RACIAL DISPARITIES IN VASCULAR FUNCTION:

THE ROLE OF OXIDATIVE STRESS

by

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DISSERTATION

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ABSTRACT

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Hypertension disproportionately affects the black population, contributing to a greater impact of cardiovascular disease in these individuals. While multiple factors underlie the development of hypertension in this population, impaired vascular function is routinely observed in this population beginning at a young age and is a likely an early contributor to the development of high blood pressure. However, the origins of this impairment have not been elucidated. Therefore, the current set of studies aimed to determine the extent to which oxidative stress plays a role in blunted vascular function in the black population. Studies 1 and 2 (Chapters 2 and 3) utilized intradermal microdialysis to mechanistically investigate the causes of blunted cutaneous microvascular function in this population. In Study 1, the enzymes NADPH oxidase and xanthine oxidase were identified as potential contributors to superoxide-mediated reductions in otherwise

healthy, young black men, whereas they did not appear to play a significant role in the blunted local heating response observed in young black women. Study 2 aimed to test whether NADPH oxidase inhibition, xanthine oxidase inhibition, or tetrahydrobiopterin supplementation could augment blunted muscarinic-mediated cutaneous dilation in black men. While the findings of that study were somewhat inconclusive, our observation of potential phenotypic differences provides important rationale for future research. Lastly, Study 3 (Chapter 4) aimed to determine the acute peripheral vascular effects of an oral antioxidant cocktail on vascular function in young black men. Utilizing a randomized, double blinded design, reactive hyperemia and brachial artery flow mediated dilation (FMD) were assessed pre- and 120 min post- antioxidant and placebo treatments. Unexpectedly, vascular function was blunted at the post timepoint across both conditions, likely due to the sedentary nature of the protocol. FMD was paradoxically reduced to a greater extent in the antioxidant condition. The work described herein is an important initial step in determining the role of oxidative stress on vascular dysfunction in black individuals. Future research building upon these findings may ultimately result in therapies to lessen the disparate burden of hypertension on the black population.

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DEDICATION

I dedicate this dissertation to my wife Amanda whose support and encouragement helped to initiate this undertaking and got me to the finish line, and for that I am incredibly grateful. Spending three years separated by two hundred miles and seeing each other a few days per month was a huge sacrifice, but we made it through stronger than ever.

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Chapter 1

INTRODUCTION

Hypertension, Race, and Vascular Dysfunction

The age-adjusted mortality rate for cardiovascular diseases (CVD) and stroke is approximately 30% greater in the black population relative to their white counterparts in the US (2). High blood pressure, or hypertension, is a primary cardiovascular risk factor and its prevalence is similarly elevated in the black population (2). Furthermore, hypertension develops more rapidly in blacks (52). Therefore, it is imperative to identify the initial factors that contribute to its development. A clear understanding of the underlying causes of this racial disparity in hypertension can improve treatment and prevention strategies and ultimately lessen the burden of disease in the black population.

While the pathogenesis of hypertension is multifactorial, vascular dysfunction occurs in populations predisposed to high blood pressure (38, 58, 59) and predicts the onset of hypertension (40). Vascular dysfunction can acutely influence peripheral resistance, a prime determinant of blood pressure, but more importantly, vascular dysfunction induces inward remodeling of resistance vessels, thus augmenting peripheral resistance (48). Nitric oxide (NO) produced in the endothelium is a key regulator of this smooth muscle cell proliferation (21, 49). In an experimental model of vascular dysfunction, chronic inhibition of nitric oxide synthase (NOS), results in vascular inflammation, thickened vessel walls, and reduced lumen diameters, thus illustrating how vascular dysfunction can lead to structural impairments (28).

There is significant evidence of blunted vasodilation in black individuals without overt disease (See Racial Differences in Vasodilation below). The aim of the current project was to determine potential mechanisms that contribute to impaired vasodilation in blacks. Contrary to

vasoconstriction, vasodilation is primarily a local phenomenon to direct blood to specific tissues for the delivery of oxygen and nutrients and the removal of metabolites. Endothelium-dependent dilation results from physical (e.g. shear stress) or chemical (e.g. acetylcholine, bradykinin) stimulation of the endothelium. Relaxation of vascular smooth muscle then follows due to endothelial release of prostaglandins and a variety of endothelium derived hyperpolarizing factors (EDHF), in addition to NO. Reductions in vasodilation due to impaired production and/or release of these substances is termed endothelial dysfunction whereas, impaired response to these substances is known as impaired endothelium-independent dilation or vascular smooth muscle dysfunction.

Assessment of Vascular Function

Multiple techniques are used to assess vascular health, and each has advantages and disadvantages. Direct arterial infusions of endothelium-dependent vasodilatory chemicals (acetylcholine, bradykinin, ATP) or endothelium-independent vasodilators such as the NO donor sodium nitroprusside (SNP) can be infused directly into the arterial circulation while changes in flow are detected via thermodilution, strain-gauge plethysmography, or Doppler ultrasound. This technique allows for interrogation of specific pathways and a clear delineation between endothelium-dependent and -independent function. However, its invasive nature limits its use. In contrast, reactive hyperemia is the substantial increase in blood flow that occurs upon cessation of an extended period (typically 5 min) of circulatory occlusion that can be measured non-invasively via strain-gauge plethysmography or Doppler ultrasound. Like arterial infusions, this provides an index of vasodilation in the resistance vessels or microvasculature. The non-invasive nature of reactive hyperemia limits its usefulness in determining mechanisms of any observed dysfunction.

Reactive hyperemia increases shear stress on the endothelial wall of the up-stream conduit artery. This shear stress, in turn, results in the endothelium-dependent release of local vasodilators, primarily NO, and elicits flow-mediated dilation (FMD). FMD is commonly assessed in the brachial artery and is used as a non-invasive index of vascular function. FMD is quantified as the minute change in arterial diameter that occurs approximately 60 s after the increase in shear due to reactive hyperemia. This technique requires a high-resolution Doppler ultrasound, specialized edge detection software, and a skilled technician (16, 61). There is also debate over how to normalize results to the reactive hyperemia stimulus (44).

Finally, the cutaneous microvasculature has recently been presented as a representative vascular bed that can be utilized to investigate mechanisms of vascular dysfunction in using minimally invasive techniques (19). The least invasive technique is iontophoresis which uses an electrical current to drive vasoactive substances, like acetylcholine or SNP into the cutaneous microcirculation, where changes in perfusion are quantified via integrated fiber laser Doppler flowmetry or laser speckle imaging (18). This technique is limited by the minimal substances that can be infused, and by the potential for results to be confounded by differences in skin electrical resistance, or non-specific, vehicle induced dilation (27, 45). In contrast, microdialysis allows for the local infusion of any liquid soluble substance via a semipermeable membrane placed in the intradermal space. Vascular function can then be quantified by the response to the direct infusion of vasodilator / vasoconstrictor substance. Additionally, local heating elicits a local increase in blood flow that is primarily NO-dependent (8, 25, 35) and is impaired in hypertensives (53), hypercholesteremics (56), chronic kidney disease patients (11), polycystic ovarian syndrome patients(55), smokers (14), and elderly individuals (36).

Racial differences in vasodilation

Blunted increases in blood flow during intra-arterial infusions of the muscarinic receptor agonist acetylcholine have been demonstrated in normotensive black subjects relative to age-matched white subjects in multiple (6, 22, 39, 57), but not all (23), investigations. Likewise, the increase in blood flow due to the acetylcholine analog, methacholine, is also reduced in healthy black subjects (57). Importantly, this racial difference is abolished when nitric oxide synthase (NO) is inhibited, thus indicating that the difference is due to a reduction in NO bioavailability in black subjects (6, 39, 57). However, this interpretation is complicated by the observation that responses to the exogenous NO donor SNP are sometimes (6, 39, 57), but not always, (22) blunted in this group. Notably, one study has shown that black subjects who did not present impaired endothelium-independent responses to exogenous NO were in their early 20s (22) while both groups that indicated impairments were around 2 decades older (6, 39, 57). Amongst blacks, early reductions in NO bioavailability may progress to impaired smooth muscle responsiveness to NO with age, however, the longitudinal studies needed to test that hypothesis have not been completed.

Impaired dilation to non-acetylcholine stimuli is also present in black individuals, as demonstrated by lower responses to intra-arterial bradykinin (39, 47) and isoproterenol (6, 26, 57) in this group relative to white subjects. In addition, the NO-mediated increase in forearm blood flow during mental stress is attenuated in black individuals (7). Taken together, these results indicate that the racial differences cannot be attributed solely to reduced muscarinic receptor number or sensitivity.

The role of NO in reactive hyperemia is limited (60), with much of the dilation occurring due to non-NO, non-prostaglandin, hyperpolarization (9, 12). Thus, any impairment in reactive hyperemia amongst blacks would suggest that racial differences extend beyond the NO pathway. Blunted reactive hyperemia in black individuals has been observed at adolescence (10), early adulthood (17, 46), and midlife (37). While not reactive hyperemia per se, Bond and colleagues demonstrated that minimal vascular resistance following a 10 min occlusion in the forearm, superimposed with handgrip exercise was greater in young, healthy black men than age-matched white men (1). The authors suggest that this technique produces a maximal microvascular dilation, and thus indicates that racial differences are structural in nature (i.e. reduced number or size of resistance vessels) and not a functional impairment. In contrast, Cardillo and colleagues observed no difference in reactive hyperemia despite the same subjects exhibiting reduced responses to the intra-arterial infusions of ACh, isoproterenol, and SNP (6).

Endothelial function of the conduit arteries, as assessed by FMD, is impaired in black individuals. Perregaux and colleagues were first to demonstrate that healthy, young black men and women exhibited reduced brachial artery FMD, yet no difference in the dilation of this vessel following sublingual nitroglycerin (41). These data indicated conduit artery vasodilatory dysfunction in blacks is confined to endothelium dysfunction while smooth muscle function was not impaired relative to white individuals. In contrast, Campia et al., found that both FMD and nitroglycerin responses were impaired in blacks (5). Notably, the age of the participants in the latter study was older and more variable. In stark contrast to both of those studies, a larger study with middle-aged individuals found no difference in FMD or nitroglycerin induced dilation of the brachial artery in black men and women relative to their white counterparts (15). Importantly, none

of these early studies using FMD accounted for the shear stimulus that elicits the FMD, and therefore any reduction in FMD may be attributable to lower reactive hyperemia (43).

Only a few studies have utilized the cutaneous microvasculature in the study of potential racial differences. Maley and colleagues observed blunted responses to acetylcholine iontophoresis in black individuals in Australia compared to white controls (30) and found that this difference is not explained by prostaglandin activity (29). Another group in South Africa demonstrated a racial difference in cutaneous microvascular dilation during iontophoresis of acetylcholine and SNP, however racial differences were removed when skin electrical resistance was considered (42). Most recently, our group has demonstrated impaired local heating induced hyperemia in healthy, young black individuals relative to age-matched white individuals (20). Utilizing microdialysis to locally deliver the NOS inhibitor L-NAME, we found the group difference was attributable to NO (20).

Mechanisms of attenuated NO-dependent vasodilation

The mechanisms involved in vascular dysfunction in black individuals have not been clearly elucidated. Oxidative stress or excessive reactive oxygen species (ROS) is a common cause of endothelial dysfunction that initially reduces NO bioavailability via direct inactivation of NO to form peroxynitrite and subsequent uncoupling of eNOS (3, 32, 50, 51). In-vitro studies utilizing human umbilical vein endothelial cells (HUVECs) provide evidence for oxidative stress as the cause of impaired vasodilation in blacks. A study by Kalinowski et al. demonstrated that when stimulated via calcium ionophore, the endothelial cells from black donors produced more superoxide and peroxynitrite while producing less NO compared to cells from white donors (24).

Together, with the observation of increased expression of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase and a paradoxical increase in endothelial NOS (eNOS), the authors speculated that vascular dysfunction occurs in black populations due to NADPH-oxidase derived superoxide and subsequent oxidation of the eNOS co-factor tetrahydrobiopterin (24). These results were supported by Feairheller and colleagues (13). Furthermore, colleagues of Kalinowski observed that when these cell from black donors were incubated in nebivolol, a β blocker with antioxidant properties, NO production was restored while superoxide and peroxynitrite were lessened (24). Within the same study, another β blocker failed to effect NO and ROS production, while a specific NADPH oxidase inhibitor elicited similar results as the nebivolol, indicating a primary role for NADPH oxidase in ROS-mediated reductions in NO bioavailability (24). Further supporting a role for greater superoxide production via NADPH-oxidase in the black population, Deo and colleagues presented elegant data showing greater expression of NADPH-oxidase expression and superoxide production in peripheral blood mononuclear cells (PBMC) of healthy, young black men relative to age-matched white men. Our lab then demonstrated that dismutation of superoxide via locally infused tempol augments cutaneous microvascular responses to local heating in college-aged blacks, further supporting the role for elevated superoxide in the impairment of vasodilation in this population (20).

Statement of the problem

Racial differences in vascular function are not well described. The evidence of a role for NADPH oxidase-induced oxidative stress is strong (13, 24, 31), but indirect, as it has not been shown to affect the arterial circulation in-vivo. Additionally, the effects of NADPH oxidases may actually be attributable to subsequent activation of xanthine oxidase (33, 54) or eNOS uncoupling

via reductions in the essential cofactor tetrahydrobiopterin (34, 62), but these pathways have not been studied. While oxidative stress and reduced NO bioavailability result in impaired vasodilation, they also lead to vascular remodeling, which can lead to chronically elevated total peripheral resistance and contribute to elevations in blood pressure (4, 48). Determination of the specific enzymatic sources of oxidative stress may facilitate the development of optimal therapies to treat or prevent the development of hypertension in black individuals. The following studies aim to a) determine the enzymatic source of elevated superoxide in the cutaneous microvasculature of black individuals (Chapters 2 and 3) and b) determine if oral antioxidants augment measures of whole-limb microvascular function and conduit artery vasodilation in young black men (Chapter 4). Taken together, these studies aim to determine the role of oxidative stress in the development of reduced vasodilatory function in young, otherwise healthy black individuals.

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Chapter 2

SEX DIFFERENCES IN THE MECHANISMS MEDIATING BLUNTED CUTANEOUS MICROVASCULAR FUNCTION IN YOUNG BLACK MEN AND WOMEN

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Abstract

The black population exhibits attenuated vasodilatory function across the lifespan; yet little is known regarding the mechanisms of this impairment. Recent evidence suggests a potential role for oxidative stress. Therefore, we tested the hypothesis that NADPH oxidases (NOX) and/or xanthine oxidase (XO) contribute to blunted nitric oxide (NO)-mediated cutaneous microvascular function in young black adults. In 30 white and black subjects (8M/7F in each group), local heating was performed while NOX and XO were inhibited by apocynin and allopurinol, respectively, via intradermal microdialysis. The plateau in cutaneous vascular conductance (CVC: red blood cell flux / MAP) during 39 °C local heating at each site was compared to a control site perfused with lactated Ringer's solution. Subsequent inhibition of NO synthase via L-NAME allowed for quantification of the NO contribution to the vasodilation during heating. Black individuals, relative to white, had a blunted CVC plateau at the control site (45±9 vs 68±13 %max, $P<0.001$) that was augmented by both apocynin (61±15 %max, $P<0.001$) and allopurinol (58±17 %max, $P=0.005$). Black men and black women had similar responses to heating at the control site ($P=0.99$), yet apocynin and allopurinol augmented this response only in black men (both $P<0.001$ vs control). NO contribution was also augmented via apocynin and allopurinol exclusively in black men. These findings suggest that cutaneous microvascular function is reduced due to NOX and XO activity in black men but not black women, identifying a novel sex difference in the mechanisms that contribute to blunted vascular responses in the black population.

Introduction

Cardiovascular diseases (CVD) are the leading causes of morbidity and mortality worldwide, and the black population experiences an even greater burden of cardiovascular disease than their white counterparts (1). Furthermore, one of the leading risk factors for CVD, hypertension, develops at an earlier age and contributes to a higher death rate amongst black individuals (1). The cause of hypertension is multifactorial, however microvascular dysfunction has been implicated in its development (32, 42). Indeed, blunted microvascular function is present in the black population as early as adolescence (11) and into adulthood (7, 17, 33). However, the mechanisms responsible for this apparent racial disparity in microvascular function have not been fully elucidated, though oxidative stress is a potential candidate.

