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Mechanisms of Cutaneous Microvascular Endothelial Dysfunction in Young Black Americans

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Mechanisms of Cutaneous Microvascular Endothelial Dysfunction in Young Black Americans

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Black Americans have an increased risk for developing a variety of cardiovascular disease (CVD) when compared to white Americans and other populations in the United States. It has also been demonstrated that the underlying impairments in black Americans manifest during early adulthood prior to any overt signs of risk, which leads to higher rates of CVD related morbidity and mortality in black Americans than other populations.

Study 1 was designed to investigate the potential mechanisms of cutaneous microvascular dysfunction in young college-age black Americans. This was assessed by measuring the skin blood flow response to local heating while various vasoactive substances were delivered into the cutaneous interstitial space by intradermal microdialysis. We demonstrated that an attenuated nitric oxide (NO) mediated vasodilation due in part to a relative deficit of L-arginine in the endothelial cells is one mechanism by which microvascular dysfunction occurs in young black Americans.

Study 2 conducted to investigate the effects of acute cocoa flavanol intake on cutaneous microvascular function in young black Americans. This was assessed by measuring the skin blood flow response to local heating and delivery of vasoactive substances (as described above) before and after consumption of a beverage high in

flavanol content. Study 2 demonstrated that acute flavanol intake improved cutaneous microvascular function in response to local heating in young black Americans relative to young white Americans.

Study 3 was designed to investigate the effects of acute flavonal intake on endothelium-dependent microvascular dilation in response to exogenous administration of methacholine (MCh) in young black Americans. This was assessed by skin blood flow responses to incremental dose of MCh, which was delivered by intradermal microdialysis, before and after consumption of a beverage high in polyphenol content. Study 3 identified that acute flavanol intake did not alter the dose-response curve of MCh-induced cutaneous vasodilation in either racial groups.

Overall, the series of studies in this dissertation may provide evidence that young black Americans have attenuated microvascular function relative to young white Americans, and that a potential mechanism of decreased microvascular function is a decrease in NO bioavailability and/or NO mediated vasodilation, which is related to a deficit of L-arginine in the endothelial cells in young black Americans. Furthermore, our findings may provide evidence that the consumption of cocoa flavanols is an effective therapeutic strategy to prevent and/or delay the development of CVD at least in young black Americans.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

Cardiovascular disease (CVD) is a class of disease, which includes hypertension, heart disease, stroke, and peripheral artery disease. Although overall mortality from CVD has continued to decrease over recent decades, CVD is still the leading cause of death in the United States. It is currently estimated that 83 million adults suffer from CVD. In 2008, the estimated annual direct and indirect costs associated with the treatment of CVD was already about 300 billion dollars [1]. Research supports the conclusion that ethnicity has an effect on the progression and the manifestation of CVD [2]. Interestingly, black Americans have an increased risk for developing a variety of CVD when compared to white Americans in the United States [3, 4]. It is also believed that underlying impairments in black Americans manifest in early adulthood prior to any overt signs of risk, which leads to higher rates of morbidity and mortality in black Americans than white Americans [5-7].

The mechanisms of elevated CVD risk in black Americans are multifactorial; however, there is abundant evidence showing that microvascular endothelial dysfunction is a major contributing factor. Endothelial cells maintain vascular tone and homeostasis through a variety of mediators ultimately resulting in a balance between vasorelaxation and vasoconstriction. Nitric oxide (NO) is a potent vasodilator which is released from endothelium. A decrease in NO bioavailability and/or a blood vessels ability to respond to NO and a decrease in vascular compliance cause the balance of vascular tone in the microcirculation to shift toward vasoconstrictive forces [8-10], causing microvascular dysfunction. Impaired microvascular function due to a decrease in NO bioavailability and/or a blood vessels ability to respond to NO is now considered a precursor to macrovascular dysfunction and is a major contributor to the development of CVD [11-13].

Diet is one of the modifiable lifestyle factors that plays a pivotal role in the primary and secondary prevention of CVD [14]. Flavonoids found in fruits and vegetables have received much attention because of their potential beneficial effects on cardiovascular health [15]. Epidemiological studies suggest that the beneficial effects of flavonoids on cardiovascular health are predominantly provided by flavanols, which is one of the subgroups of flavonoids [16-18]. The exact relationship between flavanol intake and cardiovascular health have not been fully identified. However, the findings from interventional studies suggest that the benefits of flavanol intake are linked to an improvement in endothelial function via an enhanced NO bioavailability and/or a blood vessels ability to respond to NO in healthy individuals as well as in populations with increased cardiovascular disease risk [19-22].

Despite the well-known and unfortunate disparity between black Americans and white Americans regarding cardiovascular risk, less is known how microvascular dysfunction affects increased cardiovascular risk and how flavanol intake improves endothelial health in the microvasculature in black Americans. Therefore, this dissertation investigated the underlying mechanisms of microvascular dysfunction in young black Americans and the effects of flavanol intake on microvascular function as a preventive measure to reduce cardiovascular risk in this population.

Chapter 2: Statement of the Problem

- Cardiovascular disease (CVD) is the leading cause of deaths and the financial burden associated with the treatment of CVD are estimated to increase continuously in the United States as well as worldwide, which suggests the significance of developing ways to prevent and delay the initiation of CVD.
- Black Americans are at a higher risk for developing a variety of CVD including hypertension, stroke, peripheral artery disease, and type 2 diabetes relative to white Americans [3, 4, 23].
- The underlying impairments which contribute to future disease risk in black Americans reveal in young adults prior to any overt signs of risk [5, 24].
- We have recently reported that cerebral vasodilatory capacity is blunted in college-aged black Americans relative to white Americans counterparts [25], suggesting endothelial dysfunction in young black Americans. Furthermore, we have also recently reported that impaired cerebral vasodilatory capacity in this population is reversed following acute consumption of a beverage that is high in flavanol content [26].
- The findings from interventional studies have suggested that the benefits of flavanol intake are partially linked to improved endothelial function via enhanced nitric oxide (NO) bioavailability and/or a blood vessels ability to respond to NO in healthy individuals as well as in populations with elevated cardiovascular disease risk [19-22].

Study 1 of this dissertation was designed to investigate cutaneous microvascular function in healthy young black Americans in order to determine the potential mechanisms of microvascular dysfunction as they are associated with an increased risk for CVD. Study 2 was designed to investigate the effects of acute cocoa flavanol consumption on

microvascular function in healthy young black Americans in order to determine whether the benefits of acute flavanol intake could be directly applied to restore attenuated microvascular function in young black Americans. Last, study 3 aimed to investigate the effects of flavanol intake on methacholine-induced NO-dependent microvascular function in healthy young black Americans in order to determine whether acute flavanol intake could restore attenuated microvascular function in young black Americans.

Chapter 3: Experimental Design

To accomplish the aims of this dissertation, three studies utilizing local administration of vasoactive substances into the cutaneous microcirculation via the intradermal microdiaysis technique were conducted. The measurement of cutaneous microvascular function via this technique is advantageous because it is minimally invasive and allows for the manipulation of local skin blood flow in response to a variety of vasoactive substances that can stimulate or inhibit a variety of physiological pathways and its molecular reactions. Therefore, it provides a powerful tool to mechanistically assess microvascular function.

The purpose of study 1 was to investigate cutaneous microvascular function in response to a typical local heating protocol in young black Americans in order to determine the potential mechanisms of microvascular dysfunction as they are associated with an increased risk for cardiovascular disease in this population. Healthy subjects conducted a typical local heating protocol. Cutaneous vascular conductance (CVC) was calculated as red blood cell (RBC) flux divided by mean arterial pressure (MAP) for an index of cutaneous microvascular function. All CVC data were presented as a percentage of the maximal CVC (%CVCmax) achieved during 43 °C local skin heating combined with local infusion of 28 mM sodium nitroprusside (SNP) which is an exogenous NO donor. We tested the hypothesis that CVC in response to local heating of skin would be attenuated in young black Americans relative to age-matched white Americans due to the lack of nitric oxide (NO) bioavailability and/or a blood vessels ability to respond to NO. Furthermore, we additionally tested the hypothesis that this reduced CVC in young black Americans would be restored with the intradermal infusion of L-arginine and arginase inhibitors, respectively.

The purpose of study 2 was to investigate the effects of acute cocoa flavanol intake on microvascular response to the aforementioned typical local heating of skin in young black Americans. A randomized, double-blind, and placebo-controlled cross-over design were conducted in this study. Based on the design, all subjects reported to the laboratory on two different days, separated by a minimum of 1 week, as the subjects consumed either a beverage high in flavanol content or a nutrient-matched placebo beverage with the exception that it contained no flavanols. CVC was calculated as RBC flux divided by MAP for an index of cutaneous microvascular function. All CVC data were presented as %CVCmax achieved during 43 °C local skin heating combined with local infusion of 28 mM SNP. We tested the hypothesis that acute flavanol intake would improve CVC in response to local heating of skin in the black American group while there is less of or no improvements of CVC with acute flavanol intake in the white American group.

The purpose of study 3 was to investigate the effects of acute flavonal intake on NO-dependent microvascular dilation in response to exogenous administration of incremental doses of methacholine (MCh) in young black Americans. A randomized, double-blind, and placebo-controlled cross-over design were conducted in this study. Based on the design, all subjects reported to the laboratory on two different days, separated by a minimum of 1 weak, as the subjects consumed either a beverage high in flavanol content or a nutrient-matched placebo beverage with the exception that it contained no flavanols. CVC was calculated as RBC flux divided by MAP for an index of cutaneous microvascular function. All CVC data were presented as %CVCmax achieved during 43 °C local skin heating combined with local infusion of 28 mM SNP. We tested the hypothesis that the EC50, i.e. the concentration of MCh required to elicit 50% of the maximal response, would be higher in the black Americans (indicative of vascular dysfunction). Additionally, we also tested the hypothesis that the consumption of flavanol-

containing beverage would produce a leftward shift in the dose-response curve of MCh-induced cutaneous vasodilation in young black Americans, while there would be no effects of acute flavanol intake in young white Americans.

Chapter 4: Study One

The Effects of Nitric Oxide Bioavailability on Cutaneous Microvascular Function in Healthy, Young Black Americans

4.1 ABSTRACT

Black Americans are at an increased risk for developing a variety of cardiovascular disease relative to white Americans and other populations. Underlying impairments manifest early in young adults prior to overt signs of risk. The mechanisms of this increased risk are multifactorial; however, recent evidence suggests that microvascular dysfunction in young black Americans is a primary contributor. This study tested the hypothesis that microvascular function, indexed by the cutaneous vascular conductance (CVC), in response to local heating of skin would be attenuated in young black Americans relative to age-matched white Americans due to the lack of nitric oxide (NO) bioavailability and/or an impaired vasodilatory response to NO. Furthermore, we additionally tested the hypothesis that this reduced CVC in young black Americans would be restored following intradermal infusion of L-arginine and an inhibitor of arginase, respectively.

Nine black Americans and nine white Americans adults participated in this study. Four microdialysis membranes were placed in each subject's non-dominant forearm. Each microdialysis site was randomly assigned to receive 1) lactated Ringer's solution as a control, 2) 20 mM NG-nitro-L-arginine (L-NAME) to inhibit NO, 3) 10 mM L-Arginine to locally supplement the substrate for NO synthase, or 4) a combination of 5.0 mM (S)-(2-boronoethyl)-L-cysteine-HCL (BEC) and 5.0 mM Nω -hydroxy-nor-L-arginine (nor-NOHA) to locally inhibit arginase activity at a rate of 2 μl/min. Skin blood flow (SkBF) was assessed while the skin was clamped at 33 °C for 10 min followed by 42 °C for ~ 30 min and values were normalized to a maximal value obtained during 43 °C local heating combined with infusion of 28 mM of sodium nitroprusside (SNP). CVC was calculated as SkBF/mean arterial pressure and normalized as a percentage of maximal CVC (%CVCmax). The difference in %CVCmax between the control site and L-NAME site

across the rise in skin temperature was calculated to assess the NO contribution (Δ %CVCmax).

