, (2.) all other medical interventions have failed, (3.) the patient is less than 65 years old, (4.) the patient is stable enough to survive a major surgery, and (5.) the patient has refrained from the use of tobacco products for at least six months.

[Heartcenteronline, 2005; Zaret, 1992] An ideal donor heart comes from a person less than 35 years old who sustained brain death without evidence of chest injury. The donor should be free of infection, lack a history of cardiac illness, and should not have undergone prolonged cardiopulmonary resuscitation. [Zaret, 1992] Hearts are matched on the basis of urgency of need, blood type, and size. [Heartcenteronline, 2005; Zaret, 1992]

A successful heart transplant is influenced by three main factors: (1.) careful matching of the donor and recipient, (2.) properly monitored immunosuppression, and (3.) early detection of acute rejection through numerous endomyocardial biopsies. [Cotran, 1999] Acute rejection occurs when the recipient’s immune system attacks the newly implanted heart and is characterized by lymphocytic infiltrates present in the endomyocardial biopsy specimen. Recipients are commonly treated with cyclosporin, a drug that suppresses the T-cell lymphocytes responsible for rejection. Cyclosporin is unique because it acts in immunosuppression yet allows the body to maintain its ability to fight against infection. Infection is the other major limiting factor in recipient survival. [Chok, 2002b; Cotran, 1999; Zaret, 1992] If caught in the early stages, acute rejection can be reversed by increasing immunosuppressive therapy. In advanced stages, acute rejection results in severe myocardial damage, creating an irreversible and often fatal situation. [Cotran, 1999]
Chronic rejection is often characterized by the development of transplant CAD, a condition seen in approximately half of all transplant recipients. [Heartcenteronline, 2005] It is a chronic inflammatory process characterized by a progressive and diffuse intimal thickening of the coronary arteries causing decreased blood supply to the myocardium leading to ischemia, hypoxia and dysfunction. [Holweg, 2004; Saraiva Camara, 2005] Recipients must undergo periodic cardiac catheterization to monitor for the development of transplant CAD. Advanced transplant CAD commonly results in CHF or sudden death. [Cotran, 1999]

3.4.2 The Role of Oxidant Stress in Transplant Rejection

Ischemia/reperfusion injury is the single major source of oxidative stress in cardiac allografts. Ischemia/reperfusion injury can trigger proinflammatory responses that activate T-cell lymphocytes thereby promoting the development of acute rejection. [Saraiva Camara, 2005] It has also been implicated in the pathogenesis of transplant CAD. The ROS that accumulate during ischemia/reperfusion injury can overwhelm endogenous antioxidant genes leading to the necrosis or apoptosis of endothelial cells, VSMCs, and myocytes. This activates cytokines and growth factors involved in the process of intimal thickening. [Holweg, 2004; Saraiva Camara, 2005] Endomyocardial biopsy samples from human transplant recipients reveal a high prevalence of apoptotic endothelial cells and myocytes in cases of transplant rejection. [Laguens, 1996] Exposure to a pro-oxidant environment also activates endothelial cells, resulting in vasoconstriction, leukocyte activation and adhesion, and thrombosis. [Soares, 2001]
3.4.3 The Association of HO-1 with Transplant Rejection

Vascular expression of HO-1 is associated with preventing rejection in animal models of heart transplantation. Hancock et al investigated the mechanisms behind long-term cardiac allograft survival in mice treated with CD40-ligand monoclonal antibody and donor-specific spleen cells in order to block T-cell co-stimulation. The results showed that allograft survival correlated with increased vascular expression of protective genes such as HO-1 and Bcl-xl, and that in vivo induction of HO-1 protected against the atherosclerosis associated with chronic rejection. [Hancock, 1998; Platt, 1998]

Chen et al used a model of rat aortic transplantation to examine the effect of systemic IL-10 expression on neointimal proliferation and inflammation, two features of chronic vascular rejection. IL-10 is a cytokine with anti-inflammatory, immunosuppressive, and immunostimulatory properties similar to those of HO-1. Rats pre-treated with an adenovirus vector for IL-10 displayed a significant reduction in neointimal proliferation along with reduced macrophage and lymphocyte graft infiltration. The protective effects were reversed in the presence of HO-1 inhibitors, indicating that HO-1 is an important mediator used by IL-10 to regulate the inflammatory responses involved in chronic rejection. [Chen, 2005]

Soares et al showed rejection of mouse-to-rat cardiac xenografts could be prevented through the use of a treatment regimen involving brief complement inhibition by cobra venom factor and sustained T-cell immunosuppression by cyclosporin A. This regimen results in accommodation, a situation in which incompatible heart transplants survive in the presence of circulating antibodies and complement that normally cause rejection in naïve grafts. Endothelial cells and VSMCs taken from rat xenograft
recipients displaying accommodation express anti-inflammatory genes, especially HMOX1. Transplant experiments involving hearts from mice with targeted disruption of HMOX1 revealed that HO-1 deficient hearts were unable to achieve accommodation. [Platt, 1998] Soares et al speculate that the expression of HO-1 is functionally associated with xenograft survival and that rapid expression of HO-1 in cardiac xenografts is essential to protect against long-term rejection. [Soares, 1998; Soares, 2001]

The intracellular balance between anti-apoptotic and pro-apoptotic proteins determines whether a cell undergoes apoptosis, a process that plays a significant role in transplant rejection. Chok et al examined the expression of anti-apoptotic HO-1 in endomyocardial biopsy samples from human transplant recipients. During episodes of acute rejection, HO-1 expression was reduced in the endothelial cells, cardiomyocytes and infiltrating cells of the biopsy sample. An inverse correlation was discovered between the expression of HO-1 and the degree of cellular apoptosis. These results emphasize the important role of HO-1 mediated inhibition of apoptosis in transplant survival. [Chok, 2002a; Chok, 2002b]

3.5 The Relationship Between the Heme Oxygenase and Nitric Oxide Synthase Enzyme Systems

3.5.1 The Nitric Oxide Synthase Pathway and the Cardiovascular System

Nitric oxide synthase (NOS) is an enzyme that converts L-arginine and molecular oxygen to L-citruline and nitric oxide (NO). There are three isoforms of NOS. Endothelial NOS (eNOS) is encoded by NOS3 and found bound to the membranes of numerous cell types. Neuronal NOS (nNOS) is encoded by NOS1 and located in the
cytosol. These isoforms are constitutively expressed and activated by the intracellular calcium-dependent binding of calmodulin. [Kimura, 2003] In the cardiovascular system, eNOS is located in endothelial cells, platelets, VSMCs, and cardiac myocytes. The transcription of eNOS can be upregulated by hypoxia, shear stress, oxidized LDLs, and mechanical forces. [Albrecht, 2003] Inducible NOS (iNOS), is encoded by NOS2 and is induced in a variety of cells by inflammatory signals. The induction of iNOS does not depend on intracellular calcium levels. [Kimura, 2003]

NOS plays a key role in the cardiovascular system through its production of NO. NO can directly affect biological molecules through S-nitrosylation reactions. NO also influences surrounding cells by binding to the iron in the heme moiety of sGC and activating the enzyme. [Hartsfield, 2002] This activation causes an increase in cGMP, which then triggers a cascade of transcriptional and translational responses. [Albrecht, 2003; Wang, 2000] Small amounts of NO perform the following physiologically important roles in the cardiovascular system: (1.) maintenance of VSMC relaxation, (2.) regulation of vascular tone, (3.) regulation of blood flow to tissues, (4.) regulation of blood pressure, (5.) regulation of myocardial contractility, (6.) regulation of endothelial integrity and permeability, (7.) regulation of vascular cell proliferation, (8.) regulation of endothelial-leukocyte interaction, (9.) inhibition of platelet aggregation and adhesion, and (10.) exertion of an overall antiatherogenic effect. [Albrecht, 2003; Hartsfield, 2002; Jugdutt, 2002; Kimura, 2003; Schwartz, 2001;]

Abnormal NO production has been associated with the development of ischemic heart disease, hypertension, hypercholesterolemia, atherosclerosis, diabetes mellitus, arterial restenosis, and heart failure. [Jugdutt, 2002] In turn, NOS activity and
subsequent NO production can be affected by cardiovascular pathology. Endothelial trauma, chronic hyperlipidemia, oxidized LDL, and ROS have all been shown to decrease NO production. [Cotran, 1999; Duckers, 2001; Ishikawa, 2001a; Schwartz, 2001]

### 3.5.2 The Interaction between Heme Oxygenase and Nitric Oxide Synthase

The HO and NOS enzyme systems share many similarities. Both have constitutive and inducible isoforms, require molecular oxygen and NADPH to work, and increase cGMP production through the activation of sGC. The enzyme reaction products NO and CO can act as neurotransmitters, inhibitors of platelet aggregation, and vasodilators. CO and NO can act in a synergistic, compensatory and/or counterregulatory manner depending on the microenvironment into which they are released. [Hartsfield, 2002]

There are many ways in which HO can regulate the NOS reaction. The HO reaction degrades heme, a key component in the NOS enzyme, which can then reduce the amount available for NOS production. [Galbraith, 1999; Immenschuh, 2000] Fe²⁺ can reduce NOS production by preventing its transcription, and high amounts of CO can bind to NOS inhibiting its activity. [Alcaraz, 2003; Dong, 2000; Hartsfield, 2002; Maines, 1997] Low concentrations of CO and the reduction of ROS by biliverdin and bilirubin have been shown to increase NO production. [Hartsfield, 2002; Schwartz, 2001] NOS, HO, and biliverdin reductase all require NADPH for enzymatic activity. In cases where all three enzymes are active, the competition for electrons shifts in favor of the HO pathway. [Hartsfield, 2002]

NOS also has the ability to regulate the HO reaction. Numerous *in vitro* studies in which cell cultures were treated with NO donors have shown increases in both HO-1
mRNA and protein levels. [Durante, 1997; Hara, 1999; Polte, 2000; Siow, 1999] The NO-dependent induction of HO-1 is regulated by the cGMP-dependent activation of CRE and AP-1 transcription sites on the HMOX1 promoter. [Immenschuh, 2000; Suematsu, 1999; Suematsu, 2001] NO has also been found to increase the half-life of HO-1 mRNA, therefore contributing to increased HO-1 levels. [Bouton, 2000; Hartsfield, 2002] Peroxynitrite, a compound derived from NO, has also been found to induce HO-1 activity. [Alcaraz, 2003; Hartsfield, 2002; Wagener, 2003] Figure 3.1 summarizes the regulatory effects the NOS and HO-1 enzyme systems have on each other.
3.5.3 The Combined Actions of Heme Oxygenase and Nitric Oxide Synthase in the Cardiovascular System

ROS play important roles in the cardiovascular system by participating in cellular signaling and are generated by the actions of pro-oxidant enzymes such as NOS. The levels of ROS must be carefully balanced to avoid generating oxidative stress. In the cardiovascular system, antioxidant enzymes such as HO-1 are used to maintain proper cellular redox states. Cardiovascular disease can result from an imbalance between these enzyme systems. Heritable polymorphisms in NOS3, the gene encoding pro-oxidant
eNOS, have been shown to increase ROS production and the risk of atherosclerosis. A deficiency the antioxidant activity of HO-1 favors ROS accumulation and increases the risk of vascular disease. [Leopold, 2005]

The NOS and HO-1 enzyme systems have been found to complement each other in fighting cardiovascular disease. Vascular trauma decreases eNOS activity in endothelial cells while inducing HO-1 in VSMCs, allowing for HO-1 activity to compensate for diminished NO production. [Benjamin, 1998; Christou, 2000; Duckers, 2001; Ishikawa, 2003] HO-1 modulation is inversely associated with plasma levels of nitrite and nitrate, the stable end products of NO, further illustrating the interdependence of these systems. [Ishikawa, 2001b] CO and bilirubin, products of the HO-1 enzyme reaction, have been identified as the causative mediators in the antiatherosclerotic actions of NO and cGMP. [Hoekstra, 2004; Siow, 1999] Recent work has shown that circulating endothelial progenitor cells overexpressing both eNOS and HO-1 transplanted into balloon-injured vessels contributed to the prevention of restenosis. [Abraham, 2005] The NOS and HO-1 enzymes also work together to modulate the expression of VEGF as illustrated in Figure 3.2, thereby influencing the process of angiogenesis in the cardiovascular system. [Dulak, 2002; Hartsfield, 2002; Kimura, 2003; Maines, 1997]
3.6 The Protective Role of HO-1 in the Cardiovascular System

HO-1 provides protection in the cardiovascular system through its anti-inflammatory, vasodilatory, anti-proliferative, anti-apoptotic, and antioxidant actions. This section describes how each of the HO-1 reaction products is involved in the protection of the cardiovascular system. It also highlights some of the numerous studies examining the benefits of HO-1 overexpression in the cardiovascular system.
3.6.1 The Actions of Carbon Monoxide

The majority of endogenous CO found in humans is produced by the heme oxygenase enzyme reaction. [Otterbein, 2000b] When present at low concentrations, this gaseous messenger molecule provides protection in the cardiovascular system through anti-inflammatory, vasodilatory, and anti-proliferative mechanisms. [Perrella, 2003, Suematsu, 2001] CO participates in cell signaling by binding to iron in the heme moiety of soluble guanylate cyclase (sGC) therefore inducing the enzyme to convert GTP to cGMP.

The anti-inflammatory effects of CO have been illustrated by in vitro experiments in which the CO-induced production of cGMP inhibited platelet activation and aggregation. [Morse, 2002; Otterbein, 2000b; Siow, 1999; Wagner, 1997] CO inhibits pro-inflammatory genes while augmenting anti-inflammatory cytokine production through the selective activation of several p38 MAPK signaling pathways. [Otterbein, 2000a] CO can also attenuate inflammatory responses by modulating the activity of cytochrome P450-dependent monoxygenases, an intracellular source of oxidants. [Suematsu, 1999; Suematsu, 2001] The anti-inflammatory effects of CO reduce ischemia/reperfusion injury in vivo, and suppress the pro-inflammatory response of monocytes and macrophages to oxidized lipoproteins in atherosclerotic plaques. [Otterbein, 2003; Schillinger, 2004; Yet, 2001] The ability of CO to inhibit platelet aggregation and suppress pro-inflammatory responses has also been shown to protect against the rejection of transplanted hearts. [Morse, 2002; Otterbein, 2003; Sato, 2001; Soares, 1998]
Vasoconstriction compromises the lumen size of blood vessels and can increase local hemodynamic forces that contribute to both the formation of atherosclerotic plaques and plaque fracturing. [Cotran, 1999] The risk factors for atherosclerosis have also been associated with the loss of endothelium-dependent vasoregulation. [Schwartz, 2001] CO has been shown to directly mediate peripheral vasodilation through the activation of sGC and subsequent production of cGMP. [Chen, 2003; Morse, 2002; Siow, 1999] CO also directly stimulates calcium-activated potassium channels to promote vasodilation VSMCs. [Chen, 2003; Ishikawa, 2003] CO can act in the nucleus tractus solitarii of the central nervous system to promote changes in glutamatergic neurotransmission and thereby lower systemic blood pressure. [Morse, 2002] CO induced vasodilation allows for the maintenance of blood flow under inflammatory conditions, helping to prevent the anoxia and tissue necrosis that contributes to transplant rejection. [Soares, 2001]

CO produced as a result of the local upregulation of HO-1 can block neointimal formation elicited by arterial injury, such as that resulting from PTCA. [Otterbein, 2003; Suematsu, 1999; Togane, 2000] CO produced by VSMCs acts upon endothelial cells to inhibit their expression of platelet derived growth factor (PDGF) and endothelin-1. [Morita, 1995; Morse, 2002; Perrella, 2003;) This acts as a negative feedback mechanism to inhibit VSMC growth, stopping the cells at the G1/S transition phase of the cell cycle. [Morita, 1995; Morse, 2002; Peyton, 2002] Duckers et al determined that this cell cycle arrest was connected to the expression of p21^{Cip1}, a G1 cyclin-dependent kinase inhibitor. [Duckers, 2001] In vivo experiments involving balloon injury of rat carotid arteries demonstrated that HO-1 derived CO decreased artery neointimal thickness and
medial wall thickness, thereby providing evidence of reduced VSMC proliferation. [Morse, 2002; Togane, 2000]

CO generated by HO-1 in the cardiovascular system has also been found to have anti-apoptotic effects. Work by Brouard et al showed that CO production prevents apoptosis in endothelial cells through a mechanism dependent on the activation of the p38 MAPK signal transduction pathway. The results also suggest that the CO from cells expressing HO-1 acts as an intracellular signaling molecule to prevent the apoptosis of surrounding cells lacking HO-1 expression. [Brouard, 2000] The anti-apoptotic effect of the p38 MAPK pathway on endothelial cells is mediated through interactions with NFκB resulting in the expression of NFκB dependent anti-apoptotic genes. [Soares, 2001] CO has also been shown to inhibit the apoptosis of rat aortic VSMCs in vitro, an ability that may play an important role in blocking lesion formation at sites of vascular injury. [Liu, 2002] The ability of CO to suppress endothelial cell apoptosis has been shown to prevent chronic rejection in cardiac allografts. [Morse, 2002; Soares, 1998; Soares, 2001]

3.6.2 The Actions of Biliverdin and Bilirubin

Both biliverdin and bilirubin are bile pigments that act as strong antioxidants. [Maines, 1997; Perrella, 2003; Suematsu, 2001] Bilirubin is the most abundant endogenous antioxidant in mammalian tissue and accounts for the majority of antioxidant activity in human serum. [Otterbein, 2000b] It’s ability to inhibit the oxidation of LDL and the formation of ROS provides protection against atherosclerosis, CAD, and ischemia/reperfusion injury. [Clark, 2000b; Hoekstra, 2004; Mayer, 2000; Perrella, 2003] The inhibition of lipid peroxidation by biliverdin and bilirubin has been shown to prevent monocyte chemotaxis and leukocyte adhesion to the vascular endothelium. [Hulea, 1995;
Numerous studies have found an inverse relationship between plasma bilirubin concentrations and the presence of CAD. [Breimer, 1995; Hopkins, 1996; Hunt, 1996; Schwertner, 1994; Schwertner, 1998] Several known risk factors for CAD also show an inverse correlation with plasma bilirubin concentrations, including smoking, diabetes and obesity. [Madhavan, 1997; Schwertner, 1998]

Biliverdin and bilirubin inhibit complement-dependent reactions in vitro, suggesting another possible way these bile pigments act in tissue protection. [Nakagami, 1993] Clark et al examined the dynamics of HO-1 expression and bilirubin production in VSMCs after hemin stimulation. The results showed that increased bilirubin, as a consequence of HO-1 induction, acts in protecting VSMCs from oxidant stress. [Clark, 2000a] Bilirubin has been shown to have an inhibitory effect on protein phosphorylation and protein kinase C activity, both of which can lead to the inactivation of proatherogenic factors. [Amit, 1993; Hansen, 1996; Nakagami, 1993; Willis, 1996] Exogenously applied bilirubin also has the ability to prevent ANG II-mediated endothelial cell DNA damage and cell death by suppressing pro-oxidant activities. [Abraham, 2005]

3.6.3 The Actions of Ferrous Iron

The free ferrous iron released during the HO-1 reaction plays a protective role in the cardiovascular system by modulating the binding of iron regulatory proteins to the iron-responsive elements of ferritin mRNA, thereby causing an increase in ferritin synthesis. [Galbraith, 1999; Otterbein, 2000b] Ferritin is a cellular storage system for iron, and its synthesis has been shown to increase or decrease along with HO-1 activity or inhibition, respectively. [Dong, 2000; Galbraith, 1999; Immenschuh, 2000; Otterbein, 2000b] Work by Balla et al demonstrated that HO-1 mediated enhancement of
intracellular ferritin decreased the cytotoxic effects of oxidant damage on vascular endothelial cell cultures. [Balla, 1992] Studies by Hoekstra et al utilizing endothelial cell cultures from atherosclerosis-susceptible and atherosclerosis-resistant strains of Japanese quail showed that exposure to oxidative stress caused different responses in the cell types. The resistant cells responded with increased HO-1 expression and ferritin upregulation, resulting in their cytoprotection. The susceptible cells displayed lower HO-1 activity and higher levels of intracellular catalytic iron, causing them to succumb to oxidative damage. [Hoekstra, 2003a; Hoekstra, 2003b] Ferritin is upregulated in early atherosclerotic lesions where it may function with HO-1 to protect cells from the damaging effects of iron overload. [Wang, 1998] Ferritin induction has also been found to protect against ischemia/reperfusion injury. [Perrella, 2003]

Generating high levels of ferrous iron through increased HO-1 activity can trigger the actions of an iron ATPase located in the endoplasmic reticulum. This enzyme acts as an iron pump limiting intracellular iron content. [Otterbein, 2000b; Wagener, 2003] The upregulation of this iron pump has been associated with preventing apoptosis in fibroblasts from HO-1 deficient mice. Decreased intracellular iron content reduces the amount of ROS generated through the Fenton reaction, therefore preventing the ROS-mediated induction of pro-apoptotic signal transduction pathways. [Ferris, 1999; Wagener, 2003] The sequestration and elimination of excess iron can also prevent endothelial cell apoptosis, thereby promoting the survival of cardiac allografts. [Soares, 2001]
3.6.4 The Overexpression of HO-1

The numerous protective actions of the HO-1 enzyme reaction products in the cardiovascular system prompted investigators to examine the effects of HO-1 overexpression on the pathogenesis of cardiovascular disease. The literature reviewed in this section highlights studies involving both pharmacological and transgenic HO-1 induction. The effects of HO-1 overexpression on angiogenesis, hypertension, cardiac hypertrophy, atherosclerosis, and ischemia/reperfusion injury are discussed.

