RACE-DEPENDENT MODULATION OF ENDOTHELIAL CELL RESPONSES TO SHEAR STRESS: IMPLICATIONS FOR VASCULAR HEALTH IN AFRICAN AMERICANS

A Dissertation
Submitted to
the Temple University Graduate Board

In Partial Fulfillment
of the Requirements for the Degree
DOCTOR OF PHILOSOPHY

By
Deborah L. Feairheller
January 2011

Examining Committee Members:

Michael D. Brown, Ph.D., Kinesiology, College of Health Professions & Social Work
Joon-Young Park, Ph.D., Kinesiology, College of Health Professions & Social Work
Victor Rizzo, Ph.D., Anatomy & Cell Biology, School of Medicine
Zebulon Kendrick, Ph.D., Kinesiology, & Dean of Graduate School
Stephen M. Roth, Ph.D., Kinesiology, School of Public Health, University of Maryland
ABSTRACT

Race-dependent Modulation of Endothelial Cell Responses to Shear Stress: Implications for Vascular Health in African Americans

By Deborah L. Feairheller

Doctor of Philosophy
Temple University, January 2011

Major Advisor: Dr. Michael D. Brown

It is known that African American ethnicity is an independent risk factor for exaggerated oxidative stress which is intricately intertwined with inflammation, hypertension (HT), and cardiovascular disease (CVD). The purpose of this dissertation study was to examine the racial differences that exist between African Americans and Caucasians in oxidative stress levels at the molecular level using an in vitro model of Human Umbilical Vein Endothelial Cells (HUVECs). African American HUVECs were found to have significantly higher baseline levels of oxidative stress in vitro compared to Caucasian HUVECs.

In order to establish proof of concept, three preliminary studies were conducted. The first preliminary study, an acute exercise protocol was conducted in young healthy adults in order to measure plasma oxidative stress markers in response to a single moderate intensity treadmill exercise bout. In this study, it was found that the treadmill exercise did not elicit a race-dependent responses, but that African American adults had higher level of oxidative stress at all sample times when compared to the Caucasians.
A second preliminary study was conducted using a parallel cell culture design to measure basal oxidative stress levels in African American and Caucasian HUVECs without stimulation. These data were shown in relation to the plasma levels of oxidative stress in resting African American and Caucasian adults. This was done in order to show that the common oxidative stress markers measured in human plasma can also be measured in cell culture supernatant and lysate. It was found that both African American adults and HUVECs had heightened oxidative stress and inflammatory markers when compared to their Caucasian counterparts.

The third preliminary study was conducted using tumor Necrosis Factor-α (TNF-α) as an inflammatory stimulant and measuring the oxidative stress response in both African American and Caucasian HUVECs. This was done in order to show that cells of different race respond differently to stimuli. It was found that the response to TNF-α was blunted in African American HUVECs.

The final step was to use laminar shear stress (LSS) as an exercise mimetic in order to examine whether HUVECs from different race respond differently. HUVECs from both race were harvested under static condition (no LSS), with low LSS at 5 dyne/cm², and with a moderate level of LSS at 20 dyne/cm². It was found that despite the fact that African American HUVECs had higher levels of oxidative stress under static conditions, when LSS was applied, protein expressions and oxidative stress biomarkers adjusted to levels that were similar to the Caucasian HUVEC adaptations to LSS. From this, it appears that African American HUVECs have a larger response to LSS stimulus indicating that aerobic exercise prescriptions may be valuable for this population since the potential exists for large improvements in oxidative stress levels for this population.
ACKNOWLEDGEMENTS

I have been fortunate to work with people who were willing to teach me, to learn with me, and to help me develop as a researcher, to have support of people who were interested in making sure that I succeeded, and to have the love from my family who made it all possible. Through this, there are countless people that I’d like to thank. This journey has been long, exciting, unforgettable, and very productive because of them.

First and most importantly, at home: Philip, Kayla, Kacey, and Kyle, you are my life. I could not have done this without you standing on the sidelines cheering, admiring, and helping me along the way. This has been quite an experience for all of us, and I hope that you each take something from it. I sure have. I love you all and hope to make you proud as we move onto the next chapter of our exciting book. You all make me proud every single day and my life is complete because of you.

Here at Temple: Drs. Sachs and Kendrick, thank you for bringing me to Temple. Without you believing in me from the start, it would have not been possible. Dr. Kendrick, you always had respect for me, placed me on several university-wide committees, and through this kept me in the pulse of the Temple community. Thank you for that. Dr. Libonati, even though you have moved on to another university, thank you for seeing something more in me that first year and knowing that Dr. Brown and I would have been a good fit. You were right.

Dr. Santiago, you were first my teacher, then my mentor, and now a friend. You have always had a listening ear. Thank you for your endless support and enthusiasm in
my journey. Dr. Rizzo and Dr. Park, you both inspire me. Thank you for your patience with my questions and for your support of me through this process. Dr Park, I thank you for coming to Temple at such a perfect time. You transformed our lab.

Katie, you inspire me everyday with your dedication and perseverance. Thank you for the friendship and support. It means the world to me. My HyMAP team, my peers, and my friends; this journey has been so much fun and full of memories because of you. Thank you all for everything.

And Dr. Brown, words just don’t express. You pushed me, you encouraged me, you let me learn and try on my own. All while you allowed me to take charge of your lab and run your study. I never imagined that I could learn and grow so much. Thank you for everything. Having had you as a mentor through this journey, and developing the friendship that we have, are the things that I cherish. And I am looking forward to many future collaborations. Thank you from the bottom of my heart.
# TABLE OF CONTENTS

Page  

ABSTRACT .................................................................................................................... iii  

ACKNOWLEDGEMENTS .............................................................................................. v  

LIST OF TABLES ............................................................................................................. x  

LIST OF FIGURES ......................................................................................................... xi  

LIST OF ABBREVIATIONS ........................................................................................ xiii  

CHAPTER  

1. REVIEW OF LITERATURE ........................................................................................1  

   Introduction ............................................................................................................1  
   Hypertension ..........................................................................................................4  
      Racial Differences and Hypertension .................................................................5  
      Hypertension and Exercise ...............................................................................6  
   Endothelial Function ..............................................................................................7  
      Hypertension and Endothelial Function .............................................................7  
      Endothelial Cells ...............................................................................................8  
      Exercise and the Endothelium ...........................................................................9  
      Racial Differences in Endothelial Function .....................................................10  
   Oxidative Stress ....................................................................................................11  
      Oxidative Stress and Cardiovascular Disease ................................................12  
      Superoxide Dismutase (SOD) ...........................................................................13  
      Catalase (CAT) .................................................................................................16  
      Nitric Oxide (NO) ...............................................................................................17  
      Endothelial Nitric Oxide Synthase (eNOS) .......................................................19  
      Total Antioxidant Capacity (TAC) .................................................................21  
      Oxidative Stress and Exercise Training .........................................................23  
      Racial Differences and Oxidative Stress ........................................................24  
   NADPH Oxidase ...................................................................................................25  
      NADPH Oxidase Physiology ............................................................................26  
      NOX Homologues ............................................................................................28  
      NADPH Oxidase Activation .............................................................................29  
      p22phox NADPH oxidase Subunit ....................................................................30  
      gp91phox (NOX2) NADPH oxidase ...............................................................31
2. PRELIMINARY STUDIES

Introduction ..............................................................................................................41
Preliminary Study #1: Racial Differences in the Time-Course Oxidative Stress Responses to Acute Exercise .................................................................41
  Abstract ..............................................................................................................41
  Introduction .......................................................................................................42
  Methods ............................................................................................................44
  Statistical Analysis .........................................................................................46
  Results .............................................................................................................46
  Discussion .......................................................................................................50
  Conclusion ....................................................................................................53
Preliminary Study #2: Racial Differences in Oxidative Stress and Inflammation: in vitro and in vivo ....................................................................................54
  Abstract ..............................................................................................................54
  Introduction .......................................................................................................55
  Methods ............................................................................................................56
  Statistical Analysis .........................................................................................60
  Results .............................................................................................................60
  Discussion .......................................................................................................65
  Conclusion ....................................................................................................69
Preliminary Study #3: Effects of TNF-α Stimulation on NOS Expression in Racially-derived HUVECs ....................................................................................69
  Abstract ..............................................................................................................69
  Background & Purpose ..................................................................................71
  Methods ............................................................................................................71
  Results & Conclusion .....................................................................................73

3. RESEARCH DESIGN

Methods ..............................................................................................................76
Cell Culture .........................................................................................................76
In Vitro Flow: Laminar Shear Stress .................................................................77
Protein Expression: Western Blotting ...............................................................78
4. RESULTS

NADPH Oxidase Protein Expression and Activity Results ............................................. 82
  p47phox Protein Expression by Race with LSS .................................................. 82
  NOX2 Protein Expression by Race with LSS .................................................. 83
  NOX4 Protein Expression by Race with LSS .................................................. 85
  eNOS Protein Expression and NO End-product Production by Race with LSS .... 86
Antioxidant Production Results ........................................................................... 90
  Total Antioxidant Capacity by Race with LSS ............................................. 90
  SOD2 Protein Expression and Total SOD Activity by Activity Results ........ 91
  Catalase Protein Expression by Race with LSS ......................................... 95

5. DISCUSSION

  Introduction .......................................................................................................... 97
    Supporting Evidence .......................................................................................... 98
    Main Findings .................................................................................................. 100
  NADPH Oxidase – Responses to LSS by Race ................................................. 100
    p47phox NADPH Oxidase Subunit Responses ............................................. 100
    NOX2 NADPH Oxidase Isoform Responses .............................................. 102
    NOX4 NADPH Oxidase Isoform Responses .............................................. 104
      Racial Differences in NADPH Oxidase Expression in Static Culture ... 105
  Nitric Oxide (NO) Production – Responses to LSS by Race ......................... 106
    Endothelial Nitric Oxide Synthase (eNOS) Protein Responses ............. 106
    Nitric Oxide (NO) End-Product Responses ........................................... 108
    NADPH Oxidase, eNOS, and NO Responses ............................................ 109
  Antioxidant Production – Responses to LSS by Race ................................... 110
    Total Antioxidant Capacity (TAC) Responses ........................................ 110
    Superoxide Dismutase (SOD) & Catalase (CAT) Responses ................ 111

6. CONCLUSION

  Addressing Hypotheses & Specific Aims ....................................................... 113
  Concluding Remarks & Future Research .................................................... 115

REFERENCES .............................................................................................................. 118

APPENDIXES ............................................................................................................... 139

  A. Consent Form: Oxidative Stress and Acute Exercise ......................... 140
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.</td>
<td>Subject Characteristics by Ethnic Group</td>
<td>47</td>
</tr>
<tr>
<td>2.2.</td>
<td>Total Area Under Oxidative Stress Response Curves, by Ethnic Group</td>
<td>50</td>
</tr>
<tr>
<td>2.3.</td>
<td>Subject Characteristics by Ethnic Group</td>
<td>61</td>
</tr>
<tr>
<td>2.4.</td>
<td>Relative eNOS Expression Changes with Stimulation</td>
<td>74</td>
</tr>
<tr>
<td>2.5.</td>
<td>Relative iNOS Expression Changes with Stimulation</td>
<td>74</td>
</tr>
<tr>
<td>4.1.</td>
<td>Differences in p47phox Protein Expression by Race with LSS</td>
<td>83</td>
</tr>
<tr>
<td>4.2.</td>
<td>Differences in NOX2 Protein Expression by Race with LSS</td>
<td>84</td>
</tr>
<tr>
<td>4.3.</td>
<td>Differences in NOX4 Protein Expression by Race with LSS</td>
<td>86</td>
</tr>
<tr>
<td>4.4.</td>
<td>Nitric Oxide Levels by Race with LSS</td>
<td>88</td>
</tr>
<tr>
<td>4.5.</td>
<td>Differences in eNOS Protein Expression by Race with LSS</td>
<td>89</td>
</tr>
<tr>
<td>4.6.</td>
<td>Differences in Nitric Oxide Levels by Race with LSS</td>
<td>89</td>
</tr>
<tr>
<td>4.7.</td>
<td>Total Antioxidant Capacity by Race with LSS</td>
<td>91</td>
</tr>
<tr>
<td>4.8.</td>
<td>Differences in Total Antioxidant Capacity by Race with LSS</td>
<td>91</td>
</tr>
<tr>
<td>4.9.</td>
<td>Total Superoxide Dismutase Activity by Race with LSS</td>
<td>93</td>
</tr>
<tr>
<td>4.10.</td>
<td>Differences in SOD2 Protein Expression by Race with LSS</td>
<td>94</td>
</tr>
<tr>
<td>4.11.</td>
<td>Differences in Total Superoxide Dismutase Activity by Race with LSS</td>
<td>95</td>
</tr>
<tr>
<td>4.12.</td>
<td>Differences in Catalase Protein Expression by Race with LSS</td>
<td>96</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Protein Expression Differences by Race</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>Pilot Study: Racial Differences in SOD Activity</td>
<td>15</td>
</tr>
<tr>
<td>1.3</td>
<td>Pilot Study: Racial Differences in HUVEC NO Levels</td>
<td>20</td>
</tr>
<tr>
<td>1.4</td>
<td>Time-dependent NADPH Oxidase mRNA Expression</td>
<td>34</td>
</tr>
<tr>
<td>2.1</td>
<td>Total SOD by Race</td>
<td>47</td>
</tr>
<tr>
<td>2.2</td>
<td>PC Levels by Race</td>
<td>48</td>
</tr>
<tr>
<td>2.3</td>
<td>TAC Levels by Race</td>
<td>49</td>
</tr>
<tr>
<td>2.4</td>
<td>TBARS Levels by Race</td>
<td>49</td>
</tr>
<tr>
<td>2.5</td>
<td>Racial Differences in NO Levels</td>
<td>61</td>
</tr>
<tr>
<td>2.6</td>
<td>Racial Differences in IL-6 Levels</td>
<td>62</td>
</tr>
<tr>
<td>2.7</td>
<td>Racial Differences in Total SOD Activity</td>
<td>63</td>
</tr>
<tr>
<td>2.8</td>
<td>Racial Differences in TAC Levels</td>
<td>64</td>
</tr>
<tr>
<td>2.9</td>
<td>Racial Differences in Plasma PC Levels</td>
<td>64</td>
</tr>
<tr>
<td>2.10</td>
<td>NADPH Oxidase Subunit Protein Expression by Race</td>
<td>65</td>
</tr>
<tr>
<td>2.11</td>
<td>Research Design</td>
<td>72</td>
</tr>
<tr>
<td>2.12</td>
<td>eNOS Protein Expression Levels by Race</td>
<td>75</td>
</tr>
<tr>
<td>2.13</td>
<td>iNOS Protein Expression Levels by Race</td>
<td>75</td>
</tr>
<tr>
<td>3.1</td>
<td>Dissertation Study Research Design</td>
<td>76</td>
</tr>
<tr>
<td>4.1</td>
<td>p47phox Protein Expression Levels by Race with LSS</td>
<td>82</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>4.2</td>
<td>NOX2 Protein Expression Levels by Race with LSS</td>
<td>84</td>
</tr>
<tr>
<td>4.3</td>
<td>NOX4 Protein Expression Levels by Race with LSS</td>
<td>85</td>
</tr>
<tr>
<td>4.4</td>
<td>eNOS Protein Expression and NO End-Product Production by Race with LSS</td>
<td>87</td>
</tr>
<tr>
<td>4.5</td>
<td>Total Antioxidant Capacity by Race with LSS</td>
<td>90</td>
</tr>
<tr>
<td>4.6</td>
<td>SOD2 Protein Expression and Total SOD Activity by Race with LSS</td>
<td>92</td>
</tr>
<tr>
<td>4.7</td>
<td>Catalase Protein Expression by Race with LSS</td>
<td>96</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>HT</td>
<td>Hypertension</td>
<td>iii</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
<td>iii</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human Umbilical Vein Endothelial Cells</td>
<td>iii</td>
</tr>
<tr>
<td>LSS</td>
<td>Laminar Shear Stress</td>
<td>iv</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
<td>1</td>
</tr>
<tr>
<td>AHA</td>
<td>American Heart Association</td>
<td>1</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide</td>
<td>1</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
<td>2</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
<td>3</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
<td>3</td>
</tr>
<tr>
<td>JNC</td>
<td>Joint National Committee</td>
<td>4</td>
</tr>
<tr>
<td>ACSM</td>
<td>American College of Sports Medicine</td>
<td>6</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxyl</td>
<td>12</td>
</tr>
<tr>
<td>ONOO$^-$</td>
<td>Peroxynitrite</td>
<td>12</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen Peroxide</td>
<td>12</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
<td>12</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
<td>12</td>
</tr>
<tr>
<td>GPX</td>
<td>Glutathione Peroxidase</td>
<td>12</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese SOD</td>
<td>13</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>Copper/Zinc SOD</td>
<td>13</td>
</tr>
<tr>
<td>EcSOD</td>
<td>Extracellular SOD</td>
<td>13</td>
</tr>
<tr>
<td>AngII</td>
<td>Angiotensin II</td>
<td>14</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
<td>19</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal NOS</td>
<td>19</td>
</tr>
<tr>
<td>iNOS</td>
<td>Cytokine-inducible NOS</td>
<td>19</td>
</tr>
<tr>
<td>OSS</td>
<td>Oscillatory Shear Stress</td>
<td>21</td>
</tr>
<tr>
<td>TAC</td>
<td>Total Antioxidant Capacity</td>
<td>21</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor-α</td>
<td>29</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
<td>29</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
<td>29</td>
</tr>
<tr>
<td>CYBA</td>
<td>p22phox NADPH Subunit Gene</td>
<td>30</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric Acid Reactive Substances</td>
<td>30</td>
</tr>
<tr>
<td>8-iso-PGF$_{2α}$</td>
<td>8-isoprostaneprogstaglandin F$_{2α}$</td>
<td>30</td>
</tr>
<tr>
<td>CYBB</td>
<td>gp91phox NADPH Subunit Gene</td>
<td>31</td>
</tr>
<tr>
<td>NCF-1</td>
<td>p47phox NADPH Subunit Gene</td>
<td>32</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2’-azinobis-3-ethylbenzthiazolesulfonate</td>
<td>40</td>
</tr>
<tr>
<td>PC</td>
<td>Protein Carbonyl</td>
<td>45</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
<td>46</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
<td>46</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
<td>49</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
<td>52</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
<td>54</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
<td>57</td>
</tr>
<tr>
<td>RIPAPi</td>
<td>Radio-Immunoprecipitation Assay Buffer with Protease Inhibitor</td>
<td>57</td>
</tr>
<tr>
<td>Ca2+</td>
<td>Calcium</td>
<td>107</td>
</tr>
</tbody>
</table>
CHAPTER 1

REVIEW OF LITERATURE

Introduction

African Americans exhibit higher incidence of cardiovascular disease (CVD), renal disease, disproportionate levels of hypertension (HT), as well as heightened inflammation when compared to other ethnic groups, in particular Caucasians. African Americans also have higher levels of reactive oxygen species (ROS), attenuated antioxidant capacity, and thereby favor a pro-oxidant state. These racial differences continue to be reported in the literature and defining the underlying reasons for the disparity will help in clinical diagnoses and could potentially guide therapeutic treatments.

In the United States, the latest Heart Disease and Stroke Statistics report that over 73.6 million adults have HT, with annual treatment costs estimated at close to 73 billion dollars. Furthermore, the American Heart Association (AHA) estimates an average of 1 death every 37 s from CVD. CVD is a broad term encompassing pathologies such as stroke, heart failure, arterial disease, coronary heart disease, and HT. CVD continues to be the leading cause of death for African Americans, and the prevalence of HT alone in this ethnic group continues to exceed other U.S. populations.

Oxidative stress plays a critical role in the pathology and progression of HT and CVD, and compelling evidence indicates that endothelial dysfunction is recognized as the hallmark step in the progression of overt HT. Furthermore, reactive superoxide (O$_2^-$) is a
widely accepted surrogate marker for oxidative stress and is primarily produced by the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Production of O$_2^-$ by NADPH oxidase contributes to endothelial dysfunction and thus is ultimately associated with HT and CVD.

Endothelial dysfunction is a disregulation of transcription factors and gene expression within the endothelium that leads to abnormal cell growth, vascular smooth muscle hypertrophy, and ultimately atherosclerosis. The endothelium is a large organ that exists as a monolayer of cells lining the inner surface of all blood vessels. This lining of cells serves as a dynamic interface involved in regulating passage of molecules in and out of circulating blood, in maintaining cardiovascular homeostasis, and also in the delicate balance of oxidants to antioxidant enzyme activity. As the interior lining of vessels, the endothelium encounters a fluid shear stress from the flow of blood, and this stress can modulate endothelial function. This constant blood flow and dragging frictional force creates a laminar shear stress (LSS) on the endothelial cells. Lower levels of LSS, such as that found at atherosclerotic lesions or at vessel bifurcations, have been associated with endothelial mal-adaptation, cell reorientation, and modified oxidant status. On the contrary, high levels of LSS, representative of the increased blood flow during aerobic exercise, have been associated with favorable endothelial adaptations such as reduced NADPH oxidase activity. Research continues to explore the physiological effects that varying levels of LSS have on the endothelial surface in order to understand how this LSS can modulate oxidative stress.

Several critical cell culture studies have specifically examined the effects of LSS on NADPH oxidase activity. In particular, Duerrschmidt et al. quantified the expression
of specific NADPH subunits in response to LSS, and it was found that LSS down-regulated endothelial NADPH oxidase activity\(^2\). In this study, human umbilical vein endothelial cells (HUVECs) exposed to fluid shear stress exhibited a time- and dose-dependent response.

A centerpiece to the rationale for the proposed study is the recent study by Kalinowski et al. that used also used HUVECs to determine potential race differences in NADPH-dependent \(O_2^-\) production between Caucasians and African Americans\(^3\). As seen in Figure 1.1, they suggested that African American endothelial cells had significantly greater \(O_2^-\) production than Caucasian cells, evidenced by increased expression levels of three critical NADPH oxidase subunits (p22phox, p47phox, and p67phox). Interestingly, endothelial nitric oxide synthase (eNOS) expression was also higher in African Americans than in Caucasians, indicating a compensatory overproduction of nitric oxide (NO). Therefore, this endothelial environment in Blacks that favors oxidative stress is
likely to contribute to endothelial dysfunction and, therefore, HT. No study has used LSS, as an in vitro model of exercise to examine race-specific differences in endothelial cell oxidative stress responses by comparing the expression of NADPH subunits in HUVECs from African American and Caucasian donors.

Based on the following in vitro, in vivo, and clinical evidence that: 1) there appears to be increased levels of NADPH oxidase subunits in endothelial cells obtained from African Americans; 2) high physiological levels of LSS down-regulate NADPH expression; 3) African Americans tend to have more endothelial dysfunction; and 4) disproportionate rates of HT; it is likely that endothelial NADPH oxidase activity plays a mechanistic role in the pathology of HT in African American adults. However, it is not known if increased levels of LSS, such as those seen in exercise training, can reverse the otherwise enhanced NADPH oxidase activity observed in endothelial cells from African American donors.

Hypertension

Global HT rates have reached epidemic proportions, with 15-37% of the population, or more than one billion individuals, diagnosed. Blood pressure is the force exerted by the blood on all of the vessels in the circulatory bed. Research has established that blood pressure increases with age, body weight, and inactivity. Furthermore, elevated blood pressure is a primary risk factor for CVD and peripheral arterial disease, as well as implicated in end-organ disease of the kidneys and heart. Recently, new guidelines for prevention and management of HT were released by the “Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure” (JNC). The dogma remains that through extensive translational scientific
research, a better understanding of the fundamental causes of this epidemic can be understood and this could aid in the potential prevention and treatment of HT.

**Racial Differences and Hypertension**

Globally, it is well established that African Americans report the highest percentage of HT⁷⁻⁹, and that HT contributes to higher CVD mortalities within this population¹⁰⁻¹². From 1988-1994 through 1999-2002, the prevalence of HT in African Americans increased from 35.8% to 41.4%. Compared to other ethnic groups, African Americans develop HT earlier in life, at any given age have a higher overall average blood pressure, and their progression to more severe levels of HT are more rapid¹³. Because of this, the African American population has a 1.8 times risk of stroke, 1.5 times risk of CVD death, and a 4.2 times risk of end-stage kidney disease when compared to Caucasians¹. The challenges in understanding racial differences in HT and how the socioeconomic disparity between ethnic groups contributes to said racial differences are quite substantial. Some studies have categorized socioeconomic risk factors in order to understand the potential associations that exist with higher HT and CVD mortalities within racial groups¹⁴,¹⁵. In one study, it was found that African Americans tend to have a higher frequency of elevated risk factors than Caucasians, but when adjusted for education and other socioeconomic factor differences, the groups had relatively identical rates of CVD. This indicates that the disparity in CVD is physiological and may not be socioeconomic. Despite this, HT treatment and control in the US continues to be less than optimal. The burden of HT on African Americans is extensive and identifying and understanding the physiological mechanisms associated with racial differences is vital to improving public health.
Hypertension and Exercise

Exercise remains the cornerstone recommended non-pharmacologic therapy for treatment, control and prevention of HT. Current treatment guidelines prescribed by the JNC, the AHA, and the American College of Sports Medicine (ACSM) state that newly diagnosed HT patients should undergo lifestyle modifications. These lifestyle modifications include weight reduction, adoption of an AHA recommended low fat, low salt diet, moderation of alcohol consumption, and increased aerobic exercise. It is known that increased physical activity beneficially modulates cardiac function, and that aerobic exercise places a larger volume load on the heart and vasculature than traditional resistance exercise. Blood pressure reductions following single acute aerobic exercise bouts are similar to the reductions seen with chronic exercise, systolic pressure decreases about 5-8 mmHg, and diastolic pressures decrease by 6-8 mmHg. Despite this, research has yet to define the true cause of the blood pressure response to chronic aerobic exercise.

