RACIAL DISPARITY IN CARDIOVASCULAR DISEASE AND OXIDATIVE STRESS IN AFRICAN AMERICAN WOMEN

by

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THESIS

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# TABLE OF CONTENTS

Acknowledgements...........................................................................................................3

Abstract ............................................................................................................................4

List of Figures ..................................................................................................................6

CHAPTER 1 Review of Literature........................................................................................7

CHAPTER 2........................................................................................................................17

2.1 Abstract.....................................................................................................................18

2.2 Introduction ...............................................................................................................19

2.3 Methods....................................................................................................................20

2.4 Statistical Analysis ...................................................................................................23

2.5 Results ......................................................................................................................24

2.6 Discussion ...............................................................................................................27

CHAPTER 3.......................................................................................................................29

3.1 Future Directions.......................................................................................................30

4.2 Conclusion...............................................................................................................31

Bibliography ....................................................................................................................32

Appendix A .....................................................................................................................35

Appendix B.......................................................................................................................36
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Thank you to Dr. Paul Fadel for guiding me through my master’s degree and providing me with the tools to learn and collaborate with other researchers into the farther reaches of science. I will continue to keep the education I have learned as I pursue future endeavors within and beyond the laboratory walls.

August 2018
Several studies have demonstrated that African Americans (AA) exhibit elevations in systemic oxidative stress compared to Caucasian Americans (CA). Peripheral blood mononuclear cells (PBMCs) have been identified as one of the primary contributors to systemic reactive oxygen species (i.e. oxidative stress) via the NADPH enzyme pathway and may be an underlying mechanism for the development of hypertension and cardiovascular diseases. Previous work demonstrates that young healthy AA men have elevated PBMC-derived superoxide production when compared to CA men. However, whether PBMC-derived superoxide production is also elevated in young healthy AA women remains unknown. Accordingly, this study investigated PBMC-derived superoxide production in young healthy AA and CA women. We tested the hypothesis that, relative to CA women, AA women would exhibit greater PBMC-derived intracellular superoxide with corresponding expressions of the NADPH oxidase subunit proteins. In ten normotensive AA women and twelve age-matched normotensive CA women, resting intracellular superoxide levels were assessed from freshly isolated PBMCs using dihydroethidium fluorescence within one hour of venous blood sampling. PBMCs were frozen in -80°C and protein was later extracted to assess expression of the NADPH-oxidase subunits p47\textsuperscript{phox} (cytosolic) and gp91\textsuperscript{phox} (membranous) using
Western blot analysis in a subset of subjects (AA n=7, CA= n=5). Significantly higher resting intracellular superoxide production was found in AA women compared to CA (AA 4.1±1.9 vs. CA 2.7±1.0 Relative Fluorescent Units; \( P=0.025 \)) as well as elevated protein expression of p47\(_{phox}\) (e.g., p47\(_{phox}\): 3.7 ± 1.5, AA vs. 0.5 ± 0.2, CA, \( P < 0.05 \)). Interestingly, there was no difference in gp91\(_{phox}\) expression among the AA and CA women (gp91\(_{phox}\) 5.4 ± 2.4, AA vs. 4.4 ± 0.8, CA women, \( P < 0.05 \)). These findings indicate that young AA women exhibit greater resting PBMC-derived superoxide production and suggest the NADPH-oxidase pathway may play a role in their elevation in superoxide production. Thus, PBMCs may represent a source of elevated oxidative stress in AA women.
LIST OF FIGURES

FIGURES AND TABLES

Figure 1. .........................................................................................................................9
Figure 2 .......................................................................................................................12
Figure 3. .....................................................................................................................13
Figure 4. .....................................................................................................................14
Figure 5. .....................................................................................................................15
Figure 6. .....................................................................................................................24
Figure 7. .....................................................................................................................25
Figure 8. .....................................................................................................................26