In 1998, Deramaudt et al transfected rabbit coronary endothelial cells with the human HO-1 gene. These cells showed a twofold increase in blood vessel formation, facilitated through an increase in VEGF production. The study provided evidence that the induction of HO-1 following vascular injury is an important tissue adaptive mechanism for moderating the severity of cell damage produced by inflammatory reactions. [Deramaudt, 1998]

Later work by Dulak et al revealed that rat VSMCs stimulated with hemin to increase HO-1 expression showed enhanced VEGF production. The increase in VEGF production was reversed with blockage of the HO-1 pathway. Rat VSMCs were also transfected with HMOX1 resulting in a significant increase in VEGF protein. [Dulak, 2002]

Hypertension is involved with the pathogenesis of CAD and cardiac hypertrophy. Numerous studies demonstrated that the acute or chronic treatment of spontaneously hypertensive rats with HO-1 inducers causes a normalization of blood pressure. [Chen, 2003; Perrella, 2003; Sacerdoti, 1989] A retroviral vector containing human HO-1 cDNA injected into the hearts of spontaneously hypertensive rats, resulted in transgene overexpression in the kidneys, liver, heart, lungs and brain. The human HO-1 was active
in the rats and attenuated the development of hypertension. [Sabaawy, 2001] Rats undergoing chronic hypoxia experience a sustained induction of vasoconstrictors, resulting in vasoconstriction and remodeling of pulmonary arterioles. Rats under hypoxic conditions treated with agonists to increase HO-1 expression fail to develop pulmonary hypertension or display pulmonary arteriole remodeling. [Christou, 2000]

Seki et al pharmacologically induced HO-1 mRNA to examine the effects on cardiac hypertrophy in spontaneously hypertensive rats. Treated rats showed significant decreases in their left ventricular/body weight ratio and levels of left ventricular brain natriuretic peptide mRNA, two markers of cardiac hypertrophy. These results showed that induction of HO-1 in the heart attenuates cardiac hypertrophy through a load-independent mechanism in spontaneously hypertensive rats. [Seki, 1999] Hu et al examined the effects of HO-1 overexpression on ANG II induced cardiac hypertrophy. An HO-1 inducer or a recombinant adenovirus carrying the human HO-1 gene were used to stimulate HO-1 production in rats and cultures of rat neonatal cardiomyocytes exposed to ANG II. HO-1 overexpression suppressed ANG II induced hypertrophy both in vitro and in vivo, and this suppression appeared to be the result of bilirubin inhibiting the production of ROS. [Hu, 2004]

Ishikawa et al used LDL-receptor knock out mice to examine the effect of HO-1 overexpression on the development of atherosclerotic lesions. Mice were fed a high fat diet and injected with HO-1 inducers, HO-1 inhibitors, or saline. The proximal aortas from mice treated with HO-1 inducers had significantly less atherosclerotic lesions than controls, while mice treated with HO-1 inhibitors had larger lesions than controls. The results suggested that HO-1 induced under hyperlipidemia protected against the
development of atherosclerosis, possibly by inhibiting lipid peroxidation and influencing the NO pathway. [Ishikawa, 2001b] Juan et al used an adenovirus to transfer HO-1 into arteries of apoE-deficient mice to discover if HO-1 overexpression could reduce iron overload and inhibit atherosclerosis. The aortas of treated mice contained significantly smaller atherosclerotic lesions and lower iron deposition compared to controls. The results revealed that the overexpression of HO-1 in vascular cells facilitates iron metabolism and attenuates the development of atherosclerosis in apoE-deficient mice. [Juan, 2001]

Tulis et al investigated the ability of localized adenovirus-mediated HO-1 gene delivery to modify arterial remodeling after balloon angioplasty in rat carotid arteries. Arteries treated with HO-1 had significantly reduced neointimal area, medial wall area, neointimal area/medial wall area ratio, and neointimal thickness when compared to controls. The medial wall of the treated arteries had a reduction in viable cells, a situation contributing to diminished neointimal development. The results indicate that HO-1 is an important in vivo vasoprotective mediator capable of attenuating remodeling after vascular injury. [Tulis, 2001]

Duckers et al used pig arteries transfected with HO-1 encoding vectors for vascular reactivity studies in the presence of a NOS inhibitor. The experiments showed that HO-1 overexpression significantly upregulated vascular relaxation through a mechanism mediated by sGC and cGMP yet independent of NO. Experiments were then performed in which porcine femoral arteries were injured and transfected with the HO-1 gene. The treated arteries showed significantly reduced intimal and medial cell proliferation associated with a significant reduction in intimal lesion formation. In vitro
experiments involving VSMCs transfected with HO-1 revealed that HO-1 attenuates DNA synthesis and cell proliferation through cGMP and guanylate cyclase activation. These experiments also led to the discovery that p21\textsuperscript{Cip1} contributes significantly to the antiproliferative effects of HO-1, as previously discussed in Section 3.6.1. [Duckers, 2001]

Numerous studies have examined the effects of HO-1 overexpression on ischemia/reperfusion injury. The cardiac-specific overexpression of HO-1 in mice exposed to ischemia/reperfusion injury has resulted in improved cardiac function, smaller areas of MI, reduced myocyte apoptosis, reduced inflammation, and reduced oxidative damage. [Chen, 2003; Hoekstra, 2004; Vulapalli, 2002; Yet, 2001] Melo et al pretreated rat myocardium with an adenovirus vector containing HO-1 several weeks before acute coronary artery ligation and release. The treated myocardium displayed remarkable protection against ischemia/reperfusion injury. The treated myocardium also showed decreases in the pro-apoptotic protein Bax and pro-inflammatory cytokine interleukin-1β, and an increase in the anti-apoptotic protein Bcl-2. [Melo, 2002] Pachori et al, utilizing a vector system designed to place HO-1 under ischemia-regulated expression, conducted a similar pretreatment experiment. Ischemic insult to the myocardium caused a rapid induction of HO-1 resulting in the prevention of myocardial tissue remodeling and the normalization of myocardial function. The investigators hypothesize the use of this ischemia-regulated HO-1 vector could be a future treatment option for patients at high risk for ischemia/reperfusion injury. [Pachori, 2004]
3.7 The Effect of HO-1 Inhibition or Deficiency on the Cardiovascular System

Section 2.5.1 of the previous chapter reviewed the only known case of human HO-1 deficiency. The young patient had severely damaged vascular endothelium, hypertension, and atherosclerosis. [Kawashima, 2002; Yachie, 1999] Section 2.5.2 described the first studies utilizing HO-1 knock out mice, including experiments by Yet et al in which HO-1 deficient mice developed right ventricular infarcts and damaged cardiomyocytes in response to hypoxia. [Yet, 1999] This section will highlight additional studies examining the effects of HO-1 deficiency or inhibition on the cardiovascular system.

Experiments in which normal rats were treated with metalloporphyrins, inhibitors of HO-1, resulted in increased systemic arterial pressure. [Chen, 2003; Perrella, 2003] Yang et al injected murine left ventricles with a retrovirus containing an HO-1 antisense sequence to block HO-1 expression and activity. Rats treated with the antisense retrovirus showed decreased renal HO-1 protein expression and activity compared to controls, and had significant increases in mean arterial pressure when given ANG II. These results indicate that endogenous HO-1 is required to counteract the pressor responsiveness of rats to ANG II. [Yang, 2004] Studies involving normal mice, mice heterozygous for a lack of HO-1, and homozygous HO-1 knock out mice showed no differences in systolic blood pressure at baseline between the three populations, indicating that the chronic absence of HO-1 does not lead to a sustained increase in systolic blood pressure. [Chen, 2003; Perrella, 2003]

Several studies have examined the impact of decreased HO-1 on the generation of atherosclerotic plaques. In 2001, Ishikawa et al examined the effects of HO-1 inhibition
on atherosclerotic lesion formation in Watanabe heritable hyperlipidemic rabbits, an animal model of human familial hypercholesterolemia. Rabbits treated with an HO-1 inhibitor had significantly greater atherosclerotic lesions and increased lipid peroxide levels compared to controls. [Ishikawa, 2001a] Yet et al generated mice deficient in both HO-1 and apoE and found that when fed a high fat diet, these double knock out mice had accelerated atherosclerotic lesion formation compared to mice lacking apoE alone. Yet et al also examined the influence of HO-1 deficiency on neointimal formation in a mouse model of vein graft stenosis. Vein grafts from mice lacking HO-1 developed larger neointima compared to vein grafts from normal mice, and VSMCs isolated from the knock out mice were more susceptible to oxidative stress induced death. [Yet, 2003] Duckers et al found that after injury, lesion formation was very severe in the femoral arteries of HO-1 knock out mice compared to wild type mice. VSMCs from the knock out mice had a greater proliferation rate than wild type cells. Further investigation revealed that VSMCs lacking HO-1 had reduced levels of p21$^{\text{Cip1}}$ cyclin-dependent kinase inhibitor, the mediator of HO-1’s antiproliferative effects. [Duckers, 2001]

Yoshida et al examined the effect of ischemia/reperfusion injury in mice heterozygous for HO-1 deficiency. These mice had a 40% reduction in HO-1 protein compared to wild type mice. After undergoing ischemia and reperfusion, hearts from the heterozygous mice were more susceptible to oxidative stress than wild type mice. The HO-1 deficient hearts had reduced ventricular recovery, increased creatine kinase release, and increased infarct size. Antioxidant treatment partially rescued the heterozygous hearts from ischemia/reperfusion injury, illustrating the vital role HO-1 plays as an intracellular antioxidant. [Yoshida, 2001]
HO-1 inhibition can also effect transplant rejection. Sato et al. performed mouse-to-rat cardiac transplantations accompanied by cobra venom factor and cyclosporin A treatment. The HO-1 inhibitor tin protoporphyrin was also given to the donor and recipient. HO-1 inhibition led to graft rejection in a manner similar to that seen when hearts from HO-1 knock out mice are used for transplantation. The rejection was characterized by widespread hemorrhagic infarction associated with vascular thrombosis and leukocyte infiltration. Exposure to exogenous CO suppressed graft rejection in the presence of the HO-1 inhibitor, an effect due to the ability of CO to inhibit platelet aggregation, thrombosis, MI, and apoptosis. [Sato, 2001]

3.8 The Pharmacological Interactions of HO-1

Many pharmacological agents used to treat cardiovascular disease interact with HO-1, as summarized in Table 3.2. In the vascular system, aspirin decreases platelet aggregation and thrombus formation, and has anti-inflammatory and antioxidant effects. [Grosser, 2003; Mycek, 2000] Losartan is an ANG II antagonist used in the treatment of hypertension. [Ishizaka, 1997b] Pentaerythrityl tetranitrate exerts long-term antioxidant and antiatherosclerotic effects in the cardiovascular system. [Oberle, 2002] Probucol, a drug with weak cholesterol lowering and antioxidant abilities, is best known for its ability to reduce restenosis after coronary angioplasty. [Deng, 2004; Lau, 2003] Rapamycin is an immunosuppressive agent that blocks cell cycle progression in the G1 phase, now being used to coat coronary stents to prevent restenosis. [Ishikawa, 2003] Statins are
lipid-lowering agents that also have anti-inflammatory and antioxidant actions.

Treatment with statins significantly decreases cardiovascular morbidity and morality in patients with CAD. [Grosser, 2004b; Mycek, 2000]
<table>
<thead>
<tr>
<th>Drug</th>
<th>Interaction with HO-1</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>Increases HO-1 protein and enzyme activity through an NO-dependent pathway protecting human endothelial cell cultures from oxidative stress mediated injury.</td>
<td>Grosser, 2003</td>
</tr>
<tr>
<td>Losartan</td>
<td>Blocks upregulation of HO-1 mRNA resulting from ANGII induced hypertension in endothelial cells of rat aortas; Blocks downregulation of HO-1 in VSMCs</td>
<td>Ishizaka, 1997a and 1997b</td>
</tr>
<tr>
<td>Pentaerythritol Tetranitrate</td>
<td>Increases HO-1 mRNA and protein in human endothelial cell cultures, protecting them from oxidative stress.</td>
<td>Oberle, 2002</td>
</tr>
<tr>
<td>Probucol</td>
<td>Induces HO-1 expression resulting in the inhibition of VSMC proliferation therefore preventing intimal thickening.</td>
<td>Deng, 2004</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>Increases HO-1 mRNA and protein in human pulmonary artery endothelial cells and smooth muscle cells preventing the proliferation of PDGF-stimulated smooth muscle cells.</td>
<td>Visner, 2003</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>Increases HO-1 mRNA and protein levels in cultured human endothelial cells resulting in a reduction in the NADPH-dependent production of oxygen radicals.</td>
<td>Grosser, 2004a</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>Increases HO-1 activity in cultures of human and rat aortic VSMCs resulting in anti-inflammatory and antiproliferative effects.</td>
<td>Lee, 2004</td>
</tr>
<tr>
<td>Simvastatin and Lovastatin</td>
<td>Increases HO-1 mRNA and protein levels in cultured human endothelial cell resulting in reduction of ROS and protection from oxidative stress mediated injury.</td>
<td>Grosser, 2004b</td>
</tr>
</tbody>
</table>

Table 3.2: The Pharmacological Interactions of HO-1
3.9 The HMOX1 Polymorphisms and their Relationship to Cardiovascular Disease

In the previous chapter, Section 2.6 described the promoter polymorphisms of HMOX1, the single nucleotide polymorphism at base –413 and the (GT)n microsatellite polymorphism at base –257. The following section details studies examining the relationship of these polymorphisms to cardiovascular disease.

3.9.1 The Single Nucleotide Polymorphism at Base –413 of HMOX1

As discussed in Section 2.6.1, Ono et al. were the first group to characterize the SNP-413 in the HMOX1 promoter. In the original paper describing the polymorphism, a significant association was discovered between the AA genotype and arterial hypertension in Japanese women. The authors proposed that the hypertension could be caused by an interaction between estrogen-induced NOS expression and HO-1 derived CO, which attenuates NO-induced vasodilation. [Ono, 2003]

In a separate study, Ono et al. examined the relationship between the SNP-413 polymorphism and ischemic heart disease in the Japanese population. A control population and a cohort of patients with a history of myocardial infarction or angina pectoris were genotyped. Multiple logistic regression analysis showed that gender, smoking history, diabetes mellitus, body mass index, and the SNP-413 genotype affected the occurrence of ischemic heart disease. The AA genotype was significantly associated with a reduced incidence of ischemic heart disease in the Japanese population. [Ono, 2004]

3.9.2 The HMOX1 Promoter Microsatellite Polymorphism

Multiple studies examined the relationship between the HMOX1 (GT)n microsatellite polymorphism and the development of CAD. Chen et al. screened a
Chinese population of 474 patients with angiographically verified CAD and 322 healthy controls. An association between the presence of 32 or more GT repeats and an increased risk for CAD was found in subjects with type II diabetes mellitus. [Chen, 2002] Kaneda et al screened a Japanese population consisting of 577 patients who underwent selective coronary angiography because of suspected CAD, and found that patients possessing risk factors, such as hypercholesterolemia, diabetes mellitus, and smoking, with less than or equal to 26 GT repeats were less likely to develop CAD. [Kaneda, 2002] Endler et al screened a Caucasian population of 258 patients with myocardial infarction, 180 patients with stable CAD, and 211 healthy controls, and was unable to find any significant associations between the microsatellite polymorphism and the development of CAD. These results led the investigators to speculate that the length of the microsatellite polymorphism may be related to ethnicity. Endler et al did observe that subjects with microsatellite repeats of less than 25 had higher levels of bilirubin and a more favorable lipid profile than subjects with higher repeat alleles. [Endler, 2004]

Restenosis, which can develop after balloon angioplasty or coronary stenting, involves many of the factors inhibited by HO-1, including inflammation, vascular remodeling, and VSMC proliferation. [Exner, 2004] In 2001, Exner et al examined the association between the HMOX1 microsatellite polymorphism and restenosis after peripheral percutaneous transluminal angioplasty in a Caucasian population. Patients with alleles containing less than 25 GT repeats had reduced postdilation restenosis six months after the procedure. [Exner, 2001b] Later work by the same group involving a different cohort of peripheral angioplasty patients revealed that patients with alleles of less than 25 GT repeats had lower levels of postinterventional C-reactive protein.
[Schillinger, 2004] The decreased inflammatory response in patients with the short microsatellite allele may help to prevent the development of restenosis. Chen et al examined the association between the microsatellite polymorphism and adverse events after coronary artery stenting in an Asian population. The results revealed a 3.74-fold increased risk for angiographic restenosis in patients carrying an allele with dinucleotide repeats of greater than or equal to 26. The study also showed that during the twenty month follow up period, patients homozygous for the large repeat alleles had a 3.26-fold increased risk for adverse cardiac events, such as death, non-fatal myocardial infarction, and unstable angina requiring revascularization. [Chen, 2004]

HO-1 has been shown to be an important factor in protecting against the rejection of transplanted organs. Holweg et al hypothesized that patients with the genetic capacity for decreased HO-1 or who receive a heart from a donor with decreased HO-1 are more prone to acute rejection or transplant CAD. A predominantly Caucasian cohort of 311 heart transplant recipients and 263 donors were genotyped for the HMOX1 microsatellite polymorphism. The recipients were monitored for a period of one year for signs of acute rejection or the development of transplant CAD. The study found that the repeat length did not influence patient survival, the occurrence of acute rejection, or the development of transplant CAD. [Holweg, 2005]
CHAPTER 4

GENERAL METHODS AND MATERIALS

4.1 Introduction

Analyzing the different genetic polymorphisms found in HMOX1 involves the following laboratory techniques: DNA extraction, polymerase chain reaction (PCR), gel electrophoresis and sequencing analysis. In order to avoid unnecessary repetition of the same methods in every chapter, the general methods and materials are described in this chapter. References to specific sections of this chapter will be cited in the rest of the dissertation where appropriate.

4.2 Extraction of Genomic DNA

The experiments described in this dissertation utilized DNA extracted from either a venous blood sample, a saliva sample collected in 10 ml of mouthwash, or a frozen section of the left ventricle. A standard salting out extraction method, based on that
described by Miller et al, was used to extract DNA from both the peripheral blood leukocytes of the venous blood sample and the cardiomyocytes of the left ventricle sample. [Miller, 1988] DNA was extracted from buccal cells of the saliva sample using the Puregene® DNA Purification Kit (Gentra Systems, Minneapolis, MN). After extraction, the concentration of the DNA sample was determined by measuring the absorbance at 260 nm and 280 nm. All samples were then diluted with TE buffer to a concentration of 25 ng/ul.

4.3 Polymerase Chain Reaction (PCR)

All PCR reactions were run in a total volume of 50 ul, and utilized the following reaction mixture: 5 ul of 10X PCR Buffer (2M Tris, 1M (NH₄)₂SO₄, 1M MgCl₂, and distilled water), 1.5 ul of each primer (diluted to 12 uM), 1.33 ul of dNTP mixture (containing 7.5 mM of each nucleotide), 0.5 ul MgCl₂, and 0.5 ul Taq polymerase. Each reaction used 300 ng of DNA, and was run using the following PCR program: 95 °C for 5 minutes; 35 cycles of 94 °C for 45 seconds, annealing temperature for 30 seconds, and 72 °C for 1 minute; 72 °C for 10 minutes. A different primer set, requiring a unique annealing temperature, was used to genotype each HMOX1 and eNOS polymorphism. The specific details for each primer are listed in Table 4.1 and will be described in the appropriate chapters.
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Annealing Temp.</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO DEL3</td>
<td>5'-GCG CTG CAT GGC TGG TGT A-3’</td>
<td>58 °C</td>
<td>Antisense primer HMOX1 exon 3 deletion (CH. 5)</td>
</tr>
<tr>
<td>HO DEL5</td>
<td>5'-GCC CTG GAG GAG GAG ATT G-3’</td>
<td>58 °C</td>
<td>Sense primer HMOX1 exon 3 deletion and control reaction (CH. 5)</td>
</tr>
<tr>
<td>HO 3</td>
<td>5’–GGC TGG TGT GTA GGG GAT G- 3’</td>
<td>58 °C</td>
<td>Antisense primer control reaction for HMOX1 exon 3 deletion (CH. 5)</td>
</tr>
<tr>
<td>HO 2B</td>
<td>5'-GCA ATG TTG GGG AAG GTG AA-3’</td>
<td>58 °C</td>
<td>Antisense primer Sequencing HMOX1 exon 3 (CH. 5)</td>
</tr>
<tr>
<td>GT 5</td>
<td>5'-CCA GCA GGT GAC ATT TTA GGG-3’</td>
<td>58 °C</td>
<td>Sense primer HMOX1 promoter polymorphisms (CH. 6-9)</td>
</tr>
<tr>
<td>GT 3</td>
<td>5'-ACA GCT GAT GCC CAC TTT CTG-3’</td>
<td>58 °C</td>
<td>Antisense primer HMOX1 promoter polymorphisms (CH. 6-9)</td>
</tr>
<tr>
<td>894S</td>
<td>5'-GAA ACG GTC GCT TCG ACG T- 3’</td>
<td>47 °C</td>
<td>Sense primer eNOS G894T missense mutation in exon 7 (CH. 8)</td>
</tr>
<tr>
<td>894A</td>
<td>5'-ATC CCA CCC AGT CAA TCC CT- 3’</td>
<td>47 °C</td>
<td>Antisense primer eNOS G894T missense mutation in exon 7 (CH. 8)</td>
</tr>
<tr>
<td>NOS1</td>
<td>5'-AGG CCC TAT GGT AGT GCC TTT- 3’</td>
<td>47 °C</td>
<td>Sense primer eNOS intron 4 VNTR polymorphism (CH. 8)</td>
</tr>
<tr>
<td>NOS2</td>
<td>5'-TCT CTT AGT GCT GTG GTC AC- 3’</td>
<td>47 °C</td>
<td>Antisense primer eNOS intron 4 VNTR polymorphism (CH. 8)</td>
</tr>
</tbody>
</table>

Table 4.1: PCR Primers Used for Experiments
4.4 Gel Electrophoresis

Each PCR product was screened for purity and approximate size by running it on a 2.5% agarose gel containing 0.05% ethidium bromide. A Φ174/HAE standard ladder was included on the gel to provide DNA fragments of known size for comparison to the size of the PCR product. Each lane of the gel contained 7 ul of PCR product that had been mixed with 1 ul of 3X loading dye. The gel was run in 1X TAE buffer for 45 minutes at 100 volts, and then visualized with UV light on a Fluor-S MultiImager. A PCR product was considered pure if a single clear band of the desired size was seen on the gel.