A recent meta-analysis by Cornelissen and Fagard on the effects of chronic aerobic exercise training reports that the reduction of resting blood pressure was more pronounced in HT study groups. Several mechanisms have been proposed to explain the differential responses reported with exercise in the HT population. Alterations in oxidative stress biomarkers, vasoreactivity, vascular structural adaptations, neurohumoral modifications, and genetics have all been mentioned. Despite this, little research to date has addressed racial differences within the adaptive responses to aerobic exercise training, in particular within the African American population. Nevertheless, even with many suggested theories regarding the cause of HT as well as the substantial
variation in individual responses to exercise, there is no conclusive evidence purporting
one mechanism over the others and therefore, research must continue.

Endothelial Function

The endothelium is the largest organ in the human body, located within the blood
vessels, and is a single cell layer of tissue that separates the inner and outer vessel
linings\textsuperscript{27}. The endothelium also lines the inner face of all systemic organs. It is a dynamic
autocrine and paracrine organ known to maintain circulation, coagulation, and
inflammation. The basal lamina of the endothelium sits atop a thin layer of vascular
smooth muscle and it is believed that prolonged smooth muscle contraction within the
arteriole walls leads to extensive vascular remodeling\textsuperscript{28}. Considering its location, the
endothelium is the primary regulator of vascular homeostasis by maintaining a delicate
balance between vasodilation and vasoconstriction of the smooth muscle layer, as well as
between inhibition and stimulation of smooth muscle cell proliferation\textsuperscript{29}. The
endothelium also responds to modifications in activity of transcription factors and altered
gene expression. All of these adaptations could potentially play a role in the development
of endothelial dysfunction and have been implicated in the pathological progression of
HT and other CVD\textsuperscript{30-32}.

Hypertension and Endothelial Function

Ultimately HT is determined by cardiac output and vascular resistance. Peripheral
vascular resistance is affected by changes in circulating blood volume, sympathetic
nervous activity, renal filtration, and endothelial function\textsuperscript{33}. Poiseuille’s equation states
that vascular resistance is positively related to blood vessel length and blood viscosity,
while negatively related to the fourth power of vessel radius. Small changes in radius can
have profound effects on blood flow. Therefore, the majority of the vascular resistance is not determined by large arteries or capillaries, but rather by the smooth muscle actions in smaller arterioles.

The function of the endothelium has emerged as a strong indicator of cardiovascular health because impaired endothelial function is an early event in the development of atherosclerosis and subsequently HT. Dynamic changes in the smooth muscle activity as well as alterations in molecular signal traffic define how the endothelial cell layer responds to stimuli. It is suggested that any imbalance in the regulation of circulating hormones, autacoids, cytokines, and other vasoactive factors can lead to endothelial dysfunction. The imbalance can then lead to mechanical damage in the endothelial layer which further disturbs the homeostasis, altering endothelium-dependent vasodilation, and contributing to the pathology and progression of HT. The specific mechanisms involved in the pathogenesis of endothelial dysfunction and how it is related to altered vascular resistance and overt HT remains to be elucidated. Therefore, research continues to focus on potential mechanisms involved in the pathogenesis of HT in relation to endothelial dysfunction.

**Endothelial Cells**

The single layer of the endothelium is comprised of endothelial cells. These are highly sensitive cells that can sense changes in hemodynamics, oxygen pressures, and blood-borne signals traveling in the bloodstream. Due to their location, endothelial cells facilitate all of the functions of the endothelium: regulation of vasodilation, vasoconstriction, circulation, coagulation, and inflammation. Also because of their location, endothelial cells play a pivotal role in pathophysiological processes such as
arterial disease and cancer development. Understanding the endothelial cell responses to various biochemical and mechanical stimuli will help to elucidate the relationship between endothelial function, subsequent dysfunction, and specific related pathologies.

*In vitro* study of endothelial function was not possible until techniques to culture endothelial cells were developed in the early 1970s. Recently, methods have been published describing a standard protocol that can be used to isolate and extract endothelial cells from human umbilical cord veins. Since then, HUVECs have been used to study many different biological processes because cells isolated from umbilical cords are typically free from both pathogens and pathophysiology. Experiments have used HUVECs to examine inflammation, apoptosis, oxidative stress, and cytokine responses to pharmacological stimuli. In fact, HUVECs have been one of the most studied endothelial cell types since early reports were published in shear stress effects on NO production in HUVECs. The physiological response of HUVECs to any stimuli closely mimics those responses of true *in vivo* endothelial cells, thus making *in vitro* research vital to the expansion of knowledge about *in vivo* mechanisms.

*Exercise and the Endothelium*

Using research to understand the physiological mechanisms of endothelial cell responses will improve the understanding of how the endothelium as an organ functions. Increased physical activity increases the flow of blood through vessels which causes changes in endothelial regulation. Because of its location, the endothelium is constantly exposed to hemodynamic forces and thus is sensitive to different magnitudes of blood flow. Aerobic exercise training, in particular, has a larger effect on the vasculature than resistance type exercise (e.g., weight training). This is because aerobic exercise causes a
larger blood flow volume through the vessels. Traditional weight training does not have the same influence on vascular function\textsuperscript{51, 20, 22, 31, 52}.

Over the years, numerous studies have shown improvements in endothelial function with aerobic exercise. Several recent review articles highlighted the extensive literature that exists purporting the benefits that exercise training has on endothelial function. Both Di Francescomarino et al., and Moyna and Thompson provide extensive reviews on the literature in aerobic exercise and endothelial function in humans\textsuperscript{53, 54}; while Jasperse and Laughlin do the same for animal model studies\textsuperscript{55}. It is believed that the increased blood flow accompanying an exercise training regimen stimulates an endothelial cell adaptation which can ultimately improve blood pressure; however, the underlying mechanism remains to be elucidated.

\textit{Racial Differences in Endothelial Function}

Studies have shown that racial differences exist in endothelial function and oxidative stress levels\textsuperscript{3}. African Americans have been found to have impaired endothelial function as evident by reduced NO bioavailability. The concept of NO bioavailability will be further discussed in the section that follows. Since NO is a powerful vasodilator, African Americans are then assumed to have impaired vasodilatory capabilities\textsuperscript{56}. Perregaux et al., Stein et al., and Kahn et al. studied forearm vessel function and found decreased flow mediated vasodilation in response to agonists\textsuperscript{57-60}. In particular, both Stein et al., and Kahn et al. studied forearm vascular function in response to infusion of nitroprusside, verapamil, and methacholine in both HT and normotensive African Americans and Caucasians. Separately, Cardillo et al. studied forearm blood flow responses to nitroprusside, isoproterenol, and acetylcholine in African Americans and
Caucasians. In all of these studies, it was found that the vasodilator response was significantly impaired in African Americans when compared to Caucasians. Several other studies have reported blunted responses to NO-mediated vasorelaxation in African American adults, further suggesting the association with the racial disparity in HT, CVD, and end-organ damage\textsuperscript{61,62}. Separate research has also reported that African Americans have attenuated vasodilation in response to environmental stress\textsuperscript{63}, nitroglycerin\textsuperscript{64}, and mental stress\textsuperscript{65}. And finally, Marchesi et al. confirmed that endothelial reactivity is impaired in African Americans compared to Caucasians, and they associated this with a higher level of inflammation and infective burden in the African Americans studied\textsuperscript{66}. Therefore, it has been established that African Americans tend to have more endothelial dysfunction when compared to other ethnic groups. The excess disease burden among the African American population is a public health concern and elucidating specific racial differences in endothelial function and oxidative stress could provide insight to clinical diagnoses and treatment.

Oxidative Stress

Perhaps the most abundant molecule in the biological system is oxygen. Oxygen has two unpaired electrons that reside in different orbitals and have a parallel spin. Due to this, oxygen is rather unreactive, so a univalent reduction often occurs creating a $\text{O}_2^-$ free radical. The enzymatic sources of $\text{O}_2^-$ include uncoupled eNOS, xanthine oxidase, and NADPH oxidase. $\text{O}_2^-$ can also be produced as a normal byproduct of aerobic metabolism within the mitochondrial electron transport chain at cytochrome P450 by cytochrome c oxidase\textsuperscript{67}. The $\text{O}_2^-$ anion is a powerful oxidation reduction agent, capable of both
oxidation to $O_2$ as well as reduction to $H_2O_2$. Because of its high reactivity and short half-life ($10^{-5}$ s), $O_2^-$ quickly undergoes several types of reactions.

Oxidative stress is associated with an increased production of ROS, through augmented enzymatic release of $O_2^-$, and with compensatory responses in the levels of antioxidant activity. ROS is a collective term that refers to oxygen based radicals and nonradicals that exist as oxidizing agents. Free radicals are defined as species that contain one or more unpaired electrons and are capable of an unstable highly reactive independent existence. Radicals consist of $O_2^-$, hydroxyl (OH$^-$), peroxyl, alkoxyl, carbon dioxide, carbonate, and hydroperoxyl. The primary nonradical ROS are peroxynitrite (ONOO$^-$) and hydrogen peroxide ($H_2O_2$).

In the early 1990s, Halliwell and Gutteridge defined antioxidants as ‘any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate.’ Within the body, the three primary antioxidant defense enzymes are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). Antioxidants act against oxidant damage at different levels; some scavenge species that are capable of abstracting hydrogen atom radicals like OH$^-$, some bind metal ions creating compounds that will not generate further reactive species, some remove peroxides by converting them to non-radical atoms, and others directly quench or scavenge the $O_2^-$ radical. Each of these methods will be discussed further below.

**Oxidative Stress and Cardiovascular Disease (CVD)**

In the United States, CVD causes over a million deaths a year, and evidence suggests that ROS are involved in the development of many forms of CVD.
particular, ROS are involved in CVD through an increase in O$_2^-$ levels which play a pivotal role in vascular remodeling, HT, and atherosclerosis$^{74-76}$. Furthermore, HT has been associated with an increase in NADPH oxidase activity$^{77}$ which remains the predominant vascular source of O$_2^-$ in humans$^{78,79}$. Within the vascular wall, increased oxidative stress, as evidenced by higher O$_2^-$ levels, alters several important physiological functions. It is thought to alter blood flow regulation, membrane lipid oxidation, protein oxidation, platelet aggregation, leukocyte adhesions, and control of cellular growth.

Many extensive review articles have highlighted the complex association between ROS and their causal contributions to altered physiology and subsequent CVD pathologies, in particular HT$^{35,69,80-83}$.

**Superoxide Dismutase (SOD)**

The SOD enzyme serves as the main protective antioxidant scavenging enzyme against the O$_2^-$ anion. SOD catalyzes the dismutation of O$_2^-$ into H$_2$O$_2$ and water$^{84}$. Three main isoforms of the SOD enzyme exist, manganese SOD (MnSOD) located in the mitochondria, copper/zinc SOD (CuZnSOD) located in the cytoplasm, and extracellular SOD (EcSOD) located in the vascular extracellular space. In most tissues, EcSOD exists in very small amounts, but throughout the circulation it can represent up to half the total SOD$^{85}$. Total SOD activity acts as a compensatory mechanism to minimize vascular O$_2^-$ production and thus preserve endothelial function.

These compensatory mechanisms of SOD activity can become disregulated in the presence of endothelial dysfunction, atherosclerosis, HT, and other disease states. Studies have reported that levels of SOD activity are lower in HT patients when compared to normotensives, suggesting a potential increase in O$_2^-$ scavenging$^{86-89}$. One study in
particular compared EcSOD levels in normotensive and HT African Americans and reported a lower EcSOD level in the HT subjects\textsuperscript{90}. Also, Gongora et al. studied EcSOD-/- mice and found that a lack of SOD activity played a major role in modulating blood pressure in Angiotensin II (AngII)-induced HT\textsuperscript{91}. Decreased SOD activity has also been associated with aging which also has a fundamental link to hypertension. Chehab et al. reported reduced SOD activity in elderly Tunisian adults compared to younger adults\textsuperscript{92}.

On the contrary, some studies have found SOD activity to be upregulated in certain diseases. Increased SOD activity has been reported in schizophrenia\textsuperscript{93} as well as in the adaptive response to myocardial infarction\textsuperscript{94}. These variations in the responses in SOD activity to different disease states suggest that NO-mediated O$_2^-$ scavenging may also differ since NO is the other direct O$_2^-$ scavenger. Specific oxidative stress mechanisms involved in mediating pathophysiological states and in particular, how SOD activity is associated to this defense remain the focus of intense investigation.

The response to exercise in SOD activity has yet to be consistently established. In animal studies, acute exhaustive exercise did not change SOD activity\textsuperscript{95,96}, while other studies have shown that gender and training status affected SOD responses\textsuperscript{97,98}. This variation in SOD activity responses to exercise could be attributed to differences in exercise intensity, exercise time, and exercise modality.

Whether racial differences exist in SOD activity also has yet to be consistently reported. Recently, a preliminary time-course oxidative stress study was completed and this will be described in full detail under the preliminary studies chapter. Briefly, it was found that a sub-maximal treadmill exercise bout elicited a significant increase in SOD
activity from pre-exercise to post-exercise in Caucasian adults. Also, in this study the African Americans had significantly higher SOD activity compared to the Caucasians. Only one other study has reported racial differences in SOD activity levels. Zitouni et al. reported that SOD activity was higher in diabetic patients compared to healthy controls and in their study they also reported that the African American patients had significantly higher SOD activity compared to the Caucasian patients.

Separate data from a pilot study measured SOD activity in response to a single maximal treadmill test. Blood samples were collected before exercise and immediately following the treadmill test. Figure 1.2 shows that at baseline, a higher level of SOD activity was found in African Americans compared to Caucasians. Also a decrease in SOD activity was measured after the acute maximal exercise in both groups. Taken with the results from the larger preliminary study, this pilot study confirmed a higher SOD activity in African Americans. This suggests that African Americans either have a higher level of $O_2^-$ production with compensatory increases in antioxidant enzyme release or a higher physiological SOD enzyme level. \textit{In vitro} cell culture studies could likely provide
insight to the differences in SOD activity and expression between African Americans and Caucasians.

Few studies have quantified SOD activity in cell culture. Zhang et al. measured SOD activity in HUVECs to examine the effects of an agonist on bone morphogenetic protein expression in relation to oxidative stress levels\textsuperscript{100, 101}. In both of their published studies, SOD activities were decreased in response to either AngII or to oxidized-LDL. Also, Mailloux et al. measured oxidative stress responses to Angiotensin-converting enzyme inhibitors in HUVECs and found reduced GPX and SOD activities but not malondialdehyde\textsuperscript{102}. In a separate study, Mehta and Li found that epinephrine upregulated SOD activity in human coronary artery endothelial cells\textsuperscript{103}. However, to date, no study has measured SOD activity in relation to LSS, or compared SOD activity in HUVECs from different races.

\textit{Catalase (CAT)}

As mentioned above, CAT is one of the primary antioxidant enzymes that exist in the cell. CAT is the prominent peroxisomal antioxidant enzyme that is responsible for the reduction of $\text{H}_2\text{O}_2$ into $\text{H}_2\text{O}$. Peroxisomes are known to be involved in fatty acid oxidation, catabolism of purines, and the biosynthesis of bile acids and glycerolipids\textsuperscript{104}. Oxidative metabolism in peroxisomes is complex and intricately intertwined with mitochondrial metabolism\textsuperscript{105}. Rapid scavenging of $\text{H}_2\text{O}_2$ and the maintenance of delicate $\text{H}_2\text{O}_2$ levels are vital to cell life and senescence. Extensive research exists that examines the effects of $\text{H}_2\text{O}_2$ on cell function, oxidative stress, metabolism, and regulation of $\text{H}_2\text{O}_2$.

Experimental studies have shown that CAT is upregulated in the spontaneously hypertensive rat but not in lead-induced HT\textsuperscript{106, 107}. Also, it is established that exercise
training increases antioxidant activity. Linke et al. reported a 42% increase in CAT activity with 6 months of exercise training in patients with chronic heart failure. Yet no study has examined for potential racial differences in CAT protein expression levels. Since SOD activity and protein expression will be measured in the current study, CAT protein expression levels will also be included as an indicator of H$_2$O$_2$ scavenging.

**Nitric Oxide (NO)**

NO is probably the best characterized and most important vasodilator in the vasculature. It is a volatile gas endogenously synthesized by eNOS through oxidation of L-arginine into citrulline, and causes smooth muscle relaxation. In addition to its direct vasodilatory effect on smooth muscle, NO also controls vascular tone by reducing O$_2^-$ levels. NO causes dilation of blood vessels through stimulation of guanylate cyclase to produce cyclic guanosine monophosphate. In addition, NO also exerts anti-inflammatory effects on the vasculature through inhibition of leukocyte adhesion and platelet activation. Other actions of NO that are not related to vasodilation or cardiovascular function include regulation of insulin release, neurodegeneration, memory function, apoptosis, atherosclerosis, and immune response. The important physiological role that NO serves is evident, and any impairment or reduction in the bioavailability of NO can lead to endothelial dysfunction which is associated with various disease states.

Levels of bioactive NO depend on the delicate balance between NO production and degradation/sequestration through interaction with O$_2^-$. Endothelium-derived NO rapidly inactivates the O$_2^-$ radical resulting in production of ONOO$^-$. Both O$_2^-$ and NO contain unpaired electrons in their outer shells making the reaction between them...
diffusion-limited; therefore, when concentrations of \(O_2^-\) and NO are high, the rapid formation of ONOO\(^-\) is favored. Within the vascular wall, the rate of this diffusion-limited reaction is as much as three times faster than the dismutation of \(O_2^-\) by SOD\(^{74}\). This kinetic difference is integral to the determination of the level of NO bioavailability\(^{118,119}\), and has clinical implications because NO itself is critical to the mediation of endothelial function\(^{78,120}\). In healthy vessels, a delicate balance exists between NO and \(O_2^-\). This balance favors the production of NO, therefore allowing an optimal state of basal tone. Reducing the bioavailability of NO or scavenging NO through increased ROS production both appear to be fundamental processes involved in the manifestation of endothelial dysfunction.

Investigations into the exercise training effects on NO function have focused on examining whether adaptations in the regulation of blood flow and vascular tone contribute to enhanced exercise capacity or reduced CVD risk. Based on all of the studies, it appears that increased vascular NO production is a transitory response to exercise that seemingly may progress to structural adaptations which lead to enhanced blood flow and substrate delivery through the vessel beds to active muscles and tissues. In order to do such research on humans, it becomes necessary to have means of quantifying the NO levels. Considering that NO is a highly reactive substance, measurements of NO end-products in both plasma and urine are acceptable biomarkers of total \textit{in vivo} NO bioavailability\(^{121}\).

The majority of studies in humans have reported increases in plasma NO levels with chronic exercise training, in endurance athletes\(^{122}\), in runners\(^{123}\), and in cyclists\(^{124}\). It has also been shown that incremental rhythmic forearm exercise increases plasma NO
levels in young healthy individuals\textsuperscript{125}. On the contrary, two studies have separately shown that neither 12 weeks or 6 months of aerobic exercise in older adults elicited a change in plasma levels of NO\textsuperscript{126, 127}. Animal studies have revealed that strenuous acute exercise decreases NO levels\textsuperscript{128} and chronic exercise training increases plasma NO levels\textsuperscript{129}.

**Endothelial Nitric Oxide Synthase (eNOS)**

Mammalian NO is derived from the conversion of amino acid L-arginine to L-citrulline by a family of enzymes called NO synthases (NOS)\textsuperscript{130}. Three NOS isoforms which are homologous in their primary structure have been identified: neuronal NOS (nNOS), cytokine-inducible NOS (iNOS), and eNOS\textsuperscript{131}. Both nNOS and eNOS are constitutively expressed and require high levels of calcium for activity while iNOS is inducible and acts in a calcium-independent manner\textsuperscript{132, 133}. Activation of these NOS enzymes requires several cofactors: calmodulin, tetrahydrobiopterin, NADPH, flavin mononucleotide, and flavin adenine dinucleotide. NOS enzyme function is delicately regulated and any insufficiency in the availability of these cofactors leads to reduced NOS activity, or uncoupling. When eNOS becomes uncoupled, NO release diminishes and $O_2^-$ levels are thus augmented. Specific pathologies such as HT and CVD are believed to contribute to eNOS uncoupling, impaired NO regulation, and thus lead to endothelial dysfunction\textsuperscript{74}.

Endothelial cells constitutively express eNOS, but this expression is variably regulated by numerous conditions. In vivo, eNOS expression is reduced in patients with heart failure\textsuperscript{134}, reduced in cardiac myocytes of rats after intense exercise\textsuperscript{128}, and increased in spontaneously HT rats\textsuperscript{135}. 
Similar to the manner in which \textit{in vivo} mechanisms maintain a basal NO production level in order to regulate vascular tone, cell culture studies also show a low basal NO production\textsuperscript{136}. A pilot study has been completed that compared basal NO levels between African American and Caucasian HUVECs. It was found that African American cells had a higher basal production of NO end-products as measured by the Greiss assay (Figure 1.3).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{Figure1.3.png}
\caption{Figure 1.3: Pilot Study: Racial Differences in HUVEC NO Levels. NO levels between African Americans (solid bars) and Caucasians (open bars).}
\end{figure}

\textit{In vitro} research has used NOS inhibitors to elucidate mechanisms involved in regulation of NO production through transcription factor activation and found that NO modulates gene expression of adhesion molecules\textsuperscript{137}, modulates bradykinin-induced liver damage\textsuperscript{138}, plays a role in endothelial cell proliferation and migration\textsuperscript{139}, and is not affected by homocysteine infusion, a methionine metabolite known to cause endothelial cell injury\textsuperscript{140}. Extensive amounts of \textit{in vitro} research continue to demonstrate the shear stress level-dependency eNOS expression\textsuperscript{141-143}, further indicating the vital role that NO plays in vascular function. The eNOS gene remains one of the best characterized shear stress responsive genes. It has been found that activation of eNOS occurs predominantly through Akt-dependent phosphorylation of eNOS at serine 1177\textsuperscript{142,144}. Expression of
eNOS increases in HUVECs that are exposed to high physiologic levels of LSS, and regulation of eNOS protein levels and enzyme activity involves both transcriptional and posttranscriptional modifications. In 1995, Noris et al. demonstrated that LSS dose-dependently upregulated eNOS expression and that oscillatory shear stress (OSS) did not have the same effect. A recent study was the first to provide insight into the potential molecular mechanisms involved with upregulation of NO synthesis with increased levels of shear stress. Lam et al. reported that increased blood flow stimulated an increase in eNOS protein expression and enzymatic activity of the rate-limiting enzyme for biosynthesis of BH4, GTP cyclohydrolase. Thus it appears that a LSS stimulus to HUVECs will induce an increase in eNOS expression and NO levels. Despite all of the in vitro data on eNOS expression and shear stress, no study has examined racial differences in the response to a shear stimulus. Thus this dissertations study included measuring eNOS expression in response to LSS in both African American and Caucasian HUVECs.

Total Antioxidant Capacity (TAC)

H$_2$O$_2$ created by the SOD enzyme serves as an important redox signaling molecule that stimulates molecular mechanisms involved in phenotypic changes specific to endothelial dysfunction, vascular growth, apoptosis, remodeling, and inflammation. Therefore, as an adaptation to this, biological systems have several means to remove the H$_2$O$_2$ molecule. The CAT antioxidant enzyme can reduce H$_2$O$_2$ to H$_2$O. Separately, the GPX antioxidant enzyme can reduce glutathione to glutathione disulfide yielding two H$_2$O molecules. And finally, the H$_2$O$_2$ can also be reduced through a Fe$^{2+}$/Fe$^{3+}$ catalyzed fenton reaction.

Total antioxidant capacity (TAC) measures provide a relative cumulative action
of all the antioxidants present in the plasma and body fluid at the sampling time which is inherently a measure of the ability to scavenge \( \text{H}_2\text{O}_2 \). Paradoxically, it is predominantly a measurement of the low molecular weight chain-breaking antioxidants along with metal binding antioxidant proteins. These can include urate, ascorbate, bilirubin, thiols, as well as the dietary carotenoids, flavonoids and \( \alpha \)-tocopherol. The TAC assay itself provides an integrated value rather than a mere sum total of all the quantifiable antioxidant enzymes.