With the use of intradermal microdialysis and laser Doppler flowmetry, the cutaneous microcirculation serves as an easily accessible model of the systemic microvasculature for exploration of the mechanisms underlying vascular function (20). Cutaneous microvascular responses to local heating are 50-80% nitric oxide (NO)-mediated, depending upon the protocol (3, 9, 31), and are attenuated in conditions associated with increased cardiovascular risk, including hypertension (6), polycystic ovarian syndrome (41), chronic kidney disease (12, 13), and hypercholesterolemia (19). Our lab has recently observed blunted increases in cutaneous vascular conductance (CVC) during a standard local heating protocol in a cohort of healthy, college-aged black individuals relative to an age- and sex-matched white group (22). Notably, we showed that the racial difference was mitigated with local infusion of the superoxide dismutase mimetic tempol, suggesting superoxide contributes to impairments in microvascular function in black individuals early in life (22).

While there are multiple potential sources of superoxide, there is evidence of greater expression and activity of NADPH oxidases (NOX) in endothelial (14, 24) and peripheral blood mononuclear cells in black individuals (10). Xanthine oxidase (XO) is another enzymatic source of superoxide expressed in endothelial cells, but, to our knowledge, its influence on vascular function has not been examined in black individuals. Therefore, the aim of the present study was to test the hypothesis that local inhibition of NOX and XO in black individuals would augment the blunted cutaneous microvascular dilation response to local heating. Furthermore, we hypothesized these effects would be due to a greater NO contribution following NOX and XO inhibition.

Methods

Subjects: Thirty white and black young adults (8 men and 7 women in each group) were studied (Table 1). Subjects self-reported their racial identity and that of their parents. Individuals of mixed race were excluded from the study, as were smokers and trained athletes. All subjects were normotensive and free of overt cardiovascular, metabolic, or neurological disease and not currently taking antioxidant supplements or medications, other than oral contraceptives. Women were studied during the early follicular phase of the menstrual cycle or placebo week if taking oral contraceptives (day 3 ± 1 of menses). Subjects were instructed to fast overnight and abstain from alcohol, caffeine, and strenuous activity for 24 hours prior to the study. The study protocol was approved by The University of Texas at Arlington Institutional Review Board and all subjects provided written informed consent prior to participation in accordance with the Declaration of Helsinki.

Descriptive characteristics: Upon arrival to the laboratory, height and weight were assessed using a digital scale and stadiometer (Seca 769, Seca North America, Chino, CA). Waist circumference was assessed via a flexible tape measure at the level of the naval. Blood was then drawn via venipuncture from an antecubital vein into a serum separator tube, allowed to clot for 30 min, and then centrifuged at 1900 x g for 10 min. Serum samples were then sent to a local laboratory for lipid profile and comprehensive metabolic panel assessment (LabCorp, Dallas, TX).

Instrumentation: Subjects were comfortably positioned semi-recumbent with their left arm supported at approximately heart level in an environmentally controlled laboratory (~23 ºC, 40% humidity). Using aseptic techniques, a 25-guage needle was then used to pierce the skin of the dorsal forearm so that ~2.5cm of the needle remained below the skin. A microdialysis fiber (CMA 31, Harvard Apparatus, Hollister, MA) was then advanced through the lumen of the needle and the needle was withdrawn to place the 10mm semipermeable membrane (55 kDa) in the skin. This process was repeated for a total of 3 microdialysis sites, each located a minimum of 2.5cm apart with care taken to avoid superficial veins. Each membrane was then perfused with lactated Ringer's solution at a rate of 2 µl/min via a syringe pump (Model 11, Harvard Apparatus). Laser Doppler flowmetry probes with integrated temperature sensors (VP7a/T Moor Instruments, Wilmington, DE) housed inside of local heating units (PF-450 Perimed Instruments, Stockholm, SE) were then secured directly over each microdialysis site. Cutaneous blood flow was indexed as red blood cell flux (RBF: arbitrary units) via the laser Doppler flowmetry monitor (moorVMS-LDF, Moor Instruments) throughout the protocol. Trauma-induced hyperemia was given 60 min to resolve at which time a 30 min apocynin and allopurinol wash-in period began while the control site continued to receive lactated Ringer's solution. Continuous measures of RBF and local

temperature were recorded at 400hz via a data acquisition system and saved on a laboratory computer for offline analysis (PowerLab and LabChart, ADInstruments, Colorado Springs, CO).

Pharmacological Agents: Apocynin (100µM, Sigma Aldrich, St Louis, MO) and allopurinol (10µM, Sigma Aldrich) were utilized to inhibit NOX and XO, respectively. Drug dosages were chosen based upon previously published literature (13, 30). Drugs were prepared via dilution in lactated Ringer's solution each day just prior to infusion and were infused at a rate of 0.2µl/min following a 30s period of priming at 5.2 µl/min. The order of sites (apocynin, allopurinol, or control) was randomly assigned using computer-generated randomization. The nitric oxide synthase (NOS) inhibitor L-NAME (20 mM Nω-Nitro-L-arginine methyl ester hydrochloride, Sigma Aldrich) and the NO donor SNP (28mM sodium nitroprusside dihydrate, Sigma Aldrich) were later perfused through all sites, to assess NO contribution and maximal RBF (see Protocol details below).

Protocol: We chose to use 39 °C local heating based upon the work of Choi and colleagues demonstrating that NO is responsible for a greater percentage of the plateau phase dilation at this temperature compared to 42 °C (9). An additional benefit to this protocol is that the responses to the lower temperature are less likely to be constrained by a ceiling effect since the typical plateau in skin blood flow at 39 °C is ~50-80% of max compared to 90-95% at 42 °C (9). The protocol is illustrated in Figure 1. After a 30 min drug wash-in period, local heaters were set to 33 ºC to account for inter-site and inter-individual variation in skin temperature. Following 10 min of local heating to 33 ºC, baseline RBF was assessed. The local heaters were then warmed to 39 ºC at a rate of 0.1 ºC /s (9, 22) and maintained at that temperature for approximately 70 min. Local heating results in an axon reflex mediated initial peak in RBF within 5 min, followed by a gradual NO-

mediated increase in RBF that plateaus after 30-40 min of continuous heating (9, 25, 31). Once RBF was determined to have plateaued (minimum 5 min), the respective perfusates at each site were terminated and immediately switched to L-NAME at all sites until the reduction in RBF stabilized at each site (approximately 30 min). This allowed for the quantification of the NO-mediated contribution to the plateau phase of local heating. Finally, SNP was infused with the addition of 43 ºC local heating to elicit maximal RBF at each site within 20-30 min.

Data Analysis: RBF values for each site during each phase (baseline, 39°C plateau, 39°C + NOS inhibition, max dilation) were determined by visually identifying and averaging a stable 5 min period. Additionally, RBF during the initial peak phase of local heating was averaged over 30s. Brachial blood pressure was measured via an automated sphygmomanometer (Tango M2, SunTech Medical Inc, Morrisville, NC) in triplicate during each phase and averaged for the calculation of mean arterial pressure (MAP=1/3 Systolic +2/3 Diastolic). Cutaneous vascular conductance (CVC) was then calculated as RBF / MAP. CVC for each phase was normalized to the site-specific maximal CVC obtained during the 43 ºC local heating + 28mM SNP phase and all CVC data are presented as a percentage of maximal.

Statistical Analysis: Sample size was chosen based upon our previous study in this population (22) and another that used the same pharmacological agents in a different population (13). Group comparisons of descriptive characteristics were performed via two-tailed, unpaired t-tests. CVC responses during each phase (baseline, 39°C plateau, 39°C + NOS inhibition, max dilation) and NO-contribution (plateau minus L-NAME) were compared using two-way mixed model ANOVA with group as the between factor and treatment site as the within factor. Planned pairwise comparisons across groups (at the control site) and across treatments (versus control) were made

using the Holm-Sidak method, and adjusted P-values are presented for multiple comparisons. All statistical analyses were performed using commercially available software (Prism 7, GraphPad, LaJolla, CA). Alpha was set a-priori at 0.05.

Results

As illustrated in Table 1, groups were well matched for age, BMI, waist circumference, systolic BP, heart rate, total cholesterol, and fasting glucose. Relative to the white group, black participants exhibited greater diastolic BP, although it was still within the normal range. The lipid profiles of both groups were normal however, the black group had significantly lower triglycerides and VLDL compared to the white group.

Influence of Race:

At baseline, the black group had an attenuated CVC (%max) relative to the white group at the control site (Table 2). Apocynin and allopurinol had no effect on baseline CVC in the white group (P=0.70 and 0.98, respectively), but amongst the black group, baseline CVC was augmented with apocynin (P=0.001) and allopurinol (P=0.05).

The initial peak during local heating was similarly blunted at the control site in black individuals compared to the white group (P<0.001, Figure 3A). Apocynin and allopurinol had no effect on initial peak compared to control (each P=0.93) in the white group. However, within the black group, the initial peak was augmented by both treatments (apocynin: P=0.004 and allopurinol: P=0.002, Figure 3A), suggesting a role for NOX and XO derived ROS in the blunting of this response.

As shown in Figure 3B, during the plateau phase, the black group exhibited blunted CVC at the control site relative to the white group ($P<0.001$). Apocynin and allopurinol each augmented this response in the black group ($P<0.001$ and $P=0.01$, respectively) while not affecting responses in the white group (both $P>0.92$). Upon inhibition of NOS via L-NAME, black and white groups had similar CVC at the control site ($P=0.49$). As seen in Figure 3C, the NO contribution to local heating, as determined by the difference between 39 ºC plateau and 39 ºC + NOS inhibition responses, was attenuated in the black group at the control site ($P=0.002$ vs white). Allopurinol treatment in the black group resulted in a greater NO contribution ($P=0.005$), while there was a trend toward increased NO contribution at the apocynin site ($P=0.06$). The latter comparison did not reach significance due in part to a slight increase in NO-independent dilation during L-NAME at the apocynin site relative to the control site (24±13 vs 16±8 %max, $P=0.03$). There was no effect of either drug on NO contribution in the white group (both $P>0.25$).

Maximal absolute CVC (a.u.· $mmHg^{-1}$) during SNP + 43 ºC did not differ between groups at the control site ($P=0.98$, Table 2) and was unchanged following apocynin and allopurinol treatment relative to each of the respective control sites (all comparisons $P>0.40$, Table 2) indicating similar maximal vasodilatory capacities across groups that were unaffected by either treatment.

Influence of Sex:

To probe potential sex differences, each group was divided by sex and compared within their own race. White men and women were similar at the control site across all parameters, and apocynin and allopurinol had no effect on any parameter in white men or white women (all $P>0.20$,

not shown). In contrast, multiple sex by treatment interactions were observed within the black group.

The initial peak CVC during the first 5 min of local heating was similar between black men and black women at the control site (41±11 vs 53±5 %max, P=0.18). Within black women, initial peak was unchanged with either apocynin (55±16 %max, P=0.83) or allopurinol (54±10 %max, P=0.83). Similar to the plateau response, the initial peak was augmented, relative to the control site, by apocynin (58±11 %max, P<0.001) and allopurinol (61±13 %max, P<0.001) in the black men only. There was no sex difference for initial peak response within the white group at the control site, nor was there an effect of either treatment (all comparisons P>0.57, not shown).

As shown in Figure 4A, the local heating response at the control site during the plateau phase was not different between black men and black women (P=0.99), however, apocynin augmented CVC in black men relative to the control site (P<0.001), but did not change the response in black women (P=0.55). Likewise, allopurinol also resulted in augmented CVC compared to the control site in black men (P=0.002), but not black women (P=0.56). CVC after NOS inhibition was similar among black men and women at the control site (P=0.63, not shown). Apocynin tended to augment non-NO dilation in black women (P=0.09) but not black men (P=0.49). The NO contribution to heating was similar between sexes at the control site in the black group (P=0.82, Figure 4B), but only increased following apocynin and allopurinol in black men (each P<0.001, Figure 4B).

Within the black group, women had a greater maximal CVC (a.u.· $mmHg^{-1}$) than black men (P<0.001) but there was no effect of treatment in either group (Figure 4C). Baseline CVC was

augmented following apocynin treatment relative to the control site (19±13 vs 9±6 %max, P=0.002) in black men, but not black women (P=0.26) as shown in Figure 4D.

Discussion

Herein, we observed attenuated cutaneous microvascular vasodilation in response to local heating in apparently healthy, young black adults relative to age- and sex-matched white adults. Furthermore, the present study provides novel insight regarding potentially divergent mechanisms of microvascular dysfunction between black men and black women. Collectively, our findings indicate that NADPH oxidases and xanthine oxidase contribute to impairments in microvascular function in black men, while the deficit in cutaneous microvascular function amongst black women remains to be determined.

Black individuals are at an increased risk of multiple cardiovascular diseases including heart failure, peripheral artery disease, and stroke (1). The markedly higher rate of hypertension in the black population contributes to this elevated disease burden (1). Admittedly, the genesis of hypertension is multifactorial, however impaired vascular function is present in normotensive populations at risk of high blood pressure (32, 42). In this regard, attenuated vascular responses have been observed in black, relative to white, cohorts in the cerebral vasculature (21), conduit arteries (5, 34), whole limb microcirculation (7, 11, 17, 33), and the cutaneous microvasculature (22, 35). Additionally, diminished microvascular function, determined by post occlusion reactive hyperemia, has been observed in apparently healthy cohorts of black adolescents (11) and young adults (17) suggesting that vascular dysfunction manifests early in life.

The origins of microvascular dysfunction in this population have not been elucidated. Our laboratory has recently presented evidence that scavenging superoxide augments NO-mediated vasodilation and partially restores the cutaneous microvascular response to local heating (22). Superoxide readily reacts with NO to produce the free radical peroxynitrite, which can then damage cellular proteins and lead to greater superoxide production through the uncoupling of endothelial nitric oxide synthase (eNOS) (37). Superoxide is produced during aerobic metabolism in the mitochondria and via enzymatic sources including NOX and XO (37). Indeed, peripheral blood mononuclear cells from young black men exhibit increased NADPH oxidase expression and superoxide production relative to age-matched white men (10). However, the extent to which NADPH oxidase derived superoxide contributes to impaired vascular function in the black population has not been fully elucidated.

This study, consistent with our previous finding (22) and others in the whole-limb microvasculature (7, 33), observed a racial difference in NO-mediated microvascular dilation. The NO-independent component of thermal hyperemia is ascribed to endothelium derived hyperpolarizing factors (EDHF) that activate K^{+}_{Ca} channels (4). Importantly, our data indicate that black and white responses during NOS inhibition were not different, suggesting that K^{+}_{Ca} channel activity is maintained in black individuals as has been previously demonstrated in the whole-limb microvasculature (33).

The initial peak response to heating is due to the axon reflex, however, it is somewhat NO-dependent (9, 25, 31). Choi and colleagues observed that 39 °C elicited a 44 % max initial peak (9). Notably, our black group had a similar average (46 %), but our white group demonstrated a markedly greater initial peak (~68%). The reasons for this disparity are unclear. The racial

difference we observed is likely due to the NO-component of the initial peak as inhibition of ROS-production resulted in a marked improvement within black men, consistent with the plateau and NO-contribution data. We cannot rule out an effect of ROS on $K^{+}_{Ca,}$ channel activity, as its contribution to the initial peak is similar to that of NO (9).

The family of enzymes known collectively as NOX produce superoxide by transferring electrons to oxygen (37). In humans, elevated NOX activity has been implicated in vascular dysfunction in obesity (39), chronic kidney disease (13), and atherosclerosis (40). Likewise, cultured human umbilical vein endothelial cells from black donors exhibit greater content of NOX subunits than cells from white donors (14, 24). When these endothelial cells are activated in-vitro with a calcium ionophore, the black cells produce more ROS and less NO than the white cells (24). Furthermore, NOX protein content in circulating peripheral blood mononuclear cells of college-aged black men is elevated relative to age-matched white men resulting in elevated superoxide synthesis (10). Other than one in-vitro study showing that apocynin reduced ROS production and increased NO in black endothelial cells (27), to the best of our knowledge, no studies have assessed the effects of NOX inhibition on in-vivo vascular function in this population. Therefore, in the present study we show for the first time that NO-mediated vascular function in black individuals, specifically men, was limited by NOX activity.