There was no difference in the baseline %CVCmax at 33 °C between groups at all sites (all $P > 0.05$). At 42 °C, the plateau %CVCmax was significantly lower in the black American (BA) group than the white American (WA) group at the control site (BA: 62 ± 6 vs. WA: 84 ± 12 %CVCmax; $P < 0.001$). In the BA group the plateau %CVCmax at 42 °C was significantly elevated at the L-arginine supplemented site compare with the control site (Control: 62 ± 3 vs. L-arginine: 70 ± 3 %CVCmax; $P = 0.048$) but not at the arginase inhibited site (Control: 62 ± 3 vs. Arginase inhibitors: 62 ± 3 %CVCmax; $P = 0.908$). There was no change in the plateau %CVCmax following L-arginine supplemention (Control: 84 ± 3 vs. L-arginine: 83 ± 3 %CVCmax; $P = 0.900$) or arginase inhibition (Control: 84 ± 3 vs. Arginase inhibitors: 81 ± 3 %CVCmax; $P = 0.555$) in the WA group. In addition, the BA group had an attenuated contribution of NO to cutaneous hyperemic response to the local heating relative to the WA group (BA: 44 ± 3 vs. WA: 53 ± 3 Δ %CVCmax; $P < 0.001$) with a significant interaction between group and skin temperature ($P = 0.018$).

Based on these findings we suggest that 1) cutaneous microvascular function in response to local heating of skin is blunted in young black Americans when compared to age-matched young white Americans; 2) this attenuated response is partly related to decrease in NO bioavailability in young black Americans; 3) a local infusion of L-arginine, but not arginase inhibition, improves cutaneous microvascular function in response to local heating of skin in young black Americans relative to young white Americans.

4.2 INTRODUCTION

Cardiovascular disease (CVD) is the number one cause of death in the United States [1]. Black Americans are known to be at increased risk for developing a wide variety of cardiovascular diseases including coronary artery disease, stroke, hypertension, and metabolic syndrome compared with white Americans [3, 4, 23, 27]. In addition, the underlying impairments resulting in these disease states manifest during early adulthood prior to any overt signs of risk in this population [5-7]. The mechanisms resulting in increased risk in this population are likely multifactorial; however, recent evidence suggests that endothelial dysfunction is a major contributing factor [23, 27]. Previous studies have demonstrated that the vascular dilatory capacity of resistance vessels is reduced in young black Americans relative to white Americans. For example, vasodilatory capacity of the forearm resistance vessels was attenuated in normotensive young black American men when compared to normotensive young white American men [28]. Similarly, forearm vasodilatory response to isoproterenol infusion, which is a β-adrenergic agonist, was blunted in normotensive young black American men relative to white American men [29]. Nitric oxide (NO)-mediated forearm blood flow responses to methacholine and sodium nitroprusside (SNP) administration were attenuated in healthy young black American men when compared to white American men [30]. These results suggest early onset of vascular dysfunction in young black Americans prior to any overt signs of risk compared with young white Americans.

One potential mechanism that has been implicated in attenuated endothelial function in young black Americans is a decrease in NO bioavailability. NO is formed in the endothelial cells by the reaction of endothelial NO synthase (eNOS), which divide the substrate L-arginine into NO and L-citrulline, and acts as a primary vasoactive substance that plays a critical role in vasodilation [31]. The exact mechanisms of a decreased NO

bioavailability have not been fully elucidated. However, several potential signaling pathways of a decreased NO bioavailability, including eNOS uncoupling or deregulated utilization of the substrate L-arginine, have been suggested. It has recently shown that a low concentration of L-arginine can negatively effect on the production of NO in animals [32] and human cells [33], indicating that a decreased NO bioavailability due to eNOS uncoupling, may be related to a decreased concentrations of L-arginine [34].

Arginase constitutively exists in two isoforms (arginase I and arginase II) in human cells. Both arginase isoforms convert L-arginine into L-ornithine and urea during the final step in the urea cycle [35]. In terms of vascular function, arginase I is predominant in the vasculature and is co-expressed with eNOS by sharing for the substrate L-arginine [36, 37]. Therefore, the overexpression or the increase in activity of arginase can affect eNOS coupling and NO bioavailability secondary to competition between eNOS and arginase for L-argnine [34, 38-40]. Several studies using animal models have implicated that an increased arginase activity could impair microvascular function due to decreased NO bioavailability, and this attenuated microvascular function could be restored with both acute and chronic inhibition of arginase activity [36, 37, 41].

The human cutaneous circulation serves as a surrogate vascular bed for the assessment of systemic microvascular dysfunction. It has been reported that impaired cutaneous microvascular reactivity is observed before any clinical signs of microvascular dysfunction during the early stages of diseases [42], suggesting the correlation between skin vascular reactivity and a systemic microvascular dysfunction. For this reason, skin blood flow (SkBF) response to local heating has been widely used as a means to examine microvascular dysfunction because of its ease of accessibility and non-invasive characteristics [42-46]. Additionally, the combination of laser-Doppler flowmetry (LDF) and intradermal microdialysis has been successfully used in skin reactivity tests to measure

microvascular function and determine the underlying mechanisms of microvascular dysfunction in both healthy individuals and individuals with elevated CVD risk factors including smoking [47], hypercholesterolemia [48], and hypertension [49].

To our knowledge, the racial difference in the functional roles of L-arginine and arginase in eNOS uncoupling and its potential contribution to a reduced NO-dependent vasodilation are unknown. Thus, the primary aim of this study was to test the hypothesis that microvascular function in response to local heating of skin would be attenuated in young black Americans relative to age-matched white Americans due largely to the lack of NO bioavailability. A secondary aim of this study was to test the hypothesis that an attenuated cutaneous microvascular function in young black Americans would be restored with an intradermal infusion of L-arginine and arginase inhibitors, respectively.

4.3 METHODS

Ethical Approval and Subjects

Eighteen young, healthy subjects (9 black Americans and 9 white Americans) volunteered for this study (Table 1.1). All subjects self-defined as black Americans or white Americans and were only accepted into the study if both parents were black Americans or white Americans, respectively. Before admission, all experimental procedures were explained to subjects and they were given an opportunity to ask questions. Subjects of each group gave written consent approved by the Institutional Research Board at the University of Texas at Austin and completed a questionnaire on medical history, medications, and lifestyle behaviors. All individuals were normally active with no history of cardiovascular, metabolic, or neurological disease. In addition, those currently taking any medication known to alter cardiovascular function were excluded. Given that smoking can affect the peripheral vascular response, individuals who are currently smokers or quit smoking within the prior two years were excluded [50]. All female subjects were only studied during the early follicular phase (within 1-3 days of the start of menstruation) to minimize the effects of female sex hormones [51, 52].

Instrumentation and Measurements

On each study visit all subjects reported to the laboratory in the morning in the fasted condition (~12 hr), having refrained from strenuous exercise, alcohol, and caffeine for 24 hr. On the experimental day, height and weight of subjects was assessed (Seca 763, Seca, Chino, CA). Subjects were then asked to lie down on a patient bed in the semi-supine position for the remainder of data collection. Subjects were then instrumented with five electrodes for the assessment of cardiac rhythms and heart rate (HR) measured via an electrocardiogram (HP Patient Monitor, Agilent, Santa Clara, CA) interfaced with a

cardiotachometer (CWE, Ardmore, PA), and a blood pressure cuff on an upper arm for the assessment of arterial blood pressure measured from auscultation of the brachial artery via electrosphygmomanometry (Tango+; SunTech, Raleigh, NC). Following a minimum of 20 min of rest, baseline HR and blood pressure were obtained in all subjects. Mean arterial pressure (MAP) was calculated as one-third pulse pressure plus diastolic blood pressure (DBP).

Four intradermal microdialysis membranes (CMA 31 Linear Microdialysis Probe, 55 kDa cut-off membrane; CMA Microdialysis AB, Holliston, MA) were instrumented on the ventral surface of the non-dominant forearm. For each membrane, a 25-gauge needle guide was inserted horizontally into the dermal layer of the skin such that entry and exit points on each site were ≥ 2.5 cm apart. The microdialysis membrane was threaded through the needle, which was then followed by withdrawal of the needle from the skin leaving the membrane in place. After all microdialysis membranes were inserted and taped in place, each site was continuously perfused with lactated Ringer's solution at 2 μl/min via an infusion pump (Pump 11, Harvard Appartus, Natick, MA) for a 60-90 min to allow for local hyperemia associated with the needle insertion to subside [9, 53-58]. Skin blood flow (SkBF) was indexed over each microdialysis site using a LDF probe (VP7 A/T with moorVMS-LDF2; Moor Instruments, Wilmington, DE) which was housed in the center of a local heating element (PeriFlux System 5000; Perimed, Sweden).

Experimental Protocol

Following instrumentation and cessation of the hyperemia response (~90 min), the local heating elements were clamped at 33 ºC (i.e. thermoneutral skin temperature) for a 15-min baseline data collection with each site receiving lactated ringers (2 μl/min). Following baseline data collection each experimental site was infused with its respective

vasoactive agents at a rate of 2 μl/min. All vasoactive substances were mixed and prepared just before use. Each microdialysis site was randomly assigned to receive: 1) lactated Ringer's solution as a control site; 2) 20 mM NG-nitro-L-arginine (L-NAME); 3) 10 mM L-Arginine; or 4) a combination of 5.0 mM (S)-(2-boronoethyl)-L-cysteine-HCL (BEC) and 5.0 mM Nω -hydroxy-nor-L-arginine (nor-NOHA) at a rate of 2.0 μl/min. The concentrations of L-arginine and arginase inhibitors were decided to maximize cutaneous vasodilatory responses as the minimum dose based on previous study [34]. Following an additional 30 min baseline period the temperature of the local heaters was increased to 42 ºC at a rate of 0.5 ºC every 5 sec and clamped at 42 ºC until a stable plateau was reached (approximately 30 min) (19) while each site continues to receive its respective vasoactive agent. Following this 30 min period, local temperature was increased to 43 ºC and each site was perfused 28 mM SNP (Sigma-Aldrich, St. Louis, MO) to induce maximal vasodilation [59]. This procedure induces a maximal SkBF response in the cutaneous circulation that is primarily NO dependent [56, 60-62]. Immediately following this period the local heating elements were turned off.

Data Analysis

Data were sampled at 125 Hz via a data-acquisition system (Biopac System, Santa Barbara, CA). Data from the last min of each local heating phase (i.e. 33, 42, and 43 °C) were averaged and used for analysis. Cutaneous vascular conductance (CVC) was calculated from the ratio of RBC flux to MAP. CVC was presented as a percent of each respective maximal SkBF achieved during 43 °C heating plus 28 mM SNP infusion (%CVCmax). The difference in %CVCmax between the control site and L-NAME site (Δ %CVCmax) across the rise in skin temperature was calculated to assess the NO contribution as an index of NO bioavailability.

Statistical Analysis

All data were analyzed using a statistical software package (Stata 13; StataCorp LP, College Station, TX). Unpaired Student's *t*-tests were performed to detect significant differences between the black American and white Americans group for physical characteristics and resting hemodynamic variables. A two-way repeated measure mixed model was conducted to estimate 1) the effects of group and drug site on absolute maximal CVC, 2) the effects of group and skin temperature on %CVCmax at the control site, and 3) the effect of group and skin temperature on the NO contribution to vasodilation. A three-way repeated measure mixed model was conducted to estimate the effects of group and drug sites (control vs. drug) on %CVCmax over the rise in skin temperature. Bonferroni correction were performed for post-hot analysis when a significant interaction was identified. All data were presented as means ± SE unless otherwise stated. The level of significance was set at $P < 0.05$.

4.4 RESULTS

Subject Characteristics

The physical characteristics of subjects are presented in Table 4.1. Subjects were well matched for age, height, weight, and body mass index (BMI) (all $P > 0.05$). There was no significant difference between groups for resting systolic blood pressure (SBP), diastolic blood pressure (DBP), MAP, and resting HR at the end of the insertion trauma resolution period (all $P > 0.05$).

Microvascular Function

Absolute maximal CVC. Table 4.2 shows absolute maximal CVC during combined 43 °C local heating and 28 mM SNP infusion. Absolute maximal CVC was similar between groups across all sites ($P > 0.05$), demonstrating that the maximal vasodiatory capacity of the cutaneous microvasculature was similar between groups. There was no interaction between group and each treatment site relative to the control site (all $P > 0.05$) with no main effect of group and drug site (all $P > 0.05$).