In human subjects, TAC levels have consistently been found to have an inverse relationship with age, HT, CVD, and also aortic intima-media thickness. This is similar to the relationships reported with SOD activity also suggesting a potential increase in \( \text{O}_2^- \) levels which ultimately may be associated with endothelial dysfunction. Research measuring TAC responses to exercise is limited, and the results have been conflicting. In 2005, Schneider et al. found that TAC levels increased with acute exercise at both low and high intensities but not at moderate intensity levels in healthy young men. In their study, they found that both trained and untrained men had an increase in TAC levels with a maximal graded exercise test and only untrained men had an increase in TAC with a low intensity exercise test. Neither group changed TAC levels with moderate intensity exercise test. Yet, Fatouros et al. found that 4 months of moderate intensity aerobic exercise training from 50-80\% of their heart rate maximum significantly increased TAC values in older untrained men. When examining the antioxidant adaptation to habitual physical activity, some studies report no relationship between TAC and aerobic capacity, while others have found an inverse relationship. Evidently, the variation in TAC response to an exercise stimulus is broad. Differences in
sampling, methods, training status, age, and exercise conditions all appear to contribute to the variation in reported responses. Nevertheless, while only having a measure of TAC as a global indicator of oxidative stress is inadequate, combining a range of biomarker measurements can be useful for assessing overall oxidative stress status.

While much research has reported on TAC levels in response to exercise and in relation to various physiological conditions, little research exists examining TAC in cell culture. Measuring TAC levels, in conjunction with other biomarkers in order to assess oxidative stress in cell culture, can provide a relative predictive indicator of the antioxidant defense of the cells. Therefore, in vitro studies should provide potential insight into cellular TAC activity as well as elucidate potential differences that may exist in TAC levels both between race and in response to an exercise stimulus.

**Oxidative Stress and Exercise Training**

Much research has focused on the oxidative stress responses to exercise, and many extensive review articles exist summarizing these findings. It has been shown that exercise training causes a reduction in expression of NADPH oxidase. Also aerobic exercise has been shown to increase NO production, creating a higher NO bioavailability and therefore attenuated levels of $O_2^-$. And finally, as previously discussed, exercise training also has been found to improve TAC and SOD activity levels by increasing the antioxidant scavenging capability. Taken together, overall, research has established that aerobic exercise can effectively improve oxidative stress levels.

While research has established an improvement in oxidative stress levels from exercise training, it remains to be confirmed whether the intensity level of exercise mediates these responses. Few studies have investigated how exercise intensity regulates
oxidative stress production. From these, it is hypothesized that exercise intensity may affect oxidative stress responses, where a higher level of intensity may actually contribute to increased ROS production that exceeds the antioxidant’s capacity. Goto et al. in both 2003 and 2007 reported separately that levels of exercise intensity at 75% VO2max caused an increase in oxidative stress. While in 2006, Wang et al. reported that exercise at 80% VO2max also caused higher oxidative stress. Other studies have also shown that extreme vigorous exercise causes increases in oxidative stress. These previous studies have provided strong evidence that higher intensity of exercise may actually enhance oxidative stress, and show that research must continue to examine differences in oxidative stress responses to varying exercise intensities in order to optimize the therapeutic effects of exercise.

Racial Differences and Oxidative Stress

Research in humans that examines racial disparities in oxidative stress levels between African Americans and Caucasians is limited. In one study by Zitouni et al., it was reported that African American diabetic patients had higher SOD and lower GPX activities compared to the Caucasian patients. The authors hypothesized that the reduced GPX levels could be associated with altered kidney function in the African American diabetic subjects. Another more recent study reported that circulating levels of asymmetrical dimethyl arginine, a competitive endogenous inhibitor of eNOS, were significantly higher in African Americans than they were in Caucasians. These limited data indicate the necessity for further research into the racial differences that may exist in oxidative stress.

Interestingly, it has also been shown in cell culture that ethnicity can influence the
endothelial cell oxidative steady-state balance. Kalinowski et al. compared NO, ONOO’, and O₂’ production with NADPH oxidase subunit expression in HUVECs from both Caucasians and African Americans using an electrochemical assay with nanosensors³. They reported that in African American HUVECs, expression levels of three critical NADPH subunits (p22phox, p47phox, and p67phox) significantly exceeded that of the Caucasian HUVECs by a relative two-fold difference. They also reported that basal levels of NO, ONOO’, and O₂’ varied between the ethnic groups. Specifically, African American HUVECs exhibited reduced NO and increased ONOO’, and O₂’ levels. Since African American humans also consistently show heightened oxidative stress levels compared to Caucasians, further in vitro studies are necessary to help define where specific differences exist. This knowledge can then be used to guide future mechanistic research which could then help in disease diagnoses and/or prevention.

NADPH Oxidase

Traditionally, ROS have been understood to be the harmful byproducts of aerobic metabolism; however, there also exist enzymes that produce ROS as a vital component of the host defense mechanisms. Today it is understood that the NADPH oxidase enzyme is expressed in many tissues and cells throughout the body, despite the fact that originally the enzyme was believed to only exist in phagocytes functioning in immune protection through a respiratory burst. The respiratory burst concept was initially discovered in 1933¹⁷³ by Baldridge and Gerard, but then the idea was ignored for several decades. It wasn’t until 1959 when Sbarra and Karnovsky studied the metabolic patterns of leukocytes during phagocytosis that the biochemical mechanisms of this nucleophilic oxidase were identified¹⁷⁴. Subsequently, controversy existed whether the enzyme’s main
substrate was NADH or NADPH. Rossi and Zatti, in 1964, concluded that oxidation of NADPH accounted for the respiratory burst\(^{175}\), and this was further confirmed by other studies\(^{176}\). In 1973, Babior suggested that the primary product of phagocytizing leukocytes was \(\text{O}_2^-\) and not \(\text{H}_2\text{O}_2\)^{177}. From this point forward, molecular science continued to investigate the NADPH Oxidase enzyme and its ROS producing effects.

Today it is widely accepted that NADPH oxidase-mediated ROS production plays a vital role in the pathophysiology of endothelial dysfunction, inflammation, CVD pathologies, and angiogenesis. Some current research is focused on the possibility that therapies against nonphagocytic NADPH oxidase aimed at decreasing \(\text{O}_2^-\) generation or increasing NO availability may help prevent or regress target organ damage. Thus understanding the physiological functions as well as the molecular composition of this enzyme has become exceedingly important.

**NADPH Oxidase Physiology**

The NADPH oxidase system is composed of a membrane bound flavocytochrome b558 unit composed of gp91phox and p22phox, and the cytosolic subunits p47phox, p67phox, and Rac\(^{80, 178-180, 79}\). The membrane bound flavocytochrome b558 is the main structural component, carries the non-covalently bound FAD cofactor for the enzyme function, contains two heme groups, and is comprised of \(\alpha\) (p22phox) and \(\beta\) (gp91phox) subunits.

In 1987, Henderson et al. measured the depolarization of the membrane potential associated with oxidase activity. They provided evidence for the first time that NADPH oxidase is electrogenic and contains a hydrogen conduction channel\(^{181}\). Because of this, modern science accepts that when activated, the enzyme complex catalyzes a one
electron transfer from NADPH in the cytoplasm to molecular O_2 on the other side of the lumen wall.

Within the membrane bound flavocytochrome b558, the small membrane bound subunit α, p22phox, has two or four transmembrane segments and a cytoplasmic proline-rich C-terminal tail that serves as an anchoring point for the p47phox subunit when activation occurs\textsuperscript{182}. The larger membrane spanning protein subunit β, gp91phox, is also known as NOX2. The N-terminal portion of this core region of NADPH oxidase contains six transmembrane helices that coordinate the two heme groups, while the C-terminal portion expands into the cytoplasm and contains the FAD and NADPH binding sites\textsuperscript{182}. It is believed that these particular structural features are conserved among the NOX homologues. NOX2 serves as the catalytic component of the oxidase because it contains the entire redox machinery for the enzyme. Thus, NOX2 is crucial for regulation and activation of NADPH oxidase enzyme.

NADPH Oxidase has three main cytoplasmic components; p47phox, p67phox, and Rac. P47phox is also called the “organizer” subunit because this protein is phosphorylated at Serine residues in an autoinhibitory region causing a cytosolic translocation that allows it to interact with the proline residues in the C-terminal portion of the p22phox membrane bound subunit\textsuperscript{183, 184}. It is believed that this interaction activates the enzyme, allowing the organized transfer of electrons through the NADPH core to create O\textsuperscript{2-}. The smaller cytosolic component is a GTPase protein called Rac. This molecule interacts with p67phox inducing a conformational change that makes the activation domain functional in p67phox\textsuperscript{185, 186}. P67phox, is called the “activator” because
once activated by G-protein Rac, p67phox interacts directly with the membrane bound gp91phox to transfer the electron and ultimately produce $O_2^-$.

**NOX Homologues**

Until close to the end of the 20th century, it was believed that only one ROS-generating NOX enzyme existed. However, to date, there have been reports of at least seven different homologues of the NOX enzyme with a wide range of tissue and cell distributions\textsuperscript{187}. Depending on localization of the NOX enzyme, $O_2^-$ generation occurs either within the lumen or on the outside of the plasma membrane. Also dependent on NOX localization is how ROS are able to reach the cytoplasm: either by diffusion through anion channels or by dismutation into $H_2O_2$ which can freely diffuse through membranes\textsuperscript{188}.

Two of the NOX homologues are subunit-dependent enzymes which require assembly of the subunits for enzyme activation. NOX1 was the first homologue to be described\textsuperscript{189}. It is constitutively expressed and most abundant in the colon, smooth muscle, organ endothelia, prostate, uterus, and osteoblast precursor cells. NOX1 activity is dependent on all of the cytosolic subunits as well as membrane bound p22phox. The second subunit-dependent enzyme is NOX2, also called gp91phox, and is often referred to as phagocytic NADPH oxidase\textsuperscript{190}. This homologue was first described in macrophages and neutrophils, and is most abundant in vascular endothelial cells, fibroblasts, and phagocytes. NOX2 shares 60% sequence identity with NOX1\textsuperscript{189} and also requires all of the subunits for activation. Expression of NOX2 is not constitutive, but rather inducible by many different agonists.

NOX3 is the most specific tissue-restricted homologue, found mainly in the inner
ear vestibular and auditory canals. It is thought to serve a role in the biosynthesis and maintenance of otoconia but the mechanism remains unknown. The NOX3 enzyme shares about 60% sequence identity with NOX2, and activation of the enzyme functions in a subunit-dependent manner that may be distinct from the other known NOX families.

There are four remaining NOX homologues identified. NOX4 is a constitutive enzyme widely expressed in kidney and vascular cells, localized predominately in intracellular organelles. Activation of this homologue is least understood, but it is thought that NOX4 is dependent only on p22phox, and cytosolic subunits are not required.

The last three homologues are all Ca^{2+} dependent. NOX5 is found in lymphoid tissues, testis, and fetal tissues. DUOX1 and DUOX2 are found in thyroid gland, respiratory, and digestive epithelia. Mechanisms regulating these proteins are still misunderstood, but activation of each is associated with elevations in cytosolic Ca^{2+} levels.

**NADPH Oxidase Activation**

The entire NADPH oxidase enzyme complex sits in a quiescent state within the membrane, either cell membrane or organelle membrane. It is believed that an increase in enzyme kinetics occurs during physical inactivity, with age, with HT, and with other specific CVD pathologies. Also, molecular biology studies have discovered several agonists responsible for NADPH stimulation. Studies have shown that tumor necrosis factor-α (TNF-α), endothelin-1 (ET-1), thrombin, and platelet derived growth factor (PDGF) all can mediate production of ROS through NADPH oxidase. Furthermore, research indicates that activated NADPH oxidase requires
phosphorylation of cytosolic subunit, p47phox, through stimulation by AngII\textsuperscript{202, 204-206}. AngII binds to the Angiotensin type1 receptor ultimately leading to conformational binding between cytosolic p47phox and membrane p22phox, where the p22phox subunit serves as the final electron transporter. Therefore, both the p47phox and p22phox subunit have functional relevance in NADPH NOX2 activation.

\textit{p22phox NADPH Oxidase Subunit}

The p22phox (CYBA) gene, located on the long arm of chromosome 16 (at q24), encodes the α-subunit of the membrane bound cytochrome complex, spans 8.5 kb and contains 5 introns and 6 exons\textsuperscript{207}. Association between the p22phox subunit and NADPH oxidase activation is well established in the literature\textsuperscript{180, 208-211}. Also, genetic research has reported many different CYBA variants that may be related to CVD\textsuperscript{212-219} and HT\textsuperscript{215, 219-222}. While studies have shown association of CYBA polymorphisms with disease, research on the relationship between genetic variations of the p22phox subunit and exercise training is sparse. Park et al. have reported that the A allele of CYBA A640G polymorphism may be associated with decreased plasma thiobarbituric acid reactive substances (TBARS) levels, a common indicator of lipid peroxidation, after 6 months of aerobic exercise training\textsuperscript{223}. And more recently, Feairheller et al. reported that pre-exercise levels of 8-isoprostane prostaglandin F\textsubscript{2α} (8-iso-PGF\textsubscript{2α}), a common indicator of membrane lipid peroxidation, but not post-exercise training levels may be associated with the CYBA C242T polymorphism\textsuperscript{224}. Both studies were done in adults, and together, they highlight the important role that p22phox may have in NADPH oxidase-mediated O\textsubscript{2}⁻ production as well as the necessity for further exercise training research on this particular subunit.
**gp91phox (NOX2) NADPH Oxidase**

The catalytic subunit component gp91phox (CYBB), also known as NOX2, gene is located at locus xp21. The NOX2 serves as the catalytic moiety of the NADPH oxidase membrane bound flavocytochrome b558. It is recognized as the catalytic component because it contains the entire redox machinery for the enzyme and has been found to be the main heme binding subunit of NADPH oxidase.

In 2000, Bayraktutan et al. characterized the intracellular locations and biochemical activities of the cytochrome b558 subunits and detailed their location in endothelial cells. Since then, studies in gp91phox knock-out mice have confirmed that NOX2 has a functionally important role in NADPH-mediated arterial ROS generation. Jung et al. found that renal clipping of gp91phox-/- mice tended to increase O$_2^-$ production, but renal clipping of wild-type mice caused a significant increase in O$_2^-$ production. Renal clipping also did not alter eNOS, CAT, CuZnSOD, or EcSOD protein expression in either gp91phox-/- or wild-type mice. In this same study, it was found that NADPH oxidase inhibitors had no effect on endothelium-dependent relaxation in either clipped or sham gp91phox-/- mice, but concentrations of AngII at 100nmol impaired endothelial relaxation in gp91phox-/- mice. This suggests that gp91phox-mediated O$_2^-$ production and subsequent NO scavenging may ultimately have relevance for overall NO bioavailability.

Understanding the enzymatic mechanisms that lead to vascular ROS production in relation to specific NADPH oxidase subunits are still not completely understood. Expression of NOX2 was originally thought to exist solely in granulocytes, but has since been found to be expressed in rat aortic endothelial cells and HUVECs. Showing
relationship with disease states, upregulation of NOX2 protein expression has been reported in vein and artery segments from patients with coronary artery disease\textsuperscript{231}, with atherosclerosis\textsuperscript{199}, in patients undergoing heart transplant\textsuperscript{75}, and in spontaneously HT rats\textsuperscript{232}. No study exists that measured NOX2 expression in relation to race.

\textit{p47phox NADPH Oxidase Subunit}

The \textit{p47phox} (NCF-1) gene, is located on the long arm of chromosome 7 (at q11), has 10 introns and 11 exons, and encodes the cytosolic “organizer” subunit of NADPH oxidase\textsuperscript{233}. Activation of NADPH oxidase is known to be associated with phosphorylation of the \textit{p47phox} subunit. Early studies suggested a role for phosphorylation of a serine residue at position 379\textsuperscript{234}, but subsequent research has confirmed possible multiple other serine sites between 303 and 379\textsuperscript{235, 236}. It has also been shown that \textit{p47phox} plays a role in vascular remodeling\textsuperscript{237}, obesity\textsuperscript{238}, and HT\textsuperscript{239, 240}.

Cell culture studies have reported expression of \textit{p47phox} in rat aortic endothelial cells, in HUVECs, and in neutrophils\textsuperscript{230}. Similar to NOX2, \textit{p47phox} expression is upregulated in various disease states. Increases in \textit{p47phox} protein expression have been reported in vein and artery segments obtained from patients with coronary artery disease\textsuperscript{231}, from patients undergoing heart transplant\textsuperscript{75}, and in spontaneously HT rats\textsuperscript{232, 241}. To date, Kalinowski et al. are the only group to report on \textit{p47phox} expression in HUVECs in relation to race\textsuperscript{3}.

\textit{NOX4 NAPDH Oxidase Homologue}

Recent studies have purported the importance of recognizing that the NOX4 homologue is a major source of endothelial oxidative stress. In 2004, Ago et al. were the first to suggest that NOX4 was the major catalytic component in endothelial cells\textsuperscript{230}.
Future studies confirmed this and further identified the possibility that NOX4 has a unique ROS generation and activation\textsuperscript{242}. It has been suggested that NOX4 acts independent of cytoplasmic subunits, directly produces large amounts of H\textsubscript{2}O\textsubscript{2}, and is constitutive\textsuperscript{243}. In 2008, Dikalov et al. reported that in NOX4-depleted rat smooth muscle aortic cells, the production of H\textsubscript{2}O\textsubscript{2} was significantly reduced but the production of O\textsubscript{2}\textsuperscript{-} was not affected, suggesting that NOX4 produces mainly H\textsubscript{2}O\textsubscript{2}\textsuperscript{244}. Yet, in 2009, Goettsch et al. reported that NOX4 overexpression in human coronary artery endothelial cells significantly enhanced O\textsubscript{2}\textsuperscript{-} production\textsuperscript{245}. The variation in findings between these studies indicate that NOX4 may have an oxidative stress species-dependent production.

In order to understand activation of the NOX4 homologue in relation to other NADPH isoforms, molecular studies continue to elucidate the specific redox signaling pathways involved with induction. It has been consistently shown that the NOX4 type NADPH oxidase promotes ROS generation through activation of extracellular signal-related kinase, mitogen-activate protein kinase, and receptor tyrosine kinase\textsuperscript{246, 247}. In comparison, NOX2 has been shown to be activated by Jun-N-terminal kinase\textsuperscript{248}.

Experimental studies have shown that NOX4 expression levels increase with age, are associated with TNF-\alpha induction, can be downregulated by long-term cyclic strain, and are increased in chronic HT\textsuperscript{232, 249-251}. Despite this, NOX4 activity has yet to be examined in relation to race, so this isoform was also measured in this study.

\textit{NADPH Oxidase and Exercise}

Limited studies have specifically examined exercise training effects on NADPH oxidase activity and reported that exercise reduced the expression of NADPH oxidase and thereby decreased oxidative stress\textsuperscript{167, 168}. Duerrschmidt et al. used cultured HUVECs
to quantify the expression of specific NADPH subunits in response to LSS\(^2\). Figure 1.4 shows reduced mRNA expression of NADPH oxidase subunits gp91phox and p47phox after 24 hours of LSS at 30 dyne/cm\(^2\). Since in vitro LSS is hypothesized to simulate the shear stress conditions elicited by aerobic exercise in vivo, this reported in vitro downregulation of NADPH oxidase activity may be an important contributor to the lower oxidative stress levels seen following exercise training in human subjects.

Two animal studies exist that examined the effects of exercise on expression of NADPH oxidase subunits. Levada-Pires exercised rats on treadmills for 11 weeks, 7 days a week, for 1 hr each day at 45% of the maximal running speed\(^{168}\). The purpose of their study was to examine how physical activity can enhance leukocyte function and thus reduce infections. They found that exercise training caused an increase in gp91phox, p47phox, and p22phox expression in isolated neutrophils from the sacrificed rats. In a separate study that investigated the effects of aerobic exercise on oxidative stress in rat adipose tissue, Sakurai et al. reported a reduction in expression of gp91phox\(^{252}\). Rats in this study exercised 5 days a week for 9 weeks on a 5-deg incline. These studies demonstrate that training can influence expression of NADPH oxidase subunits in a
tissue-specific manner that is potentially regulated by the volume and/or intensity of exercise.

**Shear Stress**

Molecules at the vessel endothelium have direct interaction with the circulating blood and thus respond to the action of forces by transduction of mechanical signals, regulation of genes, and alteration in release of paracrine and autocrine factors. These shear stress characteristics influence the character and progression of endothelial function, dysfunction, and subsequent atherosclerosis and CVD pathologies. Intravascular shear stress exists in two primary physiological forms: laminar and oscillatory.

**Oscillatory Shear Stress**

The OSS is irregular, and generally acts along vessel bifurcations or branch points. OSS is also called turbulent flow and has been associated with vascular pathologies typical of CVD\textsuperscript{253}. The disturbed shear regions usually correlate with vascular areas that are prone to atherosclerotic lesion formation. Evidence indicates that OSS typically occurs at about 3-5 dyne/cm\textsuperscript{2} and causes pathological adaptations such as smooth muscle hypertrophy, altered endothelial-dependent vasodilation, and potentially atherosclerosis\textsuperscript{29, 35, 221}. It has also been found that OSS is associated with higher ROS levels\textsuperscript{254, 255} and increased NADPH oxidase expression\textsuperscript{256, 257}. Hwang et al. used p47phox-/- mice to show an increase in p47phox-dependent NADPH oxidase O\textsubscript{2}\textsuperscript{-} production with OSS\textsuperscript{258}. The mechanisms regulating how OSS contributes to atherogenesis and how LSS confers an atheroprotective phenotype have been the subject of intense research for years.
Laminar Shear Stress

LSS is caused by a smooth and non-turbulent blood flow along the endothelial layer inside the vessel walls. This type of physiological shear stress is recognized as favorable to the endothelial cell environment and is known to induce the expression of a number of beneficial endothelial paracrine and autocrine products. LSS at only 10 dyne/cm$^2$ (arterial levels of flow) has also been shown to regulate at least 107 genes, all of which are consistent with a “protective” phenotype. Therefore, the association can easily be made that as systemic and cardiac blood flow increases with aerobic exercise, positive and ‘protective’ LSS increases because blood flow volume and velocity along the vessels increases. The importance of shear stress and the mechanisms underlying the athero-protective force of LSS in relation to race still remain to be fully elucidated.

In Vitro Shear Stress

Consistently over the years, research has shown that the in vitro shear effects, determined using aortic or venous endothelial cells of humans or animals, clearly represent in vivo physiological responses. Several studies have quantified shear stress levels despite the usual difficulty in measuring in vivo levels of vascular shear stress.

In 1999, Malek et al. delineated the normal magnitudes of shear stress encountered in veins, arteries, and in low-shear and high-shear pathologic states. Venous shear stress ranges from 1-6 dyne/cm$^2$ and arterial levels range from 10-70 dyne/cm$^2$, variations occurring within the vasculature over the cardiac cycle. Lower levels (<4 dyne/cm$^2$), which are typically found at sites prone to atherosclerosis, stimulate an athero-genic phenotype. While arterial levels (>15 dyne/cm$^2$) induce a athero-
protective phenotype. During mild intensity exercise, it is expected that shear stress levels along human vessels range from approximately 15 to 30 dyne/cm$^2$.

Identifying shear stress levels \textit{in vivo} is difficult, but in one study, Gnasso et al. used forearm ischemia to determine if wall shear stress in the human brachial artery was related to flow-mediated dilation, which is endothelium-dependent and largely NO-dependent$^{266}$. In this study, they found that physiological shear stress ranged from 18.8 to 63.7 dyne/cm$^2$ during flow-mediated dilation. These levels are thought to be comparable to shear stress levels during moderate to very intense exercise levels.

Furthermore, the beneficial effect from varying levels of LSS were demonstrated by Duerrschmidt et al.$^2$. In their study, they applied LSS to HUVECs at 1, 5, 10, 15, 30, and 50 dyne/cm$^2$ for 24 hours and measured the NADPH oxidase subunit mRNA and protein expression responses. At shear stress magnitudes of 1 and 5 dyne/cm$^2$, NADPH oxidase expression was higher than control HUVECs without LSS. At the 10 dyne/cm$^2$ level, NADPH oxidase expression started to reduce and was equivalent to control HUVEC expression levels. Finally, at magnitudes of 15, 30 and 50 dyne/cm$^2$, NADPH oxidase expression levels were significantly reduced to about half that of the control HUVECs. This demonstrates that shear stress levels between 15-50 dyne/cm$^2$ can potentially modulate oxidative stress.

Taken together, the fact that 5 dyne/cm$^2$ is a physiological low level of flow and 20 dyne/cm$^2$ is within the physiological arterial shear stress levels$^{267}$, the fact that LSS reduces $O_2^-$ production more than oscillatory stress does, and that mRNA expression of NADPH oxidase subunits reduces with increasing magnitudes, both 5 dyne/cm$^2$ and 20 dyne/cm$^2$ are the LSS levels that will be used for the current study to simulate a
physiological low flow and a moderate aerobic exercise flow in order to examine their affect on oxidative stress levels in both African American and Caucasian HUVECs.