Similarly, the present finding of augmented cutaneous microvascular responses to local heating following allopurinol treatment in black men provides the first evidence that XO activity contributes to racial differences in vascular function. The potential role of XO in the racial disparity in peripheral vasodilatory responses has not been elucidated, thus the present study aimed to assess the microvascular effect of XO inhibition in healthy black individuals. As uric acid is a primary

product of XO, in addition to superoxide, reports of elevated uric acid levels in the black population provide indirect evidence of elevated XO activity (28), though one study found evidence of reduced XO amongst black men and women versus whites (36). To our knowledge, only one previous study has investigated the use of allopurinol for inhibition of XO in black individuals specifically. Segal and colleagues reported a trend (P=0.059) toward a greater reduction in systolic blood pressure when allopurinol was added to diuretic treatment in black hypertensives (38). Unfortunately, no vascular function measures were included and the authors were unable to determine whether the potential benefit was due to the lowering of uric acid or a decline in ROS (38). Importantly, the improvements in vascular function observed with allopurinol in chronic heart failure patients are not related to reductions in uric acid (16). Therefore, we speculate that our observation of allopurinol-induced augmentation of NO-mediated dilation in black men is likely through a reduction in superoxide generated from XO.

There are a few potential explanations for why apocynin and allopurinol each augmented cutaneous responses to heating to a similar degree. One possibility is that NOX-derived superoxide impairs vasodilation via increases in XO activity (29). In this scenario, XO is the main source of the superoxide that reacts with NO and decreases its bioavailability, whereas inhibition of NOX results in improved dilation via reduced activation of XO. Another possible explanation is that apocynin may not exert its beneficial effects on the vasculature by inhibiting NOX, but rather by acting as a general antioxidant (18). Thus, apocynin may just increase NO bioavailability by scavenging XO-derived superoxide.

Our observation of a sex difference within our young black cohort was unexpected. Notably, black women have higher rates of hypertension than black men, whereas the opposite is

present in the white population suggesting race-by-sex interaction effects on the physiological factors that influence blood pressure (1). We can only speculate why cutaneous microvascular responses in black women, unlike black men, were not augmented by apocynin or allopurinol. Premenopausal women have lower oxidative stress than age-matched men (23), suggesting that the impairment amongst black women in the current study may not be due to ROS. In an animal model, differential expression of NOXs in the coronary arteries of male and female pigs results in NOX-mediated attenuation of vasodilation in male, but not female pigs (43). Brewster and colleagues examined isolated resistance arteries from normotensive pregnant women and found that arteries from black women exhibited greater contractility than white women despite similar arterial structure (2). In light of the higher creatine kinase measured in the arteries of black women, the authors suggested that NO bioavailability is decreased secondary to its substrate, L-arginine, being outstripped by the synthesis of creatine (2). This ROS-independent reduction in NO bioavailability could explain why cutaneous microvascular function in black women was not improved by apocynin or allopurinol in the current study. Nevertheless, further studies are warranted.

Perspectives

The black population has an increased risk of CVD compared to other racial groups (1). The present data indicate that both black men and black women have impaired microvascular function decades prior to the typical onset of CVD, suggesting that impaired vascular function early in life may contribute to this elevated risk (32, 42). Importantly, the female sex is typically associated with increased NO bioavailability (15) and a delayed age-related decline endothelium-dependent dilation (8) that coincides with a later incidence of CVD (26). The current observation

of blunted cutaneous microvascular thermal reactivity in young black women suggests that the protective effect of sex is not present in this population. Furthermore, our findings indicate that oxidative stress produced via NADPH oxidases and xanthine oxidase does not contribute to this impairment in black women. As such, future research is needed to identify the novel mechanisms underlying microvascular dysfunction in black women. Additionally, our finding that NADPH oxidases and xanthine oxidase impair microvascular function in young black men presents an opportunity for further research into the targeting of these enzymatic sources of oxidative stress to mitigate the microvascular dysfunction and increased risk of CVD in black men.

In summary, we present the first direct evidence, to our knowledge, that oxidative stress derived from NADPH oxidases and xanthine oxidase impairs cutaneous microvascular function in black men. Additionally, our results indicate that another undetermined mechanism is responsible for the impairment in young black women relative to their white counterparts. Collectively, these findings provide important information towards furthering our understanding of racial and sex differences in vascular function.

Figure Legends

Figure 1. Protocol schematic illustrating baseline, local heating, NOS inhibition, and max dilation phases. Dashed arrows indicate that the respective substance (Ringer's, apocynin, or allopurinol) was infused continuously from 30 min pre-baseline to the beginning of L-NAME infusion.

Figure 2. A) Representative local heating responses in a white man at the control site (○) and a black man at control (●), apocynin (▲), and allopurinol (■) sites. B) Representative local heating responses in a white woman at the control site (○) and a black woman at control (●), apocynin (▲), and allopurinol (■) sites. Apocynin and allopurinol sites for the white subjects are omitted for clarity.

Figure 3. Group x treatment comparisons in white (n=15) and black (n=15) individuals. A) Initial peak CVC following the onset of 39 °C local heating at control, apocynin, and allopurinol sites in white and black groups. The peak was blunted in the black group at the control site, however it was augmented in this group at apocynin and allopurinol sites. B) Plateau CVC at control, apocynin, and allopurinol sites during 39 °C local heating in white and black groups. The control site in the black group was attenuated compared to the control site in the white group. Apocynin and allopurinol augmented this response in the black group only. C) NO contribution determined by the difference in CVC between local heating response before and after NOS inhibition via L-NAME. Black group is blunted at control site relative to white group. Within the black group, allopurinol significantly augmented NO contribution versus the control site. data presented as Mean ± SD. **: $P<0.01$ vs control site in white group; ***: $P<0.001$ vs control site in white group.

Figure 4. Sex x treatment comparisons in black men (n=8) and black women (n=7). A) CVC at control, apocynin, and allopurinol sites during 39 °C local heating in black men and women. Responses were similar between sexes at the control site. Apocynin and allopurinol augmented this response in black men only. B) NO contribution determined by the difference in CVC between local heating response before and after NOS inhibition via L-NAME. NO contribution similar for black men and black women at control site however, within black men, apocynin and allopurinol both augmented NO contribution versus the control site. C) Maximal CVC (a.u.·mmHg^{-1}) was significantly greater in women relative to men; however, treatment had no effect in either sex. D) During baseline period, black men and women had a significantly blunted CVC at the control site that was augmented by apocynin in the black men only. All data presented as Mean ± SD. ***: P<0.001 vs control site in black men.

Table 1: Subject Characteristics

	White	Black	P-value
Men / Women	8/7	8/7	
Age (y)	22 ± 3	22 ± 3	0.80
BMI ($kg \cdot m^{-2}$)	23.0 ± 2.4	24.4 ± 3.3	0.21
Waist Circumference (cm)	77.7 ± 6.4	78.9 ± 9.7	0.70
Systolic BP (mmHg)	111 ± 10	115 ± 9	0.22
Diastolic BP(mmHg)	64 ± 6	71 ± 7	0.002
Heart Rate ($b \cdot min^{-1}$)	60 ± 9	64 ± 8	0.14
Glucose ($mg \cdot dl^{-1}$)	85 ± 6	86 ± 6	0.61
Total Cholesterol ($mg \cdot dl^{-1}$)	184 ± 40	159 ± 33	0.08
HDL ($mg \cdot dl^{-1}$)	57 ± 15	60 ± 6	0.49
LDL ($mg \cdot dl^{-1}$)	107 ± 27	87 ± 30	0.07
VLDL ($mg \cdot dl^{-1}$)	19 ± 8	12 ± 4	0.008
Triglycerides ($mg \cdot dl^{-1}$)	94 ± 40	59 ± 20	0.007

Mean ± SD. BP: Blood Pressure; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein; VLDL: Very Low Density Lipoprotein

Table 2: Baseline and Maximal CVC

Baseline (%CVCmax)	**Control**	**Apocynin**	**Allopurinol**
White	17 ± 8	15 ± 6	16 ± 8
Black	7 ± 5 #	14 ± 11**	11 ± 8*
Maximal CVC (a.u. ·mmHg^{-1})			
White	2.9 ± 0.8	2.7± 0.9	2.6 ± 0.8
Black	2.8 ± 1.0	2.7 ± 1.0	2.7 ± 0.9

Data presented as Mean ± SD. CVC: cutaneous vascular conductance.
#: P<0.05 vs White; *: P<0.05 vs control; **: P<0.01 vs control.

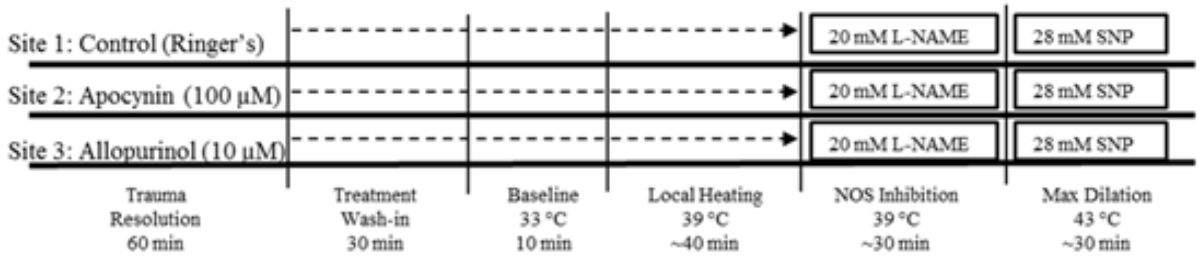
Site 1: Control (Ringer's)
Site 2: Apocynin (100 µM)
Site 3: Allopurinol (10 µM)
20 mM L-NAME
28 mM SNP
20 mM L-NAME
28 mM SNP
20 mM L-NAME
28 mM SNP
Trauma Resolution 60 min
Treatment Wash-in 30 min
Baseline 33 °C 10 min
Local Heating 39 °C ~40 min
NOS Inhibition 39 °C ~30 min
Max Dilation 43 °C ~30 min

Figure 1.

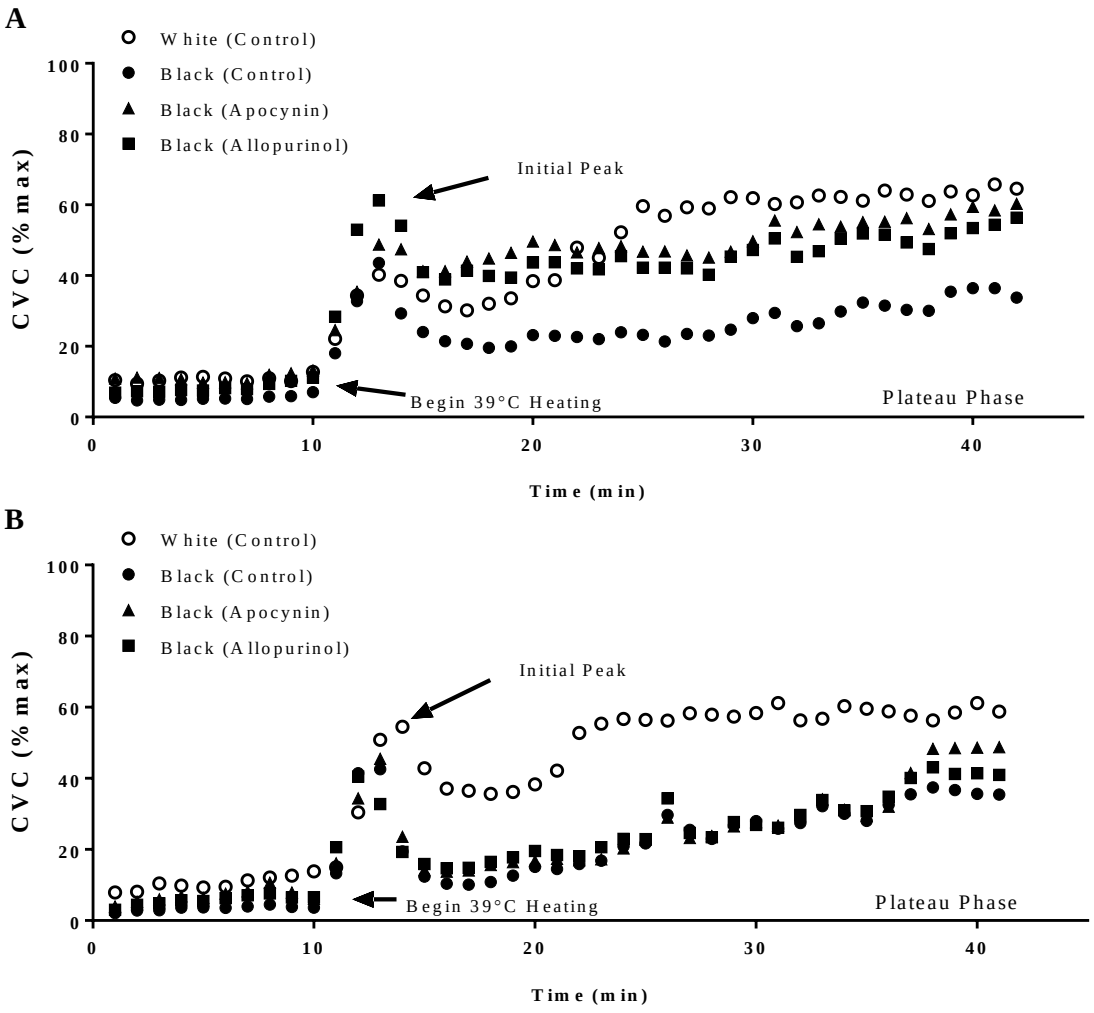
A
White (Control)
Black (Control)
Black (Apocynin)
Black (Allopurinol)
Initial Peak
Begin 39°C Heating
Plateau Phase
CVC (% max)
Time (min)
B
White (Control)
Black (Control)
Black (Apocynin)
Black (Allopurinol)
Initial Peak
Begin 39°C Heating
Plateau Phase
CVC (% max)
Time (min)

Figure 2.

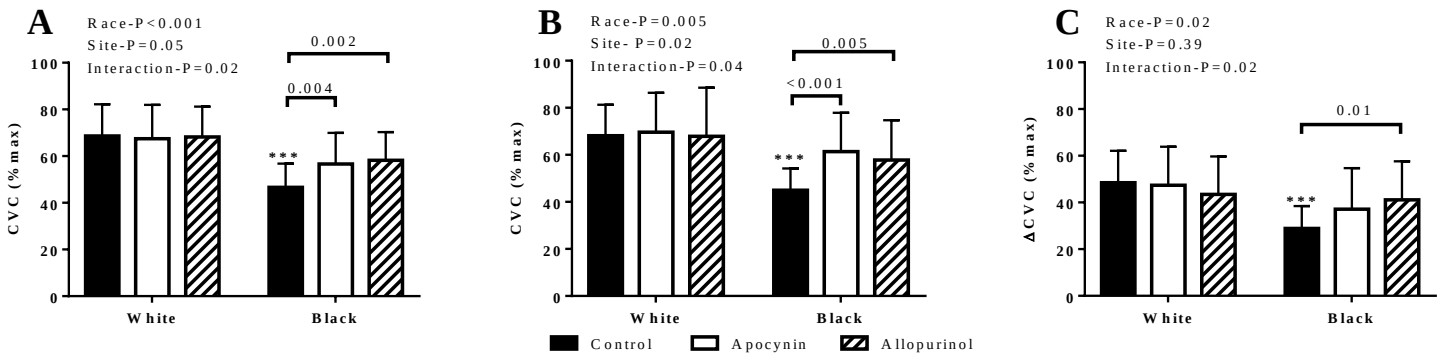
A
Race-P<0.001
Site-P=0.05
Interaction-P=0.02
0.002
0.004
***
CVC (% max)
100
80
60
40
20
0
White
Black
B
Race-P=0.005
Site- P=0.02
Interaction-P=0.04
0.005
<0.001
***
CVC (% max)
White
Black
Control
Apocynin
Allopurinol
C
Race-P=0.02
Site-P=0.39
Interaction-P=0.02
0.01
***
ΔCVC (% max)
White
Black

Figure 3.