TABLES

Table 1. .......................................................................................................................26
Chapter 1
Review of Literature
The disproportionate burden of cardiovascular diseases (CVD) on African Americans (AA) is currently among the largest public health issues in the United States. According to the 2018 update from the American Heart Association (AHA) nearly 50% of all AA adults have some form of CVD with onset of disease occurring earlier in life compared to any other race. CVD has also been reported to be responsible for nearly one-third of all mortalities in both AA men and women (Figure 1). For decades the incident rates of CVD factors such as hypertension in AA have steadily risen without sign of decline. It is clear there are wide gaps in knowledge concerning development of CVD in AA and perhaps ignored racial differences in basic physiology. Moreover, racial and sex difference studies are key to closing disparity gaps among both AA men and women. And although research on the development of CVD in AA is limited in both sexes, it well understood that AA women have been the least represented in literature
for some time. With AA women now exceeding the men statistically in CVD prevalence at 47.1% sex-specific questions within the existing CVD racial disparity gap warrant further development. [5] Considering research in AA women cardiovascular physiology is highly limited it is reasonable to refer to relevant studies on CVD development in young AA men for any racial difference interpretation before attempting sex-specific questions within race. Furthermore, it is imperative to first understand any known physiological differences at rest among AA men to begin unraveling the racial disparity among AA women. A growing body of literature has emerged highlighting racial differences among young AA men under basal-level conditions in key systems and mechanisms related to the pathogenesis of CVD that despite not being fully defined in AA women hold insights into the future development of sex-difference studies. By way of mirroring the physiological outcome measures from the AA men studies, gender-specific studies can be created to answer similar questions in AA women. Conclusively, these outcome measures involving the interplay of the sympathetic nervous system, the immune system, and oxidative stress have become a collective interest in better defining this racial and sex disparity among AA in CVD.

The sympathetic nervous system (SNS) plays a primary role in homeostasis during stressors via rapid adjustments through its ability to communicate messages among organs and cause targeted responses. [4] This communication model pattern of neural signals centers around their directions emanating from the central to peripheral components of the SNS. Importantly, this pattern contributes greatly to cardiovascular regulation at rest by continuously altering vascular resistance and blood pressure on a heartbeat-by-heartbeat basis. The end-organ response to sympathetic outflow patterns
in peripheral vasculature has been of interest in cardiovascular research due to the known fact that increased peripheral resistance over time can lead to vascular remodeling and increased risk for hypertension. In a study investigating the basal peripheral SNS response in young AA and CA men, leg vascular conductance as well as peripheral SNS was measured while subjects were supine under resting conditions for a 20-minute period. SNS outflow at rest in young AA men was found to cause an elevated vasoconstrictive effect in comparison to Caucasian American (CA) men despite having similar quantifiable sympathetic nerve activity. [3] It can thus be postulated that this exaggerated response in AA men is linked to their higher prevalence and earlier development of CVD. Another area of cardiovascular research with found differences among AA at rest is oxidative stress.

Oxidative stress, a disturbance in the balance of reactive oxygen species (ROS) and antioxidants, at chronic levels is a known a hallmark of CVD’s progression. It is hypothesized that chronic elevations in ROS can damage (i.e. oxidize) cells and other molecules such as proteins and act in signaling pathways associated in the development of atherosclerotic plaques. [2] Atherosclerosis, the leading cause of CVD is characterized by the hardening and narrowing of arteries that consequently reduce the flow and delivery of blood and oxygen throughout the body. Plaque formation in the inner coronary artery walls, consisting of bulks of low density lipoprotein (LDL) cholesterol, cellular waste and surrounding materials are caused by molecular changes induced by inflammatory factors, hormones, growth factors and oxidative species, mainly due to the interaction between endothelial cells, LDLs and macrophages (i.e. mature monocytes). Endocytosis of oxidized LDLs occurs within macrophages, an event
that is slow and, therefore, can lead to an accumulation over time in the intima resulting in the development of atherosclerosis [2] (Figure 2).

Figure 2. Atherosclerotic plaque formation process. LDLs at a high concentration inhibit endothelial cells’ (EC) endocytosis capacity, migrate and accumulate in the intima. LDLs get oxidized and induce VCAM (green square) expression in EC. Monocytes are recruited into the intima by VCAM interaction. Monocytes transform into macrophages, which take-up oxLDLs, forming foam cells. Macrophages and EC secrete chemokines and recruit T-cells. T-cells produce TNF-alpha and IFN-gamma, amplifying inflammation. Fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) stimulate smooth-muscle cells (SMC) migration and proliferation. SMCs can also accumulate lipids, migrate and proliferate. A lipid core is generated with necrotic foam cells surrounded by SMCs and a collagen fibrous cap, resulting in thrombus formation. From Gracia K., et al. *Journal of Clinical Medicine*. 2017
Monocytes, immature macrophages, have thus become of interest in cardiovascular research for their qualities that pertain to the development of CVD. Acting as major regulators of systemic inflammatory reactions, monocytes have an important role in cytokine signaling and processes of destructive (tissue infiltration, tissue damage) and constructive nature (tissue regeneration and healing) in the heart and the vascular system. Monocyte activation, infiltration into damaged tissue, and excessive inflammation has also been associated with ischemia-induced myocardial damage and remodeling [18, 19]. Low tissue oxygen levels could directly activate monocytes and macrophages, which will produce pro-inflammatory mediators and thus cause severe tissue damage. In a rat model of myocardial ischemia and reperfusion, it was shown that depletion of monocytes from the infarcted heart via neutralization of an endothelial cell growth factor (MCP1) can prevent reperfusion injury [20] Monocytes and their role in oxidative stress have also been associated with CVD development in conditions such as hypertension. In one study investigating the role of monocytes in