Summary

The present study is novel and important because it explored the capacity of LSS at 5 dyne/cm² and 20 dyne/cm², representative of resting physiological blood flow and moderate aerobic activity blood flow, to correct NADPH-mediated oxidative stress and improve antioxidant levels. Ultimately, this study provides mechanistic insights into the therapeutics of African Americans, which has important clinical significance because of the overwhelming morbidity and mortality in the African American population from both HT and CVD.

Briefly, HUVEC lines were subcultured in parallel and then subjected to LSS as the intervention, while static control samples received no LSS. After this, protein expression (NOX4, NOX2, p47phox, eNOS, SOD2, and CAT) was measured, total SOD activity and TAC measured in cell lysate, and NO levels measured in cell culture supernatant for the static and both of the LSS samples.

Statement of Purpose

Studying the different NADPH oxidase subunit, eNOS, SOD2, and CAT protein expression levels is critical because research suggests that they each play a fundamental role in NADPH oxidase activation, antioxidant activities, and NO bioavailability. Based on the above reported literature, the purpose of this study was to investigate endothelial cell NADPH-induced oxidative stress mechanisms using LSS experiments in cell culture. The specific research objectives were to characterize potential race-specific differences in
NADPH oxidase subunit and isoform expression, eNOS expression, antioxidant expressions, and cell culture antioxidant levels in response to LSS.

**Hypotheses**

Increased levels of LSS modulate the endothelial environment favoring a healthy endothelium and lower blood pressure. Physiological evidence shows that elevated LSS, comparable to blood flow levels during aerobic exercise, improves the endothelial cell phenotype. Evidence also suggests that the NADPH oxidase subunits are important factors in the modulation of oxidative stress with aerobic exercise training\(^{263}\). However, it is not known if LSS can reverse the otherwise enhanced NADPH oxidase activity observed in endothelial cells from African Americans.

It is hypothesized that compared to static levels, LSS for 24 hours at 5 dyne/cm\(^2\) will upregulate all protein expressions, while LSS for 24 hours at 20 dyne/cm\(^2\) will significantly reduce NADPH oxidase subunit expressions, increase antioxidant enzyme levels, increase eNOS expression, and lead to increased NO bioavailability in cultured HUVECs. Furthermore, due to the heightened oxidative stress levels in African Americans and the potential for existing endothelial dysfunction, it is believed that African American HUVECs will respond to LSS stimulus differently than Caucasian HUVECs.

**Specific Aims**

1. Determine NADPH oxidase subunit and isoforms; p47phox, NOX2, and NOX4; eNOS, SOD2, and CAT protein expressions, before and after exposing cultured HUVECs from African American and Caucasian donors to LSS at levels comparable to \textit{in vivo} blood flow levels at rest (5 dyne/cm\(^2\)) and during moderate aerobic exercise (20
dyne/cm$^2$). Protein expression was measured by Western Blot analysis using antibodies specific for NOX2, NOX4, p47phox, eNOS, SOD2, and CAT.

2. Determine NO bioavailability in African American and Caucasian HUVECs; NO, SOD, and TAC levels were quantified. NO measurements were performed by using the Griess reaction to measure total nitrate and nitrite in cell culture supernatant. Total SOD activity in the cell lysate was assessed using an assay which measures dismutation of O$_2^-$ radicals generated by xanthine oxidase and hypoxanthine. Finally, TAC levels were quantified using a commercial assay that measures the reduction of 2,2’-azinobis-3-ethylbenzthiazoline-sulfonate (ABTS).
CHAPTER 2

PRELIMINARY STUDIES

Introduction

Three separate studies were conducted prior to the actual dissertation research study, and these were completed in order to establish proof of concept. The first study, “Racial differences in the time-course oxidative stress responses to acute exercise”, established that basal redox differences are inherent in adults of the two races. The second study, “Racial differences in oxidative stress and inflammation: in vitro and in vivo”, used a translational experimental design to show that the common plasma markers of oxidative stress found to be higher in African American adults are also higher in HUVEC culture from African American donors. The third study, “Effects of TNF-α Stimulation on NOS Expression in Racially-derived HUVECs”, was conducted to examine whether the HUVECs from different race responded differently to pharmacological stimuli, in particular to TNF-α.

Preliminary Study #1:

Racial Differences in the Time-Course Oxidative Stress Responses to Acute Exercise

Abstract

African Americans have disproportionate levels of hypertension, cardiovascular disease, and oxidative stress. The purpose of this study was to examine racial differences between African American and Caucasian adults in time-course oxidative stress responses to a treadmill exercise test. After a 12 hr fast, 18 participants (9African
American and 9 Caucasian: 21±0.4 years) completed a Modified Bruce treadmill test and underwent serial blood draws; Pre-Exercise, Post-Exercise (within 2 min), 30, 60, and 120 min after exercise. At each time-point, SOD (U/mL), TAC (mM), protein carbonyls (PC, nmol/mg), and TBARs (µmol/L) were measured. No difference existed between groups for blood pressure, BMI, or exercise capacity. African Americans had significantly higher (p<.05) SOD (Pre: 5.45±0.4 vs. 3.69±0.69; 60 min: 8.99±0.7 vs. 4.23±0.6; 120 min: 9.69±1.6 vs. 3.52±0.7), TAC (Pre: 2.31±0.25 vs.1.16±0.3; Post: 2.39±0.2 vs.1.34±0.2; 30 min: 2.29±0.2 vs.1.09±0.2), and PC (Pre: 1.09±0.1 vs. 0.82±0.1; Post: 1.14±0.1 vs.0.81±0.1; 30 min: 1.13±0.1 vs.0.85±0.1; 60 min: 1.06±0.1 vs.0.81±0.05) compared to the Caucasian adults, but not TBARs. Between groups, only SOD exhibited a different time-course response: total SOD activity levels for the African American adults rose steadily throughout the 120 min, while levels for Caucasians peaked at 30 min and by 120 min had returned to pre-exercise value. In conclusion, race had a greater effect on oxidative stress responses than sub-maximal exercise did.

Introduction

African Americans exhibit disproportionate levels of HT, higher incidence of CVD and renal disease, and elevated levels of oxidative stress when compared to other ethnic groups, in particular Caucasians. Additionally, studies in un-stimulated endothelial cell culture have shown that these racial differences also exist in vitro by reporting heightened oxidative stress in African American cells compared to Caucasian cells.

The effects of sub-maximal acute exercise on the oxidant/antioxidant balance over a post-exercise time period are not well known. Inconsistencies are found in results from one study to the next due to differences in exercise protocol, training status, and gender.
It is also not well known whether a disparity exists between races in the oxidative stress response to exercise. Since exercise is often prescribed as a non-pharmacologic treatment of chronic diseases such as HT, and given that African Americans tend to have higher levels of oxidative stress, it becomes critical to understand the appropriate exercise intensity that will not elicit an exaggerated oxidative stress response.

Oxidative stress is an imbalance between the production of free radicals and the antioxidant system’s ability to buffer the oxidative damage. Exercise causes an increase in oxygen consumption and therefore the production of ROS, ultimately leading to increased oxidative stress if the antioxidant system’s buffering ability is insufficient. This oxidative stress response to exercise varies by biomarker. It is understood that proteins are expressed in response to exercise, their expression levels peak at different times, and the amount of time that it takes to return to baseline expression levels varies by marker. Classic pre-post exercise studies collect the post-exercise sample immediately following the exercise bout, and only a few studies exist where more than two blood samples have been collected to explore time-course responses to acute exercise. Along these lines, in 2007 Michailidis et al. investigated the time-course responses of several oxidative stress markers during a 24 hr period after one acute 45 min treadmill exercise session at 70-75% VO$_{2}$max$^{268}$. They studied 11 untrained men and found different response times for the oxidative stress markers. However, to the best of our knowledge, this type of study has not been done to examine potential racial differences.

The purpose of our study was to examine racial differences between African American and Caucasian adults in the time-course oxidative stress responses to an acute exercise bout. Because exhaustive exercise to volitional fatigue is not commonplace for
most exercise sessions among the general public, we sought to determine whether responses to a sub-maximal exercise test differed by race.

**Methods**

Young college-aged students, aged 18-25 years, were recruited through advertisements and word of mouth. All were apparently healthy and free of CVD risk factors, and this was assessed by completion of an extensive health history form during an initial laboratory visit. The study was approved by the Institutional Review Board of Temple University, Philadelphia, PA., and all qualified students provided their written, informed consent (Appendix A).

**Experimental Design**

The students were asked to refrain from vitamins for 2 weeks prior, from caffeine, alcohol, or exercise training for 24 hr prior, and to fast for at least 10 hr the night before the study. Research has suggested that the hormone fluctuations during the menstrual cycle can influence oxidative stress responses to exercise\(^{269}\). Therefore, all females were tested during days 1-5 of their menstrual cycle because hormone levels tend to be lowest early in the follicular phase.

On the morning of the study, height and weight were measured, and a pre-exercise blood sample was collected. Blood samples were collected in EDTA and Sodium-Heparin tubes, centrifuged at 2000 g for 20 min at 4 °C, and then the plasma was frozen at 80 °C until assay. Then a modified Bruce sub-max treadmill exercise test was performed. The test was terminated when the student reached 75-80% of their heart rate reserve, and regression analysis using data collected by indirect calorimetry was used to predict VO\(_{2}\)\(_{\text{max}}\) levels. Post-exercise blood samples were collected at the following time-
points: immediately following exercise (within 2 min), 30, 60, and 120 min. All students remained in the lab area throughout the 2 hr post-exercise period in order to control for food and water intake. During this time, they were instructed to sit and read, or work on the computer, and were only allowed to drink up to 1 L of water. At the completion of the test, juice and snacks were provided. Subject data was only included if 80% of the blood samples were collected.

**Measurement of Superoxide Dismutase (SOD) Activity**

Plasma samples were diluted 1:5 in sample buffer (50 mM Tris-HCl, pH 8.0). SOD activity was measured by assay (Cayman Chemical, Ann Arbor, MI, USA), as previously reported\(^{270}\). Inter-assay and intra-assay coefficients of variation were 5.9% and 12.4% respectively.

**Measurement of Total Antioxidant Capacity (TAC)**

Plasma samples were diluted 1:20 in Assay buffer (5 mM potassium phosphate, pH 7.4, containing 0.9% sodium chloride and 0.1% glucose). TAC was measured by assay (Cayman Chemical, Ann Arbor, MI, USA), as previously reported\(^{270}\). Inter-assay and intra-assay coefficients of variation were 6.7% and 9.2% respectively.

**Measurement of Protein Carbonyls (PC) Formation**

Average human plasma protein levels were determined to be 6 g/dL by using the Bradford Protein Assay prior to the measurement of PC. PC formation was determined with the Oxiselect™ Protein Carbonyl ELISA Kit (Cell Biolabs, Inc., San Diego, CA). Inter-assay and intra-assay coefficients of variation were 5.5% and 7.8% respectively.

**Measurement of Thobarbituric Acid Reactive Substances (TBARS)**

Lipid peroxidation was assessed by the measurement of TBARS in the plasma.
Briefly, the assay involves the reaction of malondialdehyde (MDA), contained in the sample, with thiobarbituric acid (TBA) under high temperature and acidic conditions to form a MDA-TBA complex that can be quantified colorimetrically. On the day of assay, plasma samples were mixed with sodium dodecyl sulfate solution and TBA reagent (530 mg thiobarbituric acid solubilized in a mixed solution containing 50 ml of sodium hydroxide and 50 ml acetic acid). Absorbance was read at 535 nm using a SpectraMax Microplate Reader (Molecular Devices, Sunnyvale, CA). All reagents were obtained from Cayman Chemical (Ann Arbor, MI). Inter-assay and intra-assay coefficients of variation were 12.9% and 15.1% respectively.

Statistical Analysis

Data are presented as means ± SE and significance was set at P < 0.05. The distribution of all variables was examined using the Shapiro-Wilk test of normality, and homogeneity of variances was determined using Levene’s test. All data were normal. Independent t-tests were used to determine if there were significant differences between ethnic groups. Two-way repeated measures ANOVA, with Huynh-Feldt or Greenhouse-Geisser correction when necessary, were conducted to assess whether significant effects of race or time existed. This was followed by post-hoc paired t-test analyses. Area under the curve (AUC) was calculated by polynomial integration and independent t-test analyses was conducted to examine for differences between ethnic groups. Statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL).

Results

Eighteen young adults participated in this study. The entire group had an average age of 21 ± 0.4 years, BMI 25.8 ± 1.1 kg/m², BP 123.8/78.4 ± 2.6/2.2 mmHg, and VO₂max
of 45.1 ± 1.9 ml/kg/min. For analyses, participants were divided into groups by race and

difference existed between the groups for any of these variables.

**Table 2.1. Subject Characteristics by Ethnic Group**

<table>
<thead>
<tr>
<th></th>
<th>African Americans (N=9)</th>
<th>Caucasians (N =9)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs</td>
<td>21.3 ± 0.5</td>
<td>20.6 ± 0.7</td>
<td>0.40</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.8 ± 1.1</td>
<td>25.2 ± 2.2</td>
<td>0.83</td>
</tr>
<tr>
<td>VO₂max. ml/kg/min</td>
<td>44.9 ± 3.4</td>
<td>44.6 ± 2.6</td>
<td>0.96</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>122.6 ± 4.4</td>
<td>126.2 ± 3.9</td>
<td>0.55</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>79.0 ± 3.8</td>
<td>78.0 ± 3.2</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE. N, sample size; BMI, body mass index; VO₂max, maximum oxygen consumption; BP, blood pressure.

**SOD Responses by Race**

Figure 2.1 shows SOD activity by race over time. There was a significant time and race effect for SOD activity (p=0.02). Analyses showed a time effect from pre-exercise to post-exercise (p=0.03), and from the 30 to 60 min (p=0.02) time points in the.

![Figure 2.1: Total SOD by Race. Comparison of SOD levels between African American (closed triangles) and Caucasian (open triangles) adults. Values are means ± SE. * Significant difference between Ethnic groups. †Significant difference from previous sample time. Significance level set at p=0.05.](image)
Caucasian group. In the African American adults, analyses showed a time effect from post-exercise to the 30 min point (p=0.01). Separate pair-wise analyses showed significant differences between race at pre-exercise (p=0.04), 60 min (p=0.00), and at the 120 min (p=0.01) time points.

*PC Responses by Race*

Figure 2.2 shows PC values by race over time. There was only a significant race effect in PC levels. African Americans had higher PC values at all time points, with statistical significance at pre-exercise (p=0.01), post-exercise (p=0.00), 30 min (p=0.01), and at 60 min (p=0.01) time points.

![Figure 2.2. PC Levels by Race. Comparison of PC levels between African American (closed triangles) and Caucasian (open triangles) adults. Values are means ± SE. * Significant difference between Ethnic groups. Significance level set at p=0.05.](image)

*TAC Responses by Race*

Figure 2.3 shows TAC values by race over time. Only a significant race effect was found in TAC responses. While the African Americans had higher TAC values at all time points, analysis showed significant difference between African American and
Caucasians at pre-exercise (p=0.01), post-exercise (p=0.00), and at 30 min (p=0.00) time points.

![Figure 2.3. TAC Levels by Race](image)

**Figure 2.3. TAC Levels by Race.** Comparison of TAC levels between African American (closed triangles) and Caucasian (open triangles) adults. Values are means ± SE. * Significant difference between Ethnic groups. Significance level set at α=0.05.

**TBARS Responses by Race**

Figure 2.4 shows TBARS values by race over time. No significant time or race effect was found.

![Figure 2.4. TBARS Levels by Race](image)

**Figure 2.4. TBARS Levels by Race.** Comparison of TBARS levels between African American (closed triangles) and Caucasian (open triangles) adults. Values are means ± SE.

**Total Area under the Oxidative Stress Response Curves (AUC):**

Table 2.2 reports the AUC differences by race. Integration was completed to analyze AUC for each oxidative stress variable. Independent t-test analysis showed
significant race differences in SOD, TAC, and PC, but not in TBARS. This data confirms that the African Americans had a higher oxidative stress load over the entire sampling time.

**Table 2.2.** Total Area Under Oxidative Stress Response Curves, by Ethnic Group

<table>
<thead>
<tr>
<th></th>
<th>African Americans (N = 9)</th>
<th>Caucasians (N = 9)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC SOD, U/ml per 120 min</td>
<td>986.0 ± 86.3</td>
<td>517.9 ± 51.8</td>
<td>0.00</td>
</tr>
<tr>
<td>AUC TAC, mM per 120 min</td>
<td>293.2 ± 31.9</td>
<td>180.7 ± 23.4</td>
<td>0.01</td>
</tr>
<tr>
<td>AUC PC, nmol/mg per 120 min</td>
<td>126.1 ± 7.7</td>
<td>96.9 ± 8.7</td>
<td>0.02</td>
</tr>
<tr>
<td>AUC TBARS, umol/L per 120 min</td>
<td>405.2 ± 74.9</td>
<td>478.8 ± 54.9</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE. N, sample size; AUC, total area under the response curve; SOD, superoxide dismutase activity; TAC, total antioxidant capacity; PC, protein carbonyls; TBARS, thiobarbituric acid reactive substances.

**Discussion**

The main findings from this study are that significant racial differences exist in oxidative stress markers, but the sub-maximal exercise intensity was only enough to elicit an SOD activity response to the exercise bout. Research has established that chronic aerobic exercise can effectively improve oxidative stress levels, but the results on the oxidative stress responses to acute exercise remain inconsistent. Reports from previous studies vary by response and by oxidative stress marker, and this discrepancy may be largely influenced by exercise intensity, by vast differences in training status, age, gender, race, and exercise modality. High intensity acute exercise has been shown to increase oxidative stress\(^{271, 272}\), while other studies report differential responses depending on exercise intensity\(^{273, 274}\). For example, Dayan et al. used a modified Balke exercise test to examine lipid peroxidation in healthy males and found no change in lipid oxidation\(^{275}\).

The sub-maximal intensity of a Balke protocol is similar to that used in our study and we also found no change in lipid peroxidation. Considering this disparity in the literature, it
appears that further research is still needed to elucidate the oxidative stress responses to acute exercise.

In young Caucasian adults, we found that an acute sub-maximal exercise bout caused a significant increase in SOD activity from pre-exercise to post-exercise with no change observed in the African American adults. Some studies have shown no change in SOD activity in animal models after acute exhaustive exercise, while other studies have shown that gender and training status influence SOD responses. No study has examined the racial differences in response to acute aerobic exercise.

We found a racial difference in SOD activity over the time-course sampling period. As mentioned, the Caucasian adults showed a significant increase in SOD activity in response to the acute exercise bout. These levels continued to rise until they peaked at 30 min, and by 60 min had returned to basal levels. On the contrary, the African American adults had a delayed response to the sub-maximal exercise stimulus. The levels of SOD activity didn’t begin to rise until the post-exercise time point and continued to trend upward for the following 2 hr. To the best of our knowledge, only one other study has reported racial differences in SOD responses, and this was in diabetic patients. Zitouni et al. reported that African American patients had significantly higher SOD activity compared to Caucasian patients which is similar to what we found in our adults. The SOD enzyme is the main antioxidant enzyme that catalyzes the dismutation of superoxide anion into hydrogen peroxide, so a higher SOD activity in African American adults suggests an existing higher level of superoxide production, and thus elevated oxidative stress.

Elevations in oxidative stress interact with proteins, lipids, and deoxyribonucleic
acid (DNA) causing protein degradation, lipid peroxidation, and DNA damage. The effect of damage from exercise, however, depends on duration and degree of exercise as well as training status of the subjects\textsuperscript{171}. ROS- induced protein modifications lead to altered protein structure or unfolding of proteins, and the most common products of protein oxidation are the PC derivatives of amino acids proline, arginine, lysine, and threonine. Carbonylation is an irreversible and non-enzymatic protein conversion and the byproduct derivatives are chemically stable and easily measurable markers of oxidative stress\textsuperscript{277}. Recent research has reported PC elevations associated with many endogenous and exogenous factors not related to exercise. Elevated PC levels have been found in patients who are critically ill\textsuperscript{278}, who have acute infection\textsuperscript{279}, who have neurodegeneration\textsuperscript{280}, and in the pathogenesis of aging\textsuperscript{281,282}. Finally, Yeh et al. recently found that PC levels were significantly higher in African Americans compared to Caucasians\textsuperscript{283}. Similar to this, in the present study, African Americans had significantly higher PC levels than Caucasians across all time points, thus having elevated oxidative stress.

We also found that acute sub-maximal exercise did not elicit a change in PC levels. Similar to these findings, Liu et al. compared the oxidative stress responses in rats to acute and chronic exercise. Their results showed that acute exercise (running to exhaustion) did not cause significant changes in PC levels, whereas chronic exercise induced a small decrease. Studies also report that the magnitude of oxidative damage is associated with intensity of the acute exercise bout\textsuperscript{284,285}. High-intensity and high-volumes of aerobic exercise elicit an increase in protein and DNA damage as measured by higher PC levels\textsuperscript{286,287}. Taken together, it appears that the intensity of a sub-maximal
treadmill test is not sufficient to elicit a change in PC levels, and this was confirmed by the present study.

The sub-maximal exercise test was also not a sufficient stimulus to elicit a response in TAC. Ashmaig et al. also observed no change in TAC in patients with ischaemic heart disease who underwent a standard Bruce treadmill test\(^{288}\). However, in 2006, Demirbag et al. measured TAC responses to a sub-maximum modified Bruce treadmill protocol and reported a decrease in TAC\(^{289}\). Participants in their study were patients with angina or angina-like symptoms. The variation in study populations may have contributed to the different responses in TAC seen compared to those in the present study. While we found no response to exercise in TAC, we did find significant differences between races in TAC levels pre-exercise, post-exercise, and at 30 minute time points. African American participants were higher at all points, suggesting elevated oxidative stress exists and therefore heightened antioxidant levels.

**Conclusion**

In conclusion, race had a greater effect on oxidative stress levels than time or exercise did. African Americans had significantly higher TAC, SOD, and PC levels when compared to Caucasians, while SOD was the only biomarker to demonstrate different responses to the sub-maximal exercise. These data confirm what has been previously reported that African Americans have a higher level of oxidative stress. The data also add to existing acute exercise literature by providing evidence that a single sub-maximal exercise bout will not elicit substantial oxidative stress responses, since the only marker to show response was SOD. Considering that exhaustive exercise to volitional fatigue is not commonplace for most exercise sessions among the general public, these findings
suggest that a moderate sub-maximal exercise intensity may be an appropriate exercise
prescription as a non-pharmacologic treatment for chronic diseases like HT, and CVD
because it does not elicit undue oxidative stress.

Preliminary Study #2:

Racial Differences in Oxidative Stress and Inflammation: *in vitro* and *in vivo*

*Abstract*

African American race is an independent risk factor for enhanced oxidative stress
and inflammation. We sought to examine whether oxidative stress and inflammatory
markers that are typically measured in human also differ by race in cell culture. We
compared levels between African American and Caucasian young adults. Separately, we
then cultured HUVECs from both races in parallel in order to compare biomarkers
between the two races. We found heightened oxidative stress and inflammation in the
African Americans both *in vitro* and *in vivo*. African American HUVECs showed higher
NO levels (10.8±0.4 vs 8.8±0.7 umol/L/mg, P=0.03), Interleukin-6 (IL-6) levels
(61.7±4.2 vs 23.9±9.0 pg/mg, P=0.02), and lower SOD activity (15.6±3.3 vs. 25.4±2.8
U/mg, P=0.04), and also higher protein expression (p<0.05) of NADPH oxidase subunit
p47phox, isoforms NOX2 and NOX4, eNOS, iNOS, as well as IL-6. African American
adults had higher plasma protein carbonyls (1.1±0.1 vs 0.8±0.1 nmol/mg, P=0.01),
antioxidant capacity (2.3±0.2 vs 1.1±0.3 mM, P=0.01), IL-6 (5.3±0.2 vs 2.4±0.3 mM,
P=0.07) and lower NO levels (27.2±4.4 vs 27.8±4.6 mM, P=0.71). This translational data
demonstrate that racial differences exist in HUVECs much like that in humans, and
suggests that the racial origin of cell model may be important to consider with *in vitro*
research.
Introduction

African American ethnicity is an independent risk factor for exaggerated oxidative stress, inflammation, hypertension, and CVD\textsuperscript{15, 17, 290}. Numerous clinical and epidemiological studies have reported significant racial differences between African American, Caucasian, and Mexican adult populations. Oxidative stress arises from either an increased production of ROS, through augmented release of the O\textsuperscript{2-} radical, or from an incapability of the antioxidant system to effectively remove the ROS. Inflammation and oxidative stress are intricately linked through a complex cycle. Consistently, increased oxidative stress and inflammation have both independently been associated with chronic disease, but it remains to be established which is the predecessor, and this may very well depend on external factors\textsuperscript{291-293}.

Over the past several decades, there has been a growing body of evidence defining the value of using cell culture as an appropriate \textit{in vitro} model in order to elucidate potential mechanisms associated with known \textit{in vivo} adaptations found in various pathophysologies\textsuperscript{294}. In particular, studies have used endothelial cell culture as a suitable model for studying \textit{in vivo} endothelial function. A very recent review by Niki highlights the importance of understanding the antioxidant capacity differences between \textit{in vitro} and \textit{in vivo} models. Niki suggests that using cultured cells for mechanistic studies may become more important as the use of animal models becomes more difficult\textsuperscript{295}.