%CVCmax at the control site. Figure 4.1 shows a SkBF tracing from a representative subject in each group. Figure 4.2 summarizes the SkBF response during the local heating protocol in the control site in both groups. There was no difference in baseline %CVCmax during 33 °C local heating between the black American (BA) group and white American (WA) group in the control site (BA: 9 ± 3 vs. WA: 11 ± 3 %CVCmax; $P = 0.456$). The plateau %CVCmax during 42 °C local heating was significantly lower in black Americans (BA: 62 ± 3 vs. WA: 84 ± 3 %CVCmax; $P < 0.001$). There was an interaction between group and skin temperature ($P < 0.001$) with a main effect of skin temperature ($P < 0.001$) and group ($P < 0.001$).

NOS inhibited site. Figure 4.3A summarizes the SkBF response during the local heating protocolin the NOS inhibited site in both groups. In the WA group, the baseline %CVCmax at 33 °C was similar compared with the control site (Control: 11± 3 vs. L-NAME: 7 ± 3 %CVCmax; $P = 0.326$). The plateau %CVCmax at 42 °C was significantly different between the control and NOS-inhibited sites (Control: 84 ± 3 vs. L-NAME: 28 ± 3 %CVCmax; $P < 0.001$). In the BA group, the baseline %CVCmax at 33 °C was similar compared with the control site (Control: 9 ± 3 vs. L-NAME: 4 ± 3 %CVCmax; $P = 0.289$). The plateau %CVCmax at 42 °C was significantly different between the control and NOS-inhibited sites (Control: 62 ± 2 vs. L-NAME: 19 ± 2 %CVCmax; $P < 0.001$). There was no difference in baseline %CVCmax between groups in L-NAME site ($P = 0.494$). During 42 °C local heating, there was a group difference in L-NAME site ($P = 0.031$). No significant interaction between group and drug site over a rise in local temperature was observed ($P = 0.134$). A significant three-way interaction was observed (group, local temperature, and drug site) ($P = 0.044$). There was a significant interaction between group and local temperature ($P < 0.001$) and between drug site and local temperature ($P < 0.001$) but not between group and drug site ($P = 0.056$). These significant interactions remained following Bonferroni correction. There was a significant main effect of local temperature, group, and drug site (all $P < 0.001$).

L-arginine supplemented site. Figure 4.3B summarizes the SkBF response during the local heating protocol in the L-arginine supplemented site in both groups. Baseline %CVCmax during 33 °C local heating was similar between the control and the L-arginine sites in the WA group (Control: 11 ± 3 vs. L-arginine: 15 ± 3 %CVCmax; $P = 0.414$) and in the BA group (Control: 9 ± 3 vs. L-arginine: 10 ± 3 %CVCmax; $P = 0.671$). No group difference in the baseline %CVCmax was observed in L-arginine supplemented site ($P = 0.298$). In the BA group, L-arginine supplementation increased the plateau %CVCmax at

42 °C when compare to the control site (Control: 62 ± 3 vs. L-arginine: 70 ± 3 %CVCmax; $P = 0.048$), while there was no effect in the WA group during 42 °C local heating with L-arginine supplementation (Control: 84 ± 3 vs. L-arginine: 83 ± 3 %CVCmax; $P = 0.900$). During 42 °C, there was a group difference in the plateau %CVCmax observed in L-arginine site ($P < 0.001$). There was a significant interaction between group and local temperature ($P = 0.002$) but not between drug site and local temperature ($P = 0.801$) or between group and drug site ($P = 0.440$). The significant interaction between group and local temperature remained following Bonferroni correction. There was a significant main effect of group ($P < 0.001$) and local temperature ($P < 0.001$) but not drug site ($P = 0.143$).

Arginase inhibited site. Figure 4.3C summarizes the SkBF response during the local heating protocol in the arginase-inhibited site in both groups. In the WA group, there was no difference between the control and arginase-inhibited sites in baseline %CVCmax during 33 °C local heating (Control: 11 ± 3 vs. Arginase inhibitors: 14 ± 3 %CVCmax; $P = 0.483$) and in the plateau %CVCmax at 42 °C (Control: 84 ± 3 vs. Arginase inhibitors: 81 ± 3 %CVCmax; $P = 0.555$). Similarly, in the BA group, there was no difference between the control and arginase-inhibited sites in the baseline %CVCmax at 33 °C (Control: 9 ± 3 vs. Arginase inhibitors: 8 ± 3 %CVCmax; $P = 0.876$) and in the plateau %CVCmax at 42 °C (Control: 62 ± 3 vs. Arginase inhibitors: 62 ± 3 %CVCmax; $P = 0.908$). No group difference in baseline %CVCmax was observed in the arginase-inhibited site ($P = 0.153$). During 42 °C local heating, a group difference in %CVCmax observed in the arginase inhibited site ($P < 0.001$). No significant three-way interaction was observed ($P = 0.426$). There was a significant interaction between group and local temperature ($P < 0.001$) but not between drug site and local temperature ($P = 0.604$) or between group and drug site ($P = 0.939$). The significant interaction between group and local temperature remained

following Bonferroni correction. There was a significant main effect of group ($P < 0.001$) and local temperature ($P < 0.001$) but not drug site ($P = 0.972$).

NO contribution. Figure 4.4 shows the NO contribution during local heating in both groups. There was no difference in the baseline NO contribution at 33 °C between groups (BA: 4 ± 3 vs. WA: 4 ± 3 %CVCmax; $P = 0.963$). NO contribution at 42 °C was significantly lower in the BA group than the WA group (BA: 44 ± 3 vs. WA: 53 ± 3 %CVCmax; $P < 0.001$). There was a significant interaction between group and local temperature ($P < 0.001$) with a significant main effect of local temperature ($P < 0.001$) and group ($P < 0.001$). The significant interaction between group and local temperature was remained with Bonferroni correction.

%CVCmax at the plateau. Figure 4.5 displays mean %CVCmax for the plateau phase at 42 °C heating of skin in all sites. The plateau %CVCmax at 42 °C heating was a significantly lower in the BA group when compare to the WA group in the control site (BA: 62 ± 4 vs. WA: 84 ± 4 %CVCmax; $P < 0.001$), in the L-arginine supplemented site (BA: 70 ± 4 vs. white Americans: 83 ± 4 %CVCmax; $P < 0.001$), and in the arginase-inhibited site (BA: 62 ± 4 vs. WA: 81 ± 4 %CVCmax; $P < 0.001$), while NOS inhibition significantly attenuated the plateau %CVCmax compare with the control site in the WA group (Control: 84 ± 4 vs. L-NAME: 28 ± 4 %CVCmax; $P < 0.001$) and the BA group (Control: 62 ± 4 vs. L-NAME: 19 ± 4 %CVCmax; $P < 0.001$).

4.5 DISCUSSION

The primary findings of this study are as following: 1) cutaneous vasodilation in response to local heating was blunted in young black Americans when compared to age-matched young white Americans, 2) this attenuated response was related to decrease in NO bioavailability in young black Americans, and 3) a local administration of L-arginine improved cutaneous microvascular vasodilation during local heating in black Americans but not in white Americans; however, contrary to our initial hypothesis, local inhibition of arginase administration had no effect on cutaneous microvascular function in either group. These findings suggest that an attenuated NO bioavailability due partly to a relative deficit of L-arginine in the endothelial cells is one of the mechanisms by which microvascular dysfunction occurs in young black Americans.

In the present study, %CVCmax was attenuated in response to 42 °C local heating in the BA group relative to the WA group while there was no different %CVCmax at 33 °C and absolute CVC at 43 °C + SNP infusion. This indicates that there was no difference in baseline vasodilatory function and that both young black Americans and white Americans achieved similar maximal vasodilatoy response to 43 °C local heating plus SNP infusion. As hypothesized, the hyperemic response in the cutaneous microvasculature following 42 °C heating in young black Americans was significantly blunted relative to young white Americans. The findings in the current study support our recent finding that college-aged black Americans (23 years) have reduced cerebral vasodilatory capacity during rebreathing-induced hypercapnia relative to age-matched white Americans [25]. These findings are also consistent with previous studies of endothelial function of conduit artery in young black Americans. Forearm blood flow responses to exercise [28], ischemia [63], and mental stress [64] were significantly lower in healthy black Americans when

compare to healthy white Americans. Thus, our findings further support that microvascular function is impaired partly in young, healthy black Americans.

NO bioavailability can be dictated based on the balance between the synthesis and degradation of NO in the vasculature, and we also found that NO contribution to local heating of skin was lower in young black Americans than white Americans in this study, suggesting a decreased NO bioavailability in young black Americans. Additionally, we found that attenuated microvascular function in young black Americans improved by the local administration of L-arginine in the current study. Studies have shown that a low concentration of L-arginine can limit the production of NO in both animals [32] and human cells [33], indicating that a decrease in the amount of L-arginine may reduce NO bioavailability. Thus, our finding suggests that the concentrations of available NO for eNOS in the endothelial cells and NO-dependent vasodilation is not enough to elicit the maximal SkBF response during local heating in young black Americans.

In addition to L-arginine deficiency, it is possible that an increase in oxidative stress in young black Americans may cause to attenuated NO-dependent vasodilation. It is well known that upregulated superoxide (O_2^-) reacts with NO and form peroxynitrite ($ONOO^-$) faster than superoxide metabolism by superoxide dismutase [65], resulting in a decrease in NO bioavailability. It is evident that single human umbilical vein endothelial cell (HUVEC) isolated from young black American individuals (22 years) have reduced release of NO and increased production of both O_2^- and $ONOO^-$ compared with age-matched white American individuals. In the same study, it has demonstrated that the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase upregulated in HUVECs from young black American individuals relative to young white American individuals [66]. These findings suggest that an increased oxidative stress in young black Americans leads to decrease in NO bioavailability.

Alternatively, it is possible that tetrahydrobiopterin (BH_4), the essential cofactor for the generation of NO by eNOS expression/activity may be attenuated in young black Americans. One possible mechanism of eNOS uncoupling related to BH_4 has suggested that the formation of $ONOO^-$ due to elevated oxidation may oxidize BH_4 [67]. Excessively produced O_2^- by NADPH oxidase and/or xanthine oxidase (XO) can also bind with NO and form $ONOO^-$. The oxidization of BH_4 by $ONOO^-$ is converted to BH_3 or BH_2 which are inactive forms of BH_4, leading to eNOS uncoupling [68-70]. Furthermore, it has been assumed that $ONOO^-$ might oxidize the zinc-thiolate cluster of eNOS and destabilize eNOS dimer so eNOS is uncoupled [71]. Thus, it is plausible that the intradermal administration of L-arginine may lead to a synergistic response to local heating so that an increase in expression/activity of BH_4, can contribute to increase in NO production and bioavailability in young black Americans.

L-arginine in human cells not only binds with eNOS, but also with arginase [34, 35, 38-40]. Arginase metabolizes L-arginine to L-ornithine and urea during the urea cycle and lead to reduce NO production by shunting the substrate from the eNOS pathway to the arginase pathway competing with eNOS for the substrate, L-arginine [72]. Thus, there is the possibility that upregulation of arginase may shunt the substrate L-arginine from eNOS pathway to arginase pathway, resulting in a decrease in NO bioavailability. Several studies has shown that arginase is upregulated in individuals with a variety diseased conditions including atherosclerosis [73], myocardial ischemia [74], and hypertension [75]. Contrary to these findings, the administration of arginase inhibitors did not affect the cutaneous microvascular response to local heating in young black Americans in the current study. Similarly, Holowatz et al. (2006) previously reported that intradermal administration of arginase inhibitors did not alter cutaneous vasodilation in young subjects [34]. The reasons for this remain unclear. It is plausible that the expression and/or activity of arginine may

be already low in young black Americans; thus, exogenous administration of arginase inhibitors did have no effects on NO bioavailability. It is also possible that the concentrations of arginase inhibitors used in the current study may be not enough to elicit maximal vasodilation. However, the concentrations of arginase inhibitors (5.0 mM BEC and 5.0 mM nor-NOHA) used in the current study have shown to be effective young and aged [34], and hypercholesterolaemic humans [48] using the same intradermal microdialysis technique and far exceed the Ki for the enzyme isoforms (BEC Ki at pH 7.5 = 0.31 μm; nor-NOHA Ki at pH 7.5 = 1.6 μm) [76].