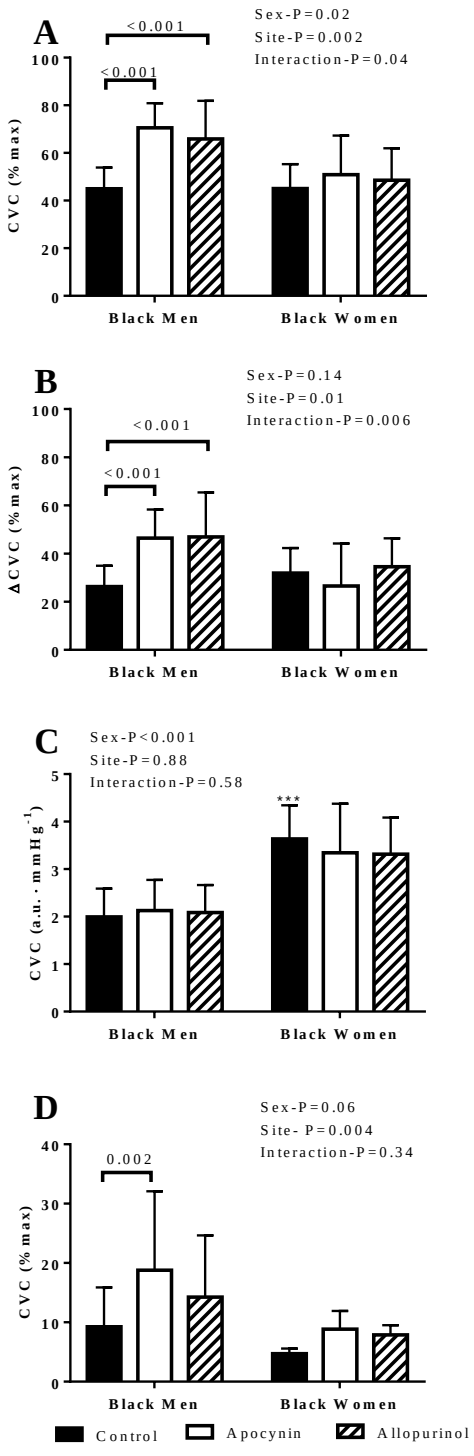
A
Sex-P=0.02
Site-P=0.002
Interaction-P=0.04
<0.001
<0.001
CVC (% max)
100
80
60
40
20
0
Black Men
Black Women
B
Sex-P=0.14
Site-P=0.01
Interaction-P=0.006
<0.001
<0.001
ΔCVC (% max)
100
80
60
40
20
0
Black Men
Black Women
C
Sex-P<0.001
Site-P=0.88
Interaction-P=0.58
***
CVC (a.u. · mmHg⁻¹)
5
4
3
2
1
0
Black Men
Black Women
D
Sex-P=0.06
Site- P=0.004
Interaction-P=0.34
0.002
CVC (% max)
40
30
20
10
0
Black Men
Black Women
Control
Apocynin
Allopurinol

Figure 4.

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Chapter 3

MECHANISMS OF BLUNTED DILATION TO INTRADERMAL METHACHOLINE ADMINISTRATION IN YOUNG BLACK MEN

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Abstract

Black individuals display blunted muscarinic receptor-mediated dilation of the peripheral limb microvasculature compared to their white counterparts. Pilot testing in our laboratory have extended these findings to the cutaneous microvasculature of healthy young black individuals. The mechanisms for this impairment are unknown. However, impaired cutaneous microvascular dilation during a non-muscarinic mediated perturbation, local heating, is related to oxidative stress black men. Therefore, the present study aimed to test the hypothesis that muscarinic-mediated dilation in the cutaneous microvasculature of 11 young black men would be enhanced following inhibition of oxidative stress produced due to NADPH oxidases, xanthine oxidases, or uncoupled endothelial nitric oxide synthase (eNOS) via apocynin, allopurinol, and tetrahydrobiopterin (BH4), respectively. Each drug was delivered for 40 min via a separate intradermal microdialysis fiber placed in the dorsal forearm followed by a co-infusion with 100μM methacholine (MCh) for 6 min. A second 6 min infusion of MCh was again performed after 60 min of 20mM L-NAME infusion to determine the NO-mediated contribution to the dilation. Vascular smooth muscle function and maximal dilation were determined using 28mM sodium nitroprusside (SNP) and combined SNP and 43° local heating, respectively. Red blood cell flux was measured at each site via laser Doppler flowmetry and subsequently reported as cutaneous vascular conductance (CVC; flux / MAP). Contrary to the hypothesis, mean CVC responses to MCh were not different at any site compared to the control site (P=0.37). Likewise, mean NO-contribution and SNP responses were also not different at any site relative to the control site (P=0.33).

Introduction

Recently, our group has demonstrated that cutaneous microvascular function is blunted in black individuals compared to their white counterparts (12, 24, 42). These observations are consistent with a larger body of literature showing that black individuals have attenuated vascular function early in life (19). Importantly, a marked racial disparity in risk of cardiovascular diseases (CVD) exists between black and white populations, with the black population having elevated rates of hypertension, stroke, and heart failure. Vascular dysfunction contributes to the development of CVD, and thus the underlying mechanisms of vascular dysfunction in black populations are potential targets for lifestyle and pharmacological interventions aimed at reducing the elevated prevalence of CVD.

Racial differences in vascular function are commonly attributed to decreased nitric oxide (NO) bioavailability in black individuals (7, 35, 39, 44). We have recently reported that healthy, college aged black individuals exhibit blunted NO-mediated increases in cutaneous microvascular conductance during local heating (24). We also established that this reduction in microvascular function is due to elevations in the reactive oxygen species (ROS) superoxide (24). During a follow-up study, we observed that inhibition of NADPH oxidases and xanthine oxidase each restored local heating responses in black men, likely through a reduction in ROS (Chapter 2). ROS produced by these oxidases may directly scavenge NO or reduce the essential cofactor tetrahydrobiopterin (BH4) which would uncouple endothelial nitric oxide synthase (eNOS), resulting in reduced production of NO in addition to further production of ROS (37). BH4 supplementation restores vascular function in aging (15), diabetes (20), and hypercholesterolemia

(1). Whether BH4 deficiency contributes to racial differences in microvascular function is not currently known.

The local heating response is largely NO-dependent (8, 28, 36), whereas muscarinic stimulation of the endothelium results in dilation due to endothelium derived hyperpolarizing factors (EDHF) and prostaglandins in addition to NO (3, 22, 29). These non-NO pathways may compensate for any reduction in NO bioavailability and maintain vascular function. Previously, the muscarinic receptor agonist acetylcholine (ACh) was shown to elicit blunted increases in forearm blood flow in black individuals relative to age- matched white participants (2, 6, 25, 39). Consistent with these findings, a pilot study in our lab indicated that healthy college-aged black individuals exhibit blunted increases in cutaneous blood flow to the acetylcholine analog methacholine chloride (MCh) at submaximal dosages (unpublished).

To the best of our knowledge, the role of oxidative stress in blunted muscarinic vasodilation in black populations has not been studied. Therefore, we tested the hypothesis that MCh-induced cutaneous microvascular dilation in young black men would be augmented via inhibition of NADPH oxidases (apocynin) and/ or xanthine oxidase (allopurinol) or via supplementation of BH4. Based upon the potential for superoxide to impair vascular smooth muscle signaling via an upregulation of phosphodiesterase 5 (PDE5), we also aimed to determine the influence of these treatments on endothelium-independent dilation in response to the exogenous NO donor sodium nitroprusside (SNP).

Methods

Ethics Approval: The study protocols were approved by the institutional review board at The University of Texas at Arlington and all subjects provided written informed consent prior to participation in accordance with the Declaration of Helsinki.

Subjects: Based upon our previous finding suggesting that cutaneous microvascular function is inhibited via ROS in black men but possibly not black women (Chapter 2 - manuscript currently in peer review), we recruited 11 healthy, college-aged black men. Subjects self-reported their racial identity and that of their parents. Individuals of mixed race were excluded from the study, as were smokers and trained athletes. All subjects were normotensive and free of overt cardiovascular, metabolic, or neurological disease and not currently taking antioxidant supplementation or medications. Subjects were instructed to fast overnight and abstain from alcohol, caffeine, and strenuous activity for 24 hours prior to the study. Height and weight were assessed upon arrival to the laboratory. Additionally, waist and hip circumferences were measured at the level of the naval and widest portion of the hips, respectively. Subject characteristics are displayed in Table 1.

Instrumentation: All vasoactive substances were delivered via intradermal microdialysis fibers (CMA 31, Harvard Apparatus, MA) as previously described (24, 40). A 25-gauge needle was inserted under the skin surface of the dorsal forearm for a distance of ~2cm. The microdialysis fiber was then advanced through the lumen and the needle was withdrawn leaving the 1cm, 55Kda membrane under the skin. This process was repeated for a total of 4 sites with each site separated by a minimum of 2.5cm. Following placement of the membranes, each fiber was perfused with lactated Ringer's solution at a rate of 2 $\mu l \cdot min^{-1}$, via electronic syringe pump (11 plus, Harvard Apparatus) for 60-90 min while trauma-induced hyperemia subsided.

Red blood cell flux (RBF) was measured via laser Doppler flowmetery (Moor) throughout the duration of the protocol. A laser Doppler flow probe with an integrated temperature sensor (Moor) housed inside of a local heating unit (PF 450 Perimed) was taped in place on the skin above each membrane. Additionally, heart rate was monitored via standard 3-lead ECG (CardioCard) and brachial blood pressure was taken throughout the protocol via an electrosphygmomanometer (Tango M2). Analog inputs were integrated and recorded at 400hz via a commercially available data acquisition system (Powerlab, ADInstruments, Colorado Springs, CO). Data was stored for offline analysis using LabChart software (ADInstruments).

Protocol: We recently observed that local heating responses in black men were restored via inhibition of NADPH oxidases and xanthine oxidase using apocynin and allopurinol, respectively (Chapter 2). Therefore, we aimed to determine if inhibition of these enzymatic sources of ROS also contributed to attenuated cutaneous microvascular responses to muscarinic stimulation via 100 µM MCh. Our pilot testing in 12 black and 11 white young adults indicated that this dose elicited approximately 50% less of an increase in CVC amongst black participants compared to the white group (unpublished). The experimental protocol is illustrated in Figure 1. 100 µM apocynin (14), 10 µM allopurinol (14), and 10mM BH4 (1) were infused alone for 30min following the trauma resolution period. Following the wash-in period, local heating units were set to 33 °C for 10 min to normalize skin temperature at all sites, and RBF in the final minute was used as baseline. MCh was then co-infused with either lactated Ringer's solution (control) or each treatment for 6 min (MCh1). 20mM L-arginine methyl ester (L-NAME) was then infused for 60 min to inhibit NOS activity. During this time apocynin, allopurinol, and BH4 were continuously

co-infused with L-NAME at their respective sites. The MCh co-infusions were then repeated as described above (MCh2).

While the local heating units remained set to 33 °C, 28mM sodium nitroprusside (SNP) was infused for 10 min to provide an index of endothelium-independent responsiveness to NO. The local heating units were then set to 43 °C and SNP infusion continued for an additional 25 min to determine maximal local vasodilatory capacity.

Drugs: All drugs were acquired from Sigma-Aldrich Corp (St. Louis, MO). Drug solutions were prepared the day of the study, covered with foil to prevent photodegradation, and filtered prior to use. At each phase of the protocol, the drugs were initially infused at a rate of 5.2 μL/min for 30 s to flush the previous drug out of the fiber.

Data analysis and Statistics: RBF was averaged during the final minute each stage of the protocol (baseline, MCh1, L-NAME, MCh2, SNP), whereas the maximal dilation was determined as the greatest one-minute average during the 43 °C + SNP stage. Cutaneous vascular conductance (CVC) was calculated as RBF / MAP and responses were normalized to the maximal CVC (%CVCmax). MCh1 and MCh2 responses are reported as the increase from baseline and L-NAME, respectively (Δ%CVCmax). NO-contribution was calculated as MCh1 – MCh2. Within-subjects one-way repeated measures analysis of variance was performed for each stage of the study and NO-contribution. The Holm-Sidak multiple comparison method was used to compare treatment sites to the control and the adjusted P values are provided. Potential responder / non-responder comparisons were made using two-tailed unpaired t-tests. All statistical analyses were

performed using commercially available statistical software (SigmaPlot 14.0, Systat Software Inc, San Jose, CA). Alpha was set a-priori at 0.05 and data are presented as mean ± SD.

Results

There was no main effect of allopurinol, apocynin, or BH4 endothelium-dependent increases in CVC following MCh1 (P=0.37, Figure 2A). In figures 2B-2D, individual data at each site are presented compared to responses at the control site. There is clear separation between 8 "low" responders to MCh 1 at the control site and 3 "high" responders (Figure 3A). The high response group had a lower waist to hip ratio (Figure 3B) but were not different in regard to BMI (Figure 3C) or waist circumference (Figure 3D). There was not a main effect of drug on NO-contribution (P=0.33, Figure 4A). Individual data show similar trends to MCh1 response (Figure 4B-D). As displayed in Figure 5A, the response to 28mM SNP at 33 °C was similar across sites. Individual data are shown with each drug compared to control (Figure 5B-D) and suggest that none of the drugs improve endothelium-independent dilation in young black men.

When expressed as %CVCmax, there was no difference in site for baseline or L-NAME stages (Table 2). Additionally, max CVC was similar across sites showing no effect of any drug on maximal vasodilatory capacity (Table 2).

Discussion:

The current study is the first, to our knowledge, to investigate the role of oxidative stress pathways in the genesis of impaired muscarinic-stimulated microvascular dilation in a cohort of healthy black individuals. Contrary to our hypothesis, we did not observe a clear improvement in the cutaneous microvascular responses to methacholine infusions following inhibition of ROS due

to xanthine oxidase, NADPH oxidases, or BH4 deficiency-induced eNOS uncoupling. However, the findings of this study, suggest that subtle increases in abdominal adiposity may contribute to impaired microvascular function in black men.

We have previously observed that local heating-induced cutaneous hyperemia is blunted in healthy young black men due to superoxide (24) produced via NADPH oxidases and xanthine oxidase (Chapter 2). The hyperemia elicited by that local heating protocol is approximately 80% NO-dependent (8). However, muscarinic receptor-mediated dilation is less dependent upon NO. Acetylcholine-induced dilation of isolated human subcutaneous arteries is primarily dependent upon hyperpolarization with only a small contribution from NO (4). Vasodilation following cutaneous infusions of ACh requires NO, prostaglandins, and EDHF to varying degrees depending upon dose (3, 22, 29). The overlapping nature of these mechanisms of dilation suggest that muscarinic-dilation may be protected from reductions in NO bioavailability in black individuals due to ROS. Whether the aforementioned oxidative stressors also inhibit muscarinic-receptor mediated microvascular vasodilation has not been investigated.

Increases in cutaneous microvascular blood flow following transdermal acetylcholine iontophoresis are reduced in black populations relative to age-matched whites, (32, 33, 42). However, interpretation of iontophoresis studies is difficult as racial differences are abolished when differences in skin resistance are taken into account (42). In contrast to iontophoresis, microdialysis allows for the continuous infusion of drugs at known dosages allowing for the mechanistic study of microvascular dysfunction (21). To our knowledge, the present study is the first to directly investigate the causes of impaired muscarinic-receptor mediated cutaneous vasodilation in black individuals.

Impaired microvascular dilation to muscarinic receptor stimulation in black individuals has been demonstrated at the whole-limb level in multiple studies measuring forearm blood flow during intra-arterial infusions of ACh (6, 25, 39, 44). NOS inhibition prior to infusion of ACh reduces the response to a lesser degree in black participants compared to white subjects, implying impaired production and/or release of NO (6, 39). Similar blood flow across groups during NOS inhibition suggests that the impairment in blacks is not due to impaired NO-independent pathways such as endothelium-derived hyperpolarization. Ozkor and colleagues inhibited K_{Ca}+ channels via tetraethylammonium and observed that forearm blood flow during ACh infusions was reduced to a similar degree in black and white participants, suggesting preserved EDHF in blacks (39).