Therefore, when the \textit{in vitro} cell culture model is used as a research tool for oxidative stress and inflammatory mechanisms, it is important that the culture samples are representative of the \textit{in vivo} model considered. HUVECs have been used to study many different biological processes because cells isolated from umbilical cords are
typically free from pathogen and pathophysiology. It is believed that the HUVEC responses to any stimuli closely mimic those responses of true in vivo endothelial cells. Research has established that racial differences exist in how humans respond to the development, progression, and treatment of disease, however the racial origins of cultured cells has commonly been ignored in the literature. Therefore, it may be plausible that the complex mechanisms mediating the in vivo racial variation could also exist in vitro between cell cultures of different races. Thus, it is critical to measure the innate oxidative stress and inflammation levels of cultured cells and determine whether these levels actually differ between racial groups.

As human clinical studies continue, translating to an appropriate in vitro model will be an important step in understanding the underlying mechanisms in the observed epidemiological differences. Therefore, we sought to examine whether common oxidative stress and inflammatory markers that are typically measured in humans also differ by race in cell culture. Using standard assays, we compared oxidative stress and inflammation levels between African American and Caucasian young adults. Separately we then used a parallel cell culture experimental design to compare oxidative stress and inflammation levels in HUVECs from both race.

Methods

Ethical Approval

The Institutional Review Board of Temple University approved this study, and qualified subjects were recruited through advertisements and word of mouth. Written, informed consent (Appendix A) was obtained from each subject prior to participation, and all protocols conformed to guidelines as set forth by the Declaration of Helsinki.
**Human Subjects**

Subjects included college-aged self-reported African American and Caucasian young adults, 18-25 years old. All were apparently healthy and free of cardiovascular risk factors as assessed by completion of an extensive health history form during an initial laboratory visit. The subjects were asked to refrain from vitamin supplements for 2 weeks prior, from caffeine, alcohol, or exercise for 24 hr prior, and to fast for at least 10 hr the night before the study. All females were tested on days 1-5 of their menstrual cycle, during early follicular phase, to eliminate hormonal interference on oxidative stress. No females were on oral contraceptives. On the morning of the study, height and weight were measured, blood was drawn from the antecubital vein into EDTA and Sodium-Heparin tubes, and plasma separated by centrifugation and stored at -80 °C until assay.

**Cell Culture**

HUVECs from both African American (N=3) and Caucasian (N=3) donors were obtained from Lonza (Walkersville, MD) and cultured in parallel, in EGM complete medium supplemented with 2% fetal bovine serum (FBS) at 37 °C in a 95 % air- 5 % CO₂ atmosphere, following methods by Lonza. HUVECs at passage 4 were used for all experiments. For all assays, control samples of culture medium were tested along with the cell culture samples in order to eliminate potential interference from culture media to the measurements. All experiments were completed three times for confirmation.

**Western Blot**

HUVECs were washed twice in ice-cold Hanks buffered saline solution and lysed in Radio-Immunoprecipitation Assay Buffer with Roche protease inhibitor (RIPA-Pi). Phenylmethylsulfonyl fluoride protease inhibitor was also added to the RIPA-Pi to
eliminate interference. At confluence, cells were collected and centrifuged at 16,000 g for 10 min at 4 °C. Quantification of protein content was measured by Bradford assay. 20 µg of protein was separated by electrophoresis through 10% SDS-polyacrylamide gel. Proteins were then transferred to nitrocellulose filter membranes. Membranes were blocked with non-fat dry milk in Tris-buffered saline and incubated overnight with primary antibodies at 4 °C. Subsequently, the membranes were washed and then incubated with secondary antibody conjugated with horseradish peroxidase. Immunoreactive proteins were detected by chemiluminescence with Thermo Scientific SuperSignal substrate systems (Pierce Biotechnology). Band densitometry analysis was performed with National Institutes of Health ImageJ software. Primary antibodies were Anti-NOX4 (ThermoScientific/Pierce Biotechnology, IL), Anti-gp91phox (NOX2) and Anti-p47phox (BD Transduction Labs, CA), Anti-SOD2 (AbFrontier), Anti-iNOS and Anti-eNOS (BD Transduction Labs, CA), Anti-IL-6 (Abcam, Inc., MA). Actin antibody (Santa Cruz Biotechnology, CA) was used as the internal control for all western blot measurements.

**Cell Harvest**

The supernatant from confluent HUVECs was removed and immediately stored at -80 °C until assay. Cell lysate was collected by cellular fractionation. Briefly, cells were washed once with ice-cold phosphate buffer solution, and resuspended in 2 mL cold HEPES buffer (Lonza, Walkersville, MD) using a rubber scraper. Cells were centrifuged at 600 g for 10 min at 4 °C, cell pellet resuspended in 500 µl cold HEPES buffer, and then transferred to a Teflon glass coupling potter. Cell solution was homogenized at 1600 rpm for 30 strokes while on ice, and then immediately centrifuged at 1500 g for 5 min at
4 °C. Aliquots of the supernatant were stored at −80 °C until assay. The remaining supernatant was then centrifuged at 10,000 g for 15 min at 4 °C. Protein concentration was measured using the Bradford method.

For all procedures, African American and Caucasian HUVECs were treated identically. Samples used for all assays were tested in duplicate, and absorbance was read using a SpectraMax Microplate Reader (Molecular Devices, Sunnyvale, CA).

**Measurement of Superoxide Dismutase (SOD) Activity**

Plasma samples were diluted 1:5 in sample buffer (50 mM Tris-HCl, pH 8.0), while HUVEC cell lysate samples were not diluted. SOD activity was measured by assay (Cayman Chemical, Ann Arbor, MI, USA), as previously reported. Inter-assay and intra-assay coefficients of variation were 5.9% and 12.4% respectively.

**Measurement of Total Antioxidant Capacity (TAC)**

Plasma samples were diluted 1:20 in Assay buffer (5 mM potassium phosphate, pH 7.4, containing 0.9% sodium chloride and 0.1% glucose), while HUVEC cell lysate samples were not diluted. TAC was measured by assay (Cayman Chemical, Ann Arbor, MI, USA), as previously reported. Inter-assay and intra-assay coefficients of variation were 6.7% and 9.2% respectively.

**Measurement of Protein Carbonyls (PC) Formation**

Average human plasma protein levels were determined to be 6 g/dL by using the Bradford Protein Assay prior to the measurement of PC. PC formation was determined with the Oxiselect™ Protein Carbonyl ELISA Kit (Cell Biolabs, Inc., San Diego, CA). Inter-assay and intra-assay coefficients of variation were 5.5% and 7.8% respectively.
Measurement of Interleukin-6 (IL-6) Levels

IL-6 concentrations were measured in human plasma and cell culture supernatants by ELISA (Thermo Scientific-Pierce Biotechnology, Rockford, IL, USA), according to manufacturer’s instructions. Inter-assay and intra-assay coefficients of variation were 8.6% and 10% respectively.

Measurement of Nitric Oxide (NO) End-products

HUVECs were grown to confluence in phenol-red free EGM medium. Human plasma samples and HUVEC supernatant were ultrafiltered through a 10,000 MWCO Amicon Ultra filter (Millipore) by micro-centrifuge at 14,000 g for 30 min at 4 °C. Levels of total NO end-products were measured using a modified Griess assay (Assay Designs, Ann Arbor, MI, USA), as previously reported\textsuperscript{270}. Inter-assay and intra-assay CVs were 7.6% and 10.6% respectively.

Statistical Analysis

Data are presented as means ± SE and significance was set at P < 0.05. The distribution of all variables was examined using the Shapiro-Wilk test of normality, and homogeneity of variances was determined using Levene’s test. All data were normal. Independent t-tests and ANOVA were used to determine if there were significant differences between ethnic groups. Statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL).

Results

Eighteen young adults participated in this study (9 African Americans and 9 Caucasians). The entire group had an average age of 21 ± 0.4 years, body mass index of 25.8 ± 1.1 kg/m\textsuperscript{2}, and blood pressure of 123.8 ± 2.6/78.4 ± 2.2 mmHg. For analyses,
participants were grouped by race. Table 2.3 shows the subject characteristics grouped by race. No significant difference existed between the groups for any of these variables.

**Table 2.3. Subject Characteristics by Ethnic Group**

<table>
<thead>
<tr>
<th></th>
<th>African Americans (N =9)</th>
<th>Caucasians (N =9)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs</td>
<td>21.5 ± 0.4</td>
<td>20.6 ± 0.6</td>
<td>0.25</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.6 ± 0.8</td>
<td>26.1 ± 2.0</td>
<td>0.85</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>121.6 ± 4.0</td>
<td>126.2 ± 3.5</td>
<td>0.41</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>80.6 ± 3.3</td>
<td>76.0 ± 2.9</td>
<td>0.32</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE. N, sample size; BMI, body mass index; BP, blood pressure.

**Racial Differences in NO production**

No difference existed between the African American and Caucasian adults (Figure 2.5A). However, African American HUVECs had significantly higher NO levels than Caucasian HUVECs (10.8 ± 0.4 vs 8.8 ± 0.7 nmol/L/mg, P=0.03) (Figure 2.5B).

**Figure 2.5. Racial Differences in NO Levels.** A. African American (AA) and Caucasian (C) human plasma. B. Basal HUVEC culture, AA and C. Values normalized to protein count. C. eNOS and iNOS protein expression levels in AA(shaded) and C(open) HUVECs, basal levels. ImageJ densitometric analysis of bands expressed in relation to β-actin. Bars show mean ±SE. *P<0.05 between ethnic group.
In order to further assess *in vitro* NO production, the protein expression levels of both eNOS and iNOS were determined by Western blot (Figure 2.5C). Protein expression of both eNOS and iNOS were significantly higher in unstimulated African American HUVECs compared to Caucasian HUVECs (p<0.01).

*Racial Differences in Interleukin-6 (IL-6)*

Because of the higher iNOS protein expression in African American HUVECs, we then measured IL-6 levels in both the adults and HUVEC supernatant to determine if a heightened inflammation level exists. No race difference existed in the adults (Figure 2.6A). However, we found higher IL-6 levels in African American HUVECs compared to Caucasian HUVECs (61.7 ± 4.2 vs 23.9 ± 9.0 pg/mg, P=0.02) (Figure 2.6B). In order to further assess the *in vitro* IL-6 levels, protein expression of IL-6 was determined by Western blot (Figure 2.6C). We measured greater protein expression of IL-6 in unstimulated African American HUVECs compared to Caucasian HUVECs (P<0.01).

**Figure 2.6. Racial Differences in IL-6 Levels.** A. African American (AA) and Caucasian (C) human plasma. B. Basal HUVEC culture, AA and C. Values normalized to protein content. C. IL-6 protein expression levels in AA (shaded) and C (open) basal HUVEC culture. ImageJ densitometric analysis of bands expressed in relation to β-actin. Bars show mean ±SE. *P<0.05 between ethnic group.
**Racial Differences in SOD Activity**

We found that African American adults had higher plasma SOD activity compared to Caucasian adults (5.4 ± 0.4 vs 3.6 ± 0.7 U/mL, P=0.04) (Figure 2.7A). However, we found lower total SOD activity in African American HUVECs (15.6 ± 3.3 vs 25.5 ± 2.8 U/mg, P=0.04) compared to Caucasian HUVECs (Figure 2.7B).

Also, we found that African American HUVECs tended to have lower levels of SOD1 activity (11.1 ± 3.9 vs 19.0 ± 5.0 U/mg, P=0.15) (Figure 2.7C). Further analysis of protein expression of the mitochondrial SOD isoform, SOD2, showed that African American HUVECs also tended to have lower SOD2 expression (P=0.31) (Figure 2.7D).
Racial Differences in TAC

Figure 2.8 shows that African American adults had higher TAC levels compared to Caucasian adults (2.3 ± 0.2 vs 1.1 ± 0.3 mM, P=0.01) and we also found higher TAC in African American HUVECS (1.3 ± 0.4 vs 0.5 ± 0.1 µM/mg, P=0.04).

![Figure 2.8. Racial Differences in TAC Levels. A. African American (AA) and Caucasian (C) human plasma. B. Basal HUVEC culture, AA and C. Values normalized to protein content. Bars show mean ±SE. *P<0.05 between ethnic group.]

Racial Differences in Plasma Protein Carbonyls (PC)

Confirming heightened oxidative stress, we found higher plasma PC levels in African American adults compared to Caucasian adults (1.1 ± 0.1 vs 0.8 ± 0.1 nmol/mg, P=0.005) (Figure 2.9).

![Figure 2.9. Racial Differences in Plasma PC Levels. PC levels in African American (AA) and Caucasian (C) human plasma. Bars show mean ±SE. *P<0.05 between ethnic group.]

Racial Differences in NADPH Oxidase Subunit Expression

To assess the potential for superoxide production, we measured protein expression of NADPH oxidase subunits and isoforms (Figure 2.10). We found higher
protein expression of p47phox subunit as well as the NOX2 and NOX4 isoforms in unstimulated African American HUVECs compared to those in Caucasian HUVECs (P<0.01).

Discussion

In this preliminary observational study we sought to examine whether the known \textit{in vivo} racial differences that exist in plasma oxidative stress and inflammatory markers also exist \textit{in vitro} in primary endothelial culture. A primary finding was that heightened oxidative stress and inflammation exist \textit{in vitro} in African Americans. Oxidative stress plays a critical role in the pathology and progression of CVD and HT, and recent evidence suggests that low grade systemic inflammation of the vasculature may also\textsuperscript{296, 297}. Here we have shown that an \textit{in vitro} racial difference exists in cultured endothelial cells in oxidative stress and inflammation levels. Recognizing that this difference exists in HUVECs could provide new directions for mechanistic and therapeutic research.

Over the past several decades, investigators have used the \textit{in vitro} cell culture...
model as a research tool, yet in vitro research on racial differences is scarce. In 1995, Frist et al. determined that African American HUVECs expressed higher levels of fibrinolytic proteins when compared to Caucasian HUVECs. But to the best of our knowledge, only one study has measured oxidative stress in African American and Caucasian HUVECs. In 2004, Kalinowski et al. reported upregulated eNOS and NADPH oxidase subunit expression for p67phox, p47phox, and p22phox in the African American HUVECs. Their data led them to conclude that the steady state NO/O$_2^-$ balance in African Americans may be kept closer to the characteristic redox state found in endothelial dysfunction. We also report that African American HUVECs have higher levels of oxidative stress than Caucasian HUVECs. Our study adds to previous findings by showing that African American HUVECs may be predisposed to inflammation more than Caucasian HUVECs are. To the best of our knowledge, this finding is novel.

Research has suggested that the observed racial differences in prevalence of CVD and HT could be attributed in part to a blunted NO-dependent vasodilation seen in vascular beds of African Americans when compared to Caucasians. It has been shown that vascular reactivity is altered in African Americans compared to Caucasians, but only a few studies have directly measured plasma NO end-products to assess potential racial differences and limited studies have done so in HUVECs of unreported origin. We found no difference between race in human plasma NO end-product levels, but this could be attributed to low sample size or could be because our human subjects were young adults. In African American HUVECs we found significantly higher level of NO end-products in cell supernatant suggesting a potentially augmented NOS production in response to high O$_2^-$.
In order to confirm the heightened NO production in African American HUVECs we measured protein expression of two of the NOS enzyme isoforms: eNOS and iNOS. In both, we found significantly higher expression in the African American HUVECs. The eNOS isoform is constitutively expressed in the endothelium, while activation of iNOS is usually cytokine induced. It has been reported that NADPH oxidase activity is associated with eNOS expression and is required for iNOS expression\textsuperscript{300, 301}. It also has been suggested that iNOS expression serves a protective role against proinflammatory cytokine conditions, with specific association to IL-6\textsuperscript{302, 303}. Taking this into consideration, we then measured IL-6 levels in the cell supernatant and confirmed a potential heightened proinflammatory state in African American compared to Caucasian HUVECs. This was further confirmed by upregulated IL-6 protein expression in the African American HUVECs. However, given the fact that IL-6 acts as both a pro-inflammatory and anti-inflammatory cytokine, further research should confirm this \textit{in vitro} racial difference in inflammation levels with mechanistic studies.

Heightened oxidative stress has been found to be associated with augmented antioxidant activity as scavenging increases to eliminate the rise in $O_2^-$ levels, and the SOD enzyme serves as the main protective antioxidant scavenging enzyme against the $O_2^-$ anion. In most tissues, SOD3 exists in very small amounts, but in the circulation it can represent up to half the total SOD activity\textsuperscript{304}. Therefore in controlled cell culture conditions, endothelial cell lysate would not have the same inherent percentages of SOD3. In HUVECs, we found African Americans had significantly lower levels of total SOD activity, while in human plasma we found African American adults to have significantly higher SOD activity when compared to Caucasians. This difference could be
attributed to the large contributions from SOD3 to the total SOD measured in plasma from the African American adults. To confirm this, we then used cell fractionation to compare isoform levels between racial groups and found that African American HUVECs tend to exhibit lower SOD1 activity and also lower SOD2 protein expression. Together, our data suggest that total SOD activity is lower in unstimulated African American HUVECs compared to Caucasian HUVECs.

Measuring TAC levels in conjunction with other biomarkers can provide a relative indicator of the antioxidant defense and therefore, in vitro studies that include TAC measures as part of their oxidative stress cascade could provide potential insight into cellular antioxidant activity. We measured TAC as the capacity of all antioxidants in the sample to prevent ABTS® oxidation and found that both African American HUVECs and African American humans had heightened antioxidant activity when compared to Caucasians. This again suggests higher in vitro and in vivo oxidative stress levels in African Americans.

NADPH oxidase-mediated radical production plays a vital role in the pathophysiology of endothelial dysfunction, inflammation, cardiovascular diseases, and angiogenesis. We found greater expression levels of p47phox, NOX2 and NOX4 in African American HUVECs than in Caucasian HUVECs. It has been reported that in primary endothelial cultures NOX2 and NOX4 serve as primary sources of NADPH-derived oxidative stress, with some reports suggesting that NOX4 is the major source. Our findings are the first report of a racial difference in NOX4 expression. Recently, in contrast to other NOX proteins, NOX4 has been suggested to directly produce large amounts of hydrogen peroxide. Taken together, we
hypothesize that the mechanisms mediating increased oxidative stress and inflammation in African American HUVECs may be very complex. This should be investigated further in order to better understand the \textit{in vivo} African American endothelial phenotype and associated mechanisms with the endothelial dysfunction that is typically observed, so that proper therapeutic interventions can be implemented.

\textit{Conclusion}

In conclusion, this preliminary observational data show that relative to Caucasian HUVECs, African American HUVECs exhibit enhanced oxidative stress and inflammation levels through increased expression of NADPH oxidase, IL-6, eNOS and iNOS, higher NO end-product production, and lower SOD activity. We have demonstrated an \textit{in vitro} racial difference in basal HUVECs much like that found \textit{in vivo}. Furthermore, it is known that racial differences exist in how humans respond to development and progression of disease. Therefore, these data suggest that the ethnicity of cell model may be important to consider with \textit{in vitro} clinical research depending on the disease or condition under study.

Preliminary Study #3:

Effects of TNF-\(\alpha\) Stimulation on NOS Expression in Racially-derived HUVECs

\textit{Abstract}

African American ethnicity is an independent risk factor for exaggerated oxidative stress, inflammation, hypertension, and cardiovascular diseases. It is well established that racial differences exist in how humans respond to the development, progression, and treatment of disease. It may also be true that the complex mechanisms mediating this \textit{in vivo} variation could also exist \textit{in vitro}. 
Previously we have shown that under basal conditions, African American HUVECs have higher oxidative stress and inflammation levels than Caucasian HUVECs. Research also has shown that TNF-α causes a disregulation in NOS mRNA and protein expression, but it is not known whether racial differences exist in this response. Separately, it has also previously been reported that HUVECs preconditioned with H$_2$O$_2$ (to mimic ischemia) have a blunted response to TNF-α stimulation$^{306}$. Therefore we aimed to determine if African American HUVECs had a blunted NOS protein expression response to TNF-α stimulation compared to Caucasian HUVECs, due to their higher basal oxidative stress and inflammatory levels.

Parallel HUVEC cultures were incubated under four separate conditions: Control cells received no stimulation, TNF-α (100 U/ml) for 4 hr, SOD (100 U/ml) for 24 hr, and SOD preincubation for 24 hr + TNF-α for 4 hr. Protein expression of eNOS and iNOS were measured by Western blotting.

We found that protein expression of both eNOS and iNOS was downregulated by TNF-α stimulation. This effect was seen in HUVECs from both races. Furthermore, we found that Caucasian HUVECs had a larger decrease in expression with TNF-α stimulation, the response was blunted in African American HUVECs.

These preliminary data suggest that the heightened basal levels of inflammation found in African American HUVECs may actually blunt the eNOS and iNOS responses to a TNF-α stimulation. Further mechanistic studies should be done to elucidate the mechanisms responsible for this disparity and whether the blunted response to TNF-α stimulation is due to the higher levels of IL-6 previously reported in un-stimulated African American HUVECs when compared to Caucasian HUVECs.
Background & Purpose

Previously, we have found that African American HUVECs actually have higher basal levels of oxidative stress and inflammation when compared to Caucasian HUVECs in a parallel culture study. Protein expression levels of IL-6 and NADPH oxidase subunits p47phox, NOX2, and NOX4 were significantly higher in African American HUVECs. Prior to this, only one other study by Kalinowski et al. has reported on basal oxidative stress differences between cells of different race.

Recently, Zahler, Kupatt & Becker\textsuperscript{306} showed that preconditioning endothelial cells with transient oxidative stress actually reduced inflammatory responses to TNF-\( \alpha \) stimulation. In their study, HUVECs were preconditioned with \( \text{H}_2\text{O}_2 \) in order to generate intracellular redox stress and mimic an ischemic condition. They found that the HUVECs pre-conditioned with the \( \text{H}_2\text{O}_2 \) had significantly blunted responses to the TNF-\( \alpha \) stimulation, but the mechanisms responsible were not resolved.

TNF-\( \alpha \) has been linked to endothelial dysfunction by depressing endothelium-dependent relaxation and NO production level. Mechanistic studies have found that TNF-\( \alpha \) alters NOS mRNA stability leading to significant downregulation of NO production\textsuperscript{307}. Therefore, we examined whether this inherent higher oxidative stress and inflammation at baseline in African American HUVECs would influence protein expression of eNOS and iNOS, and whether the response to TNF-\( \alpha \) stimulation would vary by race.

Methods

HUVECs were obtained from Lonza Inc.,(Walkersville, MD) from both African American (N=3) and Caucasian (N=3) donors. The HUVECs were cultured in parallel, in EGM medium supplemented with 2\% FBS and growth factors (Lonza) at 37 °C in a 95\%
air- 5% CO2 atmosphere, following methods by Lonza. All experiments were completed twice for confirmation.

*Experimental Procedures*

Human recombinant TNF-α was purchased from Sigma (Saint Louis, MO) and SOD was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Parallel HUVEC cultures were tested under 4 separate conditions: Control, TNF-α (100 U/ml) for 4 hr, SOD (100 U/ml) for 24 hr, and TNF-α + SOD (See the research design pictured in Figure 2.11 below).

1. Control HUVECs - No stimulation
2. 24hrs SOD (100U/mL)
3. 4hrs TNF (100U/mL)
4. 24hrs SOD (100U/mL) 4hrs TNF (100U/mL)

*Figure 2.11 : Research Design.*

100 U/ml of TNF-α was selected because it is a typical level used and falls approximately midway between the highest and lowest levels used to stimulate HUVECs in similar studies. Cell culture supernatant was obtained and immediately frozen at -80°C for NO assay. Protein concentration was measured using the Bradford method. For all procedures, African American and Caucasian HUVECs at passage 4 were treated identically. For assay, experimental samples were tested in duplicate and control samples of culture media were tested along with the cell samples in order to eliminate potential interference from culture media in measurements. Absorbance was read using a SpectraMax Microplate Reader (Molecular Devices, Sunnyvale, CA).
**Western Blot for eNOS & iNOS Protein Expression**

HUVECs were washed twice in ice-cold Hanks buffered saline solution and lysed in RIPA-Pi. Phenylmethylsulfonyl fluoride protease inhibitor was also added to the RIPA-Pi to eliminate interference. At confluence, cells were collected, centrifuged at 16,000 g for 10 min at 4 °C. Quantification of protein content was measured by Bradford assay. 20 µg of protein was separated by electrophoresis through 10% SDS-polyacrylamide gel. Proteins were then transferred to nitrocellulose filter membranes. Membranes were blocked with non-fat dry milk in Tris-buffered saline and incubated overnight with primary antibodies at 4 °C. Subsequently, the membranes were washed and then incubated with secondary antibody conjugated with horseradish peroxidase. Immunoreactive proteins were detected by chemiluminescence with Thermo Scientific SuperSignal substrate systems (Pierce Biotechnology). Anti-eNOS and Anti-iNOS (BDTransduction Labs, CA). Actin antibody (Santa Cruz Biotechnology, CA) was used as the internal control. Band densitometry analysis was performed using National Institutes of Health ImageJ software.