In the present study, the young white American group did not show any changes in cutaneous microvascular function with L-arginine supplementation and arginase inhibition. Additional studies are needed to confirm this results. However, it is likely that young and healthy white Americans individuals have low oxidative stress, low arginase activity, or high levels of L-arginine in the endothelial cells, which are responsible for the minimal or no changes in microvascular function. Taken together, these findings suggest that an attenuated NO bioavailability may related to the deficit of L-arginine concentrations in the endothelial cells and may be one of the mechanisms by which microvascular dysfunction occurs in young black Americans.

Limitations

A limitation of the present study is that we did not co-infuse L-arginine and arginase inhibitors because of limited equipment to assess RBC flux at four sites. Thus, we are not able to clarify what their combined effect would be. In addition L-NAME was not co-infused within L-arginine or arginase inhibition sites; thus we cannot determine whether

the improvement at the L-arginine supplemented site is dependent on NO bioavailability or not.

Another limitation of the present study is the use of LDF to determine cutaneous microvascular function. Although the assessment of RBC flux using LDF has been widely used in studies for microvascular function, some technical challenges including inherent variability in LDF cannot be ruled out for the precise evaluation [77]. In the present study, we observed attenuated microvascular vasodilatory response in response to local heating of skin in young black Americans relative to age-matched white Americans; however, additional studies with other techniques are needed to be conducted to confirm our findings because the use of %CVCmax, calculated by LDF and MAP, provides only a relative index of SkBF response to local heating of skin.

Conclusion

In conclusion, this study demonstrated that young black Americans have attenuated microvascular function in response to a typical local heating protocol relative to the age-matched white Americans. Furthermore, this data suggests that attenuated vasodilation is related to a decrease in NO bioavailability due to limited L-arginine availability in young black Americans. Considering attenuated NO-dependent vasodilation in young black Americans and the observed restoration of cutaneous microvascular function with the administration of L-arginine, exogenous L-arginine supplementation might be a novel potential therapeutic target to prevent racial disparities in cardiovascular disease.

4.6 TABLES AND FIGURES

Table 4.1 Subject characteristics and resting hemodynamic variables

N, no. of subjects; M, male; F, female; BMI, body mass index; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure. Values are means ± SD.

	White American (N=9)	Black American (N=9)	*P*-value
Characteristics			
Age (years)	24 ± 3	23 ± 3	0.33
Sex (M/F)	6/3	6/3	1.00
Height (cm)	173 ± 10	171 ± 10	0.79
Weight (kg)	66 ± 10	68 ± 10	0.80
BMI (kg/m^2)	22 ± 1	23 ± 2	0.35
Hemodynamics			
HR (beats/min)	57 ± 14	58 ± 7	0.82
SBP (mmHg)	113 ± 9	116 ± 7	0.43
DBP (mmHg)	70 ± 7	73 ± 9	0.46
MAP (mmHg)	84 ± 7	87 ± 7	0.39

Table 4.2 Absolute maximal cutaneous vascular conductance (CVC) across all sites

L-NAME, NG-nitro-L-arginine; arginase inhibitors, a combination of (S)-(2-boronoethyl)-L-cysteine-HCL (BEC) and 5.0 mM Nω -hydroxy-nor-L-arginine (nor-NOHA). Absolute maximal CVC was similar between groups across all sites (all $P > 0.05$). Values are means ± SE.

	White American	Black Americans
Control	3.89 ± 0.27	3.19 ± 0.27
L-NAME	3.50 ± 0.27	3.29 ± 0.27
L-arginine	3.65 ± 0.27	3.08 ± 0.27
Arginase inhibitors	3.37 ± 0.27	3.40 ± 0.27

Figure 4.1 Schematic representation of the skin blood flow (SkBF) response to local heating

LDF, laser-Doppler flowmetry. A, initial peak; B, plateau; C, maxial SkBF induced by SNP. Data selected from the last 10 min of baseline period to the end at the Ringer's site.

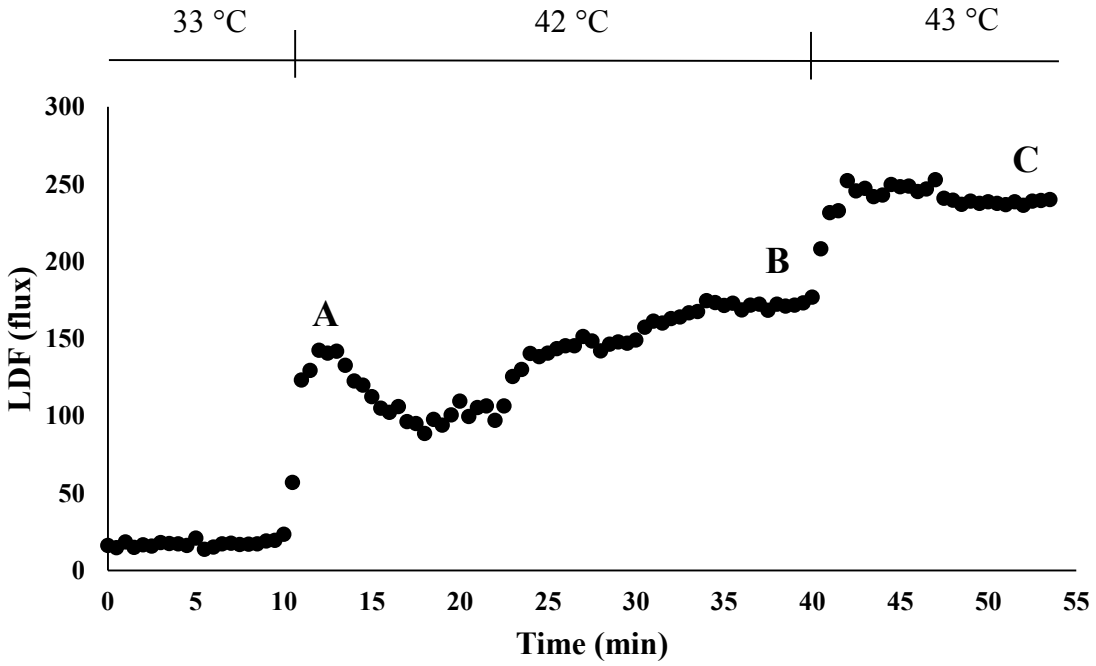


Figure 4.2 A summary of cutaneous vascular conductance as a percentage of maximal vasodilation (%CVCmax) in response to local heating between groups at the ringer's site (control)

Open bars, white American (WA) group; Filled bars, black American (BA) group. %CVCmax during the rise in local temperature (°C) was lower in the BA group than the WA group in the control site. *$P < 0.001$, significant vs. WA group; †$P < 0.001$, significant interaction (group x temperature). Values are means ± SE.

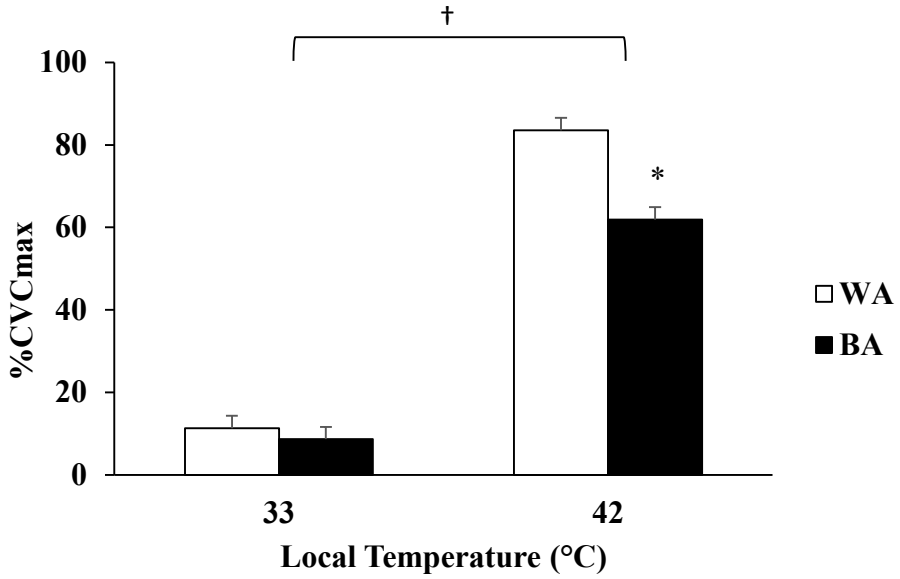


Figure 4.3 A summary of group cutaneous vascular conductance as a percentage of maximal vasodilation in response to local heating across all treatment sites

Left panels, white Americans; right panels, black Americans. A, the NOS-inhibited site. B, the L-arginine supplemented site. C, the arginase-inhibited site. Cutaneous vascular conductance during the rise in local temperature (°C) (%CVCmax) in the control site is shown for comparison. L-arginine supplementation improved %CVCmax in black Americans but not white Americans. *$P < 0.05$, significant difference vs. the control site within the groups; †$P < 0.05$, significant interaction (drug site x temperature). Values are means ± SE.

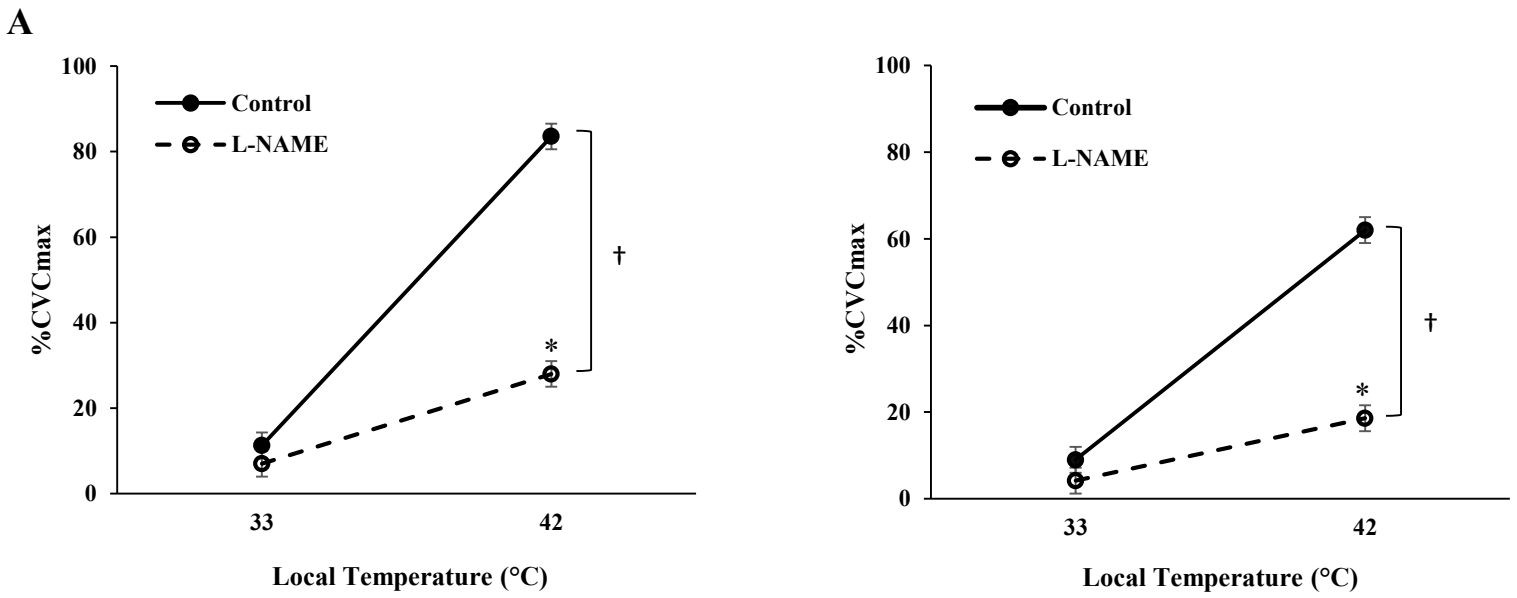


Figure 4.3 A summary of group cutaneous vascular conductance as a percentage of maximal vasodilation in response to local heating across all treatment sites (Continued)