**Figure 3.** NADPH-oxidase subunit activation and ROS generation. From J.D Lambeth, *Nature Reviews Immunology*, 2004.
hypertension monocytes were depleted in a group of mice followed by a ten-day infusion of angiotensin II (ang II). No elevations in mean arterial blood pressure (MAP) is found, however, when monocytes remained intact in the animals followed by the ang II infusion a significant rise in MAP was exhibited. [6] This suggests monocytes have a major role in CVD development through a mechanism related to hypertension. Besides monocytes, other peripheral blood mononuclear cells (PBMCs) such as T and B cells are associated with CVD and its development. It has been shown that osteopetrotic mice deficient in vascular macrophages, as well as mice deficient in T and B lymphocytes do not present hypertension and vascular remodeling in response to angiotensin II or deoxycorticosterone acetate salt. [21, 23]. Furthermore, angiotensin II, and increased sympathetic activity as well as increased oxidative stress are important modulators of T-cell activation and development of vascular inflammation [23]. T-effector cells interact with innate immune mechanisms to exaggerate, in turn, the inflammatory response via production of proinflammatory cytokines and reactive oxygen species, then contributing to the pathophysiology of hypertension and cardiovascular disease [24]. All PBMCs are primary contributors of oxidative stress with the main sources of ROS including redox enzymes such as the respiratory chain, xanthine oxidase, lipoxygenase, cyclooxygenase, and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases which produce superoxide. The NADPH-oxidase pathway consists of the catalytic subunit gp91phox (otherwise known as NOX1-NOX5 and DUOX), together with the regulatory subunits p22phox, p47phox, p40phox, p67phox found in the cytosol. The enzyme activity of gp91phox is regulated by the assembly of these cytosolic subunits with gp91phox to form an active complex. Once
the assembly proteins reach the membrane, electrons from NADPH are transferred through the enzyme gp91/NOX2 to combine with molecular oxygen extracellularly to generate superoxide (Figure 3).

Thus, quantifying intracellular superoxide generation alongside probing for the NADPH oxidase pathway subunits in PBMCs can be used as measurements of oxidative stress.

In a study comparing PBMCs from young CA and AA men greater levels of intracellular superoxide as well as gp91$^{phox}$ and p47$^{phox}$ expressions were found in the AA men. (Figures 4 and 5).[1] More intracellular superoxide inside of their PBMCs as well as greater expression of the NADPH oxidase protein subunits suggests greater capacity for oxidative stress in AA men and may be a factor in the earlier development of CVD.

![Figure 4](image)

**Figure 4.** Mean data showing peripheral blood mononuclear cells (PBMC)-derived intracellular superoxide production in young black and white men. Data are expressed as relative fluorescence units. Values are means ± SE. *P < 0.05 vs. white men. From Deo SH et al. *Am J Physiol Heart Circ*. 2015
Furthermore, it was suggested that since PBMCs have the ability to cross the blood brain barrier into areas such as the rostral ventrolateral medulla, which is known to regulate sympathetic outflow, it could be plausible for superoxide release from these cells to have a positively correlating effect on sympathoexcitation. [17]. The known elevation in superoxide levels in PBMCs of AA young men may therefore be contributing to their augmented vasoconstrictor effect from SNS output. Conclusively, outcome measurements of the SNS, PBMCs, and oxidative stress are key to CVD research and closing the racial disparity gap in CVD among AA. The ongoing body of research continues to extend important knowledge concerning AA men and CVD however these above data points, specifically PBMCs and oxidative stress, remain

\[ \text{Figure 5. A: individual contiguous Western blots side by side from 2 white and 2 black men showing protein expression of NADPH oxidase subunits (gp91}^{\text{phox}} \text{ and p47}^{\text{phox}}, \text{ angiotensin II type 1 receptor (AT}\text{1}{R}, \text{ and tubulin in PBMCs. Mean data comparing protein expression of gp91}^{\text{phox}} \text{ (B) and p47}^{\text{phox}} \text{ (C), and AT}\text{1}{R} \text{ (D) between white and black men as determined by densitometric analysis of Western blot are also shown. All protein data are expressed relative to that of tubulin. Values are means ± SE. \*} P < 0.05 \text{ vs. white men. From Deo SH et al. Am J Physiol Heart Circ.} \]
elucidated in AA women. Thus, the purpose of my study was to investigate whether PBMC-derived NADPH oxidative stress is elevated in young AA women.
Chapter 2