**Results & Conclusion**

These preliminary data show a significant response difference exists between African American and Caucasian HUVECs to stimuli. Stimulation with TNF-α caused a significant downregulation in expression of both eNOS and iNOS in both cultured African American and Caucasian HUVECs. This response was blunted in African American cells (45% compared to 71% for eNOS, and 64% compared to 66% for iNOS). Table 2.4 and 2.5 below show the expression changes with stimulation where basal level was set to 0% for comparisons. While in this preliminary study there were no significant
differences in the changes with stimulation between the groups, it seems that with further studies that include higher number of HUVEC lines, differences may exist.

Table 2.4. Relative eNOS Expression Changes with Stimulation

<table>
<thead>
<tr>
<th>Condition</th>
<th>African American</th>
<th>Caucasian</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>SOD</td>
<td>-13%</td>
<td>-59%</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>-45%</td>
<td>-71%</td>
</tr>
<tr>
<td>SOD/TNF-(\alpha)</td>
<td>-59%</td>
<td>-50%</td>
</tr>
</tbody>
</table>

Table 2.5. Relative iNOS Expression Changes with Stimulation

<table>
<thead>
<tr>
<th>Condition</th>
<th>African American</th>
<th>Caucasian</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>SOD</td>
<td>-25%</td>
<td>-5%</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>-64%</td>
<td>-66%</td>
</tr>
<tr>
<td>SOD/TNF-(\alpha)</td>
<td>-69%</td>
<td>-34%</td>
</tr>
</tbody>
</table>

Similar to Zahler’s findings, the pre-existing enhanced oxidative stress in African American cells may actually be blunting the response to TNF-\(\alpha\). We also found that pre-incubation with SOD caused significant downregulation in expression of eNOS in Caucasian HUVECs and in expression of iNOS in African American HUVECs. This variation may be related to pre-existing differences in total SOD activity between African American and Caucasian HUVECs. We have previously found that SOD activity is significantly higher in Caucasian cells. Another possibility is that mechanistic differences may also exist in NF\(_k\)B activation between the cells of different race, which needs to be further examined. Finally, it appears that pre-incubation with SOD may blunt the
response to TNF-α in HUVECs, which should also be examined further in future studies.

Again, this may be attributed to different basal SOD activity between race.

In conclusion, we have shown that the race of the cell culture model may be important to consider. We have found that African American HUVECs have enhanced oxidative stress and inflammation under basal conditions, and thus when stimulated with the inflammatory cytokine TNF-α, the response was blunted. Mechanistic studies need to be conducted in order to elucidate the proper signaling pathway that has been affected.
CHAPTER 3
RESEARCH DESIGN

The dissertation study research design is pictured in Figure 3.1 below. HUVECs from both African American and Caucasian donors were cultured in parallel. After subculture, one culture dish remained as the static control and did not receive the LSS intervention. The other two culture dishes received the LSS intervention at two magnitudes (5 and 20 dyne/cm$^2$). After the LSS intervention, measures were collected.

**HUVEC Subculture**

**Methods**

**Cell Culture**

HUVECs were purchased separately as primary cultures from African American and Caucasian donors from Lonza (Walkersville, Maryland). The cells were preserved in liquid nitrogen until subculture, and all experiments were conducted with HUVECs at
passages four or five. Briefly, HUVECs are cultured in parallel in EGM-2 medium supplemented with 2% FBS and growth factors. For NO measurement dishes, HUVECs were grown in phenol-red free EGM-2 medium. It has been reported that the addition of phenol red to medium could potentially interfere with nitrate and nitrite measurements, so for NO measurement experiments HUVECs were harvested in the phenol-red free EGM-2 \(^2\). Furthermore, it has been shown that measurement of NO is most reliable when levels are quantified from cells that have been cultured in low serum phenol-red free medium \(^1\). All cells were maintained at 37 °C, 97% humidity, and 5% CO\(_2\) in 100 mm tissue culture dishes. Cultures were examined daily for confluency, medium color, and to observe the morphology, and LSS application was done when cells reached log phase and were at 90-100% confluence.

It is recognized that EC culture methodologies have many uncontrolled confounding variables (e.g., cell density, serum lot, place in incubator, etc) that are critical to success of the project. To minimize these confounding variables a paired longitudinal experimental design was followed. Parallel cultures were processed identically for each race, two sets of cells were exposed to LSS, one set per each level of LSS, and a third set remained as the static control. After this, the resulting protein expression levels and oxidative stress biomarker measures were compared to each other (e.g. paired t-test). Three cell lines from each ethnic group were tested, and all experiments were completed twice for further confirmation.

**In Vitro Flow: Laminar Shear Stress (LSS)**

As previously explained, unidirectional LSS was used as a model for aerobic exercise-induced vascular shear stress. Confluent HUVECs grown in parallel in separate
100-mm tissue culture dishes were exposed for a period of 24 hr to LSS at two levels: low physiological flow, 5 dyne/cm$^2$, and moderate exercise flow, 20 dyne/cm$^2$, using a rotating Teflon cone (0.5 deg cone angle) according to the methods of Jo et al. Shear experiments were conducted in a humidified incubator with 5% CO$_2$ at 37 °C.

**Protein Expression: Western Blotting**

Immediately following LSS application, both the static and LSS culture dishes were harvested for protein analysis. Radio-Immunoprecipitation Assay Buffer with Roche protease inhibitor (RIPA-Pi) was used to enable cell lysis and stabilize the protein solution while protein concentration in the cell lysate is measured. Phenylmethylsulfonyl fluoride protease inhibitor was also added to the protease inhibitor-buffer cocktail to eliminate interference. Briefly, cells were washed twice with cold Hanks Buffered Saline Solution. A 400 ul vol of the RIPA-Pi cocktail was added and rubber scraper used to remove adherent cells. Cells were collected, centrifuged at 16,000 g for 10 min at 4 °C. Immediately, a Bradford Protein Assay is performed to measure the protein concentration. From there, a 5X SDS dilution is made with molecular grade H$_2$O. The protein-SDS solution is then boiled for 3 min at 95 °C and the sample immediately frozen to -80 °C for storage until Western Blot.

Aliquots of cell lysate at 15 µg of protein each were separated by SDS-PAGE on 10% gels and electrotransferred to nitrocellulose filters, and then the membrane was incubated overnight with primary antibodies at 4 °C. After washing and incubation with secondary antibody conjugated with horseradish peroxidase, total protein was detected by chemiluminescence. Band densitometry analyses were completed using ImageJ software, National Institutes of Health, to direct comparisons between experimental sets. The
primary antibodies included were Anti-NOX4 (Pierce Biotechnology), Anti-gp91phox (BD Bioscience), Anti-p47phox (BD Biosciences), Anti-SOD2 (AbFrontier), Anti-CAT (Calbiochem), and Anti-eNOS (Transduction Labs). Anti-Actin antibody (Santa Cruz Biotechnology, CA) was used for internal control.

**Nitric Oxide (NOx)**

NO levels in HUVEC culture medium supernatant were measured using a modified Griess assay. Following LSS application, cell supernatant was collected from both static and LSS HUVECs. During 24 hr of LSS some evaporation of media occurred, so at time of harvest the volume of remaining supernatant was measured and adjusted to the starting volume of 10 mL using fresh phenol-red EGM-2 media. Solution was completely mixed by pipette, and aliquots were frozen at -80 °C until assay. On the day of assay, all samples were ultrafiltrated through a 10,000 MWCO filter (Sigma-Aldrich) by micro-centrifuge at 14,000 g for 30 min at 4 °C. Briefly, the assay involves an enzymatic conversion of nitrate to nitrite by Nitrate Reductase (Aspergillus species) followed by measurement of nitrite through formation of a magenta-colored azo dye as a product of a Griess reaction. The Griess reagents used are N-(1-naphthyl)ethylenediamine in 2 M hydrochloric acid and sulfanilamide in 2 M hydrochloric acid. Absorbance was read at 540 nm using a SpectraMax Microplate Reader (Molecular Devices, Sunnyvale, CA). All reagents are from Assay Designs (Ann Arbor, MI). Inter-assay and intra-assay CVs are 7.6% and 10.6% respectively.

**Superoxide Dismutase (SOD)**

Immediately following LSS application, cell lysate was collected through cellular fractionation in one of the 100-mm tissue culture dishes. Cells were washed once with
cold phosphate buffer solution, and then collected in 2mL cold HEPES buffer using a
rubber scraper. The cell-HEPES solution was then centrifuged at 600 g for 10 min at 4
°C. Cell pellet was resuspended in 500 ul cold HEPES buffer and then transferred to a
Teflon glass coupling potter, homogenized at 1600 rpm for 30 strokes while on ice, and
then the homogenate was immediately centrifuged at 1500 g for 5 min at 4 °C. The
supernatant was then frozen in aliquots at -80 °C until assay for both total SOD and for
TAC.

Total SOD activity was determined using a commercially available kit (Cayman
Chemical, Ann Arbor, MI). SOD activity was measured by utilizing a tetrazolium salt
radical detector solution, diluted in assay buffer (50 mM Tris-HCl, pH 8.0, containing 0.1
mM diethylenetriamine-pentaacetic acid and 0.1 mM hypoxanthine), to detect superoxide
radicals generated by hypoxanthine and xanthine oxidase. One unit of SOD activity is
defined as the amount of enzyme needed to exhibit a 50% dismutation of the superoxide
radical. Absorbance was read at 450 nm using a SpectraMax Microplate Reader
(Molecular Devices, Sunnyvale, CA). The kit detection limit is 0.025 U/ml. Inter-assay
and intra-assay coefficients of variation are 5.9% and 12.4 % respectively.

Total Antioxidant Capacity (TAC)

Measurement of TAC activity in cell lysate is based on the ability of antioxidants
to inhibit the oxidation of ABTS® to ABTS®+ by metmyoglobin. The capacity of
antioxidants to prevent ABTS® oxidation is compared with that of a water-soluble
vitamin E analogue, Trolox. Absorbance was read at 750 nm using a SpectraMax
Microplate Reader (Molecular Devices, Sunnyvale, CA), and TAC activity quantified as
millimolar Trolox equivalents. TAC activity was determined using a commercially
available kit (Cayman Chemical, Ann Arbor, MI), where the detection limit is 0.044 mM. Inter-assay and intra-assay coefficients of variation are 6.7% and 9.2%, respectively.

Statistical Analysis

Data analysis began with descriptive statistics including means, medians, standard deviations, and standard error for static controls and sheared cells for each ethnic group. Within group analysis was done using paired t-tests comparing before and after LSS intervention values between each LSS level. Between racial group analysis was done using independent t-tests for before LSS, after 5 dyne/cm², and after 20 dyne/cm² LSS intervention. Following this, change values from before LSS and between both LSS levels were calculated for each variable, with t-tests done on each change value within and between groups.

Densitometric analyses was completed using the ImageJ software (NIH Freeware) to quantify protein expression levels. All protein measurements were normalized to the Actin internal control expression, and these normalized values were used for all statistical analyses.

Finally, a two way ANOVA analyses was computed to examine for any race by shear stress interaction. All statistical analysis were performed using Microsoft Excel and SPSS version 18.0 (SPSS Inc., Chicago, IL). Data are expressed as mean ±SE and significance set at P<0.05.
CHAPTER 4

RESULTS

NADPH Oxidase Protein Expression and Activity Results

*p47phox* Protein Expression by Race with LSS

Figure 4.1 shows the *p47phox* protein expression levels by race with LSS. There was a significant race effect (p=0.04) under static conditions which did not exist after application of LSS at either magnitude. African American HUVECs had higher *p47phox* protein expression. The only significant LSS effect was in African American HUVECs where *p47phox* protein expression was lower with 20 dyne/cm\(^2\) LSS (p=0.05).

![Figure 4.1](image)

**Figure 4.1.** *p47phox* Protein Expression Levels by Race with LSS. **A.** *p47phox* protein expression levels in African American (solid bars) and Caucasian (open bars) under static conditions and with LSS interventions. **B.** Representative western blot and β-actin control blot for African American (AA) and Caucasian (C) HUVECs. ImageJ densitometric analysis of bands expressed in relation to β-actin. Bars show mean ±SE. *P<0.05 from static. #P<0.05 from 5 dyne/cm\(^2\). †P<0.05 between ethnic group.
Table 4.1 reports the p47phox protein expression differences with LSS by race. There were no significant differences in changes with LSS under any condition.

Table 4.1. Differences in p47phox Protein Expression by Race with LSS

<table>
<thead>
<tr>
<th>Race</th>
<th>Static–5 dyne/cm²</th>
<th>5-20 dyne/cm²</th>
<th>Static-20 dyne/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>African American</td>
<td>0.032 ± 0.1</td>
<td>-0.263 ± 0.1</td>
<td>-0.231 ± 0.1</td>
</tr>
<tr>
<td>Caucasian</td>
<td>0.209 ± 0.2</td>
<td>-0.127 ± 0.2</td>
<td>0.082 ± 0.2</td>
</tr>
</tbody>
</table>

Data are reported in arbitrary units (band density/β-actin density), and expressed as mean ± standard error. Band density measured by ImageJ analysis software. Within groups, * indicates significant difference from static-5 dyne/cm² condition, # indicates significant difference from 5-20 dyne/cm². † indicates significant difference between racial groups. (p<0.05)

NOX2 Protein Expression by Race with LSS

Figure 4.3 shows NOX2 protein expression levels by race with LSS. Figure 4.4 shows the NOX2 protein expression differences with LSS by race. There was a significant race effect on NOX 2 protein expression under static conditions (p=0.02). African American HUVECs had higher NOX2 protein expression compared to Caucasian. There were no significant differences in response with LSS.

On the following page, Table 4.2 reports the NOX2 protein expression differences with LSS by race. There were no significant differences with LSS under any condition.
Table 4.2. Differences in NOX2 Protein Expression by Race with LSS

<table>
<thead>
<tr>
<th>Race</th>
<th>Static–5 dyne/cm²</th>
<th>5-20 dyne/cm²</th>
<th>Static-20 dyne/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>African American</td>
<td>-0.012 ± 0.1</td>
<td>-0.166 ± 0.2</td>
<td>-0.153 ± 0.1</td>
</tr>
<tr>
<td>Caucasian</td>
<td>-0.067 ± 0.1</td>
<td>0.126 ± 0.2</td>
<td>-0.059 ± 0.2</td>
</tr>
</tbody>
</table>

Data are reported in arbitrary units (band density/β-actin density), and expressed as mean ± standard error. Band density measured by ImageJ analysis software. Within groups, * indicates significant difference from static-5 dyne/cm² condition, # indicates significant difference from 5-20 dyne/cm². † indicates significant difference between racial groups. (p<0.05)
Figure 4.3 shows the NOX4 protein expression by race with LSS. There was a significant race effect under the static and 5 dyne/cm² conditions (p=0.001 and p=0.04, respectively). African American HUVECs had higher NOX4 expression under static conditions, while it appears that Caucasian HUVECs had higher NOX4 expression after LSS at 5 dyne/cm².

The difference in NOX4 protein expression from static condition to 5 dyne/cm² was significant between race (p=0.045). Protein expression in African American HUVECs decreased while in Caucasian HUVECs it increased slightly. Also, the
difference in NOX4 protein expression between 5 dyne/cm² and 20 dyne/cm² conditions was significant between race (p=0.04). African American HUVECs had a larger decrease with LSS at 20 dyne/cm².

Finally, the difference in NOX4 protein expression between the static to 5 dyne/cm² and the static to 20 dyne/cm² conditions was significant in both African American and Caucasian HUVECs (p=0.03 and p=0.05, respectively).

Table 4.3. Difference in NOX4 Protein Expression by Race with LSS

<table>
<thead>
<tr>
<th>Race</th>
<th>Static–5 dyne/cm²</th>
<th>5-20 dyne/cm²</th>
<th>Static-20 dyne/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>African American</td>
<td>-0.202 ± 0.1</td>
<td>-0.218 ± 0.1</td>
<td>-0.420 ± 0.1 *</td>
</tr>
<tr>
<td>Caucasian</td>
<td>0.092 ± 0.1</td>
<td>-0.284 ± 0.1</td>
<td>-0.191 ± 0.1 *</td>
</tr>
</tbody>
</table>

Data are reported in arbitrary units (band density/β-actin density), and expressed as mean ± standard error. Band density measured by ImageJ analysis software. Within groups, * indicates significant difference from static-5 dyne/cm² condition, # indicates significant difference from 5-20 dyne/cm². † indicates significant difference between racial groups. (p<0.05)

eNOS Protein Expression and NO End-Product Production by Race with LSS

Figure 4.4 shows the eNOS protein expression and NO end-product levels by race with LSS. There was no significant LSS or race interaction effect (p=0.45).

eNOS protein expression was significantly higher (3-fold) in both African American and Caucasian HUVECs with LSS at 20 dyne/cm² (p=0.00 and p=0.002, respectively). Likewise, eNOS protein expression was significantly higher (2-fold) in both African American and Caucasian HUVECs with LSS at 5 dyne/cm² (p=0.001 and
While there was a significant race effect under static conditions (p=0.04), after LSS there was no difference between race. There was also a significantly higher eNOS expression in both African American and Caucasian HUVECs with LSS at 20 dyne/cm² compared to LSS at 5 dyne/cm² (p=0.0002 and p=0.001, respectively).

Figure 4.4. eNOS Protein Expression and NO End-Product Production by Race with LSS. 
A. eNOS protein expression levels in African American (solid bars) and Caucasian (open bars) under static conditions and with LSS interventions. B. Representative western blot and β-actin control blot for African American (AA) and Caucasian (C) HUVECs. ImageJ densitometric analysis of bands expressed in relation to β-actin. C. Total NO end-product production with LSS application in African American (solid bars) and Caucasian (open bars) HUVEC cell culture supernatant. Bars show mean ±SE. *P<0.05 from static. #P<0.05 from 5 dyne/cm². †P<0.05 between ethnic group.
NO production was significantly higher in both African American and Caucasian HUVECs with LSS at 20 dyne/cm$^2$ compared to static conditions (p=0.00 and p=0.02, respectively). Likewise, NO production was significantly higher in both African American and Caucasian HUVECs with LSS at 5 dyne/cm$^2$ (p=0.001 and p=0.01 respectively). Interestingly, NO production was only significantly higher in African American HUVECs with LSS at 20 dyne/cm$^2$ compared to the 5 dyne/cm$^2$ condition (p=0.0003). There were no differences between races in any of the conditions.

Table 4.4. Nitric Oxide Levels by Race with LSS

<table>
<thead>
<tr>
<th>Race</th>
<th>Static</th>
<th>5 dyne/cm$^2$</th>
<th>20 dyne/cm$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>African American</td>
<td>9.36 ± 0.7</td>
<td>11.99 ± 0.4 *</td>
<td>20.16 ± 1.5 * #</td>
</tr>
<tr>
<td>Caucasian</td>
<td>7.15 ± 1.4</td>
<td>12.79 ± 0.5 *</td>
<td>17.17 ± 3.2 *</td>
</tr>
</tbody>
</table>

Data are reported in nmol/mg, and expressed as mean ± standard error. Within groups, * indicates significant difference from static condition, # indicates significant difference from 5 dyne/cm$^2$. † indicates significant difference between racial groups. (p<0.05)

The difference in eNOS protein expression from static condition to 20 dyne/cm$^2$ was significantly larger than the eNOS expression difference from static condition to 5 dyne/cm$^2$ in both African American and Caucasian HUVECs (p=0.0003 and p=0.01, respectively, Table 4.5). Also, the difference in eNOS protein expression from LSS at 5 dyne/cm$^2$ to LSS at 20 dyne/cm$^2$ was significantly larger than the eNOS expression difference from static condition to 5 dyne/cm$^2$ in both African American and Caucasian HUVECs (p=0.0002 and p=0.001, respectively). There was no significance in the differences between race.
Table 4.5. Differences in eNOS Protein Expression by Race with LSS

<table>
<thead>
<tr>
<th>Race</th>
<th>Static–5 dyne/cm²</th>
<th>5-20 dyne/cm²</th>
<th>Static-20 dyne/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>African American</td>
<td>0.638 ± 0.1</td>
<td>1.457 ± 0.2 *</td>
<td>2.096 ± 0.2 * #</td>
</tr>
<tr>
<td>Caucasian</td>
<td>0.757 ± 0.2</td>
<td>1.317 ± 0.2 *</td>
<td>2.074 ± 0.4 * #</td>
</tr>
</tbody>
</table>

Data are reported in arbitrary units (band density/β-actin density), and expressed as mean ± standard error. Band density measured by ImageJ analysis software. Within groups, * indicates significant difference from static-5 dyne/cm² condition, # indicates significant difference from 5-20 dyne/cm². † indicates significant difference between racial groups. (p<0.05)

In African American HUVECs, the difference in NO production from the static condition to the 5 dyne/cm², as well as from the 5 dyne/cm² to 20 dyne/cm² conditions were both significantly smaller than the difference in NO production from static condition to 20 dyne/cm² condition (p=0.001 and p=0.01, respectively, Table 4.6). Again, there was no significance in the differences between race.

Table 4.6. Differences in Nitric Oxide Levels by Race with LSS

<table>
<thead>
<tr>
<th>Race</th>
<th>Static–5 dyne/cm²</th>
<th>5-20 dyne/cm²</th>
<th>Static-20 dyne/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>African American</td>
<td>2.62 ± 0.6</td>
<td>8.18 ± 1.4 *</td>
<td>10.80 ± 1.6 * #</td>
</tr>
<tr>
<td>Caucasian</td>
<td>5.64 ± 1.5</td>
<td>4.37 ± 3.3</td>
<td>10.01 ± 3.5 #</td>
</tr>
</tbody>
</table>

Data are reported in nmol/mg, and expressed as mean ± standard error. Within groups, * indicates significant difference from static-5 dyne/cm² condition, # indicates significant difference from 5-20 dyne/cm². † indicates significant difference between racial groups. (p<0.05)
Antioxidant Production Results

*Total Antioxidant Capacity by Race with LSS*

Figure 4.5 shows the total antioxidant capacity measured in cell lysates and compared by race with LSS. There was no significant LSS or race interaction effect (p=0.23).

There was only a significant difference between the HUVECs from different races under the 5 dyne/cm² condition with the African American HUVECs having higher TAC levels (p=0.02, Figure 4.5). Compared to static conditions, the African American HUVECs had a significant higher TAC with LSS at both 5 dyne/cm² and 20 dyne/cm² (p=0.01 and p=0.03 respectively). Compared to the TAC levels with 5 dyne/cm² LSS, only the Caucasian HUVECs had a significantly higher TAC levels with 20 dyne/cm² LSS (p=0.04).

![Figure 4.5. Total Antioxidant Capacity by Race with LSS. Total Antioxidant Capacity (TAC) with LSS application in African American (solid bars) and Caucasian (open bars) HUVEC cell lysate. Bars show mean ±SE. *P<0.05 from static. #P<0.05 from 5 dyne/cm². †P<0.05 between ethnic group.](image-url)
Table 4.7. Total Antioxidant Capacity by Race with LSS

<table>
<thead>
<tr>
<th>Race</th>
<th>Static</th>
<th>5 dyne/cm$^2$</th>
<th>20 dyne/cm$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>African American</td>
<td>1.15 ± 0.1</td>
<td>1.90 ± 0.2 *</td>
<td>2.04 ± 0.3 *</td>
</tr>
<tr>
<td>Caucasian</td>
<td>1.24 ± 0.2</td>
<td>1.36 ± 0.1 †</td>
<td>1.65 ± 0.1 #</td>
</tr>
</tbody>
</table>

Data are reported in uM/mg, and expressed as mean ± standard error. Within groups, * indicates significant difference from static condition, † indicates significant difference from 5 dyne/cm$^2$. # indicates significant difference between racial groups. (p<0.05)

The only significance in the differences with LSS in TAC activity was in the African American HUVECs. Compared to the difference from 5 dyne/cm$^2$ to 20dyne/cm$^2$ the difference from static to 20 dyne/cm$^2$ was significantly larger (p=0.02, Table 4.8).

Table 4.8. Differences in Total Antioxidant Capacity by Race with LSS

<table>
<thead>
<tr>
<th>Race</th>
<th>Static–5 dyne/cm$^2$</th>
<th>5-20 dyne/cm$^2$</th>
<th>Static-20 dyne/cm$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>African American</td>
<td>0.75 ± 0.3</td>
<td>0.14 ± 0.2</td>
<td>0.88 ± 0.4 #</td>
</tr>
<tr>
<td>Caucasian</td>
<td>0.12 ± 0.2</td>
<td>0.29 ± 0.2</td>
<td>0.41 ± 0.2</td>
</tr>
</tbody>
</table>

Data are reported in uM/mg, and expressed as mean ± standard error. Within groups, * indicates significant difference from static-5 dyne/cm$^2$ condition, † indicates significant difference from 5-20 dyne/cm$^2$. # indicates significant difference between racial groups. (p<0.05)

*SO*2 Protein Expression and Total SOD Activity by Race with LSS

Figure 4.6 shows the SOD2 protein expression levels and total SOD activity by race with LSS. There was a significant LSS and race interaction effect (p=0.018).
There was a significant difference between HUVECs from both races under static conditions where the Caucasian HUVECs had higher SOD2 protein expression (p=0.01, Figure 4.6). The application of LSS at both magnitudes abolished this racial difference. In African American HUVECs, SOD2 protein expression with LSS at 20dyne/cm$^2$ was significantly higher (1.5-fold) than SOD2 expression under static conditions (p=0.04).