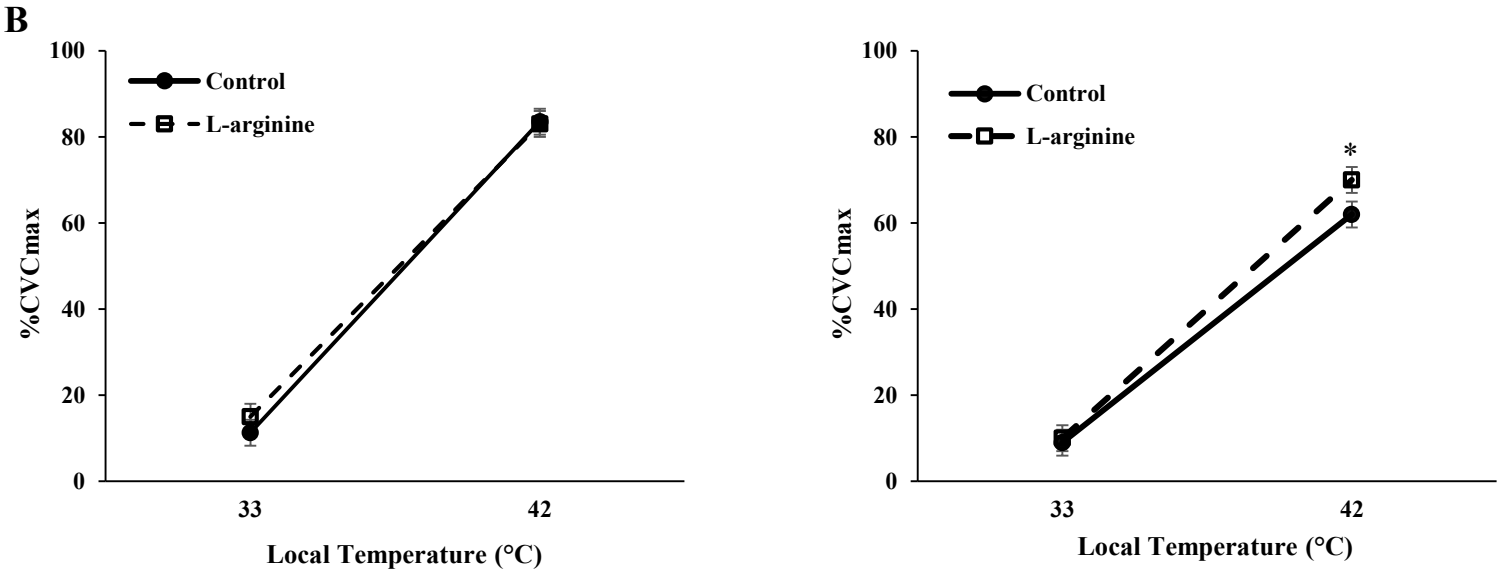


Figure 4.3 A summary of group cutaneous vascular conductance as a percentage of maximal vasodilation in response to local heating across all treatment sites (Continued)

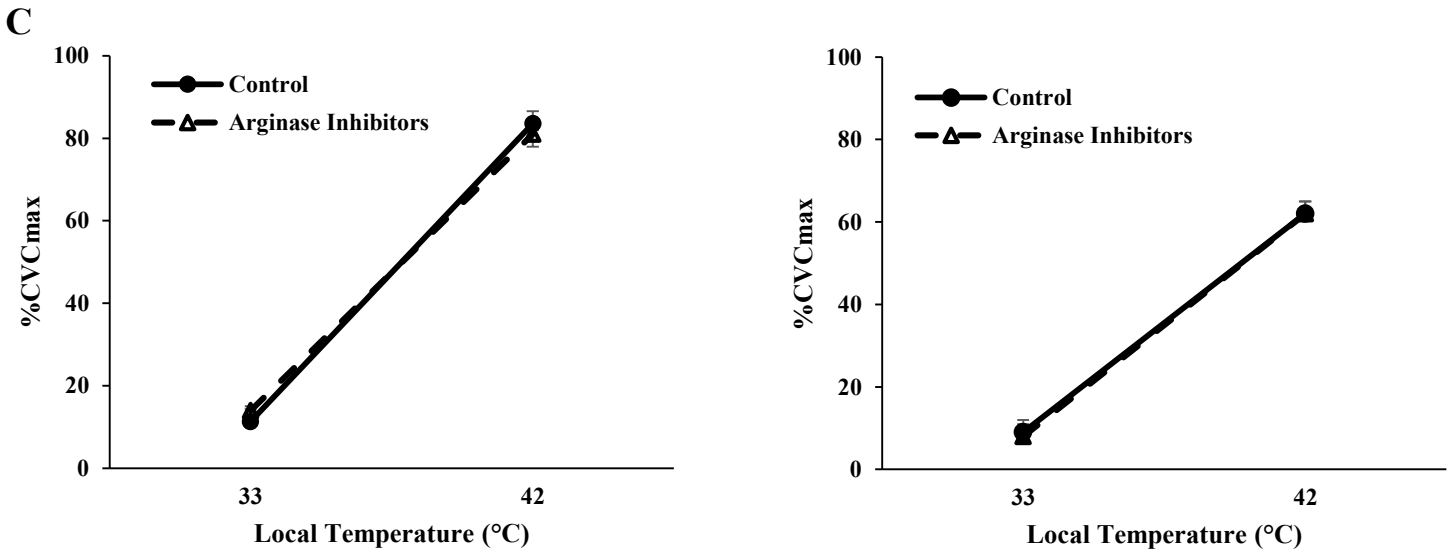


Figure 4.4 A summary of NO contribution in response to local heating of skin between groups

Open bars, white American (WA) group; Filled bars, black American (BA) group. The difference in cutaneous vascular conductance as a percentage of maximal vasodilation (%CVCmax) in response to the rise in local skin temperature (°C) between the control site and NOS-inhibited site (Δ %CVCmax) was calculated to assess NO contribution as an index of NO bioavailability. NO contribution was lower in the BA group than the WA group during local heating of skin. **P* < 0.001, significant difference vs. WA group; †*P* < 0.05, significant interaction (group x temperature). Values are means ± SE.

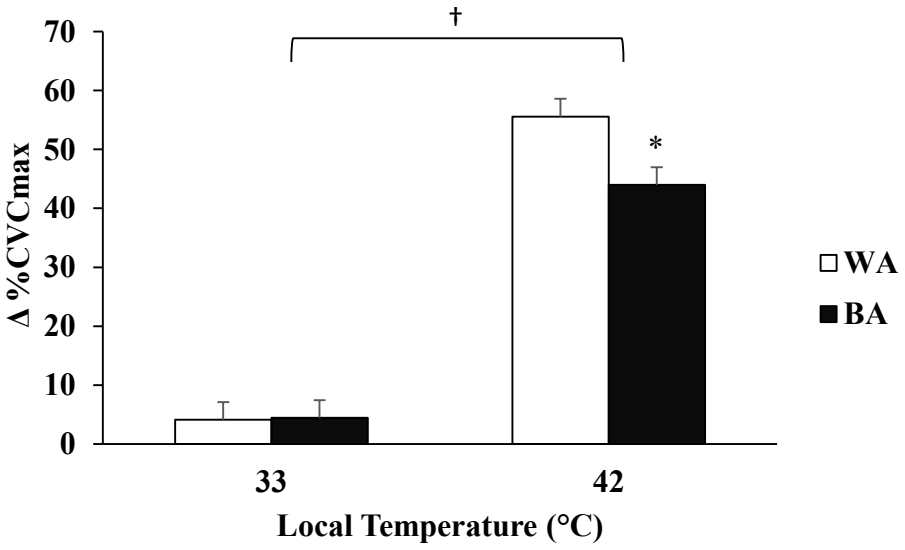
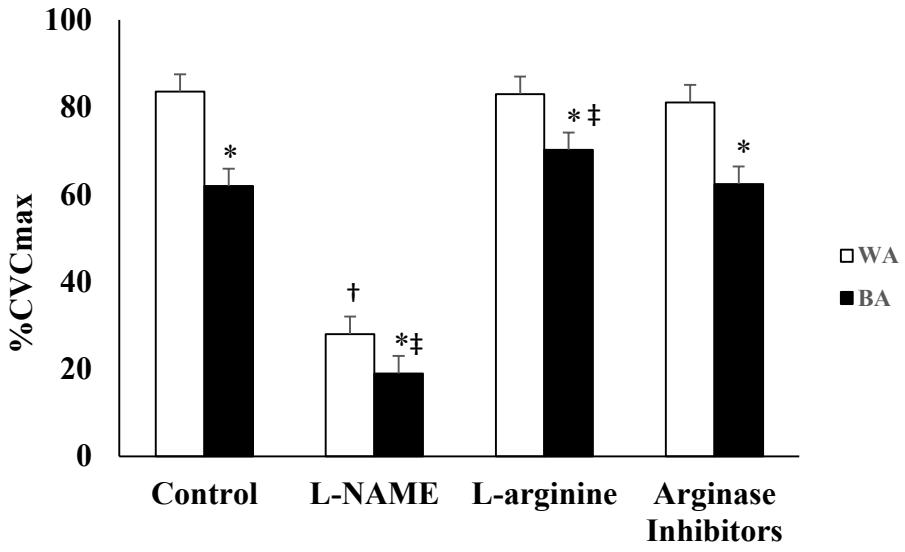


Figure 4.5 A summary of group cutaneous vascular conductance as a percentage of maximal vasodilation (%CVCmax) for the plateau phase at 42 °C heating of skin in all sites

Open bars, white American (WA) group; Filled bars, black American (BA) group. L-arginine supplementation improved %CVCmax in the BA group but not the WA group. **P* < 0.001, significant difference vs. WA group; †*P* < 0.05, significant difference vs. control site in the WA group; ‡*P* < 0.05, significant difference vs. control site in the BA group. Values are means ± SE.



Chapter 5: Study Two

The Effects of Acute Flavanol Intake on Cutaneous Microvascular Function in Healthy, Young Black Americans

5.1 ABSTRACT

Black Americans have increased risk for cardiovascular disease relative to white Americans and the underlying impairments manifest as early as the second generation prior to any overt signs of risk. We have recently demonstrated that nitric oxide (NO)-dependent microvascular function in response to local heating of skin is attenuated in healthy young black Americans relative to white Americans. Thus, we hypothesized that acute consumption of flavanol-rich cocoa would restore reduced cutaneous microvascular responses to local heating in young black Americans.

Seven black Americans and seven white Americans participated in this double-blind crossover study. Data were collected on two different days at the same time each day, separated by a minimum of one week. Two intradermal microdialysis membranes were inserted in the forearm and skin blood flow was indexed via a laser-Doppler probe housed within a local heater over the membrane sites. Each site randomly assigned to receive lactated Ringer's solution or 20 mM NG-nitro-L-arginine (L-NAME). After instrumentation, participants were randomly assigned to consume either a non-flavanol containing (NF) beverage or flavanol-rich cocoa (FC) beverage. After recovery from the insertion trauma, the local heater was clamped at 32 °C for 45 min of baseline data collection after which the temperature was increased to 39 °C for 30 min. Finally, maximal cutaneous vasodilation was achieved by a combination of infusion of 28 mM sodium nitroprusside (SNP) and 43 °C local heating. Cutaneous vascular conductance (CVC) was calculated as cutaneous blood flux/mean arterial pressure and normalized as % maximal CVC (%CVCmax). The difference in %CVCmax between the control site and L-NAME site (Δ %CVCmax) across the rise in skin temperature was calculated to assess NO contribution.

In Ringer's site, acute consumption of FC beverage improved the plateau %CVCmax at 39 °C heating in the black American group when compared to NF beverage (NF: 36 ± 4 vs. FC: 47 ± 4 %CVCmax; $P = 0.008$) while there was similar %CVCmax at 39 °C heating between beverages in the white American group (NF: 55 ± 4 vs. F: 59 ± 4 %CVCmax; $P = 0.404$). At the plateau (39 °C), NO contribution was significantly higher with FC beverage than NF beverage in black Americans (NF: 27 ± 3 vs. F: 35 ± 3 Δ%CVCmax; $P = 0.026$) while there was similar NO contribution between beverages in white Americans (NF: 42 ± 4 vs. F: 45 ± 4 Δ%CVCmax; $P = 0.363$)

The primary finding of this study is that acute consumption of FC improved cutaneous microvascular function in response to local heating in young black Americans when compare with NF. This data suggests that an attenuated microvascular function in young black Americans could be improved with acute consumption of FC.