The present data indicate no role for ROS produced from either NADPH or xanthine oxidases in the blunted response to MCh in the cutaneous microvasculature of black men. However, 7 of 11 subjects had greater responses to MCh at the allopurinol site compared to the control site, indicative of xanthine oxidase- mediated reductions in microvascular function. The 3 subjects with markedly higher CVC responses to MCh1 at the control site (i.e. function was not impaired) all had reduced responses at the allopurinol site (Figure 2B). Upon further examination, these individuals also had the lowest waist to hip ratios yet similar waist circumferences and BMI (not shown), suggesting that relative abdominal adiposity may be a key determinant of microvascular function in black men. Previous studies have demonstrated reduced muscarinic vasodilation in whole limb (45) and cutaneous microvasculature of obese individuals (11). Notably, in men, only waist to hip ratios greater than 0.90 are considered a cardiovascular risk factor (38). These data suggest that, within the black population, only men with very low waist to hip ratios are protected from vascular dysfunction. Larger studies are needed to support this

hypothesis, however, a recent study by Dass et al. found a significant relationship between waist to hip ratio and micro- and macrovascular function in young black men (10). Further research into the appropriateness of the 0.90 cut-off for black men is needed (9).

Analysis excluding the 3 individuals with significantly greater control site responses reveals a non-significant, but consistently increased CVC at the allopurinol site relative to the control site. While the present study is underpowered to detect a significant difference, this finding is directionally consistent with our previous findings using local heating as the vasodilatory stimulus suggestive of a role for xanthine oxidase-derived ROS in the blunted microvascular dilation in black men (Chapter 2). In the local heating study, we observed a similarly improved response at the apocynin site. The similar responses at allopurinol and apocynin sites are explained by previous observations that NADPH oxidase-derived superoxide activates xanthine oxidase which then produces superoxide at levels sufficient to impair NO bioavailability (13, 34). However, in the present study we did not observe an increase in dilation following NADPH oxidase inhibition via apocynin. The reasons for this discrepancy are unclear. Speculatively, the local heating technique may elicit heat shock-induced activation of NADPH oxidases and xanthine oxidase (43) that is greater in the black population than white due to greater expression of NADPH oxidase in blacks (16, 27) and thus is not as apparent during other stimuli, such as muscarinic receptor agonism. Unfortunately, the current study was not designed to test such a hypothesis.

The present study is the first, to our knowledge, to examine the role of BH4 in racial differences in microvascular function. Superoxide production in isolated human umbilical vein endothelial cells from black donors is blunted by NOS inhibition suggesting uncoupled eNOS contributes to vascular dysfunction in this population (27). Uncoupling of eNOS can occur due to

deficiency of L-arginine, related to an excess of its antagonist asymmetrical dimethyl arginine (ADMA), or the oxidation of the essential eNOS cofactor BH4 by peroxynitrite.(37) While supplemental BH4 improves NO-dependent dilation in hypercholesterolemia (1), aging (15), and diabetes (20), its affect has not been studied in black individuals. The results of the current study indicate that supplemental BH4 does not augment cutaneous microvascular dilation during methacholine infusion. The lack of an effect may suggest that uncoupled eNOS in this population, if present, is due to L-arginine deficiency or elevated circulating ADMA rather than reduced BH4. In support of this possibility are the observations that black individuals with coronary artery disease are more likely to respond positively to intra-coronary infusions of L-arginine than their white counterparts (23) and that healthy young black men have higher levels of circulating ADMA than their white counterparts (35).

Dilation in response to exogenous NO, as occurs with SNP infusion, is an index of endothelium-independent vascular smooth muscle function. However, the literature is currently equivocal on whether the endothelium-independent dilation is blunted in black populations. Multiple studies using intra-arterial infusions of SNP have found that black individuals have reduced responses (6, 39, 44) while others did not observe a difference between black and white groups (25, 26). Cutaneous microvascular responses to SNP iontophoresis are reduced in black individuals during iontophoresis with (42) and without (33) accounting for differences in skin electrical resistance. However, black groups exhibit blunted (5), maintained (41) , and augmented (18) endothelium-independent dilation of conduit arteries. Importantly, superoxide increases expression of the PDE5, resulting in reduced NO signaling in the vascular smooth muscle. The

current study identified no effect of allopurinol, apocynin, or BH4 supplementation on cutaneous microvascular responses to SNP.

Limitations: The current study had a few methodological limitations. First, we assessed microvascular function in black men only. Based upon our previous study, we did not expect any effect in white individuals or black women, however, this needs to be confirmed experimentally. Additionally, the present study is limited by a small sample size. Our previous study demonstrated clear effects of allopurinol and apocynin on local heating responses in 8 black men similar to those enrolled in the current study, therefore, we anticipated a larger effect size than what we observed in the present study. Also, we chose to use MCh as our muscarinic receptor agonist to minimize any potential effects of acetylcholinesterase activity (30). We found that MCh elicits longer lasting dilation compared to ACh (3, 17, 22), and thus, time constraints prevented us from completing multiple infusions and averaging the responses at each site as has previously been done (3, 17, 22). Doing so would have likely reduced the variability in our data and potentially exposed differential effects of the ROS-inhibiting drugs. Finally, we may be overestimating the NO-contribution to the MCh dilation as L-NAME may exert its effects by blocking muscarinic receptors rather than inhibiting NOS (31).

In conclusion, this study was the first to examine potential mechanisms of impaired muscarinic receptor-mediated dilation in black men. The current study utilized microdialysis to locally infuse allopurinol, apocynin, and BH4 to test the role of xanthine oxidase, NADPH oxidases, and BH4 deficiency, respectively, in the development of impaired microvascular function. As a whole, none of these substances had an effect on endothelium-dependent vasodilation of the cutaneous microvasculature. However, we did observe an augmented response

to muscarinic receptor stimulation at the control site in individuals with the lowest waist to hip ratio suggesting that relative abdominal adiposity may mediate microvascular function in black men. Future research is needed to clarify the mechanisms underlying the association between greater relative abdominal adiposity and impaired microvascular function in black men.

Figure Legends

Figure 1: Schematic depicting the experimental protocol. Dashed lines indicate times where each drug is being infused. MCh1, L-NAME, and MCh2 were co-infused with the drugs at each respective site.

Figure 2: A) Group average increase in %CVCmax above baseline during the first 6 min MCh infusion at all site. Data represent the final minute of the response at each site. B) The individual responses at the allopurinol site compared to the response at the control site. Note the apparent divergent responses between those who are high at control relative to those who have low %CVCmax at the control site. C) The individual responses at the apocynin site compared to the response at the control site. D) The individual responses at the BH4 site compared to the response at the control site. All $P>0.05$

Figure 3: A) Group average NO contribution to MCh-induced increases cutaneous vascular conductance (%max) calculated as MCh1 (Δ%CVCmax) – MCh2 (Δ%CVCmax). B) The individual NO-contributions at the allopurinol site compared to the response at the control site. C) The individual NO-contributions at the apocynin site compared to the response at the control site. D)The individual NO-contributions at the BH4 site compared to the response at the control site. All $P>0.05$.

Figure 4: A) Individual and average Δ%CVCmax during MCh1 of individuals who had low (<50%, n=8) and high (>60%, n=3) responses at the control site. B) Individual and average Waist to hip ratio in the low and high responders to MCh1at the control site. C) Individual and average

BMI in the low and high responders to MCh1 at the control site. D) Individual and average waist circumference in the low and high responders to MCh1 at the control site.

Figure 5: A) Group average %CVCmax following 10 min of SNP infusion at all site. Data represent the final minute of the response at each site. B) The individual responses at the allopurinol site compared to the response at the control site. C) The individual responses at the apocynin site compared to the response at the control site. D) The individual responses at the BH4 site compared to the response at the control site. All $P>0.05$

Table 1: Subject Characteristics

N	11
Age (y)	23 ± 4
BMI (kg/m^2):	25 ± 4
Systolic Blood Pressure (mmHg)	118 ± 10
Diastolic Blood Pressure (mmHg)	66 ± 7
Waist Circumference (cm)	82.8 ± 10.9
Hip Circumference (cm)	103.1 ± 9.8
Waist to Hip Ratio	0.802 ± 0.048

Table 2: CVC at Baseline, Following NOS Inhibition, and at Maximum

	Control	Allopurinol	Apocynin	BH4	ANOVA
Baseline (%CVCmax)	16 ± 15	14 ± 9	14 ± 7	21 ± 12	0.55
L-NAME (%CVCmax)	10 ± 2	13 ± 6	13 ± 7	13 ± 7	0.51
Maximal CVC (a.u. / mmHg)	2.45 ± 0.66	2.45 ± 0.51	2.1 ± 0.47	2.7 ± 0.69	0.12

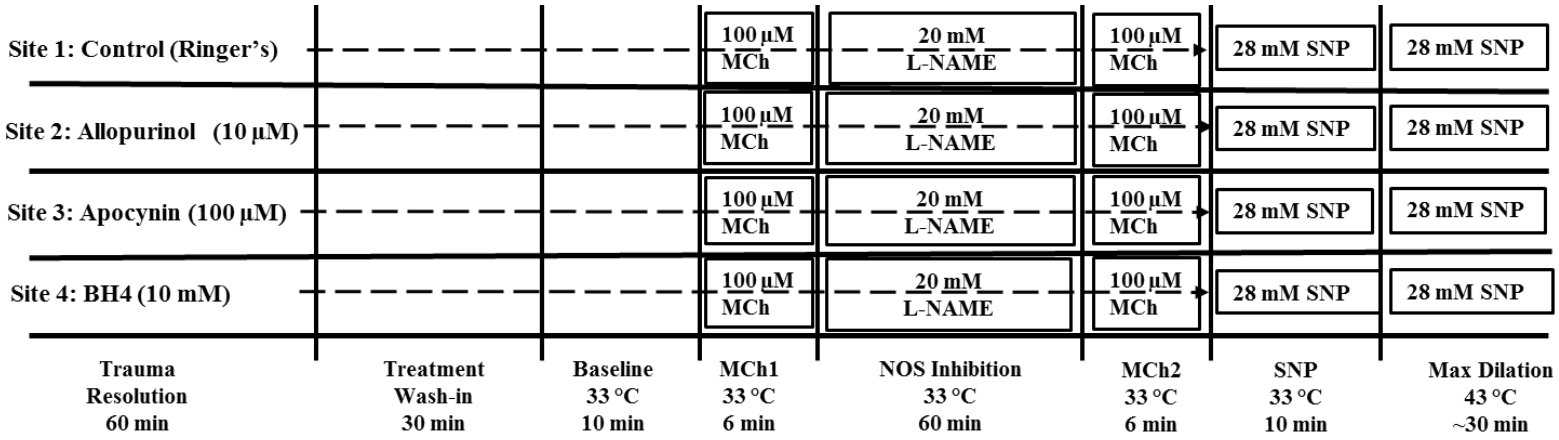
Site 1: Control (Ringer's)
Site 2: Allopurinol (10 µM)
Site 3: Apocynin (100 µM)
Site 4: BH4 (10 mM)
100 µM MCh
20 mM L-NAME
100 µM MCh
28 mM SNP
28 mM SNP
Trauma Resolution 60 min
Treatment Wash-in 30 min
Baseline 33 °C 10 min
MCh1 33 °C 6 min
NOS Inhibition 33 °C 60 min
MCh2 33 °C 6 min
SNP 33 °C 10 min
Max Dilation 43 °C ~30 min

Figure 1.

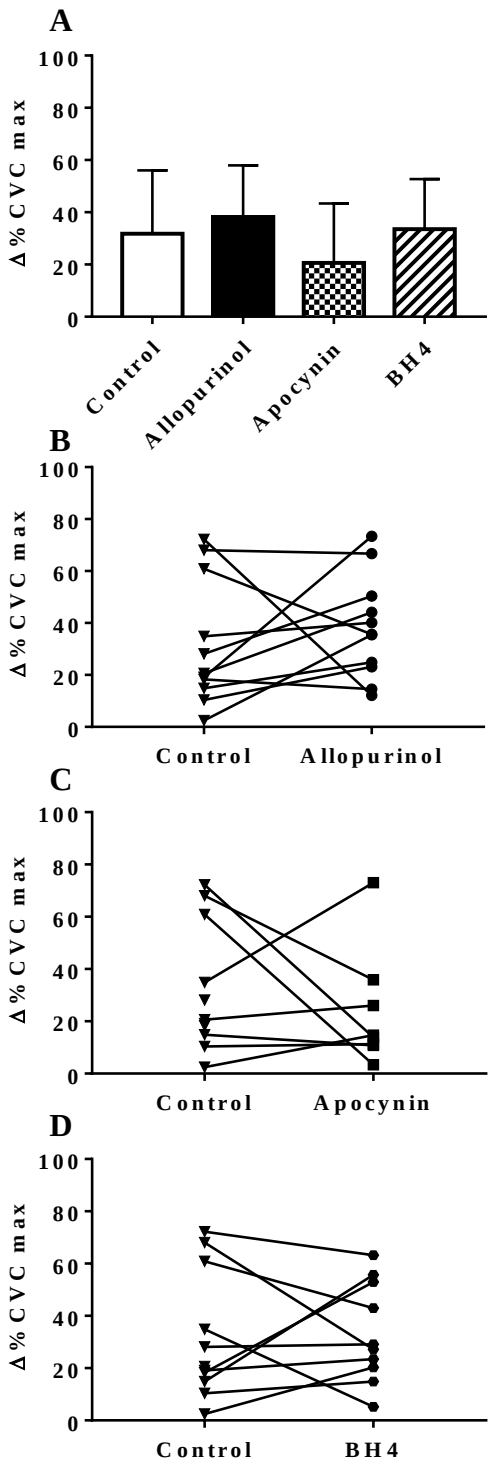
A
100
80
60
40
20
0
Δ% CVC max
Control
Allopurinol
Apocynin
BH4
B
100
80
60
40
20
0
Δ% CVC max
Control
Allopurinol
C
100
80
60
40
20
0
Δ% CVC max
Control
Apocynin
D
100
80
60
40
20
0
Δ% CVC max
Control
BH4

Figure 2.

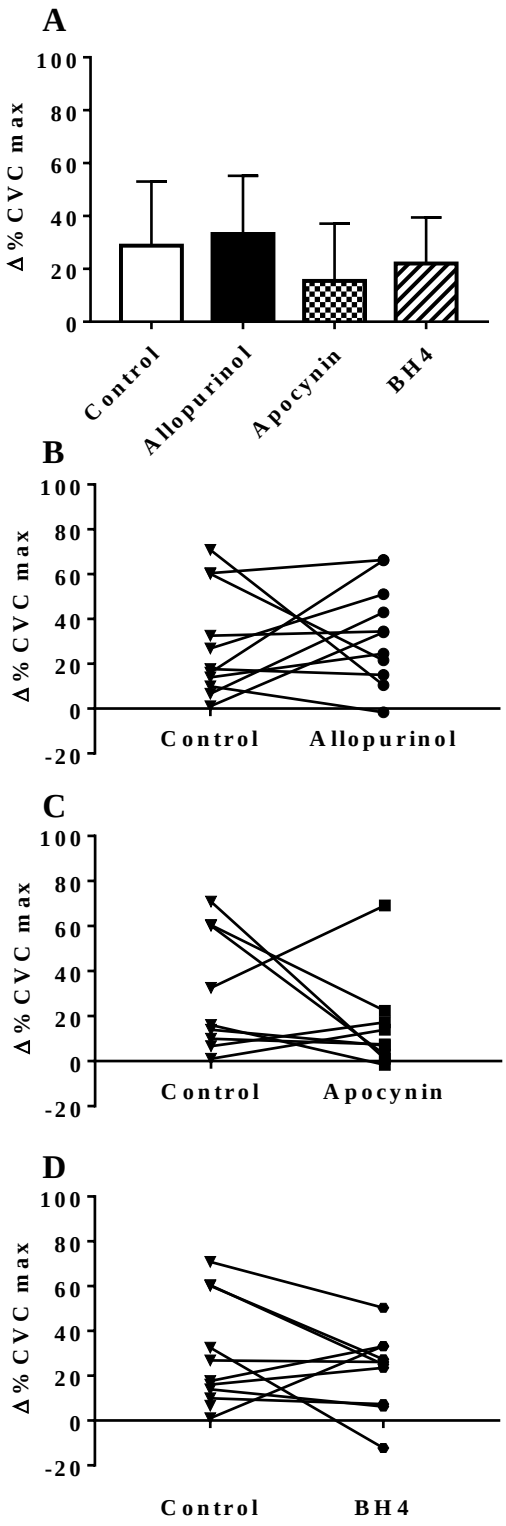
A
100
80
60
40
20
0
Δ% CVC max
Control
Allopurinol
Apocynin
BH4
B
100
80
60
40
20
0
-20
Δ% CVC max
Control
Allopurinol
C
100
80
60
40
20
0
-20
Δ% CVC max
Control
Apocynin
D
100
80
60
40
20
0
-20
Δ% CVC max
Control
BH4

Figure 3.