PBMC-derived oxidative stress in healthy young

African American women

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ABSTRACT

Previous work demonstrates that young healthy AA men have significantly higher PBMC-derived intracellular superoxide production and expression of NADPH-oxidase subunits p47phox and gp91phox compared to CA men. However, whether PBMC-derived superoxide production is also elevated in young healthy AA women remains unknown. Accordingly, we tested the hypothesis that, relative to CA women, AA women would exhibit greater PBMC-derived intracellular superoxide with corresponding expressions of the NADPH oxidase subunit proteins. In ten normotensive AA women and twelve age-matched normotensive CA women, resting intracellular superoxide levels were assessed from freshly isolated PBMCs using dihydroethidium fluorescence within one hour of venous blood sampling. PBMCs were frozen in -80°C and protein was later extracted to assess expression of the NADPH-oxidase subunits p47phox (cytosolic) and gp91phox (membranous) using Western blot analysis in a subset of subjects (AA n=7, CA= n=5). Significantly higher resting intracellular superoxide production was found in AA women compared to CA (AA 4.1±1.9 vs. CA 2.7±1.0 Relative Fluorescent Units; P=0.025) as well as elevated protein expression of (e.g., p47phox: 3.7 ± 1.5, AA vs. 0.5 ± 0.2, CA, P < 0.05). Interestingly, there was no found difference in gp91phox expression among the AA and CA women (gp91phox 5.4 ± 2.4, AA vs. 4.4 ± 0.8, CA women, P < 0.05). These findings indicate that young AA women exhibit greater resting PBMC-derived superoxide production and suggest the NADPH-oxidase pathway may play a role in their elevation in superoxide production. Thus, PBMCs may represent a source of elevated oxidative stress in AA women.
\textbf{Introduction}

Several studies have demonstrated that African Americans (AA) exhibit elevations in systemic oxidative stress compared to Caucasian Americans (CA). Peripheral blood mononuclear cells (PBMCs) have been identified as one of the primary contributors to systemic reactive oxygen species (i.e. oxidative stress) and may be an underlying mechanism for the development of hypertension (HT) and cardiovascular diseases [6]. Previous work demonstrates that young healthy AA men have elevated PBMC-derived superoxide production when compared to CA men [1]. However, whether PBMC-derived superoxide production is also elevated in young healthy AA women remains unknown. This is important because AA women are also more likely to develop hypertension and cardiovascular diseases than women of any other race. Accordingly, this study investigated PBMC-derived superoxide production in young healthy AA and CA women.
Methods

General Procedures

Ten normotensive African American women and twelve normotensive Caucasian American women were studied. All subjects were nonsmokers and were recreationally active (≤3 days per wk) but not training competitively. Exclusion criteria included hypertension (resting blood pressure > 140/90 mmHg; minimum of 5 resting measurements), smoking, and any known cardiovascular, pulmonary, metabolic, neurological disease, or irregular menstrual cycle. After receiving a detailed verbal and written explanation of the intended experimental protocol, each subject provided written informed consent. All experimental procedures and protocols conformed to the Declaration of Helsinki and were approved by the University of Texas at Arlington Institutional Review Board.

On experimental days, subjects arrived at the laboratory in the morning following an overnight fast and without alcohol and physical activity for 24 h. Subjects were positioned supine in a quiet temperature-controlled room (22 to 23°C) and rested for 10 min. Resting arterial blood pressure and heart rate were measured by auscultation of the brachial artery of the right arm using an automated sphygmomanometer. Following the measurement of cardiovascular parameters, 50 ml of blood was obtained from the antecubital vein and collected into sodium heparin tubes for the isolation of primary PBMCs via density gradient centrifugation.
Isolation of PBMCs and detection of intracellular superoxide levels