4.5 Sequence Analysis

In order to assure high quality sequencing results, each PCR product was cleaned using the QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA). The concentration of each PCR product was then determined by measuring the absorbance at 260 nm and 280 nm. PCR products were then diluted to 2ng/ul, and sequenced using standard dideoxy chain termination chemistry. [Sanger, 1977] Each sequencing reaction used one HMOX1 specific primer, diluted to 2uM, and fluorescently labeled chain terminators (BigDye™ Terminator Cycle sequencing kit, Applied Biosystems, Foster City, CA). The Ohio State University Davis Heart and Lung Institute Microarray Genetics Core or Plant-Microbe Genomics Facility ran the sequences on either an ABI Prism 3100 Genetic Analyzer or 3730 DNA Analzyer (Applied Biosystems, Foster City, CA), respectively. The author determined the genotype results by examining each individual electropherogram.
CHAPTER 5

IDENTIFICATION OF LIMITATIONS WITH THE METHODOLOGY OF THE PROTOCOL TO EXAMINE THE HMOX1 EXON 3 DINUCLEOTIDE DELETION

5.1 Introduction

Analysis of the HMOX1 gene belonging to the only known patient with HO-1 deficiency led to the discovery of two unique genetic mutations: a dinucleotide deletion in exon 3, and a complete deletion of exon 2. Yachie et al hypothesized that the combination of these mutations inhibited the production of functional HO-1 protein in the HO-1 deficient patient. [Yachie, 1999] Due to the protective role of HO-1 in oxidative injury and the evidence that mutations in the HMOX1 gene could possibly prevent enzyme function, a cohort of patients suffering from congestive heart failure were screened for the HMOX1 exon 3 dinucleotide deletion. An easily replicable mutation specific PCR to screen for the exon 3 deletion had been designed by Yachie et al, and these methods were used to test the hypothesis that the HMOX1 exon 3 dinucleotide
deletion (Figure 5.1) is associated with congestive heart failure, possibly causing a decrease in functional HO-1 protein in these patients. [Yachie, 1999]

CTGGTGATGGCCTCCCTGTACCACATCTATGTGGCCCTGGAGGAGGAGATTGAGCGCAACAAGGAGAGCCCAGTCTTCGCCCCTGTCTACTTCCCAGAAGAGCTGCACCGCAAGGCTGCCCTGGAGCAGGACCTGGCCTTCTGGTACGGGCCCCGCTGGCAGGAGGTACCATCCCCTACACACACAGCCATG

Figure 5.1: HMOX1 Exon 3 with the Dinucleotide Deletion Highlighted in Yellow
The dinucleotide deletion would disrupt the normal reading frame of HMOX1, thereby affecting the normal formation of the HO-1 protein.

5.2 Methods and Materials

5.2.1 Subjects
Patients recruited for the study were treated at The Ohio University Heart Failure Clinic and diagnosed with either idiopathic dilated cardiomyopathy or ischemic cardiomyopathy, with symptomatic congestive heart failure. The patients ranged from NYHA functional class II to IV, with the majority having such severe heart failure it necessitated heart transplantation. Patients were predominantly Caucasian, with 67 %
being male. The controls were selected anonymously from a group of blood samples provided by normal healthy subjects working at The Ohio State University. The majority of controls were Caucasian, and 46% were male. All study participants provided written, informed consent, and the protocol was approved by the Institutional Review Board for Human Subjects of the Ohio State University.

5.2.2 Extraction of Genomic DNA

Genomic DNA was extracted from a venous blood sample from each subject according to the methods in Section 4.2 of this dissertation.

5.2.3 Polymerase Chain Reaction

This study utilized the mutation specific PCR designed by Yachie et al to screen for the exon 3 dinucleotide deletion. [Yachie, 1999] Two different PCRs were run on each DNA sample. Both of these reactions were run using the PCR mixture and program described in Section 4.3. The annealing temperature for both reactions was 58 °C.

The first reaction was specific for mutation detection and used the sense primer named HO DEL5 (5’-GCC CTG GAG GAG GAG ATT G -3’) and the antisense primer named HO DEL3 (5’- GCG CTG CAT GGC TGG TGT A-3’). A PCR product would be generated in this reaction only if the dinucleotide deletion was present. In the absence of the deletion, the reaction would not work and no visible band would be seen after gel electrophoresis.

The second PCR was a control reaction using HO DEL5 as the sense primer plus an additional antisense primer named HO 3 (5’- GGC TGG TGT GTA GGG GAT G-3’). This reaction would always provide a detectable PCR product. It was run as a control to
ensure that all DNA samples used in the analysis were of good quality and able to be amplified by PCR.

A PCR was run to generate products for sequence analysis of the exon 3 deletion. This reaction also followed the protocol listed in Section 4.3. It used the HO DEL5 sense primer and the antisense primer named HO 2B (5’- GCA ATG TTG GGG AAG GTG AA-3’), which both annealed at 58 °C.

**5.2.4 Gel Electrophoresis**

To identify the presence of the exon 3 deletion, PCR products from both reactions were run side-by-side on a 7% agarose gel containing 0.05% ethidium bromide for 90 minutes at 100 volts. The use of a 7% agarose gel allowed for distinct separation to occur between the PCR products from the different reactions since they varied in size by only 7 base pairs. The first PCR reaction, specific for mutation detection, resulted in a PCR product of 160 bp. The second control PCR reaction resulted in a PCR product of 153 bp. Section 4.4 describes the rest of the gel electrophoresis protocol used in analyzing the samples.

**5.2.5 Sequence Analysis**

A subset of samples were sequenced for the exon 3 deletion in both the sense and antisense direction, using the sense primer HO DEL5 and the antisense primer HO 2B. The sequencing analysis was done according to the protocol found in Section 4.5.

**5.3 Results and Discussion**

A total of 95 subjects, 52 normal controls and 43 heart failure patients, underwent genetic analysis. The mutation specific PCR developed by Yachie et al would only yield
a PCR product of 160 bp in subjects possessing the dinucleotide deletion. The control reaction to verify the ability of the DNA sample to undergo amplification would result in a PCR product of 153 bp for every sample of quality DNA analyzed. [Yachie, 1999] A representative PCR verification gel is seen in Figure 5.2.

As illustrated by the gel in Figure 5.2, all subjects involved in the study screened positive for the exon 3 dinucleotide deletion. The fact all 95 subjects were positive for the deletion brought the validity of the results into question. The mutation should not have occurred at the frequency displayed by the genotyping results. The experiments by Yachie et al were limited in that the mutation specific PCR was only run on a total of four subjects, but the presence or absence of the exon 3 deletion had been previously identified in each subject through sequence analysis. In the Yachie study, the results of the mutation specific PCR matched the sequencing results. [Yachie, 1999]
As a means of troubleshooting the experiment, the location of the target sequences for all three PCR primers involved in the mutation specific PCR were mapped on the HMOX1 exon 3 sequence (GenBank #Z82244). The results of the mapping are in Figure 5.3 and clearly show that the location of the dinucleotide deletion corresponds to the location of the complementary sequences for both the HO DEL3 and HO 3 antisense primers. In fact, the deleted bases are actually part of the HO 3 primer sequence, and missing from the HO DEL3 sequence. The genotyping results were hypothesized to be caused by mismatched primer mutagenesis. [Strachan, 1996; Cooper, 2000] The omission of the deleted bases from the HO DEL3 primer caused the site-specific deletion of those bases in the PCR product, leading to an incorrect genotype.
In order to verify whether the deleted bases were present in the genotyped subjects, six random samples were chosen from the cohort and sequenced. The PCR that created the products for sequencing reaction used the sense primer HO DEL5 and the antisense primer HO 2B. This enabled the location of the exon 3 dinucleotide deletion to be placed in the middle of a larger (341 bp) product (Figure 5.4), assuring a quality sequencing reaction.
CTGGTGATGGCCTCCCTGTACCACATCTATGTGGCCCTGGAGGAGGAGATTGAGCGCAACAAGGAGAGCCCAGTCTTCGCCCCTGTCTACTTCCCAGAAGAGCTGCACCGCAAGGCTGCCCTGGAGCAGGACCTGGCCTTCTGGTACGGGCCCCGCTGGCAGGAGGT CATCCCCTACACACAGCCCCCATG

CAGCGCTATGGAAGCGGCTCCACGAGGTGGGGCGCACAGAGCCCGAGCTGCTGGTGGCCCA
CGCCTACACCCGCTACCTGGGTGACCTGTCTGGGGGCCAGGTGCTCAAAAAGATTGCCCAGAA
AGCCCTGGACCTGCCAGCTCAGCTCCGCTCCCCATGAAACTCCCTGGAGATGACTCCCGCAGTCAGGCAGAGGGTGATAGAAGAGGCCAAGACTGCGTTCCTGCTCAACATCCAG

Figure 5.3: HMOX1 Exon 3 with Target Sequences for PCR Primers Highlighted

The target sequence for the HO-1 DEL5 primer is highlighted in green. The target sequence for the HO-1 3 primer is double underlined, italicized and in bold font. The target sequence for the HO-1 DEL3 primer is highlighted in pink. Note that the bases involved in the dinucleotide deletion, highlighted in yellow, are not included in the sequence complementary to the HO-1 DEL3 primer, but are part of the complementary sequence for the HO-1 3 primer.

CTGGTGATGGCCTCCCTGTACCACATCTATGTGGCCCTGGAGGAGGAGATTGAGCGCAACAAGGAGAGCCCAGTCTTCGCCCCTGTCTACTTCCCAGAAGAGCTGCACCGCAAGGCTGCCCTGGAGCAGGACCTGGCCTTCTGGTACGGGCCCCGCTGGCAGGAGGT CATCCCCTACACACAGCCCCCATG

CAGCGCTATGGAAGCGGCTCCACGAGGTGGGGCGCACAGAGCCCGAGCTGCTGGTGGCCCA
CGCCTACACCCGCTACCTGGGTGACCTGTCTGGGGGCCAGGTGCTCAAAAAGATTGCCCAGAA
AGCCCTGGACCTGCCAGCTCAGCTCCGCTCCCCATGAAACTCCCTGGAGATGACTCCCGCAGTCAGGCAGAGGGTGATAGAAGAGGCCAAGACTGCGTTCCTGCTCAACATCCAG

Figure 5.4: HMOX1 Exon 3 with Target for Sequencing Primers Highlighted

The target sequence for the HO-1 DEL5 primer is highlighted in green. The complementary sequence for the Exon2 B primer is highlighted in red. Note that the bases involved in the dinucleotide deletion, highlighted in yellow, fall in the center of the PCR product.
Sequencing analysis revealed that the exon 3 dinucleotide deletion was absent from all six subjects analyzed. These sequences proved that genotypes from the mutation specific PCR were incorrect, and that the error was likely the result of mismatched primer mutagenesis. These results also suggest that the mutation specific PCR designed by Yachie et al is flawed and should not be used to screen populations for the presence of the HMOX1 exon 3 dinucleotide deletion.

The results of this study show that the best method to use in screening for the HMOX1 exon 3 deletion is sequence analysis of PCR products. The primers used in the PCR reaction to generate the products for sequencing should be designed to insure the location of the deleted bases falls in the middle of the PCR product. An alternative genotyping protocol could be developed if examination of the exon 3 sequence revealed that the presence or absence of the deleted bases created a sequence specific for a restriction enzyme. In this situation, restriction fragment length polymorphism (RFLP) analysis could be used to screen for the dinucleotide deletion.

The exon 3 deletion experiments were not pursued by this investigator because other polymorphisms were discovered in the HMOX1 promoter that were associated with cardiovascular disease. It was decided that time and resources would be better spent investigating these promoter polymorphisms instead of the exon 3 deletion in the heart failure population. The results of those experiments are reported in Chapter 8 of this dissertation.
CHAPTER 6

AN EXAMINATION OF THE ASSOCIATION BETWEEN THE HMOX1 PROMOTER POLYMORPHISMS AND THE AFRICAN AMERICAN POPULATION

6.1 Introduction

The SNP-413 and microsatellite promoter polymorphisms of the HMOX1 gene were originally characterized in Asian populations and found to be significantly associated with cardiovascular diseases in people of Asian ethnicity. [Chen, 2004; Chen, 2002; Kaneda, 2002; Ono, 2004; Ono, 2003] When similar studies examining the relationship between the HMOX1 polymorphisms and cardiovascular disease were performed in primarily Caucasian populations, the results failed to find any significant associations between the polymorphisms and cardiovascular disease. [Endler, 2004; Schillinger, 2004] The fact that the experimental results were not replicable between the Asian and Caucasian populations suggests that the frequency of the HMOX1 polymorphisms differs between ethnic groups. The experiments in this chapter were performed to examine the frequency of the HMOX1 promoter polymorphisms in both Caucasian and African American ethnic groups. This is the first study to
characterize the polymorphisms in African Americans, and it was hypothesized that the allele and genotype frequencies of the HMOX1 SNP-413 and microsatellite polymorphisms would differ between the Caucasian and African American populations.

6.2 Methods and Materials

6.2.1 Subjects

The subjects involved in these experiments included healthy controls as well as patients with cardiovascular disease. The European Collection of Cell Cultures (Sigma-Aldrich, St. Louis, MO) provided 112 normal human control DNA samples of Caucasian and African ethnicity. The remaining normal control subjects were selected anonymously from blood samples provided by the American Red Cross (Columbus, OH). One subset of patients was treated at The Ohio State University Heart Failure Clinic and diagnosed with either idiopathic dilated cardiomyopathy or ischemic cardiomyopathy, with symptomatic congestive heart failure. The other set of patients underwent percutaneous transluminal coronary angioplasty (PTCA) at The Ohio State University Hospital. This study included only subjects who racially identified themselves as African American or Caucasian. All participating subjects provided written, informed consent, and the protocol was approved by the The Ohio State University Institutional Review Board for Human Subjects.

6.2.2 Extraction of Genomic DNA

Genomic DNA was extracted from either a venous blood sample or buccal cell sample from each subject according to the methods in Section 4.2 of this dissertation.
6.2.3 Polymerase Chain Reaction

The PCR for this study utilized the sense primer GT 5 (5’-CCA GCA GGT GAC ATT TTA GGG- 3’), and the antisense primer GT 3 (5’ -ACA GCT GAT GCC CAC TTT CTG- 3’). These primers had an annealing temperature of 58 ° C. The reaction used the PCR mixture and program described in Section 4.3.

6.2.4 Gel Electrophoresis

Section 4.4 of this dissertation details the gel electrophoresis protocol followed for this study.

6.2.5 Sequencing Analysis

The PCR products generated in this study were sequenced according to the methods described in Section 4.5. The sense primer GT 5 and the antisense primer GT 3 were used in the sequencing reaction.

6.2.6 Genotyping and Statistical Analysis

The HMOX1 microsatellite polymorphism was assigned either a small (S) or large (L) allele classification based upon the number of GT repeats present in the sense and antisense sequences. The cut off point for the allele classes was determined by the median GT repeat for the total control population. An allele was classified as large (L) if the GT repeat was ≥ 26, and an allele was classified as small (S) if the GT repeat was < 26. The HMOX1 SNP-413 was genotyped according to the bases present at position – 413 of the sequences. StatView for Windows version 5.0 (SAS Institute, Inc., Cary, NC) was used for frequency distribution histograms and chi-square tests to examine the distribution of the HMOX1 polymorphisms.
6.3 Results

6.3.1 The Frequency Distribution of the HMOX1 Microsatellite Polymorphism

A total of 676 subjects, consisting of 145 African Americans and 531 Caucasians, were genotyped for the microsatellite polymorphism. The demographic information for each population is shown in Table 6.1. The subjects included 256 healthy controls (41 African Americans and 215 Caucasians) and 420 patients with known cardiovascular disease (104 African Americans and 275 Caucasians). The African American subjects, with a mean age of 54 ± 13.9 years, are significantly older than the Caucasian subjects, with a mean age of 50 ± 14.9 years (P = 0.005). Frequency distribution histograms were plotted for the number of HMOX1 promoter GT repeats found in each ethnic population (Figure 6.1). The African American population possessed a greater percentage of large microsatellite alleles, classified as greater than or equal to 26 GT repeats. The African American subjects were also found to have a significantly (P < 0.0001) greater mean GT repeat than Caucasian subjects (28.8 ± 4.5 repeats vs. 26.7 ± 3.2 repeats, respectively).
### Table 6.1: Demographic Information

<table>
<thead>
<tr>
<th></th>
<th>Total Subject Population (n = 676)</th>
<th>African American Subjects (n = 145)</th>
<th>Caucasian Subjects (n = 531)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females –no. (%)</td>
<td>276 (41)</td>
<td>72 (50)</td>
<td>204 (38)</td>
</tr>
<tr>
<td>Males – no. (%)</td>
<td>400 (59)</td>
<td>73 (50)</td>
<td>327 (62)</td>
</tr>
<tr>
<td>Age (mean ± SD) *</td>
<td>51 (± 14.8)</td>
<td>54 (± 13.9)</td>
<td>50 (± 14.9)</td>
</tr>
</tbody>
</table>

* Age vs. Ethnicity, Unpaired T-test P-value = 0.005
Figure 6.1: Frequency Distribution for Dinucleotide Repeats of the HMOX1 Microsatellite Polymorphism
Chi-square analysis of both the allele and genotype frequencies of the HMOX1 microsatellite polymorphism versus ethnicity are shown in Table 6.2. The L allele was significantly associated with the African American population (P < 0.0001), while the S allele was significantly associated with the Caucasian population (P < 0.0001). A statistically significant association was also observed between the LL genotype and all African American subjects (P < 0.0001).

Chi-square analysis of the allele and genotype frequencies vs. gender for the total population failed to find any significance (P = 0.61). When the individual racial populations were divided by gender, chi-square analysis revealed that the LL genotype was seen in 67% of African American females and 64% of African American males, compared to 39% of Caucasian females and 47% of Caucasian males (P = 0.003 and 0.004, respectively). The L allele was also significantly associated with African Americans of both genders (P = 0.007 for females and P = 0.002 for males).
<table>
<thead>
<tr>
<th></th>
<th>Total Subject Population (%)</th>
<th>African American Subjects (%)</th>
<th>Caucasian Subjects (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Allele Frequency</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>60</td>
<td>76</td>
<td>56</td>
</tr>
<tr>
<td>S</td>
<td>40</td>
<td>24</td>
<td>44</td>
</tr>
<tr>
<td><strong>Genotype Frequencies</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LL</td>
<td>48.7</td>
<td>65.5</td>
<td>44.1</td>
</tr>
<tr>
<td>LS</td>
<td>23.4</td>
<td>20</td>
<td>24.3</td>
</tr>
<tr>
<td>SS</td>
<td>27.9</td>
<td>14.5</td>
<td>31.6</td>
</tr>
</tbody>
</table>

**Table 6.2: Allele and Genotype Frequencies for the HMOX1 Microsatellite Polymorphism**

All populations are in Hardy-Weinberg equilibrium.

<sup>a</sup> Chi-square P-values < 0.0001 for both L Allele vs. Ethnicity and S Allele vs. Ethnicity

<sup>b</sup> Genotype versus Ethnicity Chi-square P-value < 0.0001
6.3.2 The Frequency Distribution of the HMOX1 Single Nucleotide Polymorphism at -413

A total of 573 subjects, consisting of 123 African Americans and 450 Caucasians, were genotyped for the single nucleotide polymorphism at base –413 of the HMOX1 promoter. Table 6.3 shows the Chi-square analysis of the allele and genotype frequencies versus ethnicity for the SNP-413. The A allele was significantly associated with the Caucasian population (P < 0.0001), while the T allele was significantly associated with the African American population (P < 0.0001). The AT genotype was most frequently seen in the Caucasian subjects, while the TT genotype was most frequent in the African American subjects (P < 0.0001).

Chi-square analysis failed to find any significance between the SNP-413 genotypes and alleles frequencies vs. gender (P = 0.69). The A allele is seen in 81.7% of Caucasian females and 81.5% of Caucasian males, compared to 59.4% of African American females and 49.2% of African American males (P = 0.0004 for females, and 0.0001 for males). Both genders of African Americans possessed the TT genotype at a greater frequency than Caucasians (P < 0.0001 and P = 0.0007, respectively).
<table>
<thead>
<tr>
<th></th>
<th>Total Subject Population (%)</th>
<th>African American Subjects (%)</th>
<th>Caucasian Subjects (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Allele Frequencies a</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>55</td>
<td>36</td>
<td>60</td>
</tr>
<tr>
<td>T</td>
<td>45</td>
<td>64</td>
<td>40</td>
</tr>
<tr>
<td><strong>Genotype Frequencies b</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>34</td>
<td>17.9</td>
<td>38.4</td>
</tr>
<tr>
<td>AT</td>
<td>41.7</td>
<td>36.6</td>
<td>43.1</td>
</tr>
<tr>
<td>TT</td>
<td>24.3</td>
<td>45.5</td>
<td>18.4</td>
</tr>
</tbody>
</table>

Table 6.3: Allele and Genotype Frequencies for the HMOX1 SNP -413

All populations are in Hardy-Weinberg equilibrium.

a Chi-square P-values < 0.0001 for both A Allele vs. Ethnicity and T Allele vs. Ethnicity

b Genotype versus Ethnicity, Chi-square p-value < 0.0001
6.3.3 The Frequency of both HMOX1 Promoter Polymorphisms in the Caucasian and African American Populations

Chi-square analysis of the genotype frequency of the HMOX1 promoter microsatellite polymorphism versus the genotype frequency of the HMOX1 SNP-413 is shown in Table 6.4. The most frequent genotype combination seen in the total population was the LL microsatellite genotype and AA SNP-413 genotype, occurring in 32% of all subjects (P < 0.0001). The least frequent genotype combination was the SS genotype and AA genotype, occurring in only 0.5% of the total subject population (P < 0.0001). In the total population, the S allele of the microsatellite polymorphism was significantly associated with the AT SNP-413 genotype, while the L microsatellite allele was significantly associated with the AA SNP-413 genotype (P < 0.0001). The L microsatellite allele and A SNP-413 allele were present together in 61% of the total subject population (P < 0.0001).