5.2 INTRODUCTION

Ethnicity is one of the major risk factors of cardiovascular disease (CVD). Research has shown that black Americans have, traditionally, experienced higher rates of mortality and morbidity from CVD than white Americans for every decennial year back to 1900 [3, 4, 78]. Black American men and women (44% and 49%, respectively) have higher prevalence of CVD than white American men and women (37% and 32%, respectively) [79]. Furthermore, CVD prevalence is higher in young black Americans than age-matched white Americans (35-44 years) and this racial disparity between young black Americans and white Americans may not be explained by clinical and socioeconomic factors [24].

The explanations for these racial differences between black Americans and white Americans adults remain unclear. However, recent studies have shown that endothelial dysfunction, because of decreased nitric oxide (NO) bioavailability, play a major role in the increased risk for CVD in this population [4, 23, 27]. Given that NO plays a pivotal role not only in vasodilation but also platelet aggregation, adhesion of leukocytes to the endothelium, and smooth muscle cell proliferation and migration [80], endothelial-derived NO acts as an important mediator of cardiovascular homeostasis and vascular tone in humans. We have previously demonstrated that NO-dependent vasodilation in microvasculature is significantly attenuated in young black American adults relative to age-matched white American adults. This finding is consistent with previous studies, showing impaired vascular function in young black American adults compared with their white counterparts [28, 30, 64]. Furthermore, there is compelling evidence that endothelial dysfunction in microvasculature is an early predictive marker of CVD [81] and its risk factors including hypertension [82] and diabetes mellitus [83].

One possible mechanism of decreased NO bioavailability observed in young black Americans is upregulation of superoxide (O_2^-). Previous studies have shown that the

administration of calcium ionophore, an endothelial NO synthase (eNOS) agonist, increases O_2^- production in human umbilical vein endothelial cells (HUVECs) more in black Americans than white Americans. In addition, upregulation of nicotinamide adenine dinucleotide phosphate (NADPH) is also observed in black Americans relative white Americans [66]. This data suggests that excessive production of O_2^- in the endothelial cells may lead to decrease in NO bioavailability in black American adults. It is also notable that O_2^- produced in the endothelial cells rapidly reacts with NO and produce a potent oxidant peroxynitrite ($ONOO^-$). $ONOO^-$ may lead to the oxidation of proteins, deoxyribonucleic acid, and lipids in vascular walls [66]. Thus, increased production of $ONOO^-$ due to upregulated O_2^- may further limit NO bioavailability.

Diet is a modifiable lifestyle factor that plays a major role in the primary and secondary prevention of CVD [84, 85]. Flavonoids are dietary plant-derived bioactive ingredients that are abundant in a variety of fruits and vegetables. It has estimated that the average daily total flavonoid intake of U.S. adults is 189.7 mg/day that is mainly from flavanols (83.5%), flavanones (7.6%), flavonols (6.8%), anthocyanidins (1.6%), flavones (0.8%), and isoflavones (0.6%) [86]. Recently, flavanols, a subgroup of flavonoids, have gained increasing attention due to its benefits on cardiovascular health. Flavanols are abundant in cocoa-containing products, green tea, red wine, and soy products [87]. The estimated mean flavanol intake of U.S. adult is in the range of 50-100 mg/day [88].

Data from a number of studies have demonstrated that flavanols independently have beneficial effects on cardiovascular health. Although the exact underlying mechanisms involved in reduced CVD risk factors on flavanol intake have not yet fully understood, recent evidence suggest that flavanol intake augments NO bioavailability in the endothelium, thus the improvement of endothelial function [89]. In the support of this hypothesis, our laboratory recently reported that cerebral vasomotor reactivity in response

to a typical rebreathing protocol is reduced in college-aged black Americans relative to age-matched white Americans, suggesting an impaired endothelial function in cerebral microcirculation [25]. This attenuated cerebral vasodilatory capacity in healthy young black Americans was improved with acute flavanol intake [26].

In addition, the majority of studies have demonstrated that flavanols are related to an improvement in larger arteries such as the brachial artery [90-92] while there is uncertainty if endothelial function might be restored in the microvasculature following flavanol ingestion. Accordingly, the current study aimed to investigate the effects of acute flavanol intake on cutaneous microvascular function in young black Americans. We tested the hypothesis that acute consumption of flavanol-rich cocoa (FC) beverage would improve cutaneous microvascular function in response to local heating in young black Americans relative to young white Americans. Furthermore, we hypothesized that the improvement of microvascular function with acute flavanol intake is related to increase in NO bioavailability in young black Americans

5.3 METHODS

Ethical Approval and Subjects

Young adults aged 18-30 were recruited for this study. The groups were compromised of seven healthy black Americans and seven healthy white Americans subjects. All subjects completed a medical history questionnaire prior to participation. Ethnicity of each subject was self-defined as black Americans or white Americans, and the subject was only accepted if both parents are black Americans or white Americans, respectively. Before participation, all experimental procedures were explained to subjects and they were given an opportunity to ask questions. All subjects were free from any known cardiovascular, neurological, and metabolic diseases, and were non-smokers. All female subjects were not on birth control and all experiments were conducted during the early follicular phase of their menstrual cycle (days 1-3) to minimalize the fluctuations of female hormone, which might affect the endothelial function [51, 52].

Instrumentation and Measurements

This study was conducted in the Environmental and Autonomic Physiology Laboratory (BEL 846) at the University of Texas at Austin. All subjects were asked to report to the temperature-controlled laboratory (22-23 °C) on two different days at the same time each day, separated by a minimum of 1 week. All data were collected in the morning following at least a 12-hour fast, having refrained from caffeine containing drinks for 12 hours and strenuous exercise and alcohol drinks for 24 hours. All subjects were asked to follow a low-flavanol diet in the three consecutive days prior to each testing day.

On the first visit, subjects read and signed the Consent to Participate form and filled out the Research Health Questionnaire. The height and weight of subjects were obtained for calculation of body mass index (BMI). Subjects were then asked to lie down on a

patient's bed in the semi-supine position for the remainder of data collection. Five electrodes were attached to the subject for the assessment of cardiac rhythms and heart rate (HR) measured via an electrocardiogram (HP Patient Monitor, Agilent, Santa Clara, CA) interfaced with a cardiotachometer (CWE, Ardmore, PA). A blood pressure cuff was put on the upper arm for the assessment of arterial blood pressure measured from auscultation of the brachial artery (Tango+; SunTech, Raleigh, NC).

Subjects were instrumented with two intradermal microdialysis membranes on the ventral surface of the non-dominant forearm (CMA 31 Linear Microdialysis Probe, 55 kDa cut-off membrane; CMA Microdialysis AB, Holliston, MA). Once each microdialysis membrane was inserted on the skin, each site continuously received lactated Ringer's solution (2 µl/min) via an infusion pump (Pump 11, Harvard Appartus, Natick, MA) [9, 53, 54]. Two laser-Dopplers (VP7 A/T with moorVMS-LDF2; Moor Instruments, Wilmington, DE) housed within 3-cm diameter local heating elements (PeriFlux System 5000; Perimed, Sweden) were placed above each microdialysis membrane. After the placement of laser-Dopplers and local heating elements, the local heaters were clamped at 32 ºC, and there was a 60-90 min waiting period to allow for the hyperemia associated with the needle insertion to subside. In the last minute of this waiting period, baseline data were obtained.

Experimental Protocol

After the 45 minutes of the 60-90 min waiting period, subjects consumed either a FC beverage or a nutrient-matched non-flavanol (NF) beverage (provided in a double blinded and randomized fashion). The macro and micronutrient composition of the beverages is provided in Table 5.1. A third party person prepared (randomized order), appropriately documented, and administered the test beverage keeping it double-blind. The

beverage was mixed in 300 ml of water, and subjects were asked to consume the beverage within 5 min. Upon completion of the beverage, a timer was immediately started to ensure that the second measures of interest are taken as close to a 2 hour post-ingestion. Upon the completion of a 60-90 min waiting period, the baseline data were obtained and the experimental sites were randomly infused either lactated Ringer's solution as control or 20 mM L-NAME to locally inhibit NO production at a rate of 2 μl/min for a 45-min drug wash-in period. Following this drug wash-in period the temperature of each local heating element to was raised to 39 ºC at a rate of 0.5 every 5 seconds and was clamped at 39 ºC for 30 min, while each site continued to receive its respective solutions. Following this 30 min period, local temperature was increased to 43ºC, and 28 mM of sodium nitroprusside (SNP) was perfused through each site to induce maximal vasodilation [11]. During the heating protocol, subjects were asked to keep their non-dominant arm stable. At the end of the heating protocol, the heaters were turned off and removed from the skin to minimalize the possibility of 'desensitization' in the skin with prolonged heating [93-95].

Data Analysis

The laser-Doppler flowmetry (LDF) (in flux units) and skin temperature data were integrated with a data acquisition system (MP150, BIOPAC Systems, Goleta, CA) and recorded on a laboratory computer for off-line analysis (Acknowledge, BIOPAC, Goleta, CA). The red blood cell (RBC) flux recorded via LDF in the final minute of each stage (baseline at 32 ºC, plateau at 39 ºC, and SNP plus 43 ºC heating) was averaged. In the last minute of each stage, blood pressure measurement was also taken via auscultation at the brachial artery to calculate mean arterial pressure (MAP) to calculate cutaneous vascular conductance (CVC) at each stage. CVC was then presented as a percentage of maximal CVC (%CVCmax) that was obtained during combined SNP infusion and 43 ºC local

heating. The difference in %CVCmax between the control site and L-NAME site (Δ %CVCmax) across the rise in skin temperature was calculated to assess the NO contribution as an index of NO bioavailability.

Statistical Analysis

All data were analyzed using a statistical software package (Stata 13; StataCorp LP, College Station, TX). A paired *t*-test showed no significant differences in physical characteristics and hemodynamic variables obtained under fasting conditions during rest on each of the experimental days, so these data were averaged. Unpaired Student's *t*-tests were then performed to detect significant differences between groups for in physical characteristics and hemodynamic variables. A three-way repeated measure mixed model was conducted to estimate; 1) the effects of group, test beverage, and drug site on absolute maximal CVC, 2) the effects of group and test beverage on %CVCmax over a rise in local temperature at each drug site, and 3) the effect of group and test beverage on NO contribution over a rise in local temperature. A two-way repeated measure mixed model was also conducted to estimate the effects of group and test beverage for the plateau %CVCmax (39 ºC heating) at the lactated Ringer's site. The post-hoc comparison with Tukey's HSD were performed when a significant interaction was identified. All data are presented as means ± SE unless otherwise stated. The level of significance was set at $P < 0.05$.

5.4 RESULTS

Subject Characteristics

As shown in Table 5.2, subjects were well matched for age, height, weight, and BMI (all $P > 0.05$). Baseline systolic blood pressure (SBP), diastolic blood pressure (DBP), MAP, HR, CVC, %CVC were not different between NF and FC in both groups (all $P > 0.05$) (Table 5.3).

The Effects of Acute Flavanol Intake on Cutaneous Microvascular Function

Absolute maximal CVC. Table 5.4 shows absolute maximal CVC during combined 43 °C heating and 28 mM SNP infusion. Absolute maximal CVC was similar between groups and test beverage across all drug sites (all $P > 0.05$). No significant interactions between group and test beverage across all site was observed ($P = 0.478$). There was no significant interaction between group and test beverage ($P = 0.476$), drug site and test beverage ($P = 0.812$), or group and drug site ($P = 0.966$). There no significant main effect of group ($P = 0.575$), test beverage ($P = 0.766$), or drug site ($P = 0.175$). These data confirms that the maximal vasodiatory capacity of the cutaneous microvasculature was not different between group and test beverage across all drug sites.