Following a 50 mL venous blood draw in sodium heparin green top tubes samples were immediately placed on ice and taken to the blood processing laboratory. Whole blood samples were diluted with phosphate-buffer saline (PBS) at a 1:1 ratio and placed in 50mL centrifuge tubes at 20mL aliquots. Ficoll-histopaque was then carefully layered over the diluted blood at a 1:1 ratio and samples were centrifuged at 300xg for 30 minutes. Following centrifugation PBMCs were visible as a milky-white layer between ficoll (below) and plasma (above). All PBMCs were pipetted out and placed in a new 50 mL centrifuge tube where 40 mL of PBS was added. PBMCs and PBS were then centrifuged 460xg for 10 minutes. Pelleted PBMCs were seen at the bottom of the centrifuge tube and PBS above can be decanted. 40 mL of PBS was added to pelleted PBMCs and centrifugation at 460xg for 10 min was repeated. The PBMCs pellet was then reconstituted with 5 mL of PBS and a 20 ul sample used for total and live cell counts using trypan blue stain. A small sample of PBMCs was counted for immediate use for dihydroethidium (DHE) staining while all other PBMCs are aliquoted into 1.5 microcentrifuge tubes for later protein expression and frozen immediately in -80ºC freezer. The same number of PBMCs were plated in triplicates on a black opaque 96-well plate for all subjects. Briefly, ∼1.5 × 10⁶ cells were incubated with 5 μM dihydroethidium for 60 min at 37ºC, and superoxide fluorescence was determined using a fluorescent plate reader at 544- and 612-nm excitation and emission wavelengths, respectively. The data are expressed as relative fluorescence units.
Assessment of protein expression in PBMCs by Western blot analysis.

As described western blot protocol PBMCs were homogenized in lysis buffer, and the cell lysates were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membrane. Subsequently, the nonspecific sites on the membrane were blocked with 5% nonfat dry milk for 60 min and then probed with primary antibodies against gp91\textsuperscript{phox} (membranous) and p47\textsuperscript{phox} (cytoplasmic) subunits of NADPH oxidase enzyme 4°C overnight. All blots were incubated with horseradish peroxidase-conjugated anti-IgG and later detected by enhanced chemiluminescence. GAPDH was used as a loading control, and all protein data are expressed relative to that of GAPDH. Eight and or six samples were run on each gel per target patterned for each sample’s equal treatment on each blot. Although gels were run separately, the protein targets were all probed at the same time. Densitometric analysis of the bands was performed with image analysis software.

SEE APPENDIX B FOR FULL DETAILS OF PROTOCOLS
Statistical Analysis

Group comparisons between CA and AA were conducted using unpaired Student’s t-tests. In addition, we also performed a simple correlation to examine the potential influence of estrogen and progesterone on PBMC-derived superoxide production. Results are presented as means ± SE. For all analyses, statistical significance was set at $P < 0.05$. 
RESULTS

Table 1. presents general characteristics for all subjects. There were no significant differences in age, body mass index, resting mean arterial pressure, or resting heart rate between groups. AA women exhibited significantly greater basal intracellular superoxide production in PBMCs compared with CA women ($P = 0.025$; Fig.6). Also, protein expression for $p47^{phox}$ was significantly upregulated in the AA women ($P < 0.05$; Fig.7, A). And No difference was found between groups for $gp91^{phox}$ expression (Fig.7, B). Also, no correlation was observed between superoxide production and estrogen and progesterone (Fig, 8.).

Figure 6. PBMC derived intracellular superoxide expression in healthy young CA and AA women. Young AA women exhibited significantly greater PBMC intracellular superoxide levels compared to CA women. *$P = 0.025$
Figure 7A. p47phox protein expression in PBMCs from young healthy CA and AA women. PBMCs from young AA women exhibited significantly greater protein expression of p47phox compared to CA women. *P < .05

Figure 7B. gp91phox protein expression in PBMCs from young healthy CA and AA women. No difference was found in gp91phox protein expression in PBMCs from young healthy AA compared to CA women. P = 0.25
Figure 8. Relationships between PBMC derived intracellular superoxide and sex hormones. No relationship was found between PBMC intracellular superoxide and estradiol (A), progesterone (B) or the progesterone/estradiol (P:E2) ratio (C).