In the Caucasian population (Table 6.4), the LL genotype of the HMOX1 microsatellite allele occurred with the AA SNP-413 genotype in 36.2% of subjects, while the SS microsatellite genotype occurred with the AA SNP-413 genotype in only 0.44% of subjects (P < 0.0001). The L microsatellite allele was significantly associated with the AA SNP-413 genotype, and the S microsatellite allele was significantly associated with the AT SNP-413 genotype in the Caucasian subjects (P < 0.0001). The A SNP-413 allele and L microsatellite allele were present together in 64% of the Caucasian population (P < 0.0001).

In the African American population (Table 6.4), the majority of subjects had the LL microsatellite genotype combined with either the AT or TT SNP-413 genotype. The
least frequent genotype combinations, seen in only 0.8% of the African American population, were the AA SNP-413 genotype paired with either the LL or SS microsatellite genotype (P = 0.02). The S microsatellite allele was associated with the TT SNP-413 genotype (P = 0.02) and the L microsatellite allele was associated with both the AT and TT SNP-413 genotype (P = 0.03). Half of the African American population had both the L microsatellite allele and A SNP-413 allele present (P = 0.007).
<table>
<thead>
<tr>
<th>Microsatellite Genotype, SNP-413</th>
<th>Total Population&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>African American Population&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>Caucasian Population&lt;sup&gt;c&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL, AA</td>
<td>32</td>
<td>16.3</td>
<td>36.2</td>
</tr>
<tr>
<td>LL, AT</td>
<td>12</td>
<td>25.2</td>
<td>8.4</td>
</tr>
<tr>
<td>LL, TT</td>
<td>6.8</td>
<td>26.0</td>
<td>1.6</td>
</tr>
<tr>
<td>LS, AA</td>
<td>1.6</td>
<td>0.8</td>
<td>1.8</td>
</tr>
<tr>
<td>LS, AT</td>
<td>15.9</td>
<td>8.1</td>
<td>18</td>
</tr>
<tr>
<td>LS, TT</td>
<td>3.7</td>
<td>8.1</td>
<td>2.4</td>
</tr>
<tr>
<td>SS, AA</td>
<td>0.5</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>SS, AT</td>
<td>13.8</td>
<td>3.3</td>
<td>16.7</td>
</tr>
<tr>
<td>SS, TT</td>
<td>13.8</td>
<td>11.4</td>
<td>14.4</td>
</tr>
</tbody>
</table>

**Table 6.4: Genotype Frequencies of HMOX1 Microsatellite Polymorphism vs. SNP-413**

<sup>a</sup> Microsatellite vs. SNP-413 Genotype for Total Population, Chi-Square P-value < 0.0001

<sup>b</sup> Microsatellite vs. SNP-413 Genotype for African American Population, Chi-Square P-value = 0.02

<sup>c</sup> Microsatellite vs. SNP-413 Genotype for Caucasian Population, Chi-Square P-value < 0.0001
The relationship between the GT repeat pairs occurring at greater than 1% frequency and corresponding SNP-413 genotypes was examined as a way to further characterize the HMOX1 promoter polymorphisms. The GT repeat pairs consist of the number of GT repeats counted on the sense and antisense sequence for each subject. Table 6.5 shows the results for the Caucasian subjects and reveals that the (23, 25) GT repeat pair occurred at the highest frequency in this population. The majority of Caucasian subjects with the AA SNP-413 genotype had GT repeats of 30. The AT and TT SNP-413 genotypes were seen most often with the (23, 25) GT repeat pair. In the Caucasian population, subjects with the AA SNP-413 genotype tended to have large GT repeat pairs while subjects with the TT genotype tended to have small GT repeat pairs.

The results for the African American population are shown in Table 6.6. The (23, 26) GT repeat pair occurred at the highest frequency in this population. The majority of African American subjects with the AA SNP-413 genotype had GT repeats of 30. Subjects with the AT SNP-413 genotype most often had the (30, 32) GT repeat pair. African American subjects with the TT SNP-413 genotype had either GT repeats of 23 or (35, 37). In the African American population, subjects with the AA SNP-413 genotype tended to have large GT repeat pairs. Subjects with the AT SNP-413 genotype had GT repeat pairs spread throughout the mid-range of counts, while the GT repeat pairs of African Americans with the TT SNP-413 genotype clustered around the smallest and largest values.
<table>
<thead>
<tr>
<th>Repeat Pairs</th>
<th>Percent Frequency for Repeat Pair</th>
<th>Percent Subjects with AA Genotype Present</th>
<th>Percent Subjects with AT Genotype Present</th>
<th>Percent Subjects with TT Genotype Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>22, 23</td>
<td>2.1</td>
<td>0</td>
<td>0.6</td>
<td>14</td>
</tr>
<tr>
<td>23, 23</td>
<td>3.4</td>
<td>0</td>
<td>1</td>
<td>21</td>
</tr>
<tr>
<td>23, 24</td>
<td>3.6</td>
<td>0</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>23, 25</td>
<td>13.0</td>
<td>0</td>
<td>24</td>
<td>25</td>
</tr>
<tr>
<td>24, 25</td>
<td>6.0</td>
<td>0</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>23, 26</td>
<td>8.9</td>
<td>0</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>24, 26</td>
<td>4.5</td>
<td>0</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>25, 26</td>
<td>1.7</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>24, 27</td>
<td>4.7</td>
<td>0</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>25, 28</td>
<td>1.1</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>26, 28</td>
<td>1.1</td>
<td>0.7</td>
<td>0.6</td>
<td>1</td>
</tr>
<tr>
<td>26, 29</td>
<td>1.1</td>
<td>0.7</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>27, 29</td>
<td>1.1</td>
<td>1</td>
<td>0.6</td>
<td>1</td>
</tr>
<tr>
<td>27, 30</td>
<td>2.1</td>
<td>27</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>28, 30</td>
<td>2.1</td>
<td>5</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>29, 30</td>
<td>8.5</td>
<td>6</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>30, 30</td>
<td>10.4</td>
<td>37</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>29, 31</td>
<td>1.1</td>
<td>14</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>30, 31</td>
<td>4.7</td>
<td>3</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>30, 32</td>
<td>4.1</td>
<td>6</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 6.5: GT Repeat Pairs with Greater than 1% Frequency in the Caucasian Population and their Relationship to the HMOX1 SNP-413 Genotypes
<table>
<thead>
<tr>
<th>Repeat Pairs</th>
<th>Percent Frequency for Repeat Pair</th>
<th>Percent Subjects with AA Genotype Present</th>
<th>Percent Subjects with AT Genotype Present</th>
<th>Percent Subjects with TT Genotype Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>23, 23</td>
<td>3.4</td>
<td>0</td>
<td>0</td>
<td>11.6</td>
</tr>
<tr>
<td>23, 24</td>
<td>3.4</td>
<td>0</td>
<td>0</td>
<td>9.3</td>
</tr>
<tr>
<td>23, 25</td>
<td>3.4</td>
<td>0</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>23, 26</td>
<td>8.3</td>
<td>0</td>
<td>12.5</td>
<td>4.7</td>
</tr>
<tr>
<td>24, 26</td>
<td>1.4</td>
<td>0</td>
<td>0</td>
<td>2.3</td>
</tr>
<tr>
<td>25, 26</td>
<td>2.8</td>
<td>0</td>
<td>0</td>
<td>9.3</td>
</tr>
<tr>
<td>24, 27</td>
<td>2.8</td>
<td>0</td>
<td>5</td>
<td>2.3</td>
</tr>
<tr>
<td>25, 27</td>
<td>1.4</td>
<td>0</td>
<td>2.5</td>
<td>2.3</td>
</tr>
<tr>
<td>26, 27</td>
<td>1.4</td>
<td>0</td>
<td>2.5</td>
<td>2.3</td>
</tr>
<tr>
<td>25, 28</td>
<td>1.4</td>
<td>0</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>26, 28</td>
<td>3.4</td>
<td>0</td>
<td>7.5</td>
<td>4.7</td>
</tr>
<tr>
<td>26, 29</td>
<td>2.8</td>
<td>0</td>
<td>5</td>
<td>4.7</td>
</tr>
<tr>
<td>27, 30</td>
<td>2.1</td>
<td>15.8</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>29, 29</td>
<td>2.1</td>
<td>5.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>28, 30</td>
<td>2.1</td>
<td>5.3</td>
<td>2.5</td>
<td>2.8</td>
</tr>
<tr>
<td>29, 30</td>
<td>4.1</td>
<td>26.3</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>30, 30</td>
<td>6.2</td>
<td>31.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>29, 31</td>
<td>2.1</td>
<td>5.3</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>30, 31</td>
<td>2.1</td>
<td>5.3</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>29, 32</td>
<td>3.4</td>
<td>0</td>
<td>7.5</td>
<td>4.7</td>
</tr>
<tr>
<td>30, 32</td>
<td>6.9</td>
<td>0</td>
<td>17.5</td>
<td>2.3</td>
</tr>
<tr>
<td>30, 33</td>
<td>7.6</td>
<td>5.3</td>
<td>20</td>
<td>4.7</td>
</tr>
<tr>
<td>35, 36</td>
<td>1.4</td>
<td>0</td>
<td>0</td>
<td>4.7</td>
</tr>
<tr>
<td>35, 37</td>
<td>3.4</td>
<td>0</td>
<td>0</td>
<td>11.6</td>
</tr>
<tr>
<td>37, 39</td>
<td>2.8</td>
<td>0</td>
<td>0</td>
<td>9.3</td>
</tr>
</tbody>
</table>

Table 6.6: GT Repeat Pairs with Greater than 1% Frequency in the African American Population and their Relationship to the HMOX1 SNP-413 Genotypes
6.4 Discussion

6.4.1 The Importance of Using Racial and Ethnic Classification in Genetic Research

Previous studies examining the association between the HMOX1 microsatellite promoter polymorphism and cardiovascular disease discovered a difference in the frequency distribution between the Asian and Caucasian populations. That discovery prompted the experiments in this dissertation to focus on characterizing ethnic differences between the association of the HMOX1 promoter polymorphisms and cardiovascular disease. The genetic variation seen in the African American and Caucasian populations, regardless of whether control or patient, was so significant that the author felt it warranted an entire chapter to discuss the differences between the populations. The relationship between the HMOX1 polymorphisms and cardiovascular disease will be examined in Chapters 7, 8 and 9.

The use of race or ethnicity as an identifier in genetic research is very controversial due to the history of prejudice and discrimination associated with such labels. The racial profile of a cohort should not be overlooked because a subject’s racial or ethnic background can be key to discovering a disease-associated mutation present at a high frequency in one population but virtually absent in another. [Cooper, 2003] In diseases that do not vary greatly between populations, the racial or ethnic profiles of patients can help to characterize unique risk-factor profiles. Investigators must be cautious and avoid overemphasizing the differences between racial and ethnic populations because that could undervalue the great diversity that exists between members of a specific population. [Burchard, 2003]
Genetic clusters have arisen due to geographical, social, and cultural barriers isolating human populations. Humans throughout the world fall into five common clusters corresponding to the following racial classifications: (1.) Africans, (2.) Europeans/West Asians, (3.) East Asians, (4.) Pacific Islanders, and (5.) Native Americans. [Tang, 2005] Ethnicity differs from race because it includes both genetic and environmental factors such as cultural traditions, common history, religion, and social background. [Burchard, 2003; Xie, 2001] Studies done without racial or ethnic labels may overlook the social, cultural, economic, behavioral, and other environmental factors that accompany genetic clustering. [Tang, 2005]

Previous research has shown that allele variants with frequencies of greater than or equal to 20% tend to occur in multiple racial groups. Variants with frequencies lower than 20% are more likely to be race specific. The African population has the highest genetic variability of all racial groups, and possesses the largest number of low-frequency alleles. The African American population remains genetically similar to the African population, despite genetic mixing with other racial groups. [Burchard, 2003] The experiments in this dissertation focused on the African American population due to its unique genetic profile and because the African American population tends to be underrepresented in genetic research. The HMOX1 promoter polymorphisms also had yet to be examined in this ethnic group.

6.4.2 A Discussion of the HMOX1 Microsatellite Polymorphism Results

Genetic analysis of the HMOX1 microsatellite polymorphism revealed that the African American population possessed a mean microsatellite GT repeat length of $28.8 \pm 4.5$ repeats, significantly larger than the Caucasian mean GT repeat of $26.7 \pm 3.2$. In
previous studies, the GT repeats have fallen into a bimodal distribution with the most frequent alleles at 23 and 30 repeats. Frequency distribution histograms for each of the ethnic groups show that the populations examined in this study also display the same bimodal distribution pattern. The histograms also show that, when compared to the Caucasian population, the African American population has a much higher percentage of repeats greater than or equal to 26.

Multiple in vitro studies involving HMOX1 promoter/luciferase fusion genes have shown that cells containing GT repeats greater than 25 GT repeats have decreased transcriptional activity, and experiments involving lymphoblastoid cell lines with known GT repeat lengths showed that HMOX1 activity was highest in cells with repeats less than 27. [Chen, 2002; Exner, 2004; Hirai, 2003; Yamada, 2000] Long sequences of GT repeats have been shown to take on the Z-DNA conformation, a situation unfavorable for transcriptional activity. [Exner, 2004; Naylor, 1990; Shibahara, 1989;] These findings, combined with the results from this study showing that the majority of the African American population possesses dinucleotide repeat lengths of greater than 25, lead the author to hypothesize that the HMOX1 transcriptional activity is reduced in African Americans. Reduced HMOX1 activity would result in decreased amounts of HO-1, thereby lowering a subject’s endogenous antioxidant activity and leaving them more susceptible to damage from oxidative stress.

For easier analysis, the author chose to classify the repeat lengths into alleles, using the median GT repeat for the total control population as the dividing point. An allele was classified as large (L) if the GT repeat was \( \geq 26 \), and as small (S) if the GT repeat was \(< 26\). This allele classification is similar to those used in previous studies.
examining the HMOX1 microsatellite polymorphism. A majority of the studies created alleles based upon the frequency distribution of the GT repeats, using the peak frequencies as cut off points. The S allele ranged from being \( \leq 25 - 27 \) repeats, and the L allele ranged from being \( \geq 27 - 33 \) repeats. [Hirai, 2003; Exner, 2001; Kaneda, 2002; Yamada, 2000; Yamaya, 2003] The remaining studies separated the large and small alleles on the basis of the HMOX1/luciferase transfection studies, resulting in an S allele of \(< 25\) and an L allele of \(\geq 25\) repeats. [Endler, 2004; Funk, 2004; Schillinger, 2004]

Because the previously described allele classifications were based primarily on findings in the Asian population, and due to the mounting evidence that the repeat size varies between ethnic groups, the author thought it best to follow the method of classifying the alleles by the median GT repeat as described by Chen et al. [Chen, 2002] The author felt this method would be best to control for any variations seen between the Caucasian and African American populations. Genetic analysis using the allele classification revealed that the L allele and LL genotype are significantly associated with the African American population, and the Caucasian population is significantly associated with the S allele and had a higher frequency of the SS genotype. The genotype analysis supports what was found by examining the GT repeat numbers: the African American population significantly possesses the large HMOX1 microsatellite polymorphism, a situation that may put them at risk for diseases resulting from oxidative damage due to decreased HO-1 activity.

6.4.3 A Discussion of the HMOX1 SNP-413 Results

In the original study characterizing the HMOX1 SNP-413, Ono et al found that the Japanese population had the following genotype frequency: AA = 22 %, AT = 46 %,
and TT = 32 %. [Ono, 2003] The current experiments show that the genotype frequency for the Caucasian population was AA = 38.4 %, AT = 43.1 %, and TT = 18.4 %. The genotype frequency for the African American population was AA = 17.9 %, AT = 36.6 %, and TT = 45.5 %. Examining the genotype frequencies of the different ethnic groups reveals that the AT genotype is most often associated with the Asian and Caucasian populations, while the TT genotype is most frequently seen in the African American population. The African American population is also least associated with the AA genotype. The results in this study show that the genotype associations are significant for each ethnic group. This study also shows that the A allele is significantly associated with the Caucasian population while the T allele is significantly associated with the African American population.

The differences in the HMOX1 SNP-413 genotype and allele frequencies are important because the presence of the AA genotype was found to be associated with a reduced incidence of CAD in the Japanese population. [Ono, 2004] In vitro promoter studies also have shown that cells containing the A allele have significantly greater promoter activity than cells containing the T allele. [Ono, 2003] The results of the current study suggest that the African American population, with its high frequency of the TT genotype and significant association with the T allele, could be at higher risk for cardiovascular disease due to inefficient HMOX1 promoter activity and subsequent lack of HO-1 activity.
6.4.4 A Discussion of the Combination of HMOX1 Promoter Polymorphisms in the African American and Caucasian Populations

Previous *in vitro* research established that the presence of the large microsatellite polymorphism decreases the transcriptional activity of HMOX1, while the presence of the A allele for the HMOX1 SNP-413 increases the transcriptional activity. These findings have been somewhat supported *in vivo* through studies in which the large microsatellite allele was found to be significantly associated with the presence of cardiovascular disease, and the AA genotype associated with a decreased risk of cardiovascular disease. The seemingly contradictory actions of these polymorphisms prompted the author to examine their genotype patterns in relation to one another to see if there is an ethnic difference between them that could result in one population having inefficient HO-1 production, therefore being at higher risk for oxidative stress related disease states.

Ono *et al* performed the only known study examining the relationship between the HMOX1 SNP-413 genotype and the GT repeat sizes of the microsatellite polymorphism. The investigators found that in the Japanese population, the AA SNP-413 genotype was most frequently seen with the (30, 30) GT repeat pair, the AT genotype with the (23, 30) repeat pair, and the TT genotype with the (23, 23) repeat pair. Two major alleles were found in this population, which the investigators called A(-413)-(GT)30 and T(-413)-(GT)23. These major alleles were used for the *in vitro* experiments in which high HMOX1 transcriptional activity was attributed to the presence of the A allele. Ono *et al* felt that the results from their *in vitro* experiments show that the A allele has more influence on transcriptional activity than the large microsatellite repeat. [Ono, 2003]
The author felt that the findings of Ono et al needed to be further explored in an
*in vivo* setting, and hypothesized that the potential influence of the polymorphisms on
each other could be determined by the genotype patterns present in a healthy control
population versus a disease population. While pursuing this investigation, the author
found a great variation between the genotype patterns in the Caucasian and African
American populations, and felt that the ethnic differences warranted a separate
discussion. Chapters 7 and 8 will detail the differences seen in a control versus a
cardiovascular disease population.

In the total subject population, the LL microsatellite and AA SNP-413 genotype
combination were seen most frequently, while the SS and AA combination were seen the
least. The Caucasian population had the same genotype results as the total population.
The most frequent GT repeats in the Caucasian population were (23,25) and (30,30).
Both the AT and TT SNP-413 genotypes were most frequently associated with the (23,
25) repeat pair, while the AA genotype was associated with the (30,30) pair. These
results closely resemble those previously described by Ono et al, and lead to the
speculation that the Caucasian population may be at less risk for diseases mediated by
oxidative stress due to properly functioning HMOX1. The proposed “bad” alleles (the
large microsatellite and T SNP-413) are grouped with “good” alleles (the A SNP-413 and
small microsatellite), which may balance out the level of HMOX1 promoter transcription.

In the African American population, the most frequent genotype combination was
the LL microsatellite genotype combined with either the AT or TT SNP-413 genotype,
and the least frequent was the AA SNP-413 combined with either the LL or SS
microsatellite genotype. The most frequent GT repeats in the African American
population were (23,26) and (30,33) which were both most frequently associated with the AT SNP-413 genotype. It is interesting to note that the AA SNP-413 genotype was most frequently associated with the (30,30) GT repeat pair, the AT genotype with the (30,32) repeat pair, and the TT genotype was split between the (23,23) and (35, 37) repeat pairs. These results suggest that the African American population may be at risk for oxidative stress mediated disease due to deficient HO-1. In this population, a large percentage of subjects have both the T SNP-413 and the large microsatellite allele, a combination that could have detrimental effects on the transcriptional activity of HMOX1.

6.4.5 A Discussion of Experimental Limitations and Future Research

The biggest limitation in this study was the small number of African American subjects. In an attempt to increase these numbers, DNA samples from subjects of African descent were obtained from the European Collection of Cell Cultures (Sigma-Aldrich, St. Louis, MO). Since it has been shown that African Americans are still genetically similar to Africans, the author felt the use of these samples would not interfere with the results of the genetic analysis. [Burchard, 2003] In fact, exclusion of the samples provided from the European source did not change the results in any way (data not shown). In order to fully verify the results, this genetic analysis should be repeated on a much larger scale. It would also be interesting to expand the study to include representatives other major racial or ethnic groups, including Hispanics and Native Americans.

Another limitation in this study was the genotyping method for the HMOX1 SNP-413. The genotypes were taken from the HMOX1 promoter sequences, but it was often only available from the sense sequence. The results included in this study should be
considered a preliminary analysis, and the SNP-413 results should be verified by some alternative means, such as a SNP kit.