Ringer's solution site. Figure 5.1 shows a skin blood flow (SkBF) tracing between dietary intervention days from a representative subject in each group. Figure 5.2 summarizes the SkBF response to the rise in local temperature between group and test beverage at the Ringer's site. There was no statistically significant difference in the baseline %CVCmax at 32 °C between NF and FC beverages in both of the white Americans group (NF: 11 ± 4 vs. FC: 10 ± 4 %CVCmax; $P = 0.735$) and the black American group (NF: 8 ± 4 vs. FC: 10 ± 4 %CVCmax; $P = 0.716$). The plateau %CVCmax at 39 °C heating with FC beverage improved %CVCmax in the black American group when compared to

NF beverage (NF: 36 ± 4 vs. FC: 47 ± 4 %CVCmax; $P = 0.008$) while there was similar %CVCmax at 39 °C between NF and FC beverages in the white American group (NF: 55 ± 4 vs. FC: 59 ± 4 %CVCmax; $P = 0.404$). No significant three-way interaction was observed ($P = 0.578$). There was a significant interaction between group and temperature ($P < 0.001$) but not between temperature and beverage ($P = 0.084$) or between group and beverage ($P = 0.208$). The significant interaction between group and temperature remained following Bonferroni correction. In the Ringer's site, there was a significant main effect of group ($P = 0.017$) and temperature ($P < 0.001$), but not beverage ($P = 0.079$).

L-NAME site. Figure 5.3 summarizes the SkBF response to the rise in local temperature between group and beverage at the NOS-inhibited site. There was no statistically significant difference in the baseline %CVCmax at 32 °C between NF and FC beverages in the white American group (NF: 7 ± 1 vs. FC: 7 ± 1 %CVCmax; $P = 0.890$) or in the black American group (NF: 5 ± 1 vs. FC: 6 ± 1 %CVCmax; $P = 0.620$). At 39 °C heating, there was no statistically significant difference in the plateau %CVCmax between NF and FC beverages in the white American group (NF: 13 ± 1 vs. FC: 13 ± 1 %CVCmax; $P = 0.919$) or in the black American group (NF: 10 ± 1 vs. FC: 12 ± 1 %CVCmax; $P = 0.158$). In the L-NAME site, there was no significant interaction between group and temperature ($P = 0.565$), between temperature and beverage ($P = 0.634$), or between group and beverage ($P = 0.283$). There was a significant main effect of temperature ($P < 0.001$), but not group ($P = 0.127$) or beverage ($P = 0.404$).

NO contribution. Figure 5.4 shows NO contribution between groups. At the baseline (32 °C), there were no differences in NO contribution between NF and FC beverages in the white American group (NF: 5 ± 3 vs. FC: 2 ± 3 Δ %CVCmax; $P = 0.688$), or in the black American group (NF: 3 ± 3 vs. FC: 4 ± 3 Δ %CVCmax; $P = 0.850$). At the plateau (39 °C), NO contribution was significantly higher with FC beverage than NF

beverage in the black American group (NF: 27 ± 3 vs. FC: 35 ± 3 Δ %CVCmax; $P = 0.028$) while there was similar NO contribution between NF and FC beverages in the white American group (NF: 40 ± 4 vs. FC: 44 ± 4 Δ %CVCmax; $P = 0.307$). No significant three-way interaction was observed ($P = 0.829$). There was a significant interaction between group and temperature ($P = 0.007$), but not between temperature and beverage ($P = 0.086$) or between group and beverage ($P = 0.419$). There was a significant main effect of temperature ($P < 0.001$) but not group ($P = 0.054$) or beverage ($P = 0.128$).

%CVCmax for the plateau at the Ringer's site. Figure 5.5A displays mean %CVCmax for the plateau phase at 39 °C heating of skin between young black Americans and white Americans at the Ringer's site. The plateau %CVmax at 39 °C heating was a significantly lower in the black American group than the white American group with NF beverage (black American (BA): 36 ± 5 vs. white American (WA): 55 ± 5 %CVCmax; $P = 0.004$). In the black American group, this attenuated %CVCmax at 39 °C heating improved to the level of the white American group by acute FC intake (BA: 47± 5 vs. WA: 59 ± 5 %CVCmax; $P = 0.082$). No interaction between group and beverage was observed ($P = 0.237$). There was a significant main effect of group ($P = 0.004$) but not beverage ($P = 0.442$).

5.5 DISCUSSION

The aim of the current study was to investigate the effects of acute flavanol intake on cutaneous microvascular function in response to local heating and to determine if the response to heating with acute flavanol intake differs between young black Americans and white Americans. The major findings of this study is that acute consumption of FC improved cutaneous microvascular function in response to local heating in young black Americans when compare to NF, and that such improvement of cutaneous microvascular function in young black Americans was related to increase in NO bioavailability.

To our knowledge, no study has specifically investigated the effects of acute consumption of FC on cutaneous microvascular function in young black Americans. However, the outcomes of this study are similar to our previous study on cerebral vasomotor reactivity, showing that attenuated cerebral vasodilatory capacity in healthy young black Americans improved with acute consumption of FC when compared to age-matched white Americans [26]. Furthermore, it is important to note that our findings are consistent with previous studies in other populations. It has been reported that acute and long-term supplementation of flavanols improve endothelial function in healthy individuals [96] and this effect is augmented individuals with endothelial dysfunction [90, 92, 97]. It has also shown that consumption of flavanol-containing food and beverages including dark chocolate, black tea, and red wine improved vasodilatory responses in individuals with coronary artery disease [98], hypertension [99], kidney disease [100], and type II diabetes [92].

NO is synthesized in the endothelial cells in the presence of the cofactor thetrahydrobiopterin (BH_4) by the reaction of eNOS, which divide the substrate L-arginine into NO and L-citrulline. NO acts as a primary vasoactive substance that plays a critical role in vasodilation [31]. NO bioavailability is detectable based on the balance between the

synthesis and degradation of NO in the vasculature. The underlying mechanisms of the improved endothelial function by the supplementation of flavanols in young black Americans has not been fully explored. However, given that endothelial dysfunction due partly to decrease in NO bioavailability is commonly observed in black Americans [23, 66, 101], it is reliable to suggest that the improvement of endothelial function is related to an increase in NO bioavailability in young black Americans. Indeed, in the current study, we found that the improvement of cutaneous microvascular function with acute flavanol intake in young black Americans was related to an increase in NO bioavailability.

The exact mechanisms by which flavanols affect NO bioavailability are unknown and require further investigation to mechanistically explain the findings in the present study; however, given that black American population commonly have higher oxidative stress levels than other racial groups, it is plausible that flavanols might scavenge free radicals including O_2^- and contribute to increase in NO bioavailability. It has been reported that reduced NO bioavailability in young black Americans (22 years) is related to the superoxide anion [66], which leads to a reduction in NO bioavailability as they react with NO to form $ONOO^-$ [69, 70]. Similarly, Manson et al. (2005) have shown that NO production is improved by a NADPH oxidase inhibitor in young black Americans [102]. Additionally, studies have demonstrated that epicatechins, primary monomer of flavanols, reduce the production of free radicals including O_2^- through of NADPH oxidase, in turn, improves NO bioavailability [103, 104]. Thus, it is possible that an elevated production of O_2^- due to upreguation of NADPH oxidase may lead to decrease in NO bioavailability and microvascular dysfunction in young black Americans, and that flavanols may affectively reduce the production of O_2^- by decreasing NADPH oxidase activity and improve NO bioavailability in young black American individuals.

Another potential mechanism of improved NO bioavailability associated with flavanols in young black Americans is the improvement of eNOS coupling by indirect pathways, including upregulation of eNOS activity, inhibition of arginase activity, and/or improvement of L-arginine availability in the endothelial cells. In vitro studies, endothelial cells incubated with flavonoid-rich red wine upregulated eNOS mRNA and protein expression, producing more bioactive NO (up to 3 times) than the control cells [105]. Plant extracts rich in flavonoids also upregulated eNOS activity in cultured endothelial cells [106, 107] and rat aorta [108]. Furthermore, cocoa flavanols also reduced arginase activity in human endothelial cells, leading to increase in local concentrations of the substrate, L-arginine [109]. Thus, we suggest the possibility that the improvement of cutaneous microvascular function associated with flavanols observed in young black Americans may be exerted via the direct reduction of free radicals including O_2^- and/or the indirect upregulation of eNOS activity.

In young white American individuals, the acute consumption of FC did not show any statistically significant improvement on cutaneous microvascular function in response to local heating compared with NF. The reasons for this can be multifactorial; however, it can be speculated that young and healthy white American individuals did not have any impairments on microvascular function such that the effects of acute flavanol intake are minimal.

Methodological consideration

Cutaneous thermal hyperemia can be observed via local heating of the skin, which leads to a rapid vasodilation. In a typical local heating protocol, thermally-induced hyperemia response to 42 °C heating of skin at a rate of 0.1 °C/s has been widely used to assess NO-dependent vasodilation in microvasculature. Local heating of skin elicits

cutaneous blood flow reactivity in two distinct phases that is marked by an initial peak in SkBF within the first 5 min of heating and is followed by a plateau which is reached within 30 min of heating [43, 62, 110-112]. However, it is important to note that the plateau phase in response to local heating of skin is dependent not only NO but also endothelium-derived hyperpolarizing factors (EDHFs). Approximately 50-60% of vasodilation on the plateau phase is dependent on NO [62, 113] through transient receptor potential vanilloid type-1 (TRPV1) channels [114], adenosine receptors [115], and reactive oxygen species [116] while the remaining 40-50% depends on EDHFs that stimulate calcium-activated potassium channels on endothelial and smooth muscle cells [110].

For this reason, we applied a 39 °C heating to elicit cutaneous thermal hyperemia, which is mostly dependent on NO but not EDHFs. Choi et al. (2014) have recently demonstrated that reducing the target temperature from 42 °C to 39 °C during a local heating protocol elicit a hyperemic response that is approximately 80% dependent on NO [117]. In the current study, the plateau %CVCmax obtained by 39 °C heating was 76% dependent on NO in the white Americans group and 72% dependent on NO in the black American group when consumed NF beverage (data not shown). Thus, we believe that the findings from the current study might be more reliable evidence than others to assess NO-dependent microvascular function.

Limitations

The present study was limited by the fact that we quantified NO contribution during heating by calculating the difference between %CVCmax at the control site and L-NAME site (Δ %CVCmax). Previously, several microdialysis studies have coinfused nonspecific NOS inhibitor L-NAME within the experimental sites to quantify NO-dependent vasodilation during a standardized local heating protocol, which the skin is needed to be

heated for a 70–90 min continuously [49, 62, 113, 118]. However, Hodges et al. (2008) have suggested that an extended length of time on local heating protocol may result in a "die-away phenomenon", attributing to a reduction in vasodilation [55]. In line with that, although we did not quantify NO contribution within each microdialysis, we did evaluate the time course of the interventions with < 60 min of local heating.

The present study was a double-blind cross-sectional study with a small sample size; thus, our results that black American improved cutaneous microvascular function with acute flavanol intake may include a type 2 error. However, we observed an increased microvascular function with flavanol intake in most of seven black American individuals, so it was not being skewed by an outlier (Fig. 5.5B). Additional data in larger groups may help to confirm our findings.

In the present study, we did not analyze blood sample including plasma flavanol metabolites, nitrate/nitrite, and oxidized low density lipoprotein to verify whether flavanols were present in the circulation or if there was an effect of flavanols on NO bioavailability. However, a 2-hour window was chosen in the present study. Several studies with flavanol ingestion have demonstrated that plasma concentration of flavanols peak after 2 hours from the ingestion [92, 119-122]. Furthermore, we believe that the flavanol content in FC beverage (792 mg) used in the present study was high enough to elicit the benefits of acute flavanol intake in microvascular function when compare to previous studies [26, 90].

Conclusion

This study demonstrated that acute consumption of FC improves cutaneous microvascular function in young black American relative to age-matched white Americans. Furthermore, this study demonstrated that the improvement of cutaneous microvascular

function with flavanols is related to an increase in NO bioavailability. Considering that young black American adults have attenuated NO-dependent vasodilation in microvasculature and that acute consumption of FC restores cutaneous microvascular function in young black American adults, flavanol-rich beverage and food could be a novel potential therapeutic target to delay onset of microvascular dysfunction and prevent the development of CVD in black American population.

5.6 TABLES AND FIGURES

Table 5.1 Product nutrition facts

Per serving	Flavanol	Non-flavanol
Calories	224	229
Fat, g (calculated)	3	0
Sat fat, g	1.5	0
Trans fat, g	0	0
Cholesterol, mg	16.5	18
Sodium, mg	546	555
Carbohydrates, g	37.5	39
Dietary fiber, g	7.5	7.5
Sugar, g	25.5	30
Protein, g	21	21
Vitamin A, IU	15	12
Vitamin C, mg	3	4.5
Calcium, mg	643.5	729
Iron, mg	1.5	0
Magnesium, mg	169.5	61.5
Potassium, mg	1170	1014
Flavanols 1-10, mg	**370.8**	0.0
Monomers	**97.2**	0.0
Catechin, mg	**26.7**	0.0
Epicatechin, mg	**70.5**	0.0
2-10 mers	**273.6**	0.0
Total flavanols, mg*	**729.0**	0.0

* Total flavanols include all flavanols that are greater than 10 polymers.