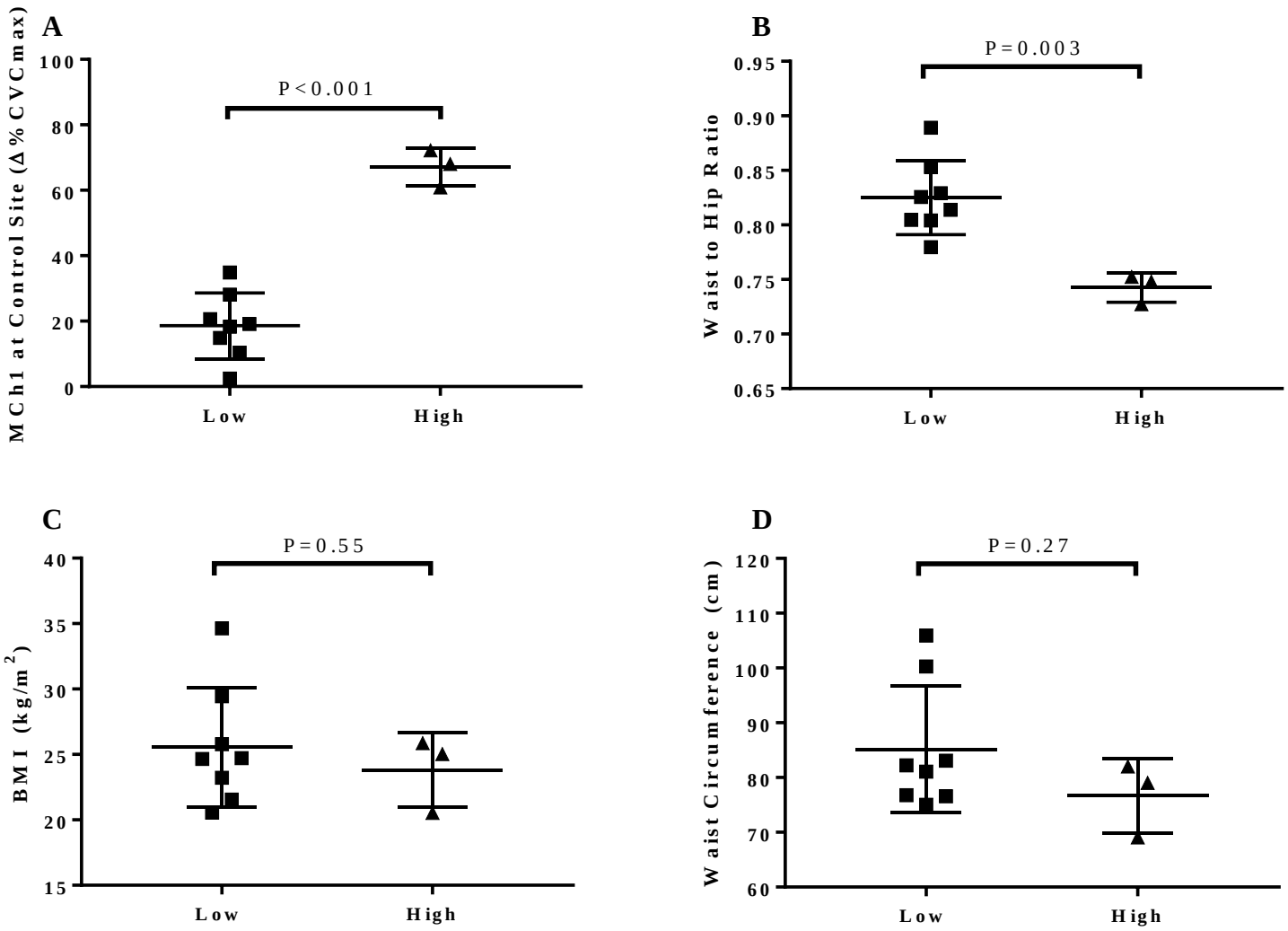
A
MCh1 at Control Site (Δ%CVCmax)
100
80
60
40
20
0
P<0.001
Low
High
B
Waist to Hip Ratio
0.95
0.90
0.85
0.80
0.75
0.70
0.65
P=0.003
Low
High
C
BMI (kg/m2)
40
35
30
25
20
15
P=0.55
Low
High
D
Waist Circumference (cm)
120
110
100
90
80
70
60
P=0.27
Low
High

Figure 4.

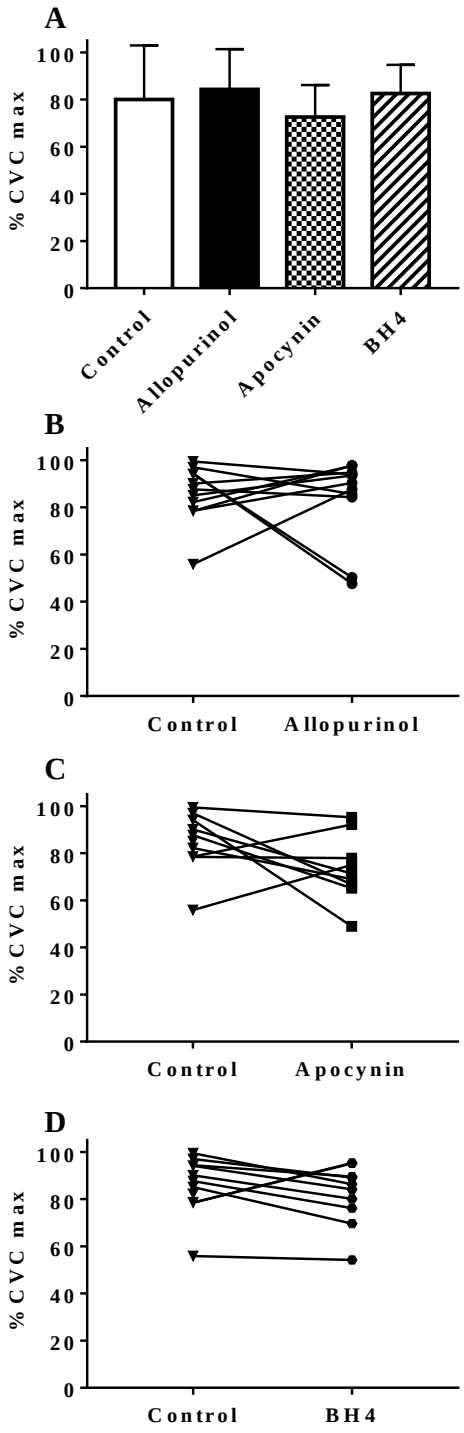
A
% CVC max
100
80
60
40
20
0
Control
Allopurinol
Apocynin
BH4
B
% CVC max
100
80
60
40
20
0
Control
Allopurinol
C
% CVC max
100
80
60
40
20
0
Control
Apocynin
D
% CVC max
100
80
60
40
20
0
Control
BH4

Figure 5.

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Chapter 4

THE EFFECTS OF AN ORAL ANTIOXIDANT COCKTAIL ON MACRO- AND MICROVASCULAR FUNCTION IN YOUNG BLACK MEN

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Abstract

Relative to their white counterparts, impaired vascular function is present in the black population across multiple vascular beds and may contribute to their increased risk of hypertension and cardiovascular disease. Recent studies suggest a role for oxidative stress in the genesis of vascular dysfunction, however, a paucity of data exists on the effectiveness of oral antioxidants on vascular responsiveness.in the black population. Therefore, the current study aimed to test the hypothesis that an oral antioxidant cocktail (AOC) would augment peripheral micro-and macrovascular function in healthy, young black men as measured by post occlusion reactive hyperemia and subsequent brachial artery flow-mediated dilation (FMD), respectively. Vascular function was determined via high resolution Doppler ultrasound measurements of brachial artery diameter and blood velocity following 5 min of forearm occlusion. Measures were made Pre and 120 min Post AOC (vitamin C, vitamin E, and coenzyme Q10) or placebo. Brachial artery baseline blood flow ($P=0.01$), peak reactive hyperemia velocity ($P<0.01$), and FMD ($P=0.01$) were reduced at Post in both conditions. In the AOC condition FMD was reduced to a greater degree than placebo ($P=0.02$). Contrary to the hypothesis, these data indicate a paradoxical reduction in conduit artery endothelial function following antioxidant supplementation.

Introduction

The black population has been repeatedly demonstrated to have impaired vascular function relative to their white counterparts (3, 4, 13, 31). This population has an increased risk of hypertension and other cardiovascular diseases compared to other races (2). Importantly, vascular dysfunction is present in other at-risk populations suggesting that it is a key contributor to the development of hypertension and cardiovascular disease (CVD) (32, 33, 37). As such, identification of the mechanisms underlying vascular dysfunction in the black population may lead to the development of treatment strategies to reduce the burden of disease in this group.

Elevated production of oxidative stress has been observed in cultured endothelial cells from black women (10, 16) and circulating peripheral blood mononuclear cells in healthy young black men (7). Furthermore, our lab has recently reported that local scavenging of superoxide ion results in augmented nitric oxide (NO) mediated vasodilation in the cutaneous microvasculature (14). Additionally, inhibition of the production of superoxide derived from NADPH oxidases and xanthine oxidase also results in restored cutaneous microvascular function in a variety of populations including black individuals (8).

Unfortunately systemic inhibition of NADPH oxidase and xanthine oxidase is not currently feasible as there is no approved NADPH oxidase inhibitor and xanthine oxidase inhibition via allopurinol has potentially serious detrimental side effects (30). Therefore, oral antioxidants are an appealing area of investigation. In isolation, oral antioxidants such as vitamin C have not proven to be effective at preventing cardiovascular disease (29). However, the use of multiple antioxidants in concert, an antioxidant cocktail, has shown some potential at improving

vascular function in populations with clear impairments (27, 39, 40). An antioxidant cocktail consisting of vitamin C, vitamin E, coenzyme Q10 has been proposed based upon the combination of lipophobic and lipophilic antioxidants (18). While no studies have assessed the effects of this cocktail on vascular function, it has been previously reported that this cocktail results in marked acute reductions in plasma superoxide, sympathetic nerve activity, and blood pressure (20). Therefore, the aim of the current study was to test the hypothesis that this oral antioxidant cocktail will acutely improve measures of systemic vascular function in young black men, ultimately implicating oxidative stress as an early cause of vascular dysfunction in this population.

Methods

Ethics Approval: All study protocols and procedures were approved by the institutional review board at the University of Texas at Arlington. Prior to participation, volunteers were provided with written and verbal descriptions of all measurements and any known risks of the study. Participants gave written informed consent in accordance with the Declaration of Helsinki.

Subjects: Eight healthy black men participated in the current study. Subject characteristics are displayed in Table 1. All subjects were free of overt cardiovascular, metabolic, or neurological disease and not currently taking medication or vitamin supplements. Smokers, competitive athletes, and bi-racial individuals were excluded from the study. Subjects reported to the laboratory in the morning (between 0700 and 0900) after an overnight (10+ h) fast on two separate occasions. Due to the extended half-life of coenzyme Q10 (~33 h), there was a minimum of 7 days between visits. Upon arrival to the laboratory, height and weight were assessed using a standard medical

scale and stadiometer. Waist and hip circumferences were measured at the level of the naval and the widest portion of the hips, respectively, with a flexible measuring tape.

Pre-Treatment Measures: Laboratory temperature was maintained at 23±1 °C for all trails and the lights were dimmed during all data collection. Following anthropometric measurements, subjects assumed a supine position for a minimum of 15 min of quiet rest. Measures of microvascular and macrovascular function, reactive hyperemia and flow-mediated dilation, respectively, were performed based upon published guidelines (12, 34). Briefly, the right arm was supported at heart level and abducted approximately 80°. The brachial artery was then imaged with a multifrequency linear array ultrasound probe (Logiq P5, GE Healthcare) in B mode. The ultrasound was then set to duplex mode allowing for concurrent imaging of the artery and Doppler measurement of the blood velocity. With the Doppler frequency set to 5 MHz, the artery was insonated at 60°, and the sample volume was set to encompass the majority of the artery without extending beyond the vessel wall. The probe was held in place via a stereotactic clamp and a 10 cm pneumatic cuff was secured around the forearm just distal to the medial epicondyle. Following 2 min of baseline data collection, the cuff was inflated to 220mmHg to occlude circulation to the forearm for 5 min. Upon cuff release, diameter and velocity were recorded for an additional 3 min. Digital video of the entire 10 min protocol was saved offline for later analysis using commercially available edge detection software (FMD Studio, Quipu). Brachial artery flow mediated dilation (FMD%) was determined as: (peak diameter – baseline diameter)/ baseline diameter x 100. Angle-corrected blood velocity was calculated as the area of the Doppler envelope each second. Shear rate (s^{-1}) was estimated as 4 x velocity / diameter. All data were assessed using a 3 sec rolling average. Following this assessment of vascular function an intravenous catheter was then placed in an antecubital vein

of the other arm for collection of a baseline (i.e. pre-treatment) blood sample (see Biochemical analysis).

Following completion of the pre-treatment vascular measures and blood sample, the subject was given either an antioxidant cocktail (AOC) consisting of two 1 g vitamin C tablets (Sundown Naturals) and a single pill containing 15O IU vitamin E and 100 mg coenzyme q10 (Qunol Ultra CoQ10) or a placebo (three capsules with 0.3 g sucrose) with 240 ml of water. The order of treatments was randomized, and the investigators were blinded until analysis was complete. The subjects rested in a semi recumbent position. During this time, they were allowed use electronic devices and change body positions, but walking was limited to a short walk (~50m) to the restroom after 75 min.

Post-treatment measures: Following the restroom break, subjects assumed a supine position and were instrumented with a standard 3-lead ECG for monitoring of heart rate, an automated blood pressure cuff (Tango M2, SunTech Medical) for periodic measurement of brachial artery blood pressure, and a pneumobelt (Model 1132 Pnuemotrace II, UFI) to monitor respiration. A 20 min resting baseline commenced 90 min after the treatment. Subjects were asked to remain quiet and relaxed while brachial blood pressure was taken every other min. Subjects were periodically reminded to stay awake if breathing patterns changed, but otherwise were not verbally engaged by the research team. Immediately after this 20 min post-treatment baseline period the aforementioned FMD protocol was repeated as described above, followed by a post-treatment blood sample.

Biochemical analysis: The pre-treatment blood sample was drawn into serum separator tubes, incubated for 30 min, centrifuged, and then sent to a commercial laboratory for metabolic and lipid

profile analysis (LabCorp). Additionally, pre- and post-treatment blood samples were collected into sodium heparin tubes for measurement of total reactive oxygen species (ROS) via the Electron Paramagnetic Resonance Spectroscopy (EPR) technique as previously described (6). Initially samples were incubated in KDD buffer (Krebs-HEPES buffer, deferoxamine methanesulfonate salt (DF), and diethyldithiocarbamic acid sodium (DETC); Noxygen Science Transfer & Diagnostics) at 37°C for 15 minutes. Samples were then incubated with 200 uM of methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH; Noxygen Science Transfer & Diagnostics) spin probe at 37°C for 30 minutes. 50 uL of each sample was loaded into a 1 ml syringe and flash frozen between two layers of KDD buffer. Samples were stored in a -80 °*C* freezer until they were analyzed.