Table 1. Subject Characteristics

<table>
<thead>
<tr>
<th></th>
<th>CA, n=12</th>
<th>AA, n=10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>23±1</td>
<td>22±1</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.4±0.7</td>
<td>24.8±0.6</td>
</tr>
<tr>
<td>Mean Arterial Pressure (mmHg)</td>
<td>79±3</td>
<td>83±2</td>
</tr>
<tr>
<td>Heart Rate (bpm)</td>
<td>63±3</td>
<td>62±3</td>
</tr>
<tr>
<td>Estradiol (pg/mL)</td>
<td>65.0±10.0</td>
<td>71.1±15.2</td>
</tr>
<tr>
<td>Progesterone (ng/mL)</td>
<td>0.31±0.07</td>
<td>1.71±1.25</td>
</tr>
</tbody>
</table>
DISCUSSION

It was demonstrated that young AA women exhibit greater basal intracellular superoxide production and protein expression for the NADPH oxidase subunit p47^phox in freshly isolated PBMCs compared with young CA women. These findings are in parallel with past findings in young AA men.

Elevations in NADPH oxidase activity and oxidative stress have been linked with the development of cardiovascular disease such as hypertension (9-16). Indeed, the greater expression of the NADPH oxidase subunit p47^phox and elevated intracellular superoxide levels in PBMCs of young AA women suggests this pathway may represent a therapeutic target to reduce oxidative stress and its associated negative cardiovascular consequences. Studies have shown elevations in oxidative stress can contribute to many cardiovascular-related detriments, including endothelial dysfunction, sympathoexcitation, apoptosis, increases in intracellular calcium, and fibrosis (9,11,12). Thus, elevated oxidative stress in young AA women may contribute to the greater predisposition for the development of cardiovascular disease in this group. Importantly, interestingly, we did not detect a correlation between PBMC-derived superoxide production and estrogen or progesterone levels. However, further research into PBMC superoxide production and the difference phases of the menstrual cycle is needed considering numbers in our groups for days 1-5 menses phase was considerably underpowered. Although higher superoxide trends were observed in the AA women in
all phases compared to CA women, more research is needed throughout the cycle phases in order to see any existing possible effects. Indeed, additional studies are warranted.

Also, possibly of interest to our findings is data collected by Feariheller et al on AA human umbilical vein endothelial cells (HUVECs). (8) An upregulation of NADPH oxidase subunits and lower mitochondrial superoxide dismutase-2 (SOD2) was found in AA compared with CA HUVECs. Importantly, this lower SOD2 in HUVECs of AA could also be present in PBMCs causing greater levels of superoxide production to be exhibited. Not studied in PBMCs, further research is needed. Furthermore, future ex vivo studies on NADPH-oxidase activation in PBMCs of AA and CA may reveal further differences in the regulation of superoxide production.

In summary, we report a greater basal intracellular superoxide production and an upregulation of the NADPH oxidase pathway in PBMCs from young AA women compared with CA women.
Chapter 3

Future Direction and Conclusion
Future Direction

A current major cause of death among African Americans (AA) is cardiovascular related diseases (CVD) with AA women at highest risk among both sexes and all other races. Our study showed that oxidative stress, a known precursor to CVD, is elevated in peripheral blood mononuclear cells (PBMCs) of young AA women compared to CA women. However, further research is needed to conclude whether estrogen and progesterone may play a role in this production of oxidative stress (i.e. superoxide). One future direction of this research will include studying women in specific phases of the menstrual phase and possibly testing a subset of women during early follicular and mid follicular of same cycle. Furthermore, considering that in AA human umbilical vein endothelial cells (HUVECs) an upregulation of NADPH oxidase subunits as well as lower mitochondrial superoxide dismutase-2 (SOD2) was found compared to CA HUVECs it could be of interest for future research that PBMCs be tested ex vivo for racial differences in their antioxidant capacity [8]. Furthermore, considering the NADPH-oxidase system was found to play a role in this superoxide production within the PBMCs of AA women it may also be of interest to conduct ex vivo studies with these PBMCs to further investigate the activation of the NADPH-oxidase subunits. To directly test NADPH oxidases role in PBMC derived superoxide production between AA and CA women, PBMCs can be placed in culture and apocynin or diphenylene iodonium can be applied to inhibit NADPH oxidase activity [25, 26].
Conclusion

In summary, the findings of this study show that AA women exhibit elevated PBMC-derived superoxide production through the NADPH-oxidase enzyme pathway compared to CA women. Although no relationship was found between PBMC derived superoxide production and sex hormones, additional studies will be needed, particularly in which the young women are studied in different phases of the menstrual cycle. The elevation in intracellular superoxide in AA women could be indicative of their higher risk for CVD and related conditions such as hypertension. This NADPH-oxidase pathway also serves as a possible target for therapeutic strategies in CVD development and related interventions considering oxidative stress in these women might be directly connected to the earlier onsets of these diseases within their race.
Bibliography


Appendix A

Autobiography

Research background:

I began research as an undergraduate in the University of Texas at Arlington (UTA) Exercise Science program within the motor control and development laboratory under the direction of Dr. Priscila Caçola in 2014 studying the effects of developmental coordination disorder (DCD) in children.