Table 5.2 Subject characteristics

N, no. of subjects; M, male; F, female; BMI, body mass index. Values are means ± SD.

	White American (N=7)	Black American (N=7)	*P*-value
Age (years)	22 ± 4	22 ± 4	0.89
Sex (M/F)	4/3	4/3	1.00
Height (cm)	171 ± 11	169 ± 10	0.82
Weight (kg)	70 ± 14	69 ± 13	0.92
BMI (kg/m^2)	24 ± 2	24 ± 2	0.87

Table 5.3 Baseline hemodynamic variables

NF, beverage without flavanols, FC, flavanol-rich cocoa beverage; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; CVC, cutaneous vascular conductance; %CVCmax: CVC as a percentage of maximal vasodilation. All data were collected in the last minute of baseline period at the Ringer's solution site. Baseline hemodynamics were not different between NF and FC in both groups (all $P > 0.05$). Values are means ± SD.

	White American		Black American	
	NF	FC	NF	FC
HR (beats/min)	59 ± 9	59 ± 8	67 ± 8	65 ± 7
SBP (mmHg)	110 ± 8	114 ± 6	120 ±12	120 ± 5
DBP (mmHg)	69 ± 4	67 ± 7	71 ± 13	71 ± 7
MAP (mmHg)	83 ± 4	82 ± 4	87 ± 10	87 ± 5
CVC (flux/mmHg)	0.34 ± 0.13	0.28 ± 0.08	0.23 ± 0.19	0.28 ± 0.14
%CVCmax	11 ± 4	10 ± 3	8 ± 7	10 ± 6

Table 5.4 Absolute maximal cutaneous vascular conductance (CVC) across all sites

L-NAME, NG-nitro-L-arginine. NF, beverage without flavanols; FC, flavanol-rich cocoa beverage. Absolute maximal CVC was similar between groups and test beverages across all sites (all $P > 0.05$). No significant interaction between group and test beverage across all site was observed ($P = 0.478$).Values are means ± SE.

	White American		Black American	
	NF	FC	NF	FC
Control site	3.21 ± 0.27	3.03 ± 0.27	2.85 ± 0.27	3.04 ± 0.27
L-NAME site	2.91 ± 0.27	2.97 ± 0.27	2.74 ± 0.27	2.81 ± 0.27

Figure 5.1 A skin blood flow tracing from a representative black American subject

LDF, laser-Doppler flowmetry. NF, beverage without flavanols; FC, flavanol-rich cocoa beverage. A, initial peak; B, plateau; C, maxial SkBF induced by SNP. Data selected from the last 10 min of baseline period to the end at the Ringer's site.

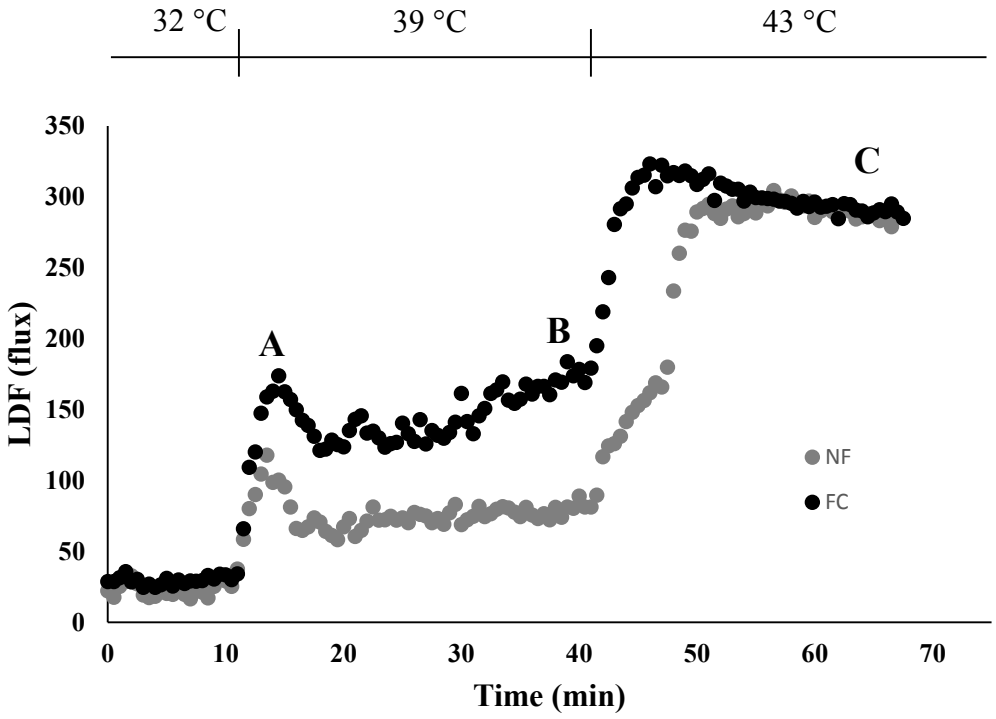


Figure 5.2 A summary of the skin blood flow response to the rise in local temperature between group and test beverage at the ringer's site

Left panel, white Americans; right panel, black Americans. NF, beverage without flavanol; FC, flavanol-rich cocoa beverage. Acute consumption of FC improved %CVCmax in black Americans but not white Americans when compare to NF. There was a significant interaction (group and temperature) ($P < 0.001$) but not (temperature and beverage) ($P = 0.084$). *P < 0.05, significant difference vs. NF within the groups. Values are means ± SE.

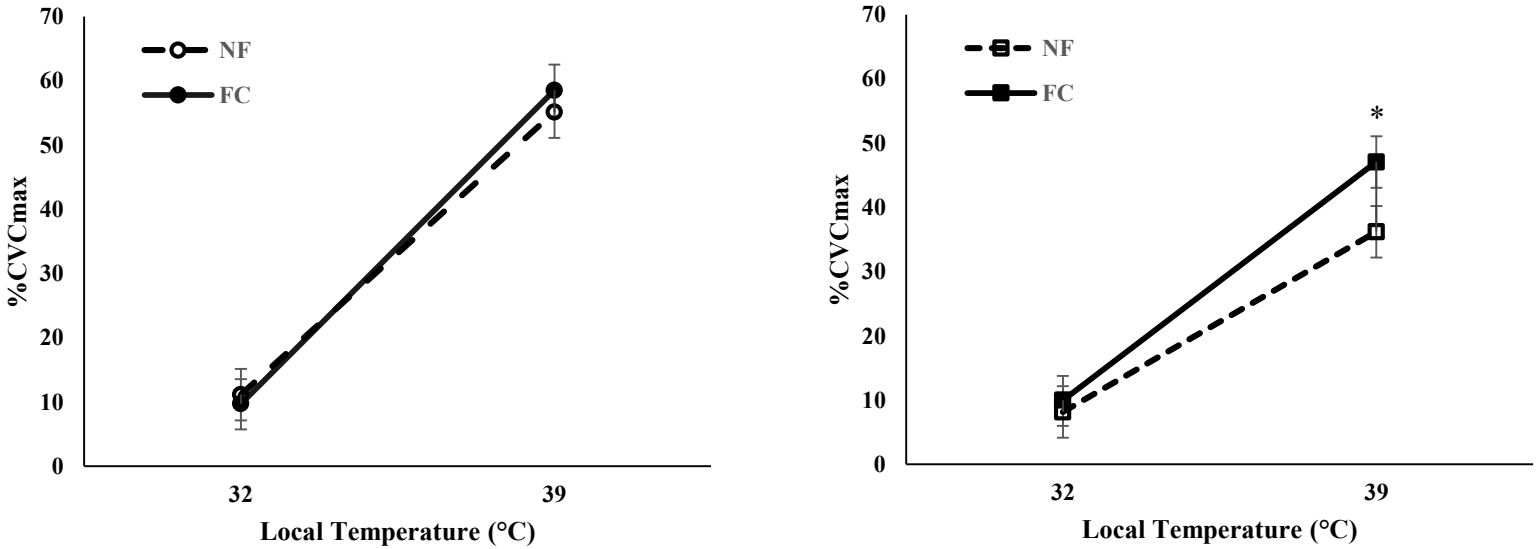


Figure 5.3 A summary of the skin blood flow response to the rise in local temperature between group and test beverage at the L-NAME site

Left panel, white Americans; right panel, black Americans. NF, beverage without flavanol; FC, flavanol-rich cocoa beverage. %CVCmax was similar between NF and FC in each group (all $P > 0.05$). There was no significant interaction between group and temperature ($P = 0.565$), between temperature and beverage ($P = 0.634$), or between group and beverage ($P = 0.283$). Values are means ± SE.

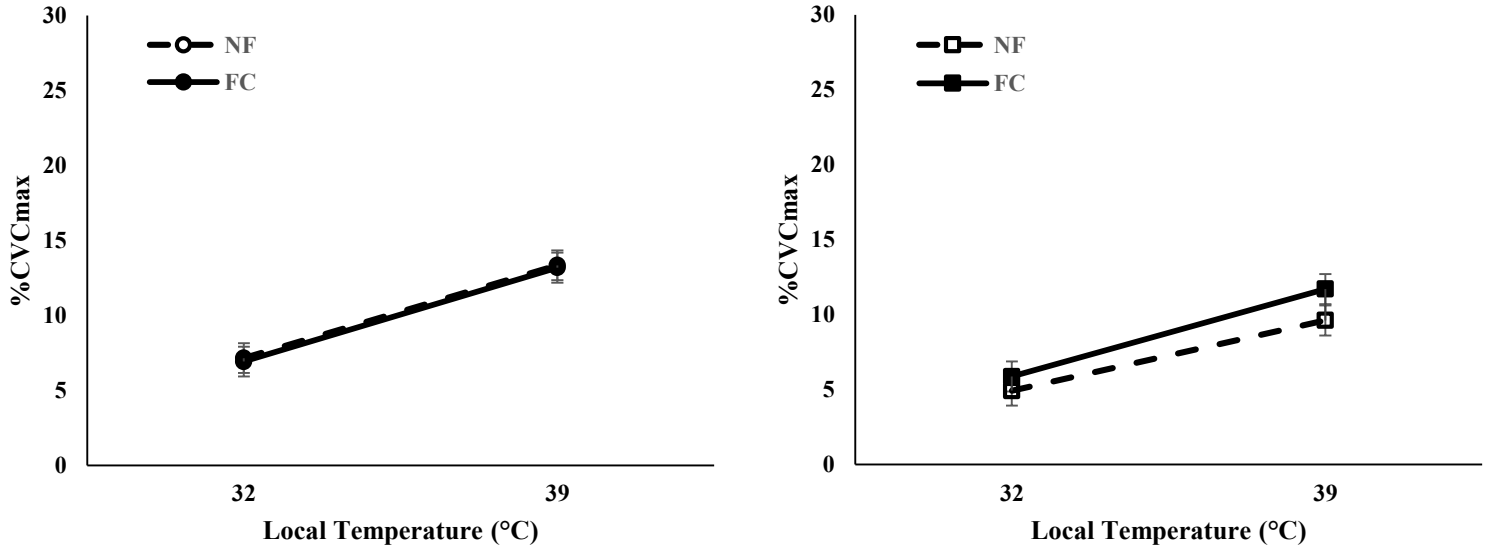


Figure 5.4 A summary of NO contribution in response to local heating of skin

Left panel, white Americans; right panel, black Americans. The difference in cutaneous vascular conductance as a percentage of maximal vasodilation in response to the rise in local skin temperature (°C) between the control site and NOS-inhibited site (Δ %CVCmax) was calculated to assess NO contribution as an index of NO bioavailability. NO contribution was lower in the black American group than the white American group during local heating of skin. There was a significant interaction between group and temperature ($P = 0.007$) but not between temperature and beverage ($P = 0.086$). *$P < 0.001$, significant difference vs. NF. Values are means ± SE.