Statistical Analysis: Descriptive characteristics were compared across visits using two-tailed paired t-tests. Repeated measures ANOVAs were used to analyze time and treatment effects on vascular function. FMD responses were also compared using the total shear to peak dilation (shear AUC) and baseline artery diameter as covariates using SPSS software (IBM). All other statistical analyses were performed using Prism 7 (Graphpad). The Holm-Sidak method for multiple comparisons was used for pairwise comparisons. Alpha was set at 0.05 and all data are presented as mean ± SD.

Results

The effect of placebo and AOC treatment on hemodynamic variables is displayed in Table 2. We observed a time dependent reduction in brachial artery diameter in the placebo condition ($P<0.01$) that did not occur during AOC ($P=0.48$). Baseline brachial blood velocity and blood flow

were also reduced post-treatment in both conditions, however there was no difference between conditions. Similarly, blood pressure was unchanged relative during AOC relative to placebo.

Macrovascular Function: Basal shear patterns are illustrated in Figure 1. Mean (time effect $P<0.01$, Figure 1A) and antegrade (time effect: $P<0.01$, Figure 1 B) shear rate were reduced at Post in both conditions. Retrograde shear rate was significantly augmented post-treatment compared to pre-treatment in the AOC condition ($P=0.01$, Figure 1C), whereas it was not different in the placebo condition ($P=0.22$, Figure 1C).

Brachial artery FMD was reduced post treatment relative to pre-treatment in placebo and AOC (main effect of time: $P=0.01$, Figure 2A). This difference remained when FMD data was co-varied to account for baseline diameter and shear AUC (main effect of time: $P<0.001$, Figure 2B). There was a greater reduction in FMD in the AOC condition compared to the placebo condition when expressed as either raw FMD (time x condition interaction: $P=0.02$, Figure 2C) or when adjusted for the aforementioned co-variates (time x condition interaction: $P<0.001$, Figure 2D). Taken together, these data indicate a reduction in conduit artery endothelial function, and this effect was exacerbated in the AOC condition.

Microvascular Variables: Similar to FMD, we observed a time-dependent reduction in reactive hyperemia when expressed as peak blood velocity ($P<0.01$, Figure 3A), peak blood flow ($P=0.01$, Figure 3B), and total hyperemia in the first 2 min following cuff release with ($P<0.01$, Figured 3C) and without ($P<0.01$, Figure 3D) inclusion of the baseline values. There were no significant interactions for these variables suggesting that AOC did not influence microvascular responses.

Total ROS: Circulating ROS levels were determined by EPR and displayed in Figure 4. Total ROS was unaffected by AOC relative to placebo (time x condition interaction: P=0.15).

Discussion

The current study presents novel findings indicating that during acute reductions in macrovascular endothelial function, an antioxidant cocktail consisting of vitamin C, vitamin E, and coenzyme Q10 amplifies the negative effects in black men. This finding occurred despite no discernible change in circulating ROS following antioxidant treatment. In contrast to the macrocirculation, time-dependent reductions in microvascular function were not exacerbated by acute oral antioxidant treatment.

The hypothesis of the current study was that oral AOC would acutely improve vasodilatory function in a population who have previously been shown to exhibit impaired micro- (4, 13, 14, 24, 31) and macrovascular dilation (3, 25). Black men, even when young and otherwise healthy, have elevated NADPH oxidase content and subsequent superoxide production in peripheral blood mononuclear cells compared to age-matched white men (7). Isolated endothelial cells from young black donors also present with greater NADPH oxidase activity and superoxide production (10, 16). As superoxide is a primary contributor to reductions in NO bioavailability and further production of free radicals, ROS are likely a contributor to racial differences in vascular function. In agreement with this hypothesis, our lab has recently observed that local reductions in superoxide via the superoxide dismutase mimetic tempol results in improved NO-mediated cutaneous dilation during local heating in black individuals (16). Furthermore, when we inhibited the production of

superoxide via NADPH oxidases and xanthine oxidase, we also abolished the difference in local heating responses amongst black and white men (Chapter 2).

The degree to which oral antioxidants can augment vascular function in black men has not been well studied. A recent study by Kappus and colleagues found no effect of an oral antioxidant treatment on FMD in healthy young black or white men (17). In contrast to the current study, the authors used a cocktail with less vitamin C (1 g vs 2 g), more vitamin E (600 IU vs 150 IU), and alpha lipoic acid rather than coenzyme Q10 (17). The antioxidant treatment utilized by Kappus and colleagues (17) has been previously shown to augment vascular function in aged (40) and diseased populations (27, 39) via a reduction in oxidative stress. As such, the lack of an effect on FMD in young, healthy black men suggests that ROS may not be solely responsible for diminished conduit artery endothelial function in black men.

Interpretation of the current study is complicated by the unexpected reduction in microvascular and macrovascular function in measures that were performed approximately 2 h apart. During this time, subjects were sedentary but not completely still. Similar to the current data, Restaino et al. demonstrated that prolonged sitting resulted in a reduction in brachial artery blood flow at two hours and reductions in reactive hyperemia after 6 h (26). The authors observed that, despite hours of reduced brachial artery blood flow and the resulting endothelial shear stress, brachial artery FMD was not reduced following 6 h of prolonged sitting (26). Our current data demonstrate that, in black men, 2 h of sedentary behavior-induced reductions brachial artery blood flow and endothelial shear stress results in a reduced FMD whether expressed unadjusted, co-varied for shear AUC alone (not shown), or co-varied for shear AUC and baseline diameter.

As extended periods of reduced shear are associated with endothelial dysfunction (21, 22, 28),the time-dependent reduction in FMD observed in both AOC and Placebo conditions is likely due to reductions in basal shear rates or altered shear patterns on the brachial artery endothelium. While basal brachial shear stress in the current study was only assessed upon arrival to the laboratory and ~2 h post treatment, popliteal artery shear rates are reduced by approximately half after just 10 min of sitting and do not decrease further over 3 h of sitting (38). We observed basal brachial artery mean shear rates were reduced post-treatment by ~60%. Based upon the findings by Vranish et al., we speculate that shear was reduced for much of the preceding 2 h due to lack of activity (38). Notably, much of the reduction in mean shear was due to decreased antegrade shear rates. Tinken et al reports that reductions in antegrade shear reduce subsequent FMD (36). However, Thijssen and colleagues report that 30 min of augmented retrograde shear induced by sub-diastolic pressure cuff inflation is adequate to elicit reductions in FMD despite no change in antegrade shear (35). Importantly, retrograde shear was significantly augmented post-treatment during the AOC trial, but not the placebo trial. This difference may explain the greater reduction in FMD after AOC.

Oscillatory shear stress on isolated cells results in superoxide production via an interaction between NADPH oxidase and xanthine oxidase (19). As superoxide readily reacts with NO and decreases its bioavailability, this is a likely mechanism through which altered shear patterns induce endothelial dysfunction. Supporting this hypothesis, acute antioxidant treatment via vitamin C prevents a blunted FMD following external compression-induced changes in shear patterns (15). Therefore, it is unclear why the AOC exacerbated the reduction in brachial artery endothelial function, rather than protect it. More research is needed to determine whether this finding was an

effect of the AOC used in the current study, or if there is an interactive effect of race and antioxidant treatment.

Consistent with previous reports (26, 38), the current data demonstrate a reduction in reactive hyperemia following a period of inactivity signifying impaired microvascular function. In contrast, to the FMD data, there was no greater reduction in reactive hyperemia with the AOC relative to the placebo trial suggesting this response is unaffected by the AOC employed in this study. This distinction suggests that the negative effect of AOC in this population is focused on NO, the primary vasodilator in brachial artery FMD (11). In contrast, AOC likely does not have an additive effect on impairment of inward rectifying potassium channels or the sodium-potassium ATPase, which are both fundamentally involved in reactive hyperemia (5). Nevertheless, blunted reactive hyperemia is an index of microvascular function that is considered predictive of future cardiovascular events (1). These data indicate the importance of taking such measurements under standardized conditions to account for differences in prior physical activity.

Limitations: The ability of this study to test our hypothesis was limited by our study design and there are other notable limitations to our interpretation of the data. While we attribute the reductions in micro-and macrovascular function to reduced activity during the study, we cannot rule out potential diurnal effects. This explanation is unlikely, however, because other studies suggest that vascular reactivity is lowest early in the morning (9, 23). Additionally, we observed no change in ROS in blood collected 2 h after AOC consumption. Without a control group, we cannot determine if this is because our cohort of young black men did not have elevated ROS, contrary to previous studies, (7) or if our AOC was ineffective. The current study utilized a novel AOC based upon currently unpublished findings from another group. This group modelled the

AOC on a study that included different dosages for each drug, different means of administration (intravenous vs oral vitamin C), and an additional component (superoxide dismutase) (18). Therefore, further studies are required to determine the efficacy of the current AOC at scavenging ROS in populations with known oxidative stress.

In conclusion, the current study presents novel evidence that a brief period of minimal activity results in reductions in microvascular and macrovascular function in otherwise healthy black men. Furthermore, these data indicate that an antioxidant cocktail of vitamin C, vitamin E, and coenzyme Q10 elicits greater decrements in conduit artery endothelial function than a placebo. Whether this impairment is specific to black men or our choice of antioxidants requires further investigation.

Figure Legends

Figure 1: A) Basal mean shear rates were reduced post treatment independent of condition. B) Basal antegrade shear rates were also lower post treatment relative to pre-treatment in both conditions. C) Retrograde shear rate was augmented post treatment relative to pre-treatment, while placebo had no effect. All data presented as individual data points and means (bars).

Figure 2: A) There was a time-dependent reduction in brachial artery FMD (%) that was driven by a significant reduction at post in the AOC. B) When brachial artery FMD was adjusted for baseline diameter and total shear rate to peak dilation (shear AUC), there was a clear effect of time ($P<0.001$) with FMD being blunted across conditions. Data for A and B presented as individual data points and means (bars). Average reduction in FMD pre-post was greater during AOC than placebo when expressed as unadjusted (C) and when adjusted (D) for baseline diameter and shear AUC. Error bars indicate SD.

Figure 3: Reactive hyperemia is expressed as A) Peak velocity, B) Peak blood flow, and C) total hyperemia in the first 120 s after cuff release. All parameters and conditions were reduced at post, however there was no difference across conditions. All data presented as individual data points and means (bars).

Figure 4: Total ROS as determined via EPR. There was no effect of time or condition. All data presented as individual data points and means (bars).

Table 1: Subject Characteristics

Age (y)	22 ± 3
BMI (kg/m^2)	25 ± 5
Systolic BP (mmHg)	116 ± 6
Diastolic BP (mmHg)	71 ± 9
MAP (mmHg)	86 ± 7
Glucose (mg/dl)	89 ± 6
Total Cholesterol (mg/dl)	156 ± 34
Triglycerides (mg/dl)	76 ± 35
HDL (mg/dl)	56 ± 13
LDL(mg/dl)	85 ± 24
VLDL (mg/dl)	15 ± 7

BMI: body mass index, BP: blood pressure, HDL: high density lipoprotein, LDL: low density lipoprotein, VLDL: very low density lipoprotein. Data from the placebo visit is displayed as there were no differences between days for any parameter.

Table 2: Baseline Brachial Artery Parameters

	Placebo		AOC		Time	Treatment	Interaction
	Pre	Post	Pre	Post		P	
Diameter (mm)	3.63 ± 0.63	3.48 ± 0.67*	3.51 ± 0.55	3.49 ± 0.63	0.15	0.22	0.04
Mean Blood Velocity (cm/s)	15 ±7	6 ±2	17 ± 8	6 ± 3	0.002	0.54	0.38
Mean Blood Flow (ml/min)	98 ± 65	35 ± 18*	109 ± 83	36 ± 32*	0.01	0.59	0.59

AOC: Antioxidant cocktail. * P<0.05 vs Pre

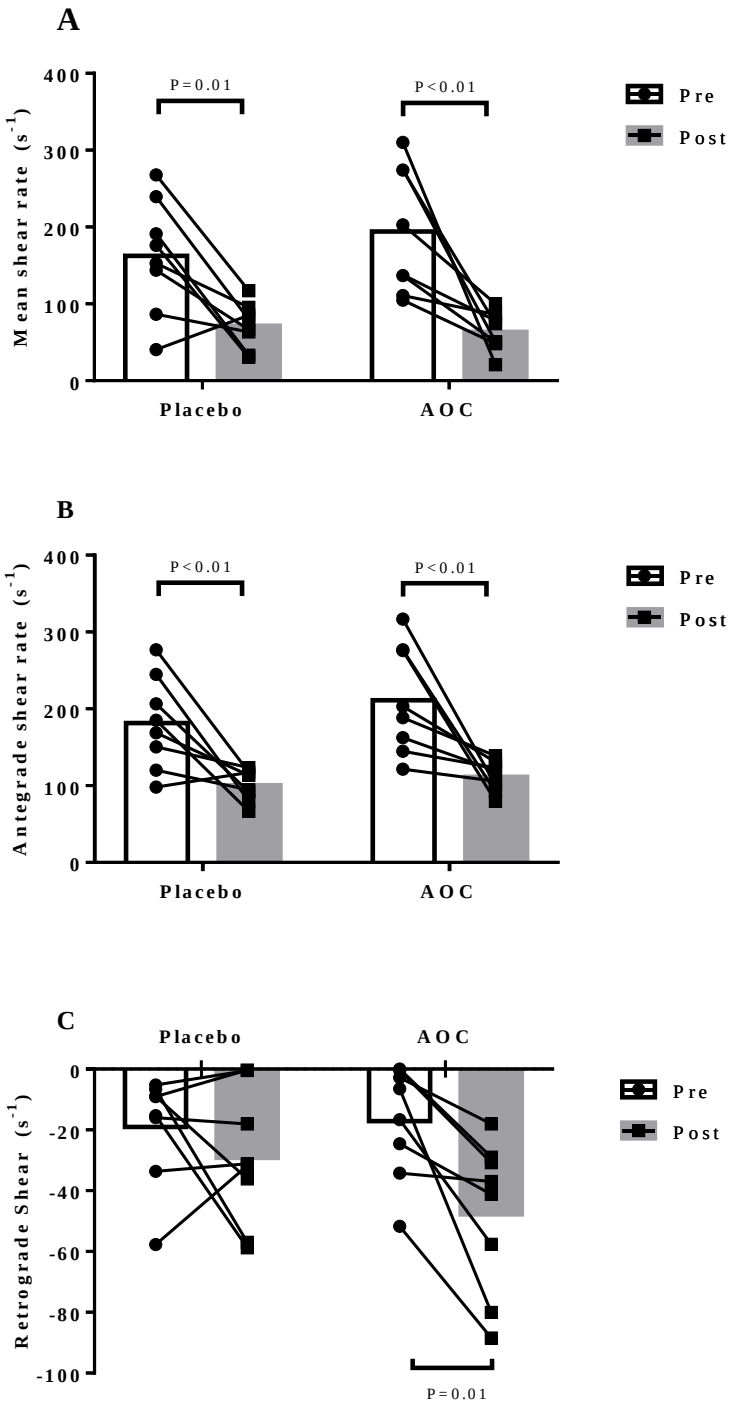
A
400
300
200
100
0
Mean shear rate (s-1)
P=0.01
P<0.01
Placebo
AOC
Pre
Post
B
400
300
200
100
0
Antegrade shear rate (s-1)
P<0.01
P<0.01
Placebo
AOC
Pre
Post
C
Placebo
AOC
0
-20
-40
-60
-80
-100
Retrograde Shear (s-1)
P=0.01
Pre
Post

Figure 1.