**Figure 4.6. SOD2 Protein Expression and Total SOD Activity by Race with LSS.**

A. SOD2 protein expression levels in African American (solid bars) and Caucasian (open bars) under static conditions and with LSS interventions. B. Representative western blot and β-actin control blot for African American (AA) and Caucasian (C) HUVECs. ImageJ densitometric analysis of bands expressed in relation to β-actin. C. Total SOD Activity levels with LSS application in African American (solid bars) and Caucasian (open bars) HUVEC cell lysate. Bars show mean ±SE. *P<0.05 from static. #P<0.05 from 5 dyne/cm$^2$. †P<0.05 between ethnic group.
Similarly, SOD2 protein expression in African American HUVECs with LSS at 5
dyne/cm² was significantly higher (2-fold) than SOD2 expression under static conditions
(p=0.002). There was no difference in Caucasian HUVECs with LSS.

Similar to SOD2 protein expression, there was a significant race effect under
static conditions, with the Caucasian HUVECs having higher total SOD activity
(p=0.004, Figure 4.6.C). This difference did not exist after application of LSS.
Furthermore, in only the African American HUVECs, total SOD activity was higher with
both LSS at 20 dyne/cm² and LSS at 5 dyne/cm² (p=003, p=0.002, respectively, Table
4.9) compared to SOD activity under static conditions. There was no difference in SOD
activity in the Caucasian HUVECs in the levels after LSS application when compared to
static conditions.

Table 4.9. Total Superoxide Dismutase Activity by Race with LSS

<table>
<thead>
<tr>
<th>Race</th>
<th>Static</th>
<th>5 dyne/cm²</th>
<th>20 dyne/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>African American</td>
<td>33.11 ± 4.7</td>
<td>99.76 ± 9.8 *</td>
<td>60.32 ± 10.4 * #</td>
</tr>
<tr>
<td>Caucasian</td>
<td>65.26 ± 7.8 †</td>
<td>81.79 ± 9.0</td>
<td>69.18 ± 6.3</td>
</tr>
</tbody>
</table>

Data are reported in U/mg, and expressed as mean ± standard error. Within groups, * indicates significant difference from static condition, # indicates significant difference from 5 dyne/cm². † indicates significant difference between racial groups. (p<0.05)

There was a significant race effect on the differences in SOD2 protein expression
from the static condition to 5 dyne/cm² condition (p=0.01, Table 4.10). African American
HUVECs had a larger increase. The decrease in SOD2 protein expression from 5 dyne to
20 dyne/cm² was significantly different than the increase from the static condition to 5
dyne/cm\(^2\) condition in HUVECs from both African Americans and Caucasians (p=0.000 and p=0.004 respectively). Also, only African American HUVECs showed a significant difference in changes between static to 5 dyne/cm\(^2\) and 5dyne to 20 dyne/cm\(^2\) (p=0.00).

Table 4.10. Differences in SOD2 Protein Expression by Race with LSS

<table>
<thead>
<tr>
<th>Race</th>
<th>Static–5 dyne/cm(^2)</th>
<th>5-20 dyne/cm(^2)</th>
<th>Static-20 dyne/cm(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>African American</td>
<td>1.525 ± 0.3</td>
<td>-0.808 ± 0.1 *</td>
<td>0.717 ± 0.3 * #</td>
</tr>
<tr>
<td>Caucasian</td>
<td>0.571 ± 0.3 †</td>
<td>-0.322 ± 0.2 *</td>
<td>0.248 ± 0.4</td>
</tr>
</tbody>
</table>

Data are reported in arbitrary units (band density/β-actin density), and expressed as mean ± standard error. Band density measured by ImageJ analysis software. Within groups, * indicates significant difference from static-5 dyne/cm\(^2\) condition, # indicates significant difference from 5-20 dyne/cm\(^2\). † indicates significant difference between racial groups. (p<0.05)

In SOD activity in cell lysate, there was a significant effect of race on both the difference from 5 dyne to 20 dyne/cm\(^2\) conditions (p=0.004) and on the difference from static to 5dyne/cm\(^2\) conditions (p=0.03, Table 4.11). The difference in SOD activity in African American HUVECs was larger for both intervals. The decrease in SOD activity from 5 dyne to 20 dyne/cm\(^2\) LSS levels was significant in both African American and Caucasian HUVECs when compared to the change from static conditions to 5 dyne/cm\(^2\) (p=0.01 and p=0.02 respectively). Only African American HUVECs had a significance in the differences from static to 20 dyne/cm\(^2\) compared to the difference from either the static condition to 5 dyne/cm\(^2\) or from 5 dyne to 20 dyne/cm\(^2\) condition (p=0.001).
Table 4.11. Differences in Total Superoxide Dismutase Activity by Race with LSS

<table>
<thead>
<tr>
<th>Race</th>
<th>Static–5 dyne/cm²</th>
<th>5-20 dyne/cm²</th>
<th>Static-20 dyne/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>African American</td>
<td>66.62 ± 12.9</td>
<td>-47.61 ± 7.6 *</td>
<td>27.20 ± 11.8 #</td>
</tr>
<tr>
<td>Caucasian</td>
<td>23.31 ± 7.7</td>
<td>-13.55 ± 6.7 * †</td>
<td>3.92 ± 7.9 *</td>
</tr>
</tbody>
</table>

Data are reported in U/mg, and expressed as mean ± standard error. Within groups, * indicates significant difference from static-5 dyne/cm² condition, # indicates significant difference from 5-20 dyne/cm². † indicates significant difference between racial groups. (p<0.05)

Catalase Protein Expression by Race with LSS

Figure 4.7 shows the Catalase protein expression by race with LSS. Catalase protein expression was 2.5-fold higher with LSS at 5 dyne/cm² in both African American and Caucasian HUVECs compared to static conditions (p=0.004 and p=0.02, respectively). While HUVECs from both races had lower Catalase expression at 20 dyne/cm² condition compared to 5 dyne/cm² condition, only the African American HUVECs had significantly lower protein expression (p=0.01).

Table 4.12 shows the differences in Catalase protein expression by race with LSS. Compared to the difference from static condition to 5 dyne/cm² condition, HUVECs from both races had a significant decrease from 5 dyne to 20 dyne/cm² (p=0.002 African American and p=0.01 Caucasian). Also, the difference from static conditions to 20 dyne/cm² LSS was significant in both African American and Caucasian HUVECs compared to the difference in expression from 5 dyne/cm² to 20 dyne/cm². However, only the African American HUVECs had a significant difference between the static conditions to 20 dyne/cm² compared to static conditions to 5 dyne/cm² (p=0.01).
Table 4.12. Differences in Catalase Protein Expression by Race with LSS

<table>
<thead>
<tr>
<th>Race</th>
<th>Static–5 dyne/cm²</th>
<th>5-20 dyne/cm²</th>
<th>Static-20 dyne/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>African American</td>
<td>1.054 ± 0.2</td>
<td>-0.616 ± 0.2 *</td>
<td>0.522 ± 0.3 * #</td>
</tr>
<tr>
<td>Caucasian</td>
<td>0.974 ± 0.4</td>
<td>-0.451 ± 0.2 *</td>
<td>0.523 ± 0.4 #</td>
</tr>
</tbody>
</table>

Data are reported in arbitrary units (band density/β-actin density), and expressed as mean ± standard error. Band density measured by ImageJ analysis software. Within groups, * indicates significant difference from static-5 dyne/cm² condition, # indicates significant difference from 5-20 dyne/cm². † indicates significant difference between racial groups. (p<0.05)
CHAPTER 5
DISCUSSION

Introduction

With respect to blood flow in human arteries, it is known that during aerobic exercise sessions, cardiac output increases as much as four to six-fold. Heart rate, coronary blood flow, and blood flow velocity increase in order to supply the extra demand for nutrients and oxygen to the working muscle beds. The purpose of the present study was to take this phenomenon to the molecular level, using an in vitro model of cell culture and LSS, and examine how endothelial cells of different races responded to an increase in flow. The central hypothesis was based on the fact that increased blood flow from aerobic exercise, modeled by in vitro shear stress, effectively modulates endothelial function toward a net anti-atherogenic and away from a net pro-atherogenic endothelial dysfunction phenotype. A pro-atherogenic phenotype is characterized by increased oxidative stress, reduced antioxidant activity, reduced endothelium-dependent vasodilation, and increased adhesion molecules and inflammation\textsuperscript{311,312}. In the present study, in vitro endothelial function was measured by NO production, antioxidant levels and oxidative stress production through NADPH oxidase subunit protein expressions. It is known that, in vivo, shear stress on endothelial cells increases NO production, modulates vascular tone, and decreases oxidative stress. An aim of the study was to examine whether a racial difference existed in HUVECs from different races in the adaptations to an in vitro application of LSS. Within the scope of this study, mechanisms
underlying the observed response were not studied, only whether racial differences existed. Based on the results of this study, future mechanistic studies can be completed to further elucidate the racial differences found.

Supporting Evidence

The effects of both acute and chronic shear stress on endothelial cells *in vitro* have been extensively studied. Acute shear stress refers to sudden exposure to shear conditions that the endothelial cells are not accustomed to and are usually measured in seconds to hours. Chronic shear stress refers to long-term exposure to shear conditions by endothelial cells in culture for several days or weeks. However, many studies investigate the responses in cells exposed to shear stress for between 1 to 48 hours, which may be considered long-term shear stress. *In vivo*, acute or long-term shear stress effects can only be observed when new flow is initiated (such as those through newly formed vessels) or when repair to an injured vessel walls occurs. Therefore, it can also be hypothesized that these acute and long-term effects are mimicked *in vivo* in vascular beds of unconditioned individuals when a new aerobic exercise training program is initiated, and thus highlights the importance of the present study. If it can be shown that LSS modulates the HUVEC oxidative stress balance towards an anti-atherogenic and away from an endothelial dysfunction phenotype, then the implications for improvement in vascular beds of untrained individuals will justify exercise prescriptions in these racial populations.

Chronic shear stress *in vivo* can refer to the repeated exposure to increased blood flow that accompanies aerobic exercise training. Chronic adaptations occur in the vasculature with aerobic exercise training which alter the blood flow and dramatically affect the pressure gradients experienced on the endothelial cell. Morphological changes
along the endothelium and smooth muscle layers of vessels alter the diameter of vessels as well as improve blood flow by increasing vessel size and diameter, and reduce plaque build-up. It is possible that endothelial cells located in different spatial regions of the vasculature are subjected to different levels of shear stress and thus may have different biological and biochemical adaptations. This makes the continued study of the effects of LSS of different magnitudes on endothelial function paramount.

It was in the 1960s and 1970s when engineering studies began to elucidate actual blood flow velocities\textsuperscript{313-315}. From there, decades of molecular research have continued to study the \textit{in vitro} effects of varying magnitudes of shear stress in attempt to understand the \textit{in vivo} effects that blood flow has on the vasculature. Recent magnetic resonance imaging studies have measured shear stress in human arteries at rest and during moderate exercise and found that during resting conditions blood flow shear stress ranges in the arteries between 10-70 dyne/cm\textsuperscript{2} while in the veins ranges between 1-6 dyne/cm\textsuperscript{2}. It has been shown that during moderate exercise the shear stress increases to between 7-50 dyne/cm\textsuperscript{2}, with blood flow shear levels depending on vessel type, size, and location\textsuperscript{316-319}.

In the present study, low LSS at 5 dyne/cm\textsuperscript{2} was used to represent a low physiological blood flow in order to measure its effects on antioxidant activity and oxidative stress levels in HUVECs compared to static cell culture. A higher LSS level of 20 dyne/cm\textsuperscript{2} was used to represent a moderate aerobic exercise blood flow and measure its effects on antioxidant activity and oxidative stress levels in HUVECs compared to static cell culture as well as compared to the low physiological blood flow level. Both conditions were applied \textit{in vitro} to cultured HUVECs from African Americans and Caucasians for 24 hours.
**Main Findings**

The main findings of this study were that overall the African American HUVECs were more responsive to LSS than the Caucasian HUVECs. African American HUVECs had significant responses to the application of LSS: increases in antioxidant production and decreases in NADPH oxidase isoforms expression. The data from this study confirm the well established upregulation of eNOS and increase in NO production that occurs with LSS and there was no effect of race. HUVECs from both races responded similarly in eNOS protein expression and NO production with application of LSS. Furthermore, racial differences existed in the HUVECs under static conditions and the application of LSS caused these differences to disappear. There were no differences between race in any of the proteins measured after LSS of 20 dyne/cm\(^2\). The data from this study provide valuable support to the prescription of exercise by physicians, because the data show that African Americans may have a larger improvement in endothelial function from the increased blood flow that accompanies aerobic exercise training, when compared to Caucasians.

**NADPH Oxidase – Responses to LSS by Race**

**p47phox NADPH Oxidase Subunit Responses**

The first reports of a racial difference in p47phox subunit protein expression were by Kalinowski et al. in 2004 where they showed increased expression in HUVECs cultured from African American donors compared to Caucasian HUVECs\(^3\). The current study found similar results. A significantly higher p47phox protein expression was found under static conditions in HUVECs from African Americans compared to Caucasians. However, when the HUVECs were subjected to LSS at 5 dyne/cm\(^2\) and 20 dyne/cm\(^2\) this
racial difference disappeared. There were no racial differences after either of the LSS conditions. Statistically, the changes with LSS were not different between races. However, observationally the trends in response appear different. It appeared that LSS at 20 dyne/cm\(^2\) caused a significant reduction in p47phox protein expression in the African American HUVECs, while both LSS magnitudes caused no change in p47phox expression of Caucasian HUVECs. These data suggest that increased shear stress modulates NADPH oxidase p47phox protein expression more in African American HUVECs than it does in Caucasian HUVECs.

In 2006, Duerrschmidt et al. reported a significant down-regulation of p47phox mRNA and protein expressions with LSS of 30 dyne/cm\(^2\) at 12 and 24 hr\(^2\). Previously, Hwang et al. reported that OSS stimulated O\(_2^-\) production in a p47phox-dependent manner by transfecting p47phox \(^{-/-}\) cells with p47phox\(^{258}\). The results of their study suggest that the major source of endothelial O\(_2^-\) in response to OSS is p47phox-dependent NADPH oxidase activity. To the best of my knowledge, no previous study has yet to measure p47phox protein expression in racially-derived HUVECs with LSS at magnitudes of 5 dyne/cm\(^2\) and 20 dyne/cm\(^2\).

Supporting the decrease in expression of p47phox seen in African American HUVECs in the present study as well as the down-regulation measured in p47phox expression by Duerrschmidt are results from a recent animal study. It has been shown in a mouse model that physical inactivity was associated with higher p47phox protein expression compared to active mice (voluntary wheel running). In the same study, vascular oxidative stress was found to be significantly increased and atherosclerotic lesion formation was accelerated in inactive compared to active mice\(^{169}\). Therefore, lower
shear rates, or low blood flow, such as that found in inactivity are associated with heightened p47phox expression and thus potentially associated with higher oxidative stress. Just as in the present study, African American HUVECs under low flow and static conditions had higher p47phox expression compared to the cells exposed to 20 dyne/cm² condition. In fact, in the present study, the application of LSS at 5 dyne/cm² for 24 hr did not cause a change in p47phox expression in HUVECs from either race. This confirms the data from Duerrschmidt et al. where they also reported no change in p47phox with 5 dyne/cm² for 24 hr².

**NOX2 NADPH Oxidase Isoform Responses**

Phagocytic NADPH oxidase is a well-characterized, multi-subunit, membrane-bound enzyme that is capable of generating large amounts of O₂⁻ in response to a variety of stimuli. Whether this enzyme is the primary source of endothelium-derived O₂⁻, in cell culture systems, remains to be consistently established. Jones et al. examined components of NADPH oxidase in HUVEC culture and concluded that although cytosolic components were present, membrane bound NOX2 levels were too low to detect and therefore this homologue of NADPH oxidase was unlikely to be the major source of endothelium-derived O₂⁻.³²⁰ Recent studies have also come to similar conclusions and suggest that NOX4 may be the primary endothelial source of ROS. This will be discussed further below.

There have been no reports of a racial difference between African Americans and Caucasians in NOX2 protein expression, this data is the first to report of a racial difference. In HUVECs under static conditions, a significant racial difference was found. African American HUVECs had higher NOX2 protein expression suggesting a higher
basal O$_2^-$ release from this NADPH oxidase homologue. However, application of LSS caused this difference to disappear. With LSS under both conditions; 5 dyne/cm$^2$ and 20 dyne/cm$^2$, it appeared that HUVECs from both races had a non-significant trend towards decreasing. This indicates that perhaps a higher magnitude of LSS may be required to significantly decrease in NOX2 protein expression. This should be examined further in HUVECs of different races by other studies. Along those lines, a study by Hwang et al. measured the flow regulation of NADPH oxidase to pulsatile shear stress at 25 dyne/cm$^2$ and OSS at ±3 dyne/cm$^2$ for 8 hr$^{256}$. They found significant down-regulation of both NOX2 and NOX4 with pulsatile flow but a significant up-regulation with OSS. In this study, Hwang et al. used bovine aortic endothelial cells instead of HUVECs. Whether there is a species-specific differential response to shear stress remains to be established, therefore the specific cell model studied must be considered when comparing results.

Furthermore, while the present study did not directly measure O$_2^-$ production responses to LSS, De Keulenaer et al. showed that 24 hr of LSS at 5 dyne/cm$^2$ didn’t cause a change in O$_2^-$ production while 24 hr of OSS at 5 dyne/cm$^2$ caused an increase$^{257}$. Duerrschmidt et al. also did not find change in NOX2 with 5 dyne/cm$^2$ LSS$^2$. Taken together, these studies provide valuable supporting evidence to the results of the present study. Through this, it appears that both 5 and 20 dyne/cm$^2$ are not sufficient magnitudes to elicit change or downregulation in NOX2 homologue protein expression. Furthermore, it may be hypothesized that NOX2 is not the primary ROS producing enzymes involved in LSS responses in HUVECS.

Finally, indirect support of these findings are also provided in the recent study by Castier et al. that showed that shear stress induced NADPH oxidase activity comprising
p47phox but not NOX2. They used surgically induced flow-loaded vessels to measure the ROS production in vessel adaptation in mice. Using p47phox \(^{-/-}\) and gp91phox \(^{-/-}\) mice, they found that NADPH oxidase with p47phox was the major generator of shear-induced ROS in adapting vessels\(^{237}\). These data combined with the findings of the present study validate a potentially reduced role for NOX2 in shear-induced NADPH oxidase responses at 5 dyne/cm\(^2\) and 20 dyne/cm\(^2\) for 24 hr.

**NOX4 NADPH Oxidase Isoform Responses**

NOX4 was first described in 2000 and believed at the time to be solely expressed in kidney, but has since been found to be expressed in many other cell types\(^{321}\). Recent studies have purported that NOX4 may be the primary ROS producing NADPH oxidase homologue in the endothelium with mRNA expression measured as much as 100-fold higher than NOX2 in HUVECs\(^{305}\). This overexpression of NOX4 is the focus of many current studies aimed at elucidating the mechanism underlying activation of this homologue. While activation of NOX2 through translocation of p47phox is well established, the mechanism of NOX4 activation remains to be discovered. Despite this, it is understood that NOX4 and NOX2 serve as the primary endothelial NADPH oxidase-derived ROS sources\(^{230,243}\).

Shear stress studies that measured NOX4 expression are limited since antibodies were not developed until recently. As mentioned above, Hwang et al. found significant down-regulation of both NOX2 and NOX4 mRNA expression with pulsatile flow but a significant up-regulation with OSS for 8 hr\(^{256}\). While, several years later Hsiai et al. found significant up-regulation of NOX4 protein expression with both pulsatile and OSS\(^{255}\) for only 4 hr. Both studies used bovine aortic endothelial cells. The only other
study that measured NOX4 protein expression with shear stress was in 2009. Goettsch et al. measured the effects of long-term cyclic strain in HUVECs and found a down-regulation in NOX4 protein expression. Cyclic strain is defined as the mechanical strain caused by vessel wall adaptations through heart beat transmissions or blood pressure amplitude changes. Taken together with the findings of the present study, it seems that with application of shear stress, whether LSS or cyclic strain, protein expression of the NOX4 NADPH oxidase homologue is decreased in HUVECs, suggesting an improvement in endothelial oxidative stress levels. No study has measured NOX4 protein expression after LSS at 5 or 20 dyne/cm² in HUVECs, therefore the present study is the first to report such findings and will thus need to be replicated by others.

In the present study, a significant racial difference existed under static conditions. African American HUVECs had higher NOX4 protein expression. Again, this racial difference did not exist after application of LSS. However, separately, African American HUVECs had a significant decrease in NOX4 protein expression with both LSS treatments: 5 and 20 dyne/cm², while the Caucasian HUVECS only had a significant decrease with 20 dyne/cm². Since no study has examined the effect of LSS on NOX4 expression, further studies are needed to confirm these observations. Also, mechanistic studies will be needed to elucidate why the differences in response exist between race.

Racial Differences in NADPH Oxidase Expression in Static Culture

A significant race difference existed in static culture for each of the NADPH oxidase subunits/homologues measured, and in each case this was abolished with LSS of 20 dyne/cm². African American HUVECs have heightened oxidative stress, through
higher NADPH oxidase expression, compared to Caucasian HUVECs under static conditions. In the African American HUVECs, each of the NADPH oxidase subunits/homologues examined in the present study showed a trend toward a decrease with LSS at 5 and 20 dyne/cm$^2$. The Caucasian HUVECs did not have as large a downward trend, except for in NOX4 expression. These observed trends provide support of the beneficial effect that increased shear stress has on ROS production. The fact that African Americans HUVECs have higher basal levels, taken together with the fact that after LSS there were no differences between race in any of the NADPH oxidase subunits/homologues, it seems that African American HUVECs have a larger response to LSS than Caucasian HUVECs. Furthermore, since the 20 dyne/cm$^2$ magnitude was used as a model for moderate aerobic exercise blood flow, when this is viewed from a translational aspect, it can be thus hypothesized that aerobic exercise training may have a more beneficial effect on endothelial oxidative stress production in African Americans than it does Caucasians.

Nitric Oxide (NO) Production – Responses to LSS by Race

*Endothelial Nitric Oxide Synthase (eNOS) Protein Responses*

Vascular endothelial cells are constantly exposed to mechanical forces from the shear stress of continuous flow of blood, and eNOS remains one of the best characterized shear-responsive proteins within the cell. It is constitutively expressed and rapidly activated in response to shear stress. Activation of eNOS causes an acute release of NO which is now recognized as the major mechanism regulating vascular tone. In 2004, Kalinowski et al. showed that African American HUVECs expressed higher levels of eNOS protein compared to Caucasian HUVECs$^3$. The present study showed similar
results. African American HUVECs in static culture had significantly higher eNOS protein expression compared to Caucasians. However, after application of LSS at both 5 and 20 dyne/cm\(^2\), the racial differences did not exist. HUVECs from both races significantly increased eNOS expression with LSS.

It is known that the most rapid cellular response to an acute shear stress involves the activation of potassium and calcium (Ca\(^{2+}\)) channels. Immediately following channel activation, rapid generation of inositol-1,4,5-trisphosphate and diacylglycerol occur. This is closely linked to intracellular Ca\(^{2+}\) mobilization and protein kinase C activation\(^{322}\). Elevations in intracellular Ca\(^{2+}\) are necessary for activation of eNOS because the Ca\(^{2+}\) binds with calmodulin in an activation domain of the enzyme\(^{323}\). Furthermore, for years it has also been assumed that activation of eNOS was through an intricate feedback loop involving transcription factor nuclear factor kappa-B, along with this Ca\(^{2+}\)/Calmodulin binding in the activation domain\(^{324}\). Recently it was shown that another transcription factor is involved in LSS induction of eNOS. Kruppel-like factor was increased in HUVECs exposed to shear stress\(^{325}\). The intricate relationship between nuclear factor kappa-B and kruppel-like factor remains to be established. Whether there are race effects on transcriptional regulation also remains to be established. The observations in the present study confirmed what was previously known, that LSS upregulates eNOS protein expression. However, what was not previously known was whether there were race-dependent responses in eNOS expression to LSS. Both African American and Caucasian HUVECs had significant increases in eNOS protein expression with 5 and 20 dyne/cm\(^2\) LSS, but the responses were different because of the racial differences that existed under static conditions. The shear-mediated signaling process is beyond the scope of this study,
but the associated mechanisms, as well as whether racial differences exist between HUVECs in transcriptional regulation, should be further investigated in order to fully understand the results presented here with the current study.