The results presented in this chapter describe the differences in the frequency distribution of the MHOX1 polymorphisms in Caucasian and African American ethnic groups. Chapters 7 and 8 expand upon this research by examining the distribution of the polymorphisms in populations of patients with cardiovascular disease. These chapters also place special emphasis on the ethnicity of the patients examined. The experiments in Chapter 9 expand upon all of the genotyping analysis by exploring the impact of the HMOX1 polymorphisms on HO-1 expression.
CHAPTER 7

AN EXAMINATION OF THE HMOX1 PROMOTER POLYMORPHISMS IN CAUCASIAN AND AFRICAN AMERICAN PATIENTS WITH CORONARY ARTERY DISEASE

7.1 Introduction

The literature reviewed in Chapter 3 describes the crucial role played by HO-1 and its reaction products in preventing the pathogenesis of CAD and details previously discovered associations between CAD and the HMOX1 promoter polymorphisms. The genetic analysis presented in this chapter examines the association between the HMOX1 promoter SNP-413 and microsatellite polymorphisms in a population of subjects with CAD, as evidenced by treatment with PTCA, and a normal control population. In an effort to further characterize the results presented in Chapter 6, the research in this chapter focuses on patients of Caucasian and African American ethnicity. The experiments in this chapter test the hypothesis that the HMOX1 promoter polymorphisms are associated with the development of CAD, especially in African Americans.
7.2 Methods and Materials

7.2.1 Subjects

This study involved a healthy control population and a population of subjects with coronary artery disease. A set of 112 normal human control DNA samples of Caucasian and African ethnicity were provided by The European Collection of Cell Cultures (Sigma-Aldrich, St. Louis, MO). The remaining normal control subjects were selected anonymously from blood samples provided by the American Red Cross (Columbus, OH). The patient population was recruited from people undergoing percutaneous transluminal coronary angioplasty (PTCA) at The Ohio State University Hospital. The subjects involved were limited to those who racially identified themselves as African American or Caucasian. The protocol was approved by the Institutional Review Board for Human Subjects of The Ohio State University, and all subjects provided written, informed consent.

7.2.2 Extraction of Genomic DNA

Genomic DNA was extracted from a venous blood sample provided by each subject according to the methods in Section 4.2 of this dissertation.

7.2.3 Polymerase Chain Reaction

The PCR for this study utilized the sense primer GT 5 (5’-CCA GCA GGT GAC ATT TTA GGG- 3’), and the antisense primer GT 3 (5’ -ACA GCT GAT GCC CAC TTT CTG- 3’). These primers annealed at 58 °C. The experiments used the PCR mixture and program described in Section 4.3 of this document.
7.2.4 Gel Electrophoresis

Section 4.4 of this dissertation details the gel electrophoresis protocol used for this study.

7.2.5 Sequencing Analysis

The PCR products generated in this study were sequenced according to the methods described in Section 4.5. The sense primer GT 5 and antisense primer GT 3 were used in the sequencing reaction.

7.2.6 Genotyping and Statistical Analysis

The HMOX1 microsatellite polymorphism was assigned either a small (S) or large (L) allele classification based upon the number of GT repeats present in the forward and reverse sequences. The cut off point for the allele classes was determined by the median GT repeat for the total control population. An allele was classified as large (L) if the GT repeat was ≥ 26, and an allele was classified as small (S) if the GT repeat was < 26. The HMOX1 SNP-413 was genotyped according to the bases present at position – 413 of the sequences. StatView for Windows version 5.0 (SAS Institute, Inc., Cary, NC) was used for frequency distribution histograms and chi-square tests to examine the distribution of the HMOX1 polymorphisms. Logistic regression was performed using STATA version 8.0 for Windows (StataCorp LP, College Station, TX).

7.3 Results

7.3.1 Demographic Information for the Patient and Control Populations

A total of 394 subjects, consisting of 138 patients who underwent PTCA and 256 normal controls, were genotyped for the HMOX1 microsatellite polymorphism. The
demographic information for each group is listed in Table 7.1. The patient population, with an average age of 60.2 ± 12.8 years, was significantly older than the control population, with an average age of 41.4 ± 12.2 years (P < 0.0001). The patient population also had a greater percentage of African American subjects (40%) than the control group (16%).

When broken down by ethnic group, the African American and Caucasian control populations had almost equal percentages of male and female subjects. The Caucasian controls were significantly (P = 0.0004) younger than the African American controls (40.2 ± 11.6 years vs. 49.7 ± 13.3 years, respectively). The African American and Caucasian patient populations were also equally split between male and female subjects and had the same mean age. Analysis of the presence of cardiovascular risk factors in the African American versus Caucasian patients revealed that hypertension was significantly associated with the African American patients (P = 0.008), while the Caucasian patients showed a trend towards having hypercholesterolemia (P = 0.13).
### Table 7.1: Demographic Information

<table>
<thead>
<tr>
<th></th>
<th>PTCA Patient Population (n = 138)</th>
<th>Control Population (n = 256)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age – mean yr (± SD)</td>
<td>60.2 (± 12.8)</td>
<td>41.4 (±12.2)</td>
</tr>
<tr>
<td>Male sex – no. (%)</td>
<td>68 (49)</td>
<td>135 (52.7)</td>
</tr>
<tr>
<td>African American Race</td>
<td>55 (40)</td>
<td>41 (16)</td>
</tr>
<tr>
<td>History of MI – no. (%)</td>
<td>62 (50)</td>
<td>NA*</td>
</tr>
<tr>
<td>History of CAD – no. (%)</td>
<td>93 (69)</td>
<td>NA</td>
</tr>
<tr>
<td>History of Hypertension – no. (%)</td>
<td>99 (75)</td>
<td>NA</td>
</tr>
<tr>
<td>History of Hypercholesterolemia – no. (%)</td>
<td>93 (78)</td>
<td>NA</td>
</tr>
<tr>
<td>History of Diabetes Mellitus – no. (%)</td>
<td>48 (37)</td>
<td>NA</td>
</tr>
<tr>
<td>Family History of CAD – no. (%)</td>
<td>69 (64)</td>
<td>NA</td>
</tr>
<tr>
<td>History of Smoking – no. (%)</td>
<td>74 (61)</td>
<td>NA</td>
</tr>
</tbody>
</table>

* PTCA Age vs. Control Age, Unpaired t-test P-value < 0.0001

* NA = Information not available for the control population
7.3.2 The Frequency Distribution of the HMOX1 Microsatellite Polymorphism

Frequency distribution histograms were plotted for the number of HMOX1 promoter GT repeats found in both the patient and control populations (Figure 7.1). The mean GT repeat for the HMOX1 microsatellite polymorphism in the control population was significantly (P = 0.002) less than the mean GT repeat for the patient population (26.9 ± 3.4 vs. 27.8 ± 3.6, respectively). Figure 7.2 shows the frequency distribution histograms for both subject populations separated on the basis of ethnicity. The African American members of both the control and patient populations had a greater percentage of large GT repeats.

In both populations, the mean GT repeat for the African American subjects was significantly larger than the mean repeat for the Caucasian subjects. The African American controls had a mean dinucleotide repeat of 28.0 ± 3.9 repeats compared to Caucasian controls having 26.7 ± 3.2 repeats (P = 0.002). The African American patients had a mean GT repeat of 29.1 ± 4.1 repeats, while the Caucasian patients had a mean of 26.9 ± 3.0 GT repeats (P < 0.0001). There was no significant difference between the mean GT repeat of the Caucasian controls versus the Caucasian patients (26.7 ± 3.2 vs. 26.9 ± 3.0 GT repeats, P = 0.60). A trend was seen in which the African American patient population had a slightly larger mean GT repeat than the African American control population (29.1 ± 4.1 vs. 28.0 ± 3.9 repeats, P = 0.07).
Figure 7.1: Frequency Distribution for Dinucleotide Repeats of the HMOX1 Microsatellite Polymorphism
Figure 7.2: Frequency Distribution for Dinucleotide Repeats of the HMOX1 Microsatellite Polymorphism Divided by Ethnicity

a.) The PTCA Patient Population

b.) The Control Population
Chi-square analysis of the allele and genotype frequencies of the HMOX1 microsatellite polymorphism is shown in Table 7.2. The control population had significantly fewer subjects with the LL genotype (44%) and more subjects with the SS genotype (27%) than the patient population (57.2% LL and 22.5% SS, \( P = 0.03 \)). The S allele was significantly associated with the controls (\( P = 0.01 \)), while the distribution of L allele in the populations was not significant (\( P = 0.29 \)).

The genotype results for the control population showed a trend in which the LL genotype was associated with the African American controls and the SS genotype was associated with the Caucasian controls (\( P = 0.10 \)). The L allele showed a trend in which it was associated with the African American controls (\( P = 0.11 \)), while the S allele was significantly associated with the Caucasian controls (\( P = 0.04 \)). Chi-square analysis of the allele and genotype frequencies versus gender for the control population failed to find any significant associations.

The chi-square analysis for the genotype frequency in the patient population revealed a significant association between the LL genotype and African American patients and the SS genotype and Caucasian patients (\( P = 0.01 \)). Logistic regression analysis found a highly significant increase in the odds of African American patients having the LL genotype (\( P = 0.009, \ OR = 2.6, \ 95\% \ CI = 1.27 – 5.40 \)). Adjusting for all other clinical variables led to even greater odds of finding the LL genotype in the African American patients (\( P = 0.009, \ OR = 3.75, \ 95\% \ CI = 1.38 – 10.16 \)). The L allele of the microsatellite polymorphism was significantly associated with the African American patients, while the S allele was significantly associated with the Caucasian patients (\( P = 0.008 \)). Chi-square analysis of the allele and genotype frequencies versus gender failed to
find any significant associations, but a trend was seen in which a greater number of female than male patients possessed the S allele (P = 0.16).

Chi-square analysis was used to examine each of the clinical variables recorded for the patient population versus the allele and genotype frequencies of the HMOX1 microsatellite polymorphism. A significant association was found between the LL genotype and a history of CAD (P = 0.04). Seventy-seven percent of the patients with the LL genotype had a known history of CAD before undergoing PTCA. This association was not significant with the L allele alone and when the patient population was split by ethnicity. Logistic regression analysis also revealed an increase odds for a patient with a history of CAD to have the LL genotype (P = 0.04, OR = 2.23, 95% CI = 1.05 – 4.68).
### Table 7.2: Allele and Genotype Frequencies for the HMOX1 Microsatellite Polymorphism

All populations are in Hardy-Weinberg Equilibrium.

<table>
<thead>
<tr>
<th>Allele Frequency</th>
<th>Total PTCA Patient Population (%)</th>
<th>African American PTCA Subjects (%)</th>
<th>Caucasian PTCA Subjects (%)</th>
<th>Total Control Population (%)</th>
<th>African American Control Subjects (%)</th>
<th>Caucasian Control Subjects (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L^b</td>
<td>67</td>
<td>80</td>
<td>59</td>
<td>58</td>
<td>71</td>
<td>56</td>
</tr>
<tr>
<td>S^c,d</td>
<td>33</td>
<td>20</td>
<td>41</td>
<td>42</td>
<td>29</td>
<td>44</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype Frequency</th>
<th>Total PTCA Patient Population (%)</th>
<th>African American PTCA Subjects (%)</th>
<th>Caucasian PTCA Subjects (%)</th>
<th>Total Control Population (%)</th>
<th>African American Control Subjects (%)</th>
<th>Caucasian Control Subjects (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL</td>
<td>57.2</td>
<td>71</td>
<td>48</td>
<td>44</td>
<td>59</td>
<td>41</td>
</tr>
<tr>
<td>LS</td>
<td>20.3</td>
<td>18</td>
<td>22</td>
<td>29</td>
<td>24</td>
<td>30</td>
</tr>
<tr>
<td>SS</td>
<td>22.5</td>
<td>11</td>
<td>30</td>
<td>27</td>
<td>17</td>
<td>29</td>
</tr>
</tbody>
</table>

^a Genotype vs. Race for PTCA subjects, Chi square p-value = 0.014

^b L Allele vs. Race for PTCA subjects, Chi-Square P-value = 0.008

^c S Allele vs. Race for PTCA subjects, Chi-Square P-value = 0.008

^d S Allele vs. Race for Control subjects, Chi-Square P-value = 0.04
7.3.3 The Frequency Distribution of the HMOX1 Single Nucleotide Polymorphism at –413

A total of 324 subjects, consisting of 210 controls and 114 patients, were genotyped for the single nucleotide polymorphism at base – 413 of the HMOX1 promoter. Chi-square analysis for the SNP-413 allele and genotype frequencies of the control and patient populations are shown in Table 7.3. A trend was seen in which the TT genotype was more prevalent in the patient population and the AA genotype in the control population (P = 0.06). A significant association was found between the A allele and the control population (P = 0.03), and a trend was seen in which the T allele occurred more in the patient population (P = 0.08).

There was a significant association between the SNP-413 genotype and the ethnicity of the control subjects (P = 0.02). The AT genotype was seen in 45% of the Caucasian controls, while only 18% had the TT genotype. The African American controls were equally associated with both the AA and TT genotypes. The A allele was also found to be significantly associated with the Caucasian controls (P = 0.01). There were no significant associations found between either the SNP-413 genotype or allele frequency when the control population was divided by gender (P = 0.80).

When the patient population was split by ethnicity, a significant association was seen between the SNP-413 genotype and ethnicity (P < 0.0001). The Caucasian patients were associated with the AA genotype, while the African American patients were equally associated with the AT and TT genotypes. The A allele was predominantly seen in the Caucasian patient population (P = 0.003), while the T allele was significantly associated with the African American patients (P < 0.0001). There were no significant associations
found between either the SNP-413 genotype or allele frequency when the patient population was divided by gender, but a trend was seen in which the female patients possessed the T allele more than male patients (P = 0.12).

Chi-square analysis was used to examine the association between the clinical variables for the patient population and the SNP-413 genotype and allele frequencies. A trend was seen between the SNP-413 genotype and the history of known CAD. The AT genotype was seen in 32% of the patients who had a history of known CAD (P = 0.11). When the patient population was split by ethnicity, the African American patients had a significant association between the AT genotype and a history of known CAD (P = 0.02). This association was not present in the Caucasian patient population (P = 0.65). The A allele was significantly associated with the history of known CAD (P = 0.05), while the T allele was not (P = 0.86).

A trend was also seen between the HMOX1 SNP-413 genotype and a history of hypertension. Patients with a history of hypertension tended to have the AT genotype and lack the AA genotype, while patients lacking hypertension tended to have the AA genotype and lack the TT genotype (P = 0.10). The T allele was significantly associated with a history of hypertension, with 77% of the patients having both (P = 0.03). There was no association between a history of hypertension and the A allele, or when the patient population was split by gender or ethnicity.
<table>
<thead>
<tr>
<th></th>
<th>Total PTCA Patient Population (%)</th>
<th>African American PTCA Subjects (%)</th>
<th>Caucasian PTCA Subjects (%)</th>
<th>Total Control Population (%)</th>
<th>African American Control Subjects (%)</th>
<th>Caucasian Control Subjects (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Allele Frequency</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (^{c,d})</td>
<td>48</td>
<td>31</td>
<td>62</td>
<td>59</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>T (^{e})</td>
<td>52</td>
<td>69</td>
<td>38</td>
<td>41</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td><strong>Genotype Frequency</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>28</td>
<td>8</td>
<td>44</td>
<td>38</td>
<td>38</td>
<td>37</td>
</tr>
<tr>
<td>AT</td>
<td>40</td>
<td>46</td>
<td>36</td>
<td>42</td>
<td>24</td>
<td>45</td>
</tr>
<tr>
<td>TT</td>
<td>32</td>
<td>46</td>
<td>20</td>
<td>20</td>
<td>38</td>
<td>18</td>
</tr>
</tbody>
</table>

**Table 7.3: Allele and Genotype Frequencies for the HMOX1 SNP –413**

All populations are in Hardy–Weinberg Equilibrium.

\(^{a}\) Genotype vs. Race for PTCA subjects, Chi Square P-value < 0.0001  
\(^{b}\) Genotype vs. Race for Control subjects, Chi Square P-value = 0.02  
\(^{c}\) A Allele vs. Race for Control subjects, Chi Square P-value =0.01  
\(^{d}\) A Allele vs. Race for PTCA subjects, Chi Square P-value = 0.003  
\(^{e}\) T allele vs. Race for PTCA subjects, Chi Square P-value < 0.0001
7.3.4 The Frequency of both HMOX1 Promoter Polymorphisms in the
Control and Patient Populations

Table 7.4 shows the chi-square analysis for the genotype frequency of the
HMOX1 microsatellite polymorphism versus the genotype frequency of the HMOX1
SNP-413. The most frequent genotype combination in the control population was the LL
microsatellite polymorphism and AA SNP-413, occurring in 35.2% of all controls (P <
0.0001). The least frequent genotype combination was the SS microsatellite and AA
SNP-413, occurring in only 0.5% of the control population (P < 0.0001). In the control
population, the most frequent allele combinations were L, A and S, T (P < 0.0001).

When the control population was divided by ethnicity, the LL and AA genotype
combination was the most frequent in both the Caucasian (34.8%) and the African
American (37.9%) subjects (P < 0.0001 and P = 0.005, respectively). The SS and AA
genotype combination was seen in only 0.6% of Caucasian controls (P < 0.0001), while
both the LS, AA and SS, AA genotype combinations were absent from the African
American controls (P = 0.005). In both the Caucasian and African American control
populations, the L allele and A allele were seen in the majority of subjects (P < 0.0001
and P = 0.01, respectively). The African American controls also had a high frequency of
the L, T allele combination (P = 0.03), while the Caucasian controls had a high frequency
of the S, T allele combination (P < 0.0001).

The LL microsatellite and AA SNP-413 genotype combination was seen most
frequently and the SS, AA genotype combination seen least frequently in the PTCA
patient population (P = 0.0005). The patient population also had a high frequency of the
L, A and L, T allele combinations (P = 0.002 and P = 0.006, respectively). When the
patient population was divided by ethnicity, the LL and AA genotype combination was most frequent in the Caucasian controls, while the SS, AA and LS, TT genotype combinations were the least frequent (P < 0.0001). A trend was seen with the frequency of the genotype combinations in the African American patients (P = 0.18). The LL microsatellite genotype and AA SNP-413 genotype were seen at the highest frequency, while the SS, AT genotype combination was absent in the African American patient population. The Caucasian patients had a high frequency of both the L, A and S, T allele combinations (P = 0.0001 and P < 0.0001, respectively). The African American patients had a high frequency of both the L, A and L, T allele combinations, but only the L and A allele combination was significant (P = 0.05, and P = 0.40, respectively).
<table>
<thead>
<tr>
<th>Microsatellite Genotype, SNP-413 Genotype</th>
<th>Control Population (%)</th>
<th>PTCA Patient Population (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (^a)</td>
<td>African American (^b)</td>
</tr>
<tr>
<td>LL, AA</td>
<td>35.2</td>
<td>37.9</td>
</tr>
<tr>
<td>LL, AT</td>
<td>7.1</td>
<td>10.3</td>
</tr>
<tr>
<td>LL, TT</td>
<td>3.8</td>
<td>17.2</td>
</tr>
<tr>
<td>LS, AA</td>
<td>1.9</td>
<td>0</td>
</tr>
<tr>
<td>LS, AT</td>
<td>20</td>
<td>10.3</td>
</tr>
<tr>
<td>LS, TT</td>
<td>3.3</td>
<td>3.4</td>
</tr>
<tr>
<td>SS, AA</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>SS, AT</td>
<td>14.8</td>
<td>3.4</td>
</tr>
<tr>
<td>SS, TT</td>
<td>13.3</td>
<td>17.2</td>
</tr>
</tbody>
</table>

**Table 7.4: Genotype Frequencies of the HMOX1 Microsatellite Polymorphism vs. SNP-413**

\(^a\) Microsatellite vs. SNP-413 for Total Control Population, Chi-Square P-value < 0.0001

\(^b\) Microsatellite vs. SNP-413 for African American Controls, Chi-Square P-value = 0.005

\(^c\) Microsatellite vs. SNP-413 for Caucasian Controls, Chi-Square P-value < 0.0001

\(^d\) Microsatellite vs. SNP-413 for Total PTCA Population, Chi-Square P-value = 0.0005

\(^e\) Microsatellite vs. SNP-413 for Caucasian PTCA Population, Chi-Square P-value < 0.0001
7.4 Discussion

7.4.1 A Discussion of the HMOX1 Microsatellite Polymorphism Results

Previous research has shown a significant association between the presence of the large HMOX1 microsatellite polymorphism and the pathogenesis of CAD. The previous studies, which primarily focused on Asian and Caucasian populations, also established that the HMOX1 microsatellite polymorphism varies between ethnic groups. The results of the experiments detailed in this chapter are significant because they further characterize the relationship between CAD and the HMOX1 microsatellite polymorphism in the Caucasian population and are the first to examine the relationship between the microsatellite polymorphism and CAD in the African American population.

The African American population has a high prevalence of hypertension and diabetes mellitus, two prominent risk factors for cardiovascular disease, and has been shown to respond unfavorably to certain medicines used in the treatment of cardiovascular disease. The use of genetic research can help to define the underlying physiological factors that predispose African Americans to cardiovascular disease. It can also provide methods to screen subjects to enable them to be proactive in preventing the pathogenesis of cardiovascular disease. [Yancy, 2003] This population is grossly underrepresented in genetic research and that is why it was chosen to be included as part of the current study.

In the patient population used for this study, a significant association was found between the African American patients and hypertension, and between the Caucasian patients and hypercholesterolemia. These results could possibly signify a difference in the etiology of CAD between the ethnic groups. Examination of the mean GT repeats for
all subjects involved in the study revealed that subjects of African American ethnicity had a significantly larger mean GT repeat than the subjects of Caucasian ethnicity. This is similar to the results reported in Chapter 6 of this dissertation. When comparing the mean GT repeats of the control and CAD patient populations, the patients had a significantly larger mean GT repeat. A trend was also seen in which the African American patients had a larger mean repeat than the African American controls. *In vitro* experiments have shown that HMOX1 transcription is decreased when the microsatellite polymorphism exceeds 25 repeats. The African American subjects, with controls having a mean GT repeat of 28 and patients having a mean GT repeat of 29, most likely have an endogenous lack of HO-1 activity due to a decrease in functioning HMOX1.