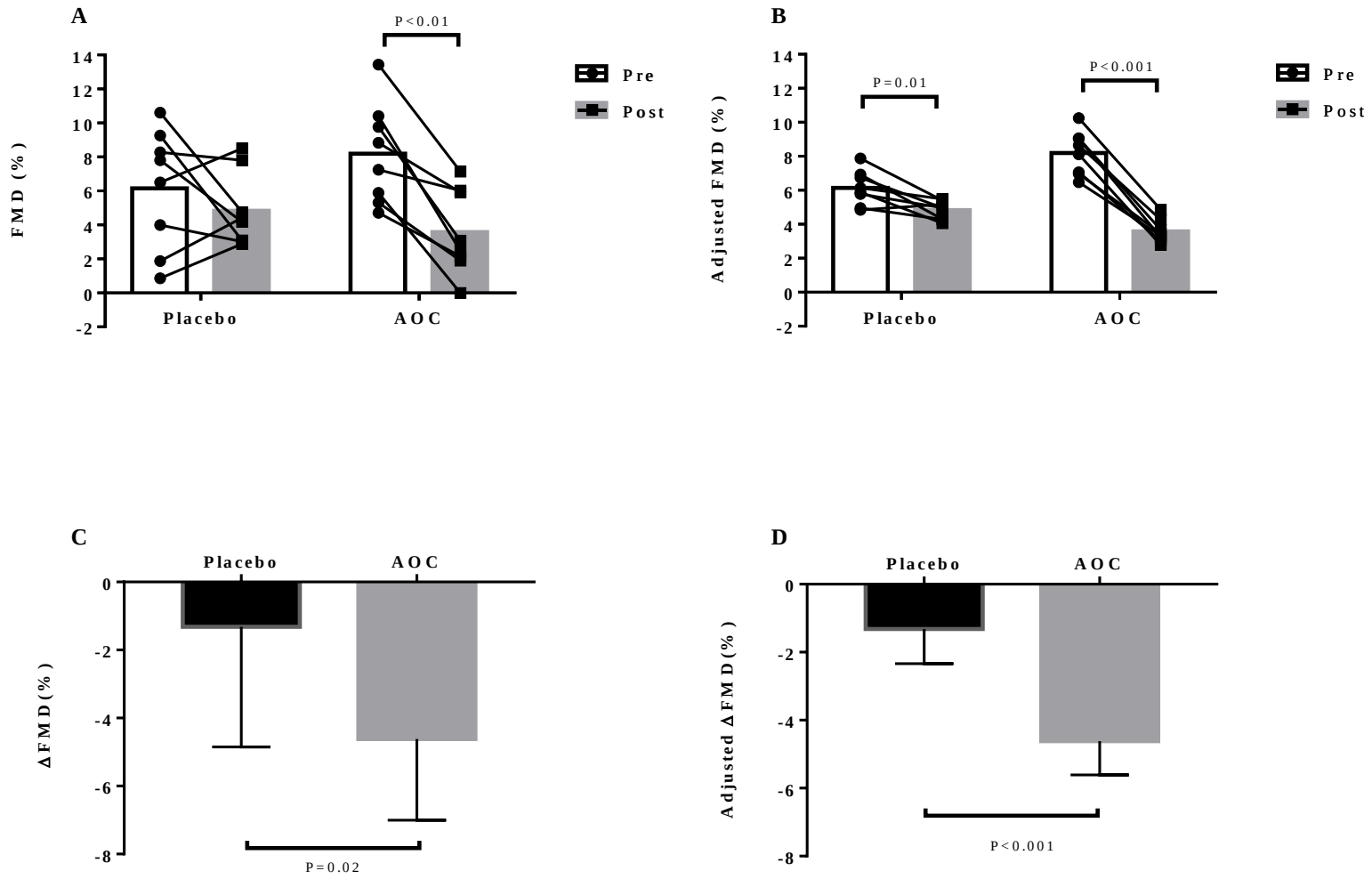
A
P<0.01
FMD (%)
14
12
10
8
6
4
2
0
-2
Placebo
AOC
Pre
Post
B
P=0.01
P<0.001
Adjusted FMD (%)
Placebo
AOC
Pre
Post
C
Placebo
AOC
ΔFMD(%)
0
-2
-4
-6
-8
P=0.02
D
Placebo
AOC
Adjusted ΔFMD(%)
P<0.001

Figure 2.

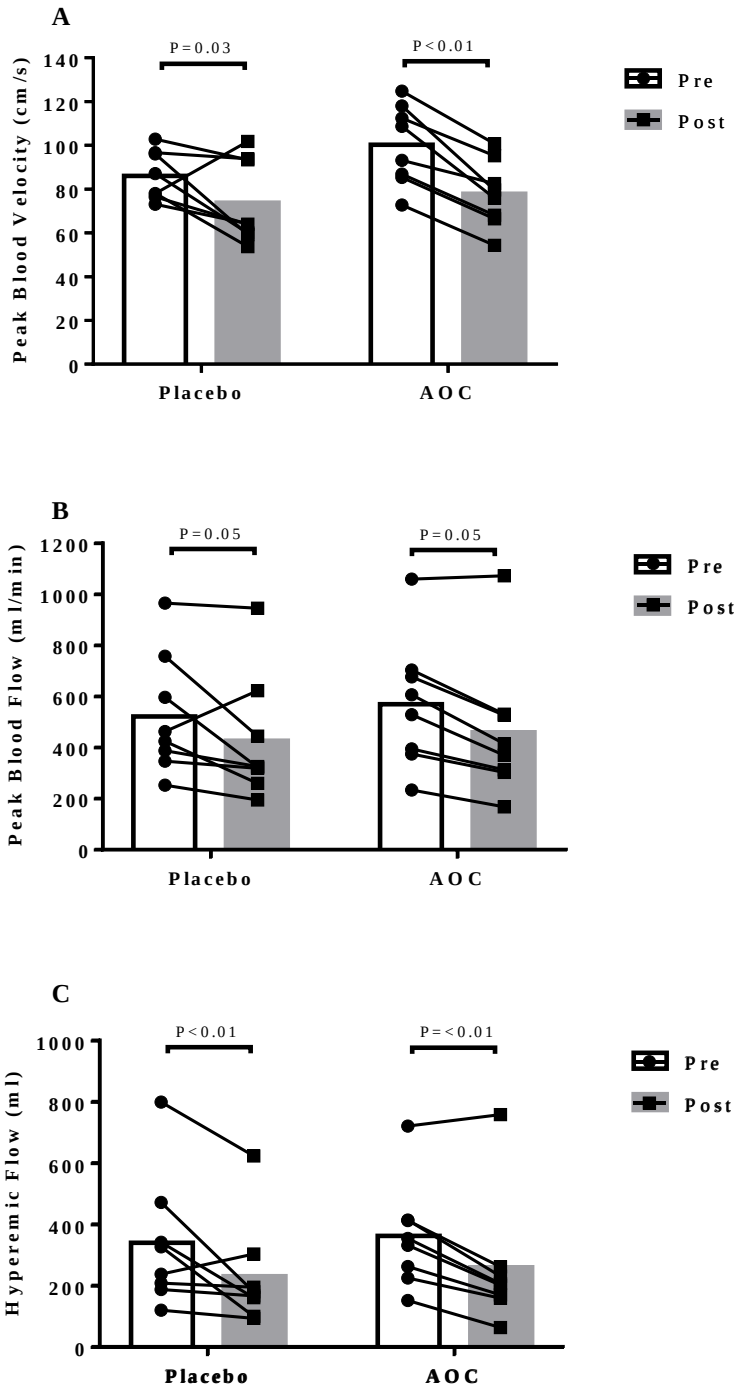
A
P=0.03
P<0.01
Peak Blood Velocity (cm/s)
140
120
100
80
60
40
20
0
Placebo
AOC
Pre
Post
B
P=0.05
P=0.05
Peak Blood Flow (ml/min)
1200
1000
800
600
400
200
0
Placebo
AOC
Pre
Post
C
P<0.01
P=<0.01
Hyperemic Flow (ml)
1000
800
600
400
200
0
Placebo
AOC
Pre
Post

Figure 3.

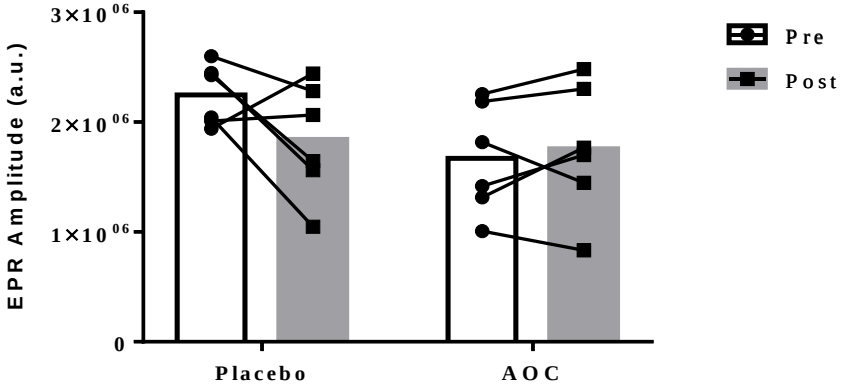

Figure 4.

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Chapter 5

FUTURE DIRECTIONS

Vascular dysfunction is an early step in the pathogenesis of hypertension (27, 32, 33) and thus may provide a therapeutic target for populations with elevated risk of hypertension, such as the black population. Impaired vascular responses are consistently observed in black individuals compared to their white counterparts in a variety of vascular beds including conduit arteries (2, 23), limb microvasculature (3, 4, 21), and the cerebral vasculature (11, 12). Our group has previously identified blunted cutaneous microvascular function in healthy young black individuals in response to both local heating (13) and direct infusions of the muscarinic agonist methacholine (manuscript in preparation). Herein, we described studies aimed at determining the role of oxidative stress in this racial disparity and the specific enzymatic sources of said oxidative stress. This work demonstrates, for the first time, that inhibition of both xanthine oxidase and NADPH oxidases abolishes the racial differences in local heating responses amongst men. Furthermore, inhibition of either enzyme does not augment cutaneous microvascular thermal reactivity in black women, suggesting a sex-difference in the mechanisms mediating microvascular dysfunction in the black population.

Future studies are needed to determine the mechanisms responsible for blunted vascular responses in black women. Relative to white women, umbilical vein endothelial cells of black women paradoxically express greater amounts of endothelial nitric oxide synthase (eNOS), while producing less nitric oxide (NO) and more reactive oxygen species in-vitro (8, 14) implicating uncoupled eNOS as a cause of impaired endothelium-dependent dilation in black women. Uncoupling of eNOS occurs due to deficiency in the eNOS cofactor tetrahydrobiopterin (BH4) or a lack of the substrate L-arginine (20). In support of the L-arginine hypothesis, supplemental L-arginine improves coronary vascular dilation during ACh infusion in black patients whereas it has

no effect in white patients (10). Likewise, in-vitro data suggest that vascular cells from black women exhibit greater creatinine kinase activity than white women which may outstrip the available L-arginine for the production of creatinine (1). To test this hypothesis, future studies should assess vascular responses in black women after supplementation of L-arginine and inhibition of creatinine kinase, both separately and in-concert. Unfortunately, such a study may be limited to isolated cells as creatine kinase inhibition is not currently available in-vivo. Alternatively, asymmetrical dimethyl arginine (ADMA) competes with L-arginine and inhibits eNOS (5). While ADMA is elevated in black men compared to white men and associated with reduced FMD (19), it is reduced in black women relative to white women (29). Thus, it is less likely that ADMA contributes to uncoupled eNOS in black women, however there is a paucity of research on this topic.

Supplementation of BH4, improves vascular function in populations with underlying impairments when administered locally (9, 31) or systemically (7, 17, 30, 34) via recoupling of eNOS. In Chapter 3, we observed no clear effect of local BH4 supplementation in black men, however its effects have not been studied in black women. Notably, BH4 deficiency is secondary to oxidation via peroxynitrite or hydrogen peroxide, thus an initial source of ROS is required (20). The data presented by Kalinowski and colleagues suggests that NADPH oxidase is that initial source (14), however its inhibition via apocynin did not result in improved cutaneous vasodilation in the current study, suggesting that NAPDH oxidase does not contribute to racial differences in vascular function. Alternatively, the dose we chose, based upon previous work, may not have been adequate to inhibit NADPH oxidase activity in this population. A follow-up study is needed to

determine if greater concentrations of apocynin are effective. Such a finding would drastically alter the interpretation of the current data.

While the isolated endothelial cell studies provide evidence of impaired endothelial production of NO in black women, it is possible that blunted vascular smooth muscle responsiveness to NO is the culprit behind racial differences in vascular function. Mixed-sex groups of black individuals exhibit blunted forearm blood flow responses to intra-arterial infusions of the NO donor sodium nitroprusside (SNP) relative to their white counterparts. Similarly, the increase in cutaneous microvascular dilation during SNP infused via iontophoresis is also blunted in black participants (24). The current data did not demonstrate a blunted maximal dilation during SNP infusion and local heating in the black women, suggesting no structural explanation for the racial differences in microvascular function. However, the possibility that the SNP dose-response relationship is blunted in the black population, similar to what we have demonstrated in obese young adults (22), warrants investigation. While the mechanisms of smooth muscle dysfunction are less commonly studied than the mechanisms of endothelial dysfunction, elevated phosphodiesterase-5 (PDE5) activity attenuates NO signaling within the vascular smooth muscle (15), and thus is an obvious initial direction to investigate. Impaired vasodilation is restored via PDE5 inhibition in men with increased cardiovascular risk (16, 26). To our knowledge the effect of PDE5 inhibitors on vascular function in black populations has not been investigated and warrants future study.

The second and third studies of this dissertation (Chapters 3 and 4) focused exclusively on black men. Our pilot data for Chapter 3 indicated blunted cutaneous vasodilation during MCh infusions in black women as well as black men, compared to their white counterparts. Likewise,

the literature that formed the basis for Chapter 4, illustrating blunted FMD and reactive hyperemia in black populations, included mixed-sex cohorts (2, 4, 6). Therefore, these studies also need to be repeated in women. While the results of the study described in Chapter 4 were potentially complicated by the sedentary nature of the protocol, the possibility of sex differences in the effect of extended sitting needs to be determined in the black population. Vranish et al. demonstrated that female sex protected conduit artery function from sitting induced dysfunction, yet microvascular function was similarly impaired across sexes in white participants (35). Whether black women are similarly protected from conduit artery dysfunction is unclear. Furthermore, it is important to determine if the antioxidant cocktail utilized in Chapter 4 exaggerates the negative effect of inactivity on conduit artery vascular function in black women like it did in black men. Valuable insight into the mechanisms through which sedentary behavior impacts health could be gained if these responses differ across race and/or sex.

While the current set of studies left many unanswered questions regarding vascular dysfunction in black women, future studies are also needed to expand upon the current findings in black men. For instance, the study described in Chapter 2 observed similar beneficial effects of inhibition of xanthine oxidase and NADPH oxidases. Based upon previous findings we suspect that NADPH oxidases and xanthine oxidases operate along a similar pathway with xanthine oxidase ultimately producing the superoxide that results in blunted NO-mediated dilation (18, 28). Simultaneous infusion of NADPH oxidase and xanthine oxidase inhibition, via apocynin and allopurinol, is needed to confirm that they do not have additive effects.

Notably, the study described in Chapter 3 directly contrasts with our hypothesis that superoxide derived from NADPH oxidases are required to activate the xanthine oxidase, as we did

not observe an effect of NADPH oxidase inhibition via apocynin, yet xanthine oxidase inhibition via allopurinol augmented MCh responses in black men with low cutaneous microvascular vasodilatory responses at the control site. The reasons for this finding are unclear, thus future research is needed to ascertain the differences between local heating and muscarinic mediated dilation.

Finally, as mentioned above, the interpretation of the findings of the study described in Chapter 4 is complicated by the time-dependent reduction in basal shear, reactive hyperemia, and FMD. We speculate that this finding was due to the inactivity imposed upon the subjects in the time between pre- and post-treatment measures. Our finding of blunted upper limb reactive hyperemia was consistent with that observed by Restaino et al., however, that study did not demonstrate an effect of inactivity on upper body conduit artery FMD when the response was normalized for shear stress (25). The current study demonstrated a clear reduction in FMD, controlled for shear stress, that was significantly exaggerated via an antioxidant cocktail. Our interpretation of this finding is that inactivity resulted in activation NADPH oxidases and xanthine oxidase which blunted NO-mediated dilation (18, 28). We speculate that the antioxidant cocktail amplified this reduction in FMD via a reduction in hydrogen peroxide mediated dilation. Direct measurement of brachial artery endothelial hydrogen peroxide production is unlikely, however a potential study design to test this hypothesis would involve the infusion of catalase to reduce local hydrogen peroxide formation.

The studies described in this dissertation are three of the first investigations to examine a role for oxidative stress in the vascular dysfunction observed in black populations. These studies

provide important insight to steer future research into this topic with the ultimate goal of reducing the disparate impact of hypertension on the black population.

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