**Nitric Oxide (NO) End-Product Responses**

NO production in response to flow-dependent shear stress on the endothelial cell surface is the fundamental mechanism involved in the regulation of vascular tone. Some scientists suggest that this is the primary molecule mediating endothelial function and any stimuli that causes reduction in NO bioavailability is therefore closely associated to endothelial dysfunction. With induction of shear stress, rapid release of NO occurs, and over the past several decades studies continually report *in vitro* increases in NO with shear stress. In the present study this was again confirmed, HUVECs from both African Americans and Caucasians had significant increases in NO production with both levels of LSS: 5 and 20 dyne/cm². The increase with 20 dyne/cm² LSS was about 2-fold higher than the increase with 5 dyne/cm². Similar results were shown in 2005 by Tao et al. They reported a dose-dependent increase in levels of NO with about a 2 fold difference in NO production between 5 and 25 dyne/cm².

In the present study there were no differences between race in NO levels for any condition. Kalinowski et al. conducted the only study so far that reports on *in vitro* racial differences in eNOS expression and NO release. No study has reported racial differences in NO levels from cell supernatant. Considering the diffusion-limited reaction rate of NO with O₂⁻ and thus the fact that NO release and NO end-product formation may differ, correlating the NO release measurements from Kaliowski’s study with the NO end-product measurements from the present study becomes difficult. As mentioned
previously, in pilot studies it was found that African American HUVECs had significantly higher NO levels than Caucasian HUVECs in static culture. While in the present study, it also seems that the African American HUVECs released more NO end-products, but the difference did not reach significance. This could be attributed to slight differences in the HUVEC cultures used or it could be due to the differences in cell culture media used. For pilot studies, EGM media was used, while for this present study, EGM-2 media was used. The main difference between the two types of media is that EGM-2 has additional growth factors. In this case, the presence of growth factors may have affected the production and release of NO through eNOS activation. This should be further examined in future studies.

**NADPH Oxidase, eNOS, and NO Responses**

NAPDH oxidase activity and NO production are intricately intertwined. In 2006, Duerrschmidt et al. concluded that the LSS downregulation of NADPH oxidase subunit expression was NO-dependent. Using both NOS inhibition and NO donors, they showed that NOS inhibition prevented NADPH oxidase downregulation, while NO donors increased the NADPH oxidase downregulation in HUVECs exposed to LSS. Also Kalinowski et al. used tandem NO/O$_2^-$/ONOO$^{-}$ nanosensors to quantify the concurrent release of NO, O$_2^-$, and ONOO$^{-}$. They observed racial differences between HUVECs from African Americans and Caucasians in the kinetics of NO, O$_2^-$, and ONOO$^{-}$ release. They further confirmed that this was associated with eNOS activation and uncoupling by using NOS and NADPH oxidase inhibitors. Both of these studies explicitly demonstrate that vascular endothelial function and NO release is associated with NADPH oxidase activity and that racial differences in this process may exist.
The observations in the present study confirm previous findings. Increases in eNOS protein expression and NO production, as well as decreases in NADPH oxidase activity were seen with the application of LSS. Racial differences were observed in HUVECs before LSS, and in response to LSS the cells of different race seemed to adjust to similar levels. The mechanisms underlying how LSS causes the protein expressions and oxidative stress biomarkers in African American HUVECs to adjust to levels that were similar to the Caucasian HUVEC adaptations remain to be discovered.

Antioxidant Production – Responses to LSS by Race

*Total Antioxidant Capacity (TAC) Responses*

Recently, it has been suggested that the determination of antioxidant capacity may be considered as a valuable tool for aiding medical treatment and diagnoses. TAC measures give a relative quantified amount of all antioxidants present in the sample at time of test, and limited *in vitro* studies have measured TAC levels. In the present study, TAC measures were included in order to provide a global assessment of the LSS effects on antioxidant capacity. The beneficial effects of LSS on antioxidant levels have been consistently reported and the results of the present study in African American HUVECs support this. Significant increases in TAC, SOD and CAT were all seen with both 5 and 20 dyne/cm² LSS. Responses were different for the Caucasian HUVECs.

In African American HUVECs, the increase in TAC with LSS was larger than the change with LSS in Caucasian HUVECs. TAC levels rose with LSS in each race, but only significantly in African American HUVECs. Furthermore, because of the larger increase with LSS of 5 dyne/cm² in African American HUVECs, there was a significant racial difference at the 5 dyne/cm² level in TAC levels and the African American
HUVECs had higher levels of TAC with this magnitude of LSS stimulation. Considering that there was no race difference in HUVECs before LSS, this larger increase in TAC with 5 dyne/cm² suggests a compensatory antioxidant response to application of low shear stress in African American HUVECs. Considering the higher levels of NADPH oxidase activity in static African American HUVECs compared to Caucasian HUVECs, and the fact that NADPH oxidase subunit expressions were similar after the application of LSS, as well as the fact that LSS at 5 dyne/cm² did not modulate expression of NADPH oxidase as much as the LSS at 20 dyne/cm² did, it is clear that a compensatory increase in antioxidant scavenging would need to occur in the African American HUVECs in order to bring the ROS balance to the same level as Caucasian HUVECs after LSS.

**Superoxide Dismutase (SOD) & Catalase Responses**

Total SOD activity and SOD2 protein expression were the only markers in the current study to show a significant interaction between race and shear stress. Under static conditions, Caucasian HUVECs had both higher total SOD activity and higher SOD2 protein expression levels than African American HUVECs. However, Caucasian HUVECs did not demonstrate a significant change in either total SOD activity or SOD2 protein expression with LSS treatment, whereas African American HUVECs had significant increases in both total SOD activity and SOD2 protein expression levels with both magnitudes of LSS.

In support of the data from this present study is the De Keulenaer et al’s research. As mentioned above, they reported that 24 hr of LSS at 5 dyne/cm² elicited a non-significant reduction in O$_2^-$ production. The results from their study also showed that
steady LSS caused a concomitant increase in CuZnSOD protein expression\textsuperscript{257}. There was no mention of the ethnicity of the HUVECs used in De Keulenaer’s study. In the present study, only the African American HUVECs had a significant increase in total SOD and SOD2 protein expression. Given this disparity in results using similar cells and shear stress magnitudes, perhaps ethnicity of cell line is important to consider. No study has measured SOD activity or expression after 20 dyne/cm\textsuperscript{2} of LSS.

Active scavenging of O\textsubscript{2} by the SOD enzyme leads to increases in intracellular H\textsubscript{2}O\textsubscript{2}, therefore it then becomes necessary to further investigate mechanisms for H\textsubscript{2}O\textsubscript{2} removal. Thus in the present study, CAT protein expression levels were measured in all HUVECs. No racial difference existed under any condition, but similar response trends were seen in CAT expression as seen in SOD activity and expression. African American HUVECs had a significant increase in CAT expression with LSS at 5 dyne/cm\textsuperscript{2} and a non-significant increase in CAT expression with LSS at 20 dyne/cm\textsuperscript{2}. The data show that, despite the increases seen in SOD activity and SOD2 protein expression, there is likely not an inherent build-up of H\textsubscript{2}O\textsubscript{2} levels within the cells because the increases in SOD are paralleled by increases in CAT. These data also further support the belief that LSS modulates endothelial cells in an antioxidative manner. Increases in SOD activity appear to be closely coupled with increases in CAT activity, which is also globally represented by the increases in TAC.
CHAPTER 6

CONCLUSION

Addressing Hypotheses and Specific Aims

**Hypotheses:** It was hypothesized that compared to static levels, LSS for 24 hours at 5 dyne/cm² would upregulate all protein expressions, while LSS for 24 hours at 20 dyne/cm² would significantly reduce NADPH oxidase subunit expressions, increase antioxidant enzyme levels, increase eNOS expression, and lead to increased NO bioavailability in cultured HUVECs. Furthermore, due to the heightened oxidative stress levels in African Americans and the potential for existing endothelial dysfunction, it was believed that African American HUVECs would respond to LSS stimulus differently than Caucasian HUVECs.

**Findings:** The results of the present study showed that African American HUVECs did indeed respond to LSS differently than Caucasian HUVECs. Larger changes were seen in the African American HUVECs due to the differences between race in static cells. However, addressing the first hypothesis, it is clear that LSS at 5 dyne/cm² for 24 hours does not upregulate all protein expressions. The NADPH oxidase subunit p47phox and NOX2 were not affected by this level of LSS while NOX4 expression actually decreased in the African American HUVECs. All of the antioxidant enzymes and also eNOS expressions increased with LSS at 5 dyne/cm².

**Specific Aims:** As listed in Chapter 1 the specific aims for this study were as follows:
1. Determine NADPH oxidase subunit and isoforms; p47phox, NOX2, and NOX4; eNOS, SOD2, and CAT protein expressions, before and after exposing cultured HUVECs from African American and Caucasian donors to LSS at levels comparable to in vivo blood flow levels at rest (5 dyne/cm$^2$) and during moderate aerobic exercise (20 dyne/cm$^2$). Protein expression was measured by Western Blot analysis using antibodies specific for NOX2, NOX4, p47phox, eNOS, SOD2, and CAT.

The study showed that LSS at 5 dyne/cm$^2$ caused a down-regulation in NOX4 protein expression in African American HUVECs, but not in Caucasian HUVECs. LSS at 5 dyne/cm$^2$ did not cause a change in p47phox, NOX2 protein expressions for either race. The study also showed that LSS at 20 dyne/cm$^2$ caused a down-regulation in p47phox protein expression in African American HUVECs, but not in Caucasian HUVECs, and also caused a down-regulation in NOX4 protein expression in HUVECs from both races. LSS at 20 dyne/cm$^2$ did not cause a change in NOX2 protein expression in HUVECs from either race.

The study showed that LSS at 5 dyne/cm$^2$ caused an up-regulation in eNOS protein expression in both races, and also caused an up-regulation in both SOD2 and CAT protein expressions in African American HUVECs but not Caucasian HUVECs. The study also showed that LSS at 20 dyne/cm$^2$ caused an up-regulation in eNOS protein expression in both race, and up-regulation of SOD2 protein expression in African American HUVECs but not Caucasian HUVECs. No change was found in CAT protein expression in Caucasian HUVECs under the 20 dyne/cm$^2$ condition. Furthermore, no change was found in SOD2 protein expression in the Caucasian HUVECs under any LSS condition.
2. Determine NO bioavailability in African American and Caucasian HUVECs; NO, SOD, and TAC levels were quantified. NO measurements were performed using the Griess reaction to measure total nitrate and nitrite in cell culture supernatant. Total SOD activity in cell lysate were assessed using an assay which measures dismutation of $O_2^-$ radicals generated by xanthine oxidase and hypoxanthine. Finally, TAC levels were quantified using a commercial assay that measures the reduction of 2,2’-azinobis-3-ethylbenzthiazoline-sulfonate (ABTS).

The study showed that NO levels were higher under both conditions of LSS in HUVECs from both races. The study also showed that both total SOD activity and TAC levels were higher under both conditions of LSS in the African American HUVECs but not in the Caucasian HUVECs.

Concluding Remarks & Future Directions

In the present study, endothelial function was measured by NO production, antioxidant capacity, and oxidative stress production through measuring NADPH oxidase subunit protein expressions. Overproduction of ROS leads to uncoupling of the eNOS enzyme, reduced NO production, and impaired antioxidant defenses which are all associated with endothelial dysfunction. The central hypothesis examined by this study was based on the observation that increased blood flow from aerobic exercise, modeled by in vitro shear stress, effectively modulates endothelial function toward a net anti-atherogenic and antioxidant phenotype, and away from a net oxidative stress, pro-atherogenic, and endothelial dysfunction phenotype. The results from this study confirmed that application of LSS to HUVECs caused a decrease in oxidative stress levels, increase in antioxidant activity, and an increase in NO production. Each of these
adaptations are indicators of improved endothelial function. The data provide valuable evidence suggesting that this in vitro model of aerobic exercise, using shear stress to represent the increased blood flow, may be an effective tool for studying aerobic exercise adaptations of the endothelial cell layer.

Furthermore, given the findings of this present study, it becomes apparent that static African American HUVECs may be a representative model for the existing endothelial dysfunction that is known to exist in vivo. Higher protein expressions of NADPH oxidase subunit p47phox and isoforms NOX2 and NOX4, as well as higher eNOS expression, and lower total SOD activity and SOD2 protein expression, all suggest that more O$_2^-$ is being produced. Most likely then, this O$_2^-$ is rapidly converted to ONOO$^-$ by the increased NO production from higher eNOS activity. Couple this with the reduced availability of SOD and the entire picture represents an in vitro model of endothelial dysfunction. Finally, given that there were no racial differences in CAT or TAC in HUVECs under static conditions, this further supports a heightened oxidative state in the African American HUVECs. Future studies can continue to compare HUVECs of different races and continue identifying where the racial differences exist in oxidative stress levels and antioxidant activity. Also, future studies can examine the racial differences in oxidative stress levels and antioxidant activity in different cell types. It should be examined whether African American vascular smooth muscle cells have higher oxidative stress when compared to Caucasian vascular smooth muscle cells.

With respect to shear stress, considerable attention is currently focused on the fundamental question of the identity, function, and mechanisms of actions of the endothelial flow-mediated mechanotransducers. Researchers look to identify the
molecules that are rapidly activated in response to fluctuations in shear stress levels, understand secondary messengers, and elucidate the intricate regulation of transcription factors. From the results of the present study, it is clear that such research could also now focus on identifying race-dependent associations in mechanotransduction.

Finally, if we were to consider the findings of the present study from a genetic viewpoint. If endothelial cell responses have true molecular and genetic racial differences in their inherent response mechanisms, then all of the physiological in vivo racial differences observed could be the result of primitive endothelial cells and thus regulated at the gene level. The larger responses seen in African American HUVECs in the present study could be the result of slight variations in the genetic makeup. This could also be examined.

Taking the findings of the present study and using them to guide future genetic, molecular and mechanistic studies aimed at understanding racial differences could lead to new frontiers.
REFERENCES


Ashmaig ME, Starkey BJ, Ziada AM, Amro A, Sobki S, Ferns GA. Changes in serum concentration of antioxidants following treadmill exercise testing in


APPENDIXES

Appendix A:

Consent Form: Oxidative Stress and Acute Exercise: Differentiating Time-Course Race-Related Responses. A Pilot Study.
1. PURPOSE OF THE STUDY

This is a student research pilot study that will be conducted by Temple University graduate students.

Temple University is not being compensated for performing this study.
Free radicals are highly reactive molecules that are made inside the human body. Because they are highly reactive, these free radicals interact with many proteins in the body, such as DNA, which are important for our health and proper functioning. This interaction ultimately results in the breakdown of these important proteins causing damage to the cells of the body. Many free radicals are oxygen centered; therefore the damage that they cause to the proteins in our body is more often called oxidative stress. Because of the ability of free radicals to breakdown important proteins in the body, oxidative stress has been connected to a number of diseases including heart disease, hypertension, cancer, and Alzheimer’s disease. Fortunately, our body has a natural defense system against free radicals called antioxidants. These antioxidants serve as a protective mechanism by neutralizing the free radicals present in the body. When there is an imbalance between the production of free radicals and the ability of the antioxidants to neutralize them, oxidative stress occurs. For healthy individuals in a resting state, the antioxidants made by the body are enough to protect against oxidative stress. However, during exercise there is an increase in oxidative stress levels. At the present time, the amount of time it takes for oxidative stress levels to rise and fall after exercise is not fully known.

This is a research study and you are being asked to join this study because you are a Temple University student and a healthy individual between the ages of 18 to 25.

The purpose of this research study is to understand the time-course of how oxidative stress levels rise and fall after one bout of exercise.

2. DESCRIPTION OF THE STUDY

If you qualify for the study you will be enrolled for a total of about 1 month. During this time period you will visit the HyMAP laboratory in the Department of Kinesiology at Temple University on two occasions. You will be one of approximately 40-48 people participating in this study at Temple University.

Screening

During your first visit to the HyMAP laboratory, you will meet with laboratory personnel and will be given a consent form to read (this document). After Dr. Brown has thoroughly explained the consent form, you will be given time to read the consent form and ask any questions you may have about participating in the study. After all of your questions have been answered and if you choose to sign this informed consent form and participate in the study, you will be asked to complete a form to determine whether you qualify for the study. The form which you will complete is a health history questionnaire used to identify any risks that you may have that would make it unsafe for you to exercise at a moderate intensity without medical supervision. If any medical risks are identified you will be excluded from the study at this point in time. This form will also be used to
determine your habitual level of physical activity. This is not an exercise training study, therefore qualifying participants are not exercise trained and are asked to refrain from exercise during the course of the study. The information which you provide on this questionnaire will be used solely for study qualification purposes and will not be used for any other reason. The total time for this visit is approximately 1 hour.

**Testing**

As part of an experimental study, you will be randomly placed into a control group. This control group will not undergo a treadmill exercise test and will only participate in the blood sample collection and body composition measurement. Which group you will be randomly assigned to will not be known to you until you arrive on the testing day. This will not impact your benefits or risks at all. Nor will it influence your compensation.

During your second visit to the HyMAP laboratory you will undergo the testing. If you are in the experimental group, you will complete a treadmill exercise test and have five blood samples taken and body composition measured. If you are in the control group, you will not complete the treadmill test, but will still have your blood samples collected and body composition measured. For this visit you will be fasted for 10 hours, meaning that no food or drinks (water is allowed) will be consumed for 10 hours prior to this visit. You will also be asked to refrain from the following: medications or vitamins 2 weeks prior, alcohol or caffeine 24 hours prior, and exercise 24 hours prior to the test. The total amount of time for the visit is approximately 6 hours.

**Body composition:** On this same day, you will have your body composition measured. This measurement will tell the investigators what percentage of your body is fat, muscle and bone. The instrument that measures your body composition is called bioelectrical impedance (BIA). The machine will cause a very small electrical current to go through your body for 2-3 seconds, but you will not feel anything. To do this test, you will lie on a patient table on your back with your left foot exposed. A technician will place two electrodes on your left foot and two electrodes on your left hand.

**Exercise Test:** Exercise tests are often used to determine fitness levels. By measuring maximal oxygen consumption ($\text{VO}_{2\text{max}}$), which is the amount of oxygen that your body can use to do physical work such as walking or climbing stairs, your present fitness levels can be determined. For this study, investigators are interested in how moderate levels of exercise affect oxidative stress levels, therefore investigators will measure your $\text{VO}_{2}$ and heart rate as you walk/run on a treadmill to make sure that you exercise at a moderate intensity. Understand that this test is not a maximal exercise test and you will not exercise to exhaustion.

Prior to starting the exercise test, a technician will apply small sticky pads known as electrodes to the skin of your upper body. At the location where the sticky pads are placed, your skin will be rubbed with an alcohol
pad prior to placement. These electrodes will allow the technician to monitor your heart while you exercise. During the exercise test you will also wear a clip on your nose and will breathe through a mouthpiece that is connected to a machine that will measure oxygen and carbon dioxide.

The exercise test will begin with a warm-up where you will walk slowly on the treadmill for 3-4 minutes to allow you to become comfortable with walking on the treadmill. The test will be easy at first and get more difficult every 3 minutes. The exercise test will become more difficult because the speed of the treadmill will be increased and the angle will go up making it seem like you are walking uphill. The exercise test will continue to increase in speed and angle every 3 minutes until you reach a moderate level of intensity or about 75% of your maximal capacity. At this point the technician will modify the speed and angle of the treadmill to keep you at this intensity for a total of 3 minutes. If you become uncomfortable or short of breath you can ask the technician to stop the test at any time. However, it is natural to become “winded” when performing moderate intensity exercise. During this exercise test your blood pressure and heart rate will be monitored every 3 minutes. At certain times during the test a technician will ask you to point to a chart to indicate how difficult the exercise is feeling. The total time to complete the entire exercise test is approximately 8-12 minutes, not including the warm-up.

**Blood Drawing:** On the same day as the exercise test you will also have blood samples taken five times so that investigators can measure how your oxidative stress levels change with exercise. Your blood will be taken before beginning the exercise test, immediately after, 30 minutes after, 1 hour after, and 2 hours after the exercise test. Because it is important that you remain fasted for the 2 hour time period after exercise for blood draws, we ask that you remain in the lab area during this time.

Your blood will be drawn from your arm by a staff member fully certified in phlebotomy. You will be asked to lie down and the staff member will tighten a band around your upper arm, wipe your arm with an alcohol pad, and then insert a small butterfly needle into a vein of your arm. Three tubes of blood will be filled for each of the five blood draws, which is less than 1 ounce (2 tablespoons) of blood. A total of less than 5 ounces (10 tablespoons) of blood will be taken during the entire study.

### 3. POSSIBLE RISKS RELATED TO PARTICIPATION IN THIS STUDY

The following risks, although low, are related to participation in this research study.

**Exercise Testing:** The risk of maximal exercise testing is that out of 10,000 tests, someone has a medical problem. In 1 out of every 70,000 exercise tests, a person will die from heart problems. In medical terms, doctors call this a rare event. Because the exercise test which you will
complete is not a maximal test, and because you are between the ages of 18 to 25 with no known risks factors, the risks of a medical problem are even lower. The investigators will make sure it is as safe as possible for you to do this test. The staff is trained in CPR and AED, and an emergency procedure plan is implemented in the laboratory which all staff reviews every 3 months.

**Giving Blood:** The research staff will take your blood in exactly the same way as when you have your blood taken at the doctor’s office. There is a small risk of bruising and rarely infection. These risks will be lowered by using sterile procedures and by having trained personnel collect all blood samples. There is also some discomfort associated with needle sticks and sometimes, people have been known to faint during needle sticks and blood drawing. Staff members will take your blood while you are lying down which helps to prevent fainting.

**Body Composition:** There are no known risks of having the amount of fat measured in your body. There are no needles and no pain. Sticky pads are placed on your foot and hand and the tests only takes about 5 minutes.

4. **POSSIBLE BENEFITS OF PARTICIPATING IN THIS STUDY**

   Exercise testing is a procedure that provides you with valuable information concerning your health and fitness status that many people often do not have access to. Typically, many individuals undergo an exercise test at a hospital when they reach middle to late adulthood when they are suspected to have a medical condition. Some endurance athletes also pay to undergo an exercise test at a sports performance laboratory in order to gain information that will enhance their training program’s effectiveness. By participating in this study you will be gaining information about your health that most adults don’t receive until much later when it may be too late to take preventive measures for your health. You also will be receiving information that may be beneficial for individualizing an exercise training program to suit your current fitness levels. Whether these benefits will occur for you cannot be guaranteed.

5. **ALTERNATIVE PROCEDURES**

   There are no alternative procedures available for the exercise test and blood draw. You have the choice to not participate in this study.

6. **CONFIDENTIALITY STATEMENT**
All documents and information about this study will be kept confidential in accordance with federal, state, and local laws and regulations. The investigators will use a coding system consisting of a combination of letters and numbers to maintain confidentiality. Your name will not appear on these samples. After researchers have completed the study all blood samples will be destroyed. You understand that medical records and data generated by the study may be reviewed by Temple University’s Institutional Review Board and the Office for Human Research Protections to assure proper conduct of the study and compliance with federal regulations. You understand that the results of this study may be published. If the results are published, you will not be identified by name.

7. VOLUNTARY PARTICIPATION STATEMENT
   You understand that participation in this study is entirely voluntary, and that refusal to participate will involve no penalty or loss of benefits to you. You may discontinue your participation at any time without penalty or loss of benefits.

8. COMPENSATION STATEMENT
   You understand that you will receive a $20 Visa gift card if you complete this study. Additionally, you will be placed in a raffle with the other participants for a 2GB Ipod Shuffle. You will receive compensation for your participation at the end of your testing period and you will be notified if you are the raffle winner of the Ipod Shuffle after the entire study is completed and the drawing occurs.

9. INSTITUTIONAL CONTACT
   If you have any questions about your rights as a research participant, you may contact the Institutional Review Board Coordinator, Richard Throm at (215) 707-8757.
   If you have questions about research-related injuries, you may contact the Principle Investigator, Deb Feairheller, in the Department of Kinesiology at (215) 204-0084.

10. STANDARD INJURY STATEMENT
   You understand that if you sustain an injury as a result of participation in this study, the physician’s fees and medical expenses that result will be billed to your insurance company or you in the usual manner. You understand that financial compensation for such injuries is not
available. You understand that you have not waived any legal rights that you would otherwise have as a participant in an investigational study.

11. COSTS STATEMENT
You understand that tests required by the study will be provided at no cost to you. You understand that you are responsible for transportation to the study site and parking.

12. TERMINATION STATEMENT
The investigators have the right to terminate your participation without regard to your consent. This could occur if you cannot make your appointments or experience a change in your medical condition during the course of the study.

13. STATEMENT OF NEW SIGNIFICANT FINDINGS
You will be informed in a timely manner of any new information regarding this study that may have an affect on your willingness to participate, continue your participation, or after your participation that may have an affect on your future medical care. You may be asked to sign a revised informed consent form that contains this new information.

14. FINAL STATEMENT AND SIGNATURE
This study has been explained to me, I have read the consent form and I agree to participate. I have been given a copy of this consent form.

Participant’s signature _______________________________ Date __________

Principal Investigator’s Signature ____________________________ Date __________

Witness’s Signature ____________________________ Date __________