Previous research has shown that the frequency distribution of the HMOX1 microsatellite polymorphism is bimodal with peaks seen at 23 and 30 repeats. The frequency distribution histograms from this study indicate that the total control group, the Caucasian controls, and the Caucasian patients all show the same bimodal distribution. The total PTCA patient group and the African American patients have a distribution containing only one peak corresponding to 30 repeats. The African American controls have a unique trimodal distribution, with peaks corresponding to 23, 26, and 30 repeats. The differences in the distribution patterns shows that the patient group, especially the African American patients, has a higher frequency of possessing only large GT repeats, thereby putting them at increased risk for having decreased HO-1 activity.

Genotype analysis revealed that the control group had significantly more SS genotypes and less LL genotypes than the patient group. The S allele was also found to be significantly associated with the control population. When the patient and control
populations were divided by ethnicity, the African American subjects from both groups were found to have more of the LL genotype and L allele, while the Caucasian subjects from both groups possessed more of the SS genotype and S allele. Logistic regression analysis showed an odds ratio of 2.6 for African American patients having the LL genotype, with the odds increasing to 3.75 after adjusting for clinical variables. These results show that the African American population consistently has the large microsatellite polymorphism and this polymorphism is significantly associated with the presence of CAD in this ethnic group.

Examination of the HMOX1 microsatellite polymorphism and its relationship to the clinical variables of the patient population revealed that the LL genotype was significantly associated with patients who had a known history of CAD. Logistic regression analysis revealed that patients with a history of CAD have 2.23-fold greater odds of also having the LL genotype. These results further establish the connection between the large microsatellite polymorphism of the HMOX1 promoter and CAD.

7.4.2 A Discussion of the HMOX1 SNP-413 Results

One major study previously investigated the role of the HMOX1 SNP-413 promoter in cardiovascular disease and discovered that the AA genotype is related to a decreased incidence of CAD in the Japanese population. In vitro studies have also shown that the A allele is associated with increased HMOX1 promoter activity. The results from the current study are important because they are the first to examine the relationship between the SNP-413 and CAD in the Caucasian and African American populations. Genotype analysis revealed a trend in which the TT genotype and T allele were associated with the patient population, while the AA genotype was associated with the
control population. A significant association was seen between the A allele and the control group. These results show that the control population has a greater frequency of the A allele, therefore possibly having increased HMOX1 activity and ample HO-1 production.

In the Caucasian control population, the AT genotype was seen most frequently and the TT genotype seen least frequently. The Caucasian controls were also associated with the A allele. The African American controls had high frequencies of both the AA and TT genotypes. In the patient population, the Caucasian subjects had the highest percentage of the AA genotype, while the African American patients had high frequencies of both the AT and TT genotypes. The Caucasian patients were also associated with the A allele, while the African American patients were associated with the T allele. The association between the Caucasian patients and the A allele may cause them to experience less severe CAD due to possible increased protective actions of HO-1.

An examination of the relationship between the HMOX1 SNP-413 and the clinical variables possessed by the patients revealed a trend in which the AT genotype was associated with a history of CAD. The A allele was also found to be significantly associated with a history of CAD. When the patient population was divided by ethnicity, a significant association was found between the AT genotype and a history of CAD in the African American patients. These results seem to contradict those of Ono et al in which an association was found between the AA genotype and a decreased incidence of CAD. [Ono, 2004] In the current study, the fact that the A allele is associated with a history of CAD may signify that the protective actions of the A allele are specific for the Asian
ethnic group or that the presence of the T allele counteracts any protection the A allele may provide. Further research is needed to fully explore this result.

A trend was also found in which the AT allele was associated with a history of hypertension. The presence of the AA genotype was also decreased in patients with hypertension. Patients without hypertension tended to have the AA genotype and lack the TT genotype. The T allele was also significantly associated with the presence of hypertension. Once again these results seem to contradict the findings of Ono et al in which the AA genotype was associated with hypertension in Japanese females. [Ono, 2003] The results presented in this study show that the AA genotype is present more often in patients lacking hypertension. In the current study, there was no significant association between the SNP-413 genotype and hypertension when patients were divided by gender. Further research is needed to determine whether the differing results are due to ethnicity or some other factor.

7.4.3 A Discussion of the Relationship Between the HMOX1 Promoter Polymorphisms

The LL microsatellite and AA SNP-413 genotype combination and L, A allele combination were seen in the largest percentage of subjects in both the patient and control populations. This remained true when the populations were separated by ethnicity. It is interesting to note that the SS, AA and LS, AA genotype combinations were absent from the African American control population, and the SS, AT genotype combination absent from the African American patient population. A large percentage of African American subjects from each group also had the L, T allele combination, while Caucasians tended to have the S, T allele combination. The African American subjects
seem to possess the combination of the L microsatellite allele and the T SNP-413 allele more frequently than the Caucasian subjects. This combination could negatively affect HMOX1 promoter function in African Americans, leaving them susceptible to diseases resulting from oxidative stress.

It is very interesting that the LL and AA genotypes, or L and A alleles, tend to associate with each other because they are reported to have opposite effects on HMOX1 transcription. It is possible that the combination of the A allele, which supposedly promotes transcription, and the L allele, which supposedly prevents transcription, may balance each other and provide seemingly normal levels of transcription. In vitro and in vivo studies are needed to further characterize the interaction between these polymorphisms and their effects on HMOX1 transcription.

7.4.4 A Discussion of Experimental Limitations and Future Research

A discussed in the previous chapter, this study was limited due to the small number of African American subjects. The experiments should be repeated in a much larger cohort to verify the results. The genotyping results for the SNP-413 should be considered preliminary and verified by other genotyping methods because it was often difficult to get an accurate genotype off of both the sense and antisense sequence. The difficulty in determining the SNP-413 genotype was the second limiting factor for this study.

It would be interesting to expand upon the results in this study and investigate the association between the HMOX1 promoter polymorphisms and restenosis in the African American population. Work has been done investigating the microsatellite polymorphism and restenosis in Asian and Caucasian populations, but a study involving
the African American population has yet to be undertaken. The effect of the HMOX1 SNP-413 polymorphism on restenosis has yet to be determined.

Section 3.8 of this dissertation describes numerous cardiovascular drugs that upregulate HO-1 in order to facilitate their protective actions. HO-1 has been linked to the activity of many drugs commonly used in the treatment of CAD, including aspirin and statins. It has also been associated with drugs involved in preventing restenosis. The results from this study could be expanded into a pharmacogenetic study examining the relationship between drug response and the HMOX1 promoter polymorphisms. If the large microsatellite polymorphism is present and decreases HO-1 activity, it can reduce the efficacy of the drugs. Pharmacological therapy may have to be adjusted in patients carrying the large microsatellite polymorphism in order to provide the most effective treatment regimen. This also could provide insight into the varied drug responses shown by different ethnic groups.
CHAPTER 8

AN EXAMINATION OF THE HMOX1 PROMOTER POLYMORPHISMS IN CAUCASIAN AND AFRICAN AMERICAN PATIENTS WITH CONGESTIVE HEART FAILURE

8.1 Introduction

The experiments in Chapter 6 showed that the HMOX1 promoter polymorphisms associated with decreased promoter activity occur more frequently in the African American population than the Caucasian population. Chapter 7 revealed that these detrimental polymorphisms are associated with CAD, especially in African American patients. This chapter expands upon the previous results to examine the association between the HMOX1 promoter polymorphisms and the pathogenesis of congestive heart failure (CHF). It is important to focus on the African American and Caucasian ethnic groups because of the unique ways in which CHF manifests itself in these populations. African Americans with CHF tend to have earlier onset and more severe disease than Caucasians. The pathogenesis of CHF in African Americans also tends to be more hypertension related, while Caucasians tend to have more CAD related CHF.
The experiments in this chapter test the hypothesis that the HMOX1 promoter polymorphisms are present more frequently in patients with CHF, especially in the African American population.

8.2 Methods and Materials

8.2.1 Subjects

This study involved a patient population treated at The Ohio State University Heart Failure Clinic and diagnosed with either idiopathic dilated cardiomyopathy or ischemic cardiomyopathy, with symptomatic congestive heart failure. The European Collection of Cell Cultures provided a set of 112 normal human control DNA samples of Caucasian and African ethnicity (Sigma-Aldrich, St. Louis, MO). The remaining normal control samples were selected anonymously from blood samples provided by the American Red Cross (Columbus, OH). Subjects were limited to those who racially identified themselves as African American or Caucasian. All study subjects provided written, informed consent, and the protocol was approved by the Institutional Review Board for Human Subjects of the Ohio State University.

8.2.2 Extraction of Genomic DNA

Genomic DNA was extracted from either peripheral blood leukocytes or from a buccal cell sample using the methods described in Section 4.2 of this dissertation.

8.2.3 Polymerase Chain Reaction

The PCR to screen for the HMOX1 polymorphisms utilized the sense primer GT 5 (5’- CCA GCA GGT GAC ATT TTA GGG-3’) and antisense primer GT 3 (5’- ACA GCT GAT GCC CAC TTT CTG- 3’), which both annealed at 58 °C. The PCR for the
eNOS Glu298Asp variant used the sense primer 894S (5’-GAA ACG GTC GCT TCG ACG T- 3’) and the antisense primer 894A (5’- ATC CCA CCC AGT CAA TCC CT-3’), which anneal at 47 °C. The PCR to screen for the eNOS intron 4 variant used the sense primer NOS1 (5’-AGG CCC TAT GGT AGT GCC TTT- 3’) and the antisense primer NOS2 (5’-TCT CTT AGT GCT GTG GTC AC- 3’), which also anneal at 47 °C. The PCR reaction mixture and program used for the analysis is described in Section 4.3.

8.2.4 Gel Electrophoresis

Section 4.4 of this dissertation describes the gel electrophoresis protocol used for this study.

8.2.5 Genotyping the eNOS Polymorphisms

The eNOS Glu298Asp polymorphism is the result of a SNP at base 894 of exon 7 in which a G is changed to a T. This creates a site specific for the restriction enzyme MboI. In the restriction digestion reaction, 8 ul of PCR product was added to 1 ul of MboI and 1 ul 10X buffer. The samples were then incubated at 37 °C for 3 hours. After the incubation, the samples were run on gel electrophoresis according to the protocol in Section 4.4. The bands visualized on the gel corresponded to the following genotypes: GG = 248 bp; GT = 248, 158, and 90 bp; TT = 158 and 90 bp.

The eNOS intron 4 polymorphism is a variable number of tandem repeats (VNTR) polymorphism. The VNTR consists of a 27 bp repeat, and is found in groupings of either 4 repeats, known as the a allele, or 5 repeats, known as the b allele. To genotype for the intron 4 polymorphism, 10 ul of PCR product was run on a 4% agarose gel for 1.5 hours according to the gel electrophoresis protocol in Section 4.4. The bands visualized
on the gel corresponded to the following genotypes: aa = 393 bp; ab = 393 and 420 bp; bb = 420 bp.

8.2.6 Sequencing Analysis

The PCR products from the HMOX1 polymorphism reaction were sequenced according to the methods described in Section 4.5. The sense primer GT 5 and the antisense primer GT 3 were used in the sequencing reaction.

8.2.7 HMOX1 Genotyping

The HMOX1 microsatellite polymorphism was assigned either a Small (S) or Large (L) allele classification based upon the amount of dinucleotide repeats present in the sense and antisense sequences. The cut off point for the allele classes was determined by the median allele for the control population. An allele was classified as S if it consisted of a GT repeat of less than 26 and L if the GT repeat was greater than or equal to 26. The HMOX1 SNP-413 was genotyped according to the bases present at position –413 of the sequences.

8.2.8 Statistical Analysis

StatView for Windows version 5.0 (SAS Institute Inc., Cary, NC) was used for frequency distribution histograms and chi-square tests to examine the distribution of all genotypes. Logistic regression was performed to adjust odds ratios for the presence or absence of the large (LL) genotype for differing clinical covariates. Specifically, a dichotomized variable indicating presence or absence of the LL genotype constituted the independent variable. Ethnic background and clinical variables characterizing the study groups were included in the logistic model as the independent variables. A stepwise regression with forward selection was used to identify variables independently associated
with presence or absence of the polymorphism. Logistic regression was performed using
STATA version 8.0 for Windows (StataCorp LP, College Station, TX).

8.3 Results

8.3.1 Demographic Information for the Patient and Control Populations

A total of 537 subjects, consisting of 256 controls and 281 heart failure patients,
were genotyped for the HMOX1 microsatellite polymorphism. The demographic
information for each group is listed in Table 8.1. The patient population, with a mean
age of 52.1 ± 14 years, was significantly older than the control population, with an
average age of 41.4 ± 12 years (P < 0.0001). Patients with CHF resulting from an
ischemic etiology were significantly older than patients with CHF from an nonischemic
etiology (58 ± 11 years vs. 47 ± 14 years, P < 0.0001). The patient population also had a
greater percentage of male subjects (70%) than the control population (53%).

The control and patient populations had similar percentages of African American
subjects. When broken down by ethnic group, the African American and Caucasian
control populations had almost equal percentages of male and female subjects. The
Caucasian controls were significantly (P = 0.0004) younger than the African American
controls (40 ± 12 years vs. 50 ± 13 years, respectively). The African American patient
population had a greater percentage of male subjects (61%) than female subjects (39%).
The Caucasian patient population also contained a greater percentage of male subjects
(72%) than female subjects (28%). A trend (P = 0.09) was seen in the CHF population in
which the Caucasian patients were older than the African American patients (52 ± 14
years vs. 49 ± 14 years, respectively). Analysis of the presence of cardiovascular risk

132
factors in the African American versus Caucasian CHF patients revealed that the African American patients were significantly associated with CHF from a nonischemic etiology, while the Caucasian patients were associated with CHF from an ischemic etiology (P = 0.01). A trend was also seen in which the Caucasian patients were associated with a history of hypercholesterolemia (P = 0.14).
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients (N = 281)</th>
<th>Controls (N = 256)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age – mean yr (± SD) a</td>
<td>52 (± 14)</td>
<td>41 (± 12)</td>
</tr>
<tr>
<td>Male sex – no. (%)</td>
<td>196 (70)</td>
<td>135 (53)</td>
</tr>
<tr>
<td>African American Race – no. (%)</td>
<td>49 (17)</td>
<td>41 (16)</td>
</tr>
<tr>
<td>Ischemic Etiology for CHF – no. (%)</td>
<td>121 (43)</td>
<td>NA b</td>
</tr>
<tr>
<td>History of Hypercholesterolemia – no. (%) (n = 249)</td>
<td>111 (45)</td>
<td>NA</td>
</tr>
<tr>
<td>History of Hypertension – no. (%) (n = 254)</td>
<td>122 (48)</td>
<td>NA</td>
</tr>
<tr>
<td>History of Diabetes – no. (%) (n = 274)</td>
<td>81 (30)</td>
<td>NA</td>
</tr>
<tr>
<td>History of Smoking – no. (%) (n = 249)</td>
<td>141 (57)</td>
<td>NA</td>
</tr>
<tr>
<td>Normalized Ejection Fraction – no. (%) (n = 281)</td>
<td>34 (12)</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Table 8.1: Demographic Information**

a Unpaired t-test, p-value < 0.001

b NA = This information was not available for the control population
8.3.2 The Frequency Distribution of the HMOX1 Microsatellite Polymorphism

Frequency distribution histograms were plotted for the number of HMOX1 promoter GT repeats found in both the CHF patient and control populations (Figure 8.1). The mean GT repeats for the HMOX1 microsatellite polymorphism in the control and patient populations were not significantly different (26.9 ± 3.4 repeats vs. 27.1 ± 3.7 repeats, respectively; P = 0.53). Figure 8.2 shows the frequency distribution histograms for both subject populations separated on the basis of ethnicity. The African American members of both the control and patient populations have a greater percentage of large GT repeats. In both populations, the mean GT repeat for the African American subjects was significantly larger than the mean GT repeat for the Caucasian subjects. The African American controls had a mean dinucleotide repeat of 28.0 ± 3.9 repeats compared to the Caucasian controls with 26.7 ± 3.2 repeats (P = 0.002). The African American CHF patients had a mean GT repeat of 29.3 ± 5.1 repeats compared to the Caucasian patients with a mean of 26.6 ± 3.2 repeats (P < 0.0001). There was no significant difference between the mean GT repeats of the Caucasian controls versus the Caucasian patients (P = 0.51). A significant difference was seen between the mean GT repeats of the African American controls and the African American patients, with the patients having the larger mean (P = 0.05).
Figure 8.1: Frequency Distribution for Dinucleotide Repeats of the HMOX1 Microsatellite Polymorphism
Figure 8.2: Frequency Distribution for Dinucleotide Repeats of the HMOX1 Microsatellite Polymorphism Divided by Ethnicity

a.) The CHF Patient Population

b.) The Control Population
Chi-square analysis of the allele and genotype frequencies of the HMOX1 microsatellite polymorphism is shown in Table 8.2. When comparing the genotype frequencies of the control and patient populations, the majority of subjects in both groups had the LL genotype. The LS genotype appeared more in the control population, while the SS genotype appeared more in the CHF population ($P = 0.05$). The distribution of the L and S alleles was not significant when comparing the populations.

The genotype results for the control population showed a trend in which the LL genotype was associated with the African American controls and the SS genotype was associated with the Caucasian controls ($P = 0.10$). The L allele showed a trend in which it was associated with the African American controls ($P = 0.11$), while the S allele was significantly associated with the Caucasian controls ($P = 0.04$). Chi-square analysis of the allele and genotype frequencies versus gender for the control population failed to find any significant associations.

Chi-square analysis for the genotype frequency in the CHF patient population revealed a significant association between the LL genotype and the African American patients. The Caucasian patients were significantly associated with the SS genotype ($P = 0.02$). Logistic regression analysis found that African American heart failure patients had greater odds of possessing the LL genotype compared to Caucasian heart failure patients ($P = 0.01$, OR = 2.26, CI = 1.19-4.29). The L allele was significantly associated with the African American patients, while the S allele was significantly associated with the Caucasian patients ($P = 0.01$).

In a forward stepwise logistic regression model that included race, age, history of hypercholesterolemia, ischemic etiology of congestive heart failure, history of
hypertension, gender, smoking history, and history of diabetes, only race was retained as
a variable significantly associated with the presence or absence of the LL genotype. This
therefore indicated that race was independently associated with the presence of the LL
genotype and its association was not due to collinearity or passive association with other
clinical variables. To adjust for the above covariates, the full logistic model
incorporating these variables and race was constructed. The resultant adjusted odds ratio
for the presence of the LL genotype in African American subjects was 3.0 with a
confidence interval of 1.4 – 6.7 and was highly significant with a P value = 0.006.

A subset of 34 patients, 7 African American and 27 Caucasians, recovered normal
ventricular systolic function as a result of standard medical therapy. Genetic analysis
revealed that the LL genotype of the HMOX1 microsatellite polymorphism was
significantly associated with the presence of a normalized ejection fraction (P = 0.03).
Sixty-two percent of the patients who possessed a normalized ejection fraction also had
the LL genotype. The L allele was also significantly associated with a normalized
ejection fraction, appearing in 88% of the patients having a normalized ejection fraction
(P = 0.009). A trend was seen in which the S allele was found more in the patients who
did not recover normal ventricular function (P = 0.11).
<table>
<thead>
<tr>
<th></th>
<th>Total CHF Patient Population (%)</th>
<th>African American CHF Subjects (%)</th>
<th>Caucasian CHF Subjects (%)</th>
<th>Total Control Population (%)</th>
<th>African American Control Subjects (%)</th>
<th>Caucasian Control Subjects (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Allele Frequency</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>59</td>
<td>74</td>
<td>55</td>
<td>58</td>
<td>71</td>
<td>56</td>
</tr>
<tr>
<td>S&lt;sup&gt;c&lt;/sup&gt;</td>
<td>41</td>
<td>26</td>
<td>45</td>
<td>42</td>
<td>29</td>
<td>44</td>
</tr>
<tr>
<td><strong>Genotype Frequency</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LL</td>
<td>49</td>
<td>65.3</td>
<td>45.2</td>
<td>44</td>
<td>59</td>
<td>41</td>
</tr>
<tr>
<td>LS</td>
<td>20</td>
<td>18.4</td>
<td>20.3</td>
<td>29</td>
<td>24</td>
<td>30</td>
</tr>
<tr>
<td>SS</td>
<td>31</td>
<td>16.3</td>
<td>34.5</td>
<td>27</td>
<td>17</td>
<td>29</td>
</tr>
</tbody>
</table>

Table 8.2: Allele and Genotype Frequencies for the HMOX1 Microsatellite Polymorphism

All populations are in Hardy-Weinberg Equilibrium.

<sup>a</sup> L Allele vs. Ethnicity and S allele vs. Ethnicity for CHF subjects, Chi Square P-value = 0.01

<sup>b</sup> Genotype vs. Ethnicity for CHF subjects, Chi Square P-value = 0.02

<sup>c</sup> S Allele vs. Ethnicity for Control subjects, Chi-Square P-value = 0.04
8.3.3 The Frequency Distribution of the HMOX1 Single Nucleotide Polymorphism at –413

A total of 458 subjects, consisting of 210 controls and 248 CHF patients, were genotyped for the single nucleotide polymorphism at base –413 of the HMOX1 promoter. Chi-square analysis for the SNP-413 allele and genotype frequencies for the control and CHF patient population is shown in Table 8.3. There were no significant associations found between the genotype and allele frequencies of the control and patient populations.