My graduate research began in 2016 under the direction of Dr. Paul J. Fadel in the Human Neural Cardiovascular Control Lab where my master's thesis work on peripheral blood mononuclear cells as they relate to oxidative stress in African American women was originated.

Conferences attended:

ACSM Texas 2017

ACSM Texas 2018

Experimental Biology 2017

Experimental Biology 2018
Appendix B

Protocols

1. Isolation of PBMCs
2. PBMC intracellular superoxide probing with dihydroethidium
3. Protein isolation from PBMCs
4. Western Blot analysis – protein analysis of NADPH-oxidase subunit expression
Isolation of Human Peripheral Blood Mononuclear cells (PBMCs) from Whole Blood

- Blood draw (~50 ml) using 23G needle in sodium heparin tubes, taking an initial ~3 ml of waste blood.
- Gently dilute whole blood 1:1 with PBS (~20 ml of whole blood mixed with ~20 ml) of PBS in two 50 ml tubes.
- Add 20 ml of ficoll to 4 separate 50 ml tubes and label. Ensure the XL pipette setting is on slow/slow and gently layer 20 ml of diluted blood over 20 ml ficoll in each tube.
- Centrifuge the tubes at 300 X g for 30 min with acceleration set to 1 and deceleration set to 0 (without brakes) at room temperature.
- Examine the separation of RBCs at the bottom, clear layer of middle ficoll, cream to yellow colored plasma up top and hazy layer containing PBMCs is between ficoll and plasma.
- Pipette out the hazy layer in another 50 ml tube and add 15 to 20 ml of DPBS (1:10 dilution; 50 ml of 10X DPBS + 450 ml of double distilled water).
- Centrifuge at 460 X g for 10 min with acceleration set to 9 and deceleration set to 9 (with brakes) at room temperature.
- Decant the supernatant and reconstitute the pellet in 15 to 20 ml of DPBS and centrifuge at 460 X g for 10 min with acceleration set to 9 and deceleration set to 9 (with brakes) at room temperature.
- Decant the supernatant and reconstitute the pellet in 5 ml of DPBS. Count the cells.
- To count the cells:
  - Add 1:1 volume of sample and trypan blue in a microcentrifuge tube (20 ul sample + 20 ul trypan blue).
  - Turn on Countess II automated cell counter and transfer 20 ul of mixed sample to slide.
    - Insert slide to Countess II and adjust focus of picture. Count viable cells (cell that take up blue stain are dead cells. Do not count them). Reinsert slide and repeat count. Take average of 1st and 2nd counts.
    - Countess II will give number of cells per ml. Calculate for 5 ml.
- Aliquot at least ~1.0 ml for western blot in separate labeled 1.5 ml tubes.
- See Protein extraction protocol.
Superoxide measurement using Dihydroethidium (DHE) fluorescence

- Superoxide Measurement:
  - DHE stock: We need 5uM and MW: 315.42
    - To make 10mM solution: We have 1mg of DHE (commercial) + 317.03ul of DMSO
    - Make 1:400 dilution of 10.a. to make 25uM solution: 7ul from 10.a. + 2793ul of DPBS = 2800ul of 25uM DHE solution
    - By adding 50ul of DHE (25uM) to the total volume of 250ul in each well will make the final concentration of 5uM for DHE.
  - Make concentration of PBMCs in separate tube: $2.5 \times 10^6$ cells/ml
  - Plate $0.5 \times 10^6$ cells/well in black opaque 96-well plate. Perform in duplicates.
  - Plating:
    - A1-A2 (200 ul PBMCs) + (50 ul DHE)
    - C1-C2 (200 ul PBS) + (50 ul DHE)
    - E1-E2 (200 ul PBMCs) + (50 ul PBS)
  - Place clear plate sealer on well plate and incubate at 37°C, 5% CO₂ for 60min.
  - Centrifuge the plates @ 500Xg for 5min and decant the supernatant.
  - Re-suspend the cells in 250ul of DPBS.
  - Read the plate in Spectramax fluorescent plate reader.
    - Excitation filter at 530-560nm (544nm)
    - Emission filter at 590-650nm (612nm)
Pelleting protein and cell lysis for Western Blot protocol - 1 mL Aliquot of PBMCs

- Spin down 1 mL sample of PBMCs on highest speed setting for 10 min. at room temperature to get pellet.
- Decant supernatant, add cell lysis buffer (350uL) + (3.5uL PMSF)
- Vortex mix well
- Centrifuge on highest speed setting for 20 min. at 4°C.
- Aliquot supernatant into 50 uL samples in microcentrifuge tubes.
- Label and save 50 uL samples in -80°C freezer.
Western Blot Protocol

- Get ice.
- Gather protein samples from -80 freezer and place over ice.