There was a significant association between the SNP-413 genotype and the ethnicity of the control subjects (P = 0.02). The AT genotype was seen in 45% of the Caucasian controls, while only 18% had the TT genotype. The African American controls were equally associated with both the AA and TT genotypes. The A allele was also found to be significantly associated with the Caucasian controls (P = 0.01). There were no significant associations found between either the SNP-413 genotype or allele frequency when the control population was divided by gender (P = 0.80).

When the CHF population was split by ethnicity, a significant association was found between the SNP-413 genotype and the different ethnic populations. The TT genotype was seen in the majority of African American patients, while the AA and AT genotypes were seen more in the Caucasian patients (P < 0.0001). The A allele was significantly associated with the Caucasian patients (P < 0.0001), while the T allele was associated with the African American patients (P = 0.007). Genetic analysis based on gender or any of the clinical variables failed to find any significant associations with the SNP-413 genotype.
<table>
<thead>
<tr>
<th></th>
<th>Total CHF Patient Population (%)</th>
<th>Total CHF Subjects (%)</th>
<th>Total Control Population (%)</th>
<th>Total Control Subjects (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>African American CHF Subjects a (%)</td>
<td>Caucasian CHF Subjects (%)</td>
<td>African American Control Subjects b (%)</td>
<td>Caucasian Control Subjects (%)</td>
</tr>
<tr>
<td>Allele Frequency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A c, d</td>
<td>55</td>
<td>33</td>
<td>59</td>
<td>59</td>
</tr>
<tr>
<td>T e</td>
<td>45</td>
<td>67</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td>Genotype Frequency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>33.5</td>
<td>16</td>
<td>37</td>
<td>38</td>
</tr>
<tr>
<td>AT</td>
<td>42.3</td>
<td>34</td>
<td>44</td>
<td>42</td>
</tr>
<tr>
<td>TT</td>
<td>24.2</td>
<td>50</td>
<td>19</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 8.3: Allele and Genotype Frequencies for the HMOX1 SNP –413

All populations are in Hardy –Weinberg Equilibrium.

a Genotype vs. Ethnicity for CHF subjects, Chi-square P-value < 0.0001

b Genotype vs. Ethnicity for Control subjects, Chi Square P-value = 0.02

c A Allele vs. Ethnicity for Control subjects, Chi Square P-value =0.01

d A Allele vs. Ethnicity for CHF subjects, Chi-square P-value < 0.001

e T Allele vs. Ethnicity for CHF subjects, Chi-square P-value = 0.007
8.3.4 The Frequency of both HMOX1 Promoter Polymorphisms in the Control and Patient Populations

The Chi-square analysis of the genotype frequency of the HMOX1 microsatellite polymorphism versus the genotype frequency of the HMOX1 SNP-413 is shown in Table 8.4. The most frequent genotype combination in the control population was the LL microsatellite polymorphism and AA SNP-413, occurring in 35.2% of all controls (P < 0.0001). The least frequent genotype combination was the SS microsatellite and AA SNP-413, occurring in only 0.5% of the control population (P < 0.0001). In the control population, the most frequent allele combinations were L, A and S, T (P < 0.0001).

When the control population was divided by ethnicity, the LL and AA genotype combination was the most frequent in both the Caucasian (34.8%) and the African American (37.9%) subjects (P < 0.0001 and P = 0.005, respectively). The SS and AA genotype combination was seen in only 0.6% of Caucasian controls (P < 0.0001), while both the LS, AA and SS, AA genotype combinations were absent from the African American controls (P = 0.005). In both the Caucasian and African American control populations, the L allele and A allele were seen in the majority of subjects (P < 0.0001 and P = 0.01, respectively). The African American controls also had a high frequency of the L, T allele combination (P = 0.03), while the Caucasian controls had a high frequency of the S, T allele combination (P < 0.0001).

The LL microsatellite and AA SNP-413 genotype combination was seen most frequently in the CHF patient population, and the SS, AA genotype combination did not appear in the patients (P < 0.0001). The allele combinations in the patient population were also significant (P < 0.0001), with the L,A and S,T combinations appearing at the
highest frequency. The Caucasian patient population had the LL and AA genotype present at the highest frequency and lacked the SS, AA genotype combination (P < 0.0001). The L and A allele combination was seen most frequently in the Caucasian patients, followed by the S and T allele combination (P < 0.0001). In the African American patient population, the genotype combinations for the HMOX1 promoter polymorphisms were not significant (P = 0.36). The LL, TT genotype combination was seen most frequently, while the SS, AA and LS, AA were absent from the African American patients. The S and T allele combination was the only one significantly associated with the African American patients (P = 0.04).
<table>
<thead>
<tr>
<th>Microsatellite Genotype, SNP-413 Genotype</th>
<th>Control Population (%)</th>
<th>CHF Population (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total °</td>
<td>African American b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LL, AA</td>
<td>35.2</td>
<td>37.9</td>
</tr>
<tr>
<td>LL, AT</td>
<td>7.1</td>
<td>10.3</td>
</tr>
<tr>
<td>LL, TT</td>
<td>3.8</td>
<td>17.2</td>
</tr>
<tr>
<td>LS, AA</td>
<td>1.9</td>
<td>0</td>
</tr>
<tr>
<td>LS, AT</td>
<td>20</td>
<td>10.3</td>
</tr>
<tr>
<td>LS, TT</td>
<td>3.3</td>
<td>3.4</td>
</tr>
<tr>
<td>SS, AA</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>SS, AT</td>
<td>14.8</td>
<td>3.4</td>
</tr>
<tr>
<td>SS, TT</td>
<td>13.3</td>
<td>17.2</td>
</tr>
</tbody>
</table>

Table 8.4: Genotype Frequencies of the HMOX1 Microsatellite Polymorphism vs. SNP-413

° Microsatellite vs. SNP-413 for Total Control Population, Chi-Square P-value < 0.0001

b Microsatellite vs. SNP-413 for African American Controls, Chi-Square P-value = 0.005

c Microsatellite vs. SNP-413 for Caucasian Controls, Chi-Square P-value < 0.0001

d Microsatellite vs. SNP-413 for Total CHF Population, Chi-Square P-value < 0.0001

e Microsatellite vs. SNP-413 for Caucasian CHF Patients, Chi-Square P-value < 0.0001
8.3.5 The Frequency of the eNOS Glu298Asp and Intron 4 Polymorphisms

8.3.5.1 The eNOS Glu298Asp Polymorphism

A total of 398 subjects, 121 controls and 277 CHF patients, were genotyped for the eNOS Glu298Asp polymorphism. Table 8.5 shows the genotype frequency for this eNOS polymorphism. The CHF patient population had the highest frequency of the GG genotype, while the control population had the highest frequency of the GT genotype. The patient population also had a higher percentage of the TT mutant genotype than the control population (P = 0.05). The genotype frequency for the eNOS Glu298Asp polymorphism was not significant when the control group was divided by ethnicity (P = 0.90). A significant association was found when the CHF patient population was separated by ethnicity. The African American patients had a higher frequency of the GG genotype compared to the Caucasian patients, while the Caucasian patients had a higher frequency of the TT mutant genotype than the African American patients (P = 0.02).

Chi-square analysis was used to examine the relationship between the genotype frequencies of the HMOX1 promoter polymorphisms and the eNOS Glu298Asp polymorphism. In the control population, no significant relationships could be found between the polymorphisms. The GG eNOS genotype and LL microsatellite genotype combination and the GG eNOS and AT SNP-413 genotype combination were seen at the highest frequency in the controls. In the CHF patient population, the GT, LL and GG, AT genotype combinations were seen most frequently, but the association was not significant. For both groups, the lack of significance was seen in the total population and when the population was divided into ethnic groups.
<table>
<thead>
<tr>
<th>eNOS Glu298Asp Polymorphism</th>
<th>CHF Patient Population</th>
<th>Control Population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Patient Population (%)</td>
<td>African American (%)</td>
</tr>
<tr>
<td>GG</td>
<td>53.8</td>
<td>72.3</td>
</tr>
<tr>
<td>GT</td>
<td>37.2</td>
<td>23.4</td>
</tr>
<tr>
<td>TT</td>
<td>9.0</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Table 8.5: Genotyping Frequency for the eNOS Glu298Asp Polymorphism

* Genotype vs. Ethnicity for the CHF population, Chi-Square P-value = 0.02

* Genotype vs. Control and CHF population, Chi-Square P-value = 0.05

8.3.5.2 The eNOS Intron 4 Polymorphism

A total of 330 subjects, consisting of 62 controls and 268 CHF patients, were genotyped for the eNOS Intron 4 polymorphism. Table 8.6 shows the genotype frequency for this eNOS polymorphism. A trend is seen in which the patient population had a higher frequency of both the ba and aa Intron 4 genotypes than the control population (P = 0.07). The frequency of the eNOS Intron 4 genotype was not significant between the African American and Caucasian control populations (P = 0.74). It should be noted that only 2 African American controls had been genotyped for the Intron 4 polymorphism, a factor that greatly influenced the genotyping analysis. The CHF population did show a significant association when divided by ethnicity. The African American patients possessed both the bb and ba genotypes at the highest frequency, while
the Caucasian patients had the bb genotype at the highest frequency (P = 0.04). The aa mutant genotype was equally found in both patient groups.

Chi-square analysis of the frequency of the eNOS Intron 4 polymorphism versus the HMOX1 microsatellite polymorphism in the control population failed to find a significant association between the genotypes. The LL microsatellite and bb Intron 4 genotype combination was seen at the highest percentage in the control group. Analysis of the SNP-413 versus the Intron 4 genotypes in the control group also failed to find significance. The AA SNP-413 and bb Intron 4 genotype were seen at the highest percentage in the control group. The lack of genotyping results for the African American controls negated the need to run any ethnic analysis.

In the CHF patient population, analysis of the microsatellite polymorphism versus the Intron 4 polymorphism showed a trend in which the LL, bb genotype combination was seen at the greatest frequency (P = 0.13). The African American patients did not show any significant associations between the microsatellite and Intron 4 genotypes. It is interesting to note that the LL, bb genotype combination was seen in the highest percentage of African American patients, while the LS, aa and SS, aa combinations were absent in these subjects. The Caucasian patients showed a trend in which the LL, bb genotype was seen at the highest frequency in the population (P = 0.12). A significant association was found between the HMOX1 SNP-413 genotype and the eNOS Intron 4 genotype. The AT SNP-413 and bb Intron 4 genotype combination was seen at the highest frequency in the patient population (P = 0.04). When the patient population was divided by ethnicity, a trend was also seen in which the Caucasian patients were associated with the AT SNP-413, bb Intron 4 genotype combination (P = 0.08). No
significant associations were seen in the African American patients, but it is interesting to note that the TT, ba and TT, bb genotype combinations were present in the highest percentage of subjects

<table>
<thead>
<tr>
<th>eNOS Intron 4 Polymorphism</th>
<th>CHF Patient Population</th>
<th>Control Population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Patient Population</td>
<td>African American</td>
</tr>
<tr>
<td></td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>bb</td>
<td>62.7</td>
<td>47</td>
</tr>
<tr>
<td>ba</td>
<td>31.3</td>
<td>47</td>
</tr>
<tr>
<td>aa</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 8.6: Genotype Frequency for the eNOS Intron 4 Polymorphism

*a Genotype vs. Ethnicity for the CHF population, Chi-Square P-value = 0.04

8.4 Discussion

8.4.1 A Discussion of Ethnicity and Congestive Heart Failure

Caucasian and African American CHF patients differ in disease manifestation and response to pharmacological therapy. African Americans tend to develop a very severe form of CHF that is most often related to a history of hypertension. Caucasians develop a milder form of CHF that is most often related to a history of CAD. CHF manifests itself
in the African American population at a much younger age than in the Caucasian population. [Goldstein, 2004; Vaccarino, 2002; Yancy, 2001] African Americans with heart failure are also at increased risk for disease progression and death compared to Caucasians. [Dries, 2002] Numerous studies have shown that African American patients have decreased responses to certain drug therapies when compared to Caucasian patients. [Dries, 1999; Exner, 2001; Julius, 2004]

The patient populations used in this study fit the traditional ethnic profile. The African American patients were significantly associated with CHF of a nonischemic etiology, such as that resulting from long term hypertension, while the Caucasian patients were significantly associated with CHF of an ischemic etiology, such as that resulting from CAD. The Caucasian patients, with a mean age of 52 ± 14 years, were significantly older than the African American patients, who had a mean age of 49 ± 14 years. Information on patient survival rates and response to drug therapy were not recorded as clinical variables for this study.

8.4.2 A Discussion of the HMOX1 Microsatellite Polymorphism Results

African American subjects in both the control and CHF patient populations had a mean GT repeat that was significantly larger than the mean GT repeat of the Caucasian subjects. The African American CHF patient population also had a mean GT repeat that was significantly larger than the mean GT repeat of the African American control population. In both populations, the African American subjects were significantly associated with the LL genotype and L allele, while the Caucasian subjects were associated with the SS genotype and S allele. These results are consistent with those reported in Chapters 6 and 7.
Logistic regression analysis revealed that the African American patients had 2.26-fold greater odds of having the LL genotype. A forward stepwise logistic regression model for all clinical variables established that race was the only variable significantly associated with the LL genotype. This model led to an adjusted odds ratio in which the African American patients had 3.0-fold greater odds of having the LL genotype. The author hypothesizes the LL genotype in the African American patients may contribute to their reduced drug response. HO-1 and NOS actively regulate each other, and both enzymes act as mediators for drugs used in the treatment of cardiovascular disease. The presence of the large dinucleotide repeat in the HMOX1 microsatellite polymorphism may decrease the amount of active HO-1, thereby negatively influencing NOS activity and drug efficacy.

The genotype analysis also revealed a unique finding. Both the LL genotype and L allele were significantly associated with the recovery of normal ventricular systolic function due to medical therapy. This is a very interesting result because the LL genotype is thought to be related to decreased HO-1 activity and therefore should negatively effect the response to pharmaceutical agents. Further research is needed to fully characterize the relationship between HO-1 activity and normalized ventricular function.

**8.4.3 A Discussion of the HMOX1 SNP-413 Results**

A significant association was seen between the AT SNP-413 genotype and the Caucasian control population, and the AA and TT SNP-413 genotypes in the African American control population. The A allele was also significantly associated with the Caucasian control population. In the CHF patient population, the TT genotype and T
allele were significantly associated with the African American subjects, while the A allele and AT and AA genotypes were associated with the Caucasian subjects. These results were consistent with those reported in Chapters 6 and 7. The genotype of the HMOX1 SNP-413 was not associated with any of the clinical variables seen in the CHF patients.

8.4.4 A Discussion of the Combination of the HMOX1 Promoter Polymorphisms

The LL microsatellite and AA SNP-413 genotype combination and the L, A allele combination were seen most frequently in the control population and the Caucasian CHF patient population. The African American CHF patients had the LL, TT genotype combination as the most frequent, but this was not shown to be a significant association. The entire CHF population lacked the SS, AA genotype combination. Once again, the combination of the supposed detrimental L microsatellite allele and protective A SNP-413 was the most frequent, further emphasizing the need for an investigation to thoroughly examine the effects of this combination on HMOX1 promoter activity in both an *in vitro* and *in vivo* setting. The experiments in Chapter 9 hope to gain insight into the relationship between the HMOX1 promoter polymorphisms by examining their effects on HMOX1 mRNA and protein expression.

8.4.5 A Discussion of the Presence of the eNOS Polymorphisms and HMOX1 Polymorphisms in the CHF Population

8.4.5.1 The eNOS Glu298Asp Polymorphism

The Asp298 variant in exon 7 of eNOS, caused by the T allele, has been linked to decreased NO activity in endothelial cells. It also is a risk factor for CAD, hypertension
and stroke, and has been associated with a poorer transplant-free survival rate for patients with CHF. The Asp298 variant is more frequently associated with the Caucasian population than the African American population. Subjects having the Asp298 variant may respond better to treatment with ACE inhibitors. [McNamara, 2004]

Because of the connection between the Asp298 variant and cardiovascular disease, it was hypothesized that the presence of the Asp298 variant would be related to the presence of the HMOX1 promoter polymorphisms. Genetic analysis of the eNOS Glu298Asp polymorphism revealed that the control population was significantly associated with the GT genotype, meaning the majority of the controls were heterozygous for the Asp298 variant. The results were not significant when the controls were separated by ethnicity.

The CHF patient population was significantly associated with the GG genotype, meaning the majority of the patients were homozygous for the Glu298 variant. When separated by ethnicity, the African American patients were associated with the GG genotype and the Caucasian patients were associated with the TT genotype. This means the majority of African American patients were homozygous for the Glu298 variant, while the majority of the Caucasian patients were homozygous for the Asp298 variant. These findings suggest that the Caucasian patients would have a better response to being treated with an ACE inhibitor. Unfortunately, the comparison of the HMOX1 polymorphisms and the eNOS3 Glu298Asp polymorphism failed to find any significant results.
8.4.5.2 The eNOS Intron 4 Polymorphism

In previous studies examining the relationship between the eNOS Intron 4 VNTR polymorphism and cardiovascular disease, the results have been split as to whether or not the a allele is associated with CAD and hypertension. The author chose to include it in this study because it has been associated with plasma nitrate and nitrite levels. Subjects with the a allele or aa genotype have been shown to have decreased nitrate and nitrite plasma levels. [Tsukada, 1998] HO-1 is inversely associated with plasma nitrate and nitrite levels, so the interaction between the HMOX1 polymorphisms and Intron 4 polymorphism would be interesting to explore.

The genotyping analysis for the Intron 4 polymorphism was hindered by the fact that only two African American controls were genotyped for this polymorphism. The analysis of the total control population failed to find any significant results. In the CHF patient population the African American patients were associated with the bb and ba genotypes, while the Caucasian patients were associated with the bb genotype. The aa genotype was equally found in both patient populations. Analysis of the HMOX1 polymorphisms and the Intron 4 polymorphism revealed a trend in which the Caucasian patients were associated with the LL microsatellite and bb Intron 4 genotypes. A significant association was found between the AT SNP-413 genotype and the bb Intron 4 genotype in the Caucasian patients. The lack of results for the control population makes it difficult to fully interpret these results. It would be best to repeat the investigation, making sure to increase the number of control subjects.
8.4.6 A Discussion about the HMOX1 Microsatellite Polymorphism and the Results of the A-HeFT Trial

A lack of properly functioning HO-1 may contribute to the findings of the A-HeFT trial, which showed a significant reduction in the mortality of African American heart failure patients treated with a combination of isosorbide dinitrate and hydralazine. A lack of HO-1 enzyme activity may also have significant interaction with abnormal eNOS activity in patients having CHF. Taylor et al suggested that hydralazine administered to the A-HeFT participants augmented the patients’ ability to combat oxidative stress. Isosorbide dinitrate acts as a vasodilator through the production of NO, and hydralazine is an antioxidant with the ability to prevent the degradation of NO. [Taylor, 2004]

Previous studies have established that a lack of proper eNOS activity is found in heart failure, as well as in the African American population, resulting in a decrease in functional NO and increased oxidative stress. [Calderone, 2003; Kalinowski, 2004; Linke, 2003; McNamara, 2003] The HO-1 and NOS systems share many similarities and are greatly dependent on each other. Many feel these enzyme pathways may act as redundant systems that assure protection against oxidant stress. [Clark, 2000; Hara, 1999; Hartsfield, 2002; Polte, 2000; Poss, 1997b; Siow, 1999;] The drug combination used in the A-HeFT trial may compensate for deficits in the NOS and HO-1 systems of African American heart failure patients, allowing them to fend off the adverse effects of oxidative stress.

The results of this investigation contribute further understanding to the mechanisms that may account for the differences in drug response in populations of
different ethnic backgrounds. The BEST, LIFE, and SOLVD trials are examples of studies in which Caucasian patients responded favorably to drug treatments, while African Americans did not. [Dries, 1999; Exner, 2001; Julius, 2004] It has been suspected that ethnic based genomic differences, which are an expected consequence of differing geographic and cultural associations, may account for differences in drug response. The current findings may illustrate such so-called race-oriented pharmacogenomics, with African American patients requiring drug therapies targeted at a genome based susceptibility to oxidative stress that exceeds that in Caucasian populations.

McNamara et al provided evidence for the importance of race-oriented pharmacogenomics in heart failure with the results from the Genetic Risk Assessment of Cardiac Events (GRACE) study. They demonstrated that African American heart failure patients had a lower frequency of the Asp298 variant of eNOS and speculated that the lack of this variant may explain the ineffectiveness of ACE inhibitors in that population. [McNamara, 2003; McNamara, 2004] Further research, such as that of the Genetic Risk Assessment of Heart Failure in African Americans (GRAHF), is needed to establish whether the function of HO-1 is truly compromised in African Americans, and whether this combines with a lack of properly functioning NOS to contribute to the pathophysiology of heart failure in this population. [McNamara, 2004] It is hoped that these results will lead to a discovery of therapies with greater therapeutic benefit based on the understanding of genomic differences.
9.1 Introduction

The results reported in Chapter 8 revealed that a significant association exists between the HMOX1 promoter polymorphisms and CHF in African Americans. In order to expand upon the results in Chapter 8 and explore the role of the HMOX1 promoter polymorphisms in gene expression, left ventricle samples from the explanted hearts of CHF patients who underwent heart transplantation were screened for HO-1 mRNA and protein production. Previous in vitro experiments involving HMOX1 promoter/luciferase fusion genes showed that the presence of the large microsatellite allele and the SNP-413 T allele were associated with lowered transcriptional activity. [Chen, 2002; Ono, 2003; Yamada, 2000] The experiments reported in this chapter tested the hypothesis that the HMOX1 promoter polymorphisms affect both gene and protein expression in patients suffering from CHF,
allowing them to be more susceptible to oxidative damage and therefore exacerbating disease progression.

9.2 Methods and Materials

9.2.1 Subjects

Patients involved in the study were treated at The Ohio University Heart Failure Clinic and diagnosed with either idiopathic dilated cardiomyopathy or ischemic cardiomyopathy, with symptomatic congestive heart failure. The patients were NYHA functional class III or IV, and all underwent heart transplantation. Left ventricle tissue samples were collected from the explanted hearts of a subgroup of these patients. All study participants provided written, informed consent, and the protocol was approved by the Institutional Review Board for Human Subjects of the Ohio State University.

9.2.2 Extraction of Genomic DNA

Genomic DNA was extracted from either a venous blood sample or left ventricle tissue sample from each subject according to the methods in Section 4.2 of this dissertation.

9.2.3 Polymerase Chain Reaction

The PCR for this study utilized the sense primer GT 5 (5’-CCA GCA GGT GAC ATT TTA GGG- 3’), and the antisense primer GT 3 (5’ -ACA GCT GAT GCC CAC TTT CTG- 3’). These primers had an annealing temperature of 58 °C. The reaction used the PCR mixture and program described in Section 4.3.
9.2.4 Gel Electrophoresis

Section 4.4 of this dissertation details the gel electrophoresis protocol used in this study.

9.2.5 Sequencing Analysis

The PCR products generated in this study were sequenced according to the methods described in Section 4.5. The sense primer GT 5 and the antisense primer GT 3 were used in the sequencing reaction.

9.2.6 Genotyping

The HMOX1 microsatellite polymorphism was assigned either a small (S) or large (L) allele classification based upon the number of GT repeats present in the sense and antisense sequences. The cut off point for the allele classes was determined by the median GT repeat for the control population. An allele was classified as large (L) if the GT repeat was ≥ 26, and an allele was classified as small (S) if the GT repeat was < 26. The HMOX1 SNP-413 was genotyped according to the bases present at position –413 of the sequences.

9.2.7 Protein Extraction

Protein was extracted from frozen left ventricle sections using the protocol provided with the StressXpress™ ELISA kits from Stressgen Biotechnologies Corp. (Victoria, BC). Tissue sections were placed inside a mortar, covered with liquid nitrogen, and ground to powder with a pestle. HO-1 Extraction Reagent (1 ml), containing a protease inhibitor, was then added to the samples. The pulverization continued until a homogenous tissue suspension was created. This suspension was transferred to a polypropylene tube and centrifuged at 14,000 x g for 10 minutes at 4 °C.
The supernatant, containing the newly extracted protein, was then transferred to another polypropylene tube.

In order to determine the concentration of each sample, a BCA assay was run. A 2 ul aliquot of the protein sample was mixed with 98 ul 0.1 N NaOH and 2 ml of a copper (II) sulfate:BCA solution (1:50 volume to volume). A serial dilution of albumin was also prepared to create a standard curve of known protein concentrations. The samples were incubated at 37 °C for 30 minutes and analyzed on a spectrophotometer. The protein samples were then diluted with Laemmli sample buffer to a stock concentration of 20 ng and stored at ~20 °C.

**9.2.8 Western Blotting**

A 12% separating gel (5.25 ml distilled water, 3.75 ml 1.5 M Tris-HCL, 6 ml 30% Acrylamide/Bis, 150 ul 10% SDS, 50 ul 10% ammonium persulfate, 10 ul TEMED) was poured and allowed to polymerize for 1 hour. A 7.5% stacking gel (3.75 ml distilled water, 1.88 ml 1.5 M Tris-HCL, 1.88 ml 30% Acrylamide/Bis, 75 ul 10% SDS, 25 ul 10% ammonium persulfate, 5 ul TEMED) was then poured on top of the separating gel and allowed to polymerize for 45 minutes. The gels were loaded with 20 ug of the stock protein sample/dye mixture, 3 ul of a protein standard ladder, and 0.7 ug of HO-1 peptide (Stressgen Biotechnologies Corp, Victoria, BC) and run at 200 volts for 1 hour. The samples were then transferred onto a nitrocellulose membrane using a semi-dry system and blocked in 3% BSA solution (pH 7.5) for 2 hours at room temperature. The membrane was incubated overnight at room temperature with an antibody solution (1.% BSA in blotting buffer, pH 7.5) containing 1.0 ug/mL of mouse anti-HO-1 monoclonal antibody (Stressgen Biotechnologies Corp, Victoria, BC). The membrane was washed
with 5 or more changes of blotting buffer (25 mM Tris pH 7.5, 0.15 M NaCl, 0.05% Tween 20) for 1 hour at room temperature. A secondary antibody solution, consisting of a 1:2000 dilution of HRP-conjugated goat anti-mouse antibody, was incubated with the membrane for 3 hours at room temperature. The washing procedure was then repeated.

Supersignal West Dura Chemiluminescent substrate (Pierce Biotechnology Inc., Rockford, IL) was added to the membrane, and the membrane exposed to X-ray film. The developed films were scanned into a computer and Optimas 6.5 (Media Cybernetics, LP) was used to quantify the signal strength of each protein sample.

9.2.9 ELISA

A StressXpress™ human HO-1 ELISA kit (Stressgen Biotechnologies Corp, Victoria, BC) was used to examine the amount of HO-1 protein in the left ventricle tissue samples. All reagents were provided as part of the kit. The reagents were brought to room temperature, and a serial dilution of the recombinant HO-1 standard in sample diluent was prepared. Five ul of each sample was diluted in 250 ul of sample diluent, and 100 ul of this mixture was loaded onto the immunoassay plate. Each sample was run in triplicate. The plate was covered and incubated at room temperature for 30 minutes. After washing 6 times with 1X wash buffer, 100 ul of anti-human HO-1 was added to each well. The plate was covered and incubated at room temperature for 1 hour. After another washing cycle, 100 ul of anti-rabbit IgG:HRP conjugate was added to each well. The plate was covered and incubated for 30 minutes at room temperature. The wells were washed again, and 100 ul of the TMB substrate was added to each well. The plates were incubated in the dark at room temperature for 15 minutes, and 100 ul of acid stop solution was added to each well. An ELX808 plate reader (Biotek Instruments Inc.,
Winooski, VT) was used to measure the absorbance of the samples at 450 nm, and KC junior v 1.21 (Biotek Instruments Inc., Winooski, VT) was used to establish the protein concentrations of each sample.

9.2.10 RNA Extraction

RNA was extracted from sections of frozen left ventricle using the RNA-Bee™ isolation protocol. Fifty mg of tissue was homogenized in a metal biopulverizer previously cooled with liquid nitrogen, and placed into a 1.5 ml eppendorf tube. The crushed tissue was suspended in 1 ml of RNA-Bee and vortexed vigorously. Chloroform (200 ul) was added to the tube, and it was shaken vigorously for 15-30 seconds. It was centrifuged at 12,000 x g for 15 minutes at 4 °C. After the centrifugation, the colorless aqueous phase, containing the RNA, was removed and placed into a clean 1.5 ml eppendorf tube. Cold 100% isopropanol (500 ul) was added, and the tube was stored for 1 hour at –20 °C. It was then centrifuged at 12,000 x g for 30 minutes at 4 °C. An RNA pellet should have been detectable at the bottom of the tube. If the pellet did not form, the tube was placed in –20 °C overnight and centrifuged at 12,000 x g for 1 hour at 4 °C. If a pellet still was still not detectable, the tube was kept at –80 °C for one hour and centrifuged at 12,000 x g for 30 minutes at 4 °C. Once a pellet formed, the supernatant was removed and the pellet carefully washed with 1 ml of cold 75 % ethanol. The tubes were centrifuged at 8,000 x g for 5 minutes at 4 °C, the supernatant carefully removed, and the washing procedure repeated. The pellet was allowed to dry briefly and dissolved in 15 ul DEPC treated water. The RNA was analyzed with a spectrophotometer to determine its quantity and quality.
9.2.11 Quantitative PCR

An Assays-On-Demand™ quantitative PCR assay (Applied Biosystems, Foster City, CA) was used to examine HMOX1 gene expression. A master mix containing the following reagents was prepared on ice and kept in the dark: Rnase free water (18 ul per sample), primer/probe (2.5 ul per sample), Beta actin control primer (2.5 ul per sample), Taq master mix (25 ul per sample). A 96 well Microamp plate was loaded with 2 ul of cDNA template, with each sample being run in triplicate. 48 ul of the master mix was then added to each well, with all reagents being kept in the dark. After preheating the thermocycler for at least 30 minutes, the plate was run on an ABI Prism 7700 (Applied Biosystems, Foster City, CA) according to the conditions listed in Table 9.1.

<table>
<thead>
<tr>
<th>Step:</th>
<th>Time:</th>
<th>Temperature:</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmpErase UNG Activation</td>
<td>2 minutes</td>
<td>50 °C</td>
</tr>
<tr>
<td>Amplitaq Gold Enzyme</td>
<td>10 minutes</td>
<td>95 °C</td>
</tr>
<tr>
<td>Activation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR (40 Cycles)</td>
<td>Denature = 15 seconds</td>
<td>95 °C</td>
</tr>
<tr>
<td></td>
<td>Anneal/Extend = 1 minute</td>
<td>60 °C</td>
</tr>
</tbody>
</table>

Table 9.1: Program for Quantitative PCR Reaction
9.2.12 Microarray Analysis

A GEArray® Q Series Human Nitric Oxide Gene Array (Superarray Bioscience Corporation, Frederick, MD) was used to examine the expression of the HMOX1, along with 95 other genes whose expression is controlled by or involved in signaling by NO. RNA samples were transformed into biotin-labeled cRNA and hybridized overnight at 60 °C with the microarray. The array was washed multiple times and blocked with GEA blocking Solution Q for 40 minutes at room temperature. After another series of washes, the array was incubated with alkaline phosphatase-conjugated streptavidin for 10 minutes at room temperature. The array was washed and incubated with CDP-Star chemiluminescent substrate for 5 minutes. The array was exposed to X-ray film and developed. The films were scanned into a computer and analyzed using the GEArray Expression Analysis Suite (Superarray Bioscience Corporation, Frederick, MD).

9.2.13 Statistical Analysis

StatView for Windows version 5.0 (SAS Institute, Inc., Cary, NC) and Microsoft Excel 2000 (Microsoft Corporation, Redmond, WA) were used for statistical analysis.

9.3 Results and Discussion

9.3.1 Genotyping of the HMOX1 Promoter Polymorphisms

A total of 71 heart transplant recipients were genotyped for this study, and their demographic information is reported in Table 9.2. A significant age difference was seen between the patients with CHF of an ischemic etiology versus patients with CHF of a nonischemic etiology (53.7 ± 7.8 vs. 45.5 ± 15.3 years, P = 0.0006). A trend was also seen in which the Caucasian patients were older than the African American patients (50.6
± 12 vs. 41.9 ± 16.1 years, P = 0.07). There were no other significant differences between the clinical variables and the Caucasian and African American patient population. The study involved only eight African Americans, and this small sample size may have affected the statistical analysis.
<table>
<thead>
<tr>
<th></th>
<th>Total Subject Population (n = 71)</th>
<th>Tissue Sample Population (n = 49)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender – no. (%)</td>
<td>51 (72)</td>
<td>34 (69)</td>
</tr>
<tr>
<td>African American Race – no. (%)</td>
<td>8 (11)</td>
<td>7 (14)</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>49.6 (± 13)</td>
<td>51.2 (± 12)</td>
</tr>
<tr>
<td>Ischemic Etiology – no. (%)</td>
<td>36 (51)</td>
<td>26 (53)</td>
</tr>
<tr>
<td>History of Hypertension – no. (%)</td>
<td>(n = 60), (n = 41)</td>
<td>31 (52)</td>
</tr>
<tr>
<td>History of Diabetes – no. (%)</td>
<td>(n = 65), (n = 45)</td>
<td>20 (31)</td>
</tr>
<tr>
<td>Acute Rejection – no. (%)</td>
<td>(n = 60), (n = 41)</td>
<td>26 (43)</td>
</tr>
<tr>
<td>Transplant CAD – no. (%)</td>
<td>(n = 57), (n = 38)</td>
<td>36 (63)</td>
</tr>
<tr>
<td>History of Hypercholesterolemia – no. (%)</td>
<td>(n = 60), (n = 42)</td>
<td>25 (42)</td>
</tr>
<tr>
<td>History of Smoking – no. (%)</td>
<td>(n = 60), (n = 41)</td>
<td>39 (65)</td>
</tr>
</tbody>
</table>

Table 9.2: Demographic Information
Holweg et al had previously examined the relationship between the HMOX1 microsatellite polymorphism and acute transplant rejection or transplant coronary artery disease in a Caucasian population. Acute rejection was defined as having one or more endomyocardial biopsies with rejection of greater than or equal to 3A during the first year post transplant. Transplant CAD was defined as any or all abnormalities of the epicardial as well as intramyocardial arteries diagnosed at one year post transplant by coronary angiogram. The investigators found that the length of the microsatellite polymorphism was not associated with transplant survival, acute rejection or transplant CAD. [Holweg, 2005]

Acute rejection and transplant CAD were also examined in the current study to see if the microsatellite polymorphism may be associated with those clinical variables in the African American population. All 71 patients were genotyped for the HMOX1 microsatellite polymorphism. No significant associations were found between the genotype frequency and any of the clinical variables, including the presence of acute rejection or transplant CAD (P = 0.72 and P = 0.84, respectively). The genotype frequency was also not significantly different between the Caucasian and African American populations (P = 0.28). It is believed that the extremely small African American sample size could have influenced the results.

The HMOX1 SNP-413 promoter polymorphism was genotyped for 59 patients, 6 African Americans and 53 Caucasians. A significant association was found between the genotype and ethnicity. The African American patients had a higher frequency of the TT genotype, while the Caucasian population had higher frequencies of the AA and AT genotypes (P = 0.04). These results were consistent with those from the genetic analysis
in Chapter 8. A trend was seen in which the AA genotype was associated with a history of hypertension (P = 0.16). Patients lacking hypertension had a higher frequency of the TT genotype. This trend was not seen in the genetic analysis of the CHF patients in Chapter 8.

The results of the analysis of the genotype and allele frequencies for the HMOX1 microsatellite versus SNP-413 polymorphisms also corresponded to those in Chapter 8. The LL microsatellite and AA SNP-413 genotype combination was present at the highest frequency in the population, while the SS, AA combination was absent from the population (P < 0.0001). The L, A and S, T allele combinations were seen most frequently in the population (P = 0.0002 and P < 0.0001, respectively). Analysis of the relationship of the polymorphisms in the different ethnic populations was not possible due to the small size of the African American population.

**9.3.2 HMOX1 Expression in Quantitative PCR**

Quantitative PCR was used to measure HMOX1 expression in RNA extracted from 38 left ventricle samples. There was no significant difference seen between the mean relative expression values when the population was split by ethnicity or gender (P = 0.90 and P = 0.40, respectively). When HMOX1 expression was compared to the presence of clinical variables, two trends were seen. Patients lacking hypercholesterolemia had a lower mean relative expression than patients with hypercholesterolemia (0.563 ± 1.0 vs. 1.53 ± 2.6, P = 0.19). Patients with a positive smoking history had a larger mean relative expression than nonsmokers (1.08 ± 1.5 vs. 0.353 ± .45, P = 0.14). The presence of hypercholesterolemia or smoking can increase levels of oxidative stress in the cardiovascular system. The elevated HMOX1 expression
seen in patients possessing these clinical variables could be the result of increased oxidative stress.

Figure 9.1a shows plots of the mean relative HMOX1 expression values for the different genotypes of the microsatellite polymorphism. The mean relative expression values for the HMOX1 microsatellite polymorphism genotypes were as follows: LL = 0.571 ± 0.58, LS = 0.901± 1.5, and SS = 1.80 ± 3.2. A trend was seen in which the mean relative expression for patients with the LL genotype was less than the mean relative expression for patients with the SS genotype (P = 0.17). The difference between the mean relative expressions of the other genotypes was not significant. When the population was split by ethnicity, a trend was seen in which the mean relative expression of African American subjects with the LS genotype was larger than the mean relative expression of African American subjects with the LL genotype (2.40 ± 2.1 vs. 0.271 ± 0.18, respectively; P = 0.09).

Figure 9.1b shows the mean relative HMOX1 expression values for the SNP-413 genotypes. The mean relative expression values for the SNP-413 genotypes were as follows: AA = 0.64 ± 0.64, AT = 1.17 ± 1.9, and TT = 1.95 ± 3.3. There were no significant differences between the mean relative expression values for the different genotypes. Figure 9.1c plots the mean relative HMOX1 expression values seen when the genotypes of the HMOX1 polymorphisms were combined. It appears that the size of the microsatellite allele has a greater influence on HMOX1 expression than the genotype of the SNP-413 polymorphism. When categorized by both genotypes, the sample size became too small to provide any significant information from an unpaired T-test.
Figure 9.1 Relative HMOX1 Expression vs. Genotypes of Promoter Polymorphisms
9.3.3 HO-1 Protein Expression

HO-1 protein expression was measured by two means, Western blotting and ELISA. Thirty patients were analyzed by Western blot. There were no significant differences between the mean gray protein values of the Caucasian and African American populations. There were no significant differences seen between the mean gray protein values of the different microsatellite polymorphism genotypes: LL = 171 ± 44, LS = 164 ± 41, and SS = 167.6 ± 56. The mean gray protein values of the SNP-413 genotypes were as follows: AA = 164 ± 43, AT = 137 ± 60, and TT = 192 ± 40. A trend was seen in which the protein expression in patients with the TT genotype was greater than in patients with the AT genotype (P = 0.06). A trend was also seen in which Caucasian patients with the TT SNP-413 genotype had greater protein expression than Caucasian patients with the AT genotype (190 ± 41 vs. 137 ± 60, P = 0.10). These results show that the SNP-413 genotype may influence HO-1 protein expression.

ELISAs were used to examine protein expression in order to provide a more reliable measurement of protein concentration. The protein expression of 49 patients was analyzed by ELISA. The average protein concentrations between the African American and Caucasian samples were not significantly different. Many trends were seen when the average protein concentration was examined in terms of each of the clinical variables. Patients with hypercholesterolemia had a lower HO-1 protein expression than patients without this variable (4.1 ± 2.5 vs. 5.1 ± 2.3, P = 0.19). Patients with CHF resulting from ischemic etiology had lower HO-1 protein expression than patients with CHF of a nonischemic origin (4.1 ± 2.5 vs. 5.1 ± 2.3, P = 0.15). Patients with diabetes mellitus had lower HO-1 protein expression than patients without diabetes (4.0 ± 2.3 vs. 5.0 ± 2.4, P = 0.19).
These trends appear to coincide since hypercholesterolemia and diabetes mellitus are primary risk factors for the development of atherosclerosis, the root of ischemic cardiomyopathy.

A significant difference was seen between the average HO-1 protein concentrations of patients with and without acute rejection. Patients who displayed signs of acute rejection had a significantly larger amount of HO-1 protein than patients not experiencing rejection (5.3 ± 2.6 vs. 3.8 ± 1.9, P = 0.04). These results are consistent with previous investigations in which high amounts of HO-1 protein accompanied episodes of acute rejection.

Figure 9.2 shows plots of the average protein concentrations for the genotypes of each of the HMOX1 promoter polymorphisms. Figure 9.2a shows the results for the HMOX1 microsatellite polymorphism. There was no significant difference between the genotypes, and their average protein concentrations were as follows: LL = 4.4 ± 2.5, LS = 4.6 ± 1.9, and SS = 4.7 ± 2.9. The results for the SNP-413 genotypes are found in Figure 9.2b. There was also a lack of significance between the HO-1 expression of the SNP-413 genotypes: AA = 4.3 ± 2.3, AT = 4.4 ± 2.6, and TT = 4.6 ± 2.7. A trend was seen in which African American patients with the TT genotype had greater HO-1 protein expression than African American patients with the AA genotype (6.3 ± 2.6 vs. 2.5 ± 0.80, P = 0.15). Figure 9.2c shows the average HO-1 protein expression for the genotype combinations of the HMOX1 polymorphisms. The LL, TT genotype combinations appeared to have much higher expression levels than the other genotypes, but this could not be verified through the use of the unpaired t-test due to extremely small sample sizes.
Figure 9.2: Average HO-1 Protein Concentration vs. Genotypes of Promoter Polymorphisms
9.3.4 Microarray Analysis

Samples from eight Caucasian patients were run on microarrays, but only 4 arrays were clean enough to quantify gene expression. One patient had an LL genotype, two patients had an LS genotype, and one patient had the SS microsatellite genotype. The SNP-413 polymorphism was not able to be genotyped in these patients. Gene expression was considered significant if a greater than 2-fold change was seen when comparing the different microsatellite genotypes. Unfortunately, there were no genes that showed a greater than 2-fold change. This analysis was limited by the small number of patients involved, the lack of African American subjects, and by the fact that the SNP-413 genotypes were unavailable.

9.3.5 A Discussion of Experimental Limitations and Future Research

This was the first study to examine the relationship between the HMOX1 promoter polymorphisms and how they affect HMOX1 promoter activity. These experiments were the first step in following up on all of the previous in vitro HMOX1 promoter/luciferase experiments. The results were not statistically significant, but suggest that the microsatellite polymorphism may be influential in HMOX1 mRNA expression, while the SNP-413 polymorphism is more important in regulating HO-1 protein expression. Further research is needed to completely characterize the relationship.

The results presented in this chapter are excellent preliminary data warranting a more in depth study to further characterize the results. This experiment was limited by the small number of subjects, especially in the African American ethnic group. If these experiments were repeated in a larger population, there would be a better chance of
finding significant results. One way of increasing the sample size would be to organize a multicenter trial in order to gain access to additional tissue samples.

The study was also limited by the fact that there were no healthy control samples available for comparison. It is extremely difficult to get a sample of left ventricle from a healthy heart because most are used in cardiac transplantation. If this study were to be expanded, control RNA samples could be extracted from venous peripheral blood samples in order to provide a control group for comparison. The results presented in this paper may have been quite significant if a control population had been available.