Protein Estimation using BSA (Pierce) colorimetric assay for Western Blot

- Mix two reagents supplied in the kit
  - Reagent A (9.8ml) + Reagent B (0.2ml) in a 15ml tube. (The kit is located in the cabinet on the left wall as you enter the VA lab)
- Pipette out 200ul in each well.
- Pipette 10ul of standards (nine (A to H): 0, 0.125, 0.25, 0.5, 0.75, 1, 1.5, 2) in duplicates in the well plate. [The standards are in freezer (4°C)]
- Pipette 10ul of unknown sample in duplicates and cover the plate with plastic cover.
- Shake the plate for 2min (1000rpm per min)
- Incubate the plate at 37°C for 30min.
- Take off the plastic cover and read the plate.
- How to operate SPECTRAMAX plate reader; Follow the steps below:
  - Login
  - Open new experiment
  - Protocols
  - Protocol manager
  - Protocol library
  - Protein quant
  - BCA
  - Click on Plate 1: Plate layout – Standards and Unknowns
  - Read Plate
  - Save
  - Ok
  - Export to excel

Western Blot

Day 1

- Calculate the amount of sample(s) to load. See excel sheet for calculations. And dilute samples accordingly with laemmli buffer.
- Boil the samples for 10 minutes.
- Samples are then chilled in ice for 10 minutes.
- Open the gel package and rinse wells out with ddH2O.
  - Take green comb out of the wells and then peel off the green tape from the bottom of the gel.
  - Put the gel in electrophoresis box with the wells facing inward and use the buffer dam on the other side if you are only running 1 gel.
  - Fill the middle full with 1x running/working buffer (1:10 TGS = 100ml of 1XTGS=SDS buffer + 900ml of H2O in a measuring cylinder) and let it fill up to the fill mark on the outside of the box.
• Load your ladder and samples.
• Close the box connect the red wire to red electrode and black wire to black electrode.
• Run at 70 V for 10 minutes and then switch it to 120 V for 45 min to an hour.
  o If the samples start to frown, turn the voltage up. If they start to smile, turn the voltage down.
  o When run is almost complete get pre-made blot membrane from 4 degree.
• When the run is complete, crack open the gel and cut across the teeth of the gel.
• Using the Trans-Blot Turbo:
  1. Once PVDF blot sandwich is made and placed in cassette, close twist/lock it and place in A or B slot.
  2. Select Turbo > select gel size and quantity > 7 min run > select drawer A or B.
• While running the membrane, start dissolving blocking buffer on rocker plate. 5% milk in TBST [40ml of TBST + 2g of non-fat milk powder].
• Once the run is complete, soak the membrane in blocking buffer for 1 hour at room temperature.
• If you’d like to check your membrane you may pour some Ponceau S dye (P7170-1L Sigma) on it and then rinse it clear with DDH2O. Your protein will dye red.
• Dilute your primary antibodies p22, gp91, p47, and GAPDH (37Kd) in 5% nonfat milk in TBST and incubate blot in boxes overnight at 4˚C on the rocking plate.

Day 2
• Take the boxes out in the room temperature on the rocker for 10min.
• Wash 3x’s with 5% nonfat milk in TBST (to reduce background) for 5 minutes each.
• Prepare the secondary antibody containing HRP conjugate in the dilution of 1:5000 in TBST with 5% non-fat milk powder.
• Incubate with secondary antibodies for 1 hour at room temperature on the rocker.
• Wash 3x’s with TBST for 5 minutes each.
• Wash 3x’s with TBS for 5 minutes each.
• ECL (1:1 ratio) using the Femto ECL (#34095 Pierce) and image on the chemidoc.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Company</strong></td>
<td><strong>Target</strong></td>
</tr>
<tr>
<td>Mouse anti-gp91phox</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Mouse anti-p22phox</td>
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<td>Goat anti-p47phox</td>
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<tr>
<td>GAPDH</td>
<td>ThermoFisher</td>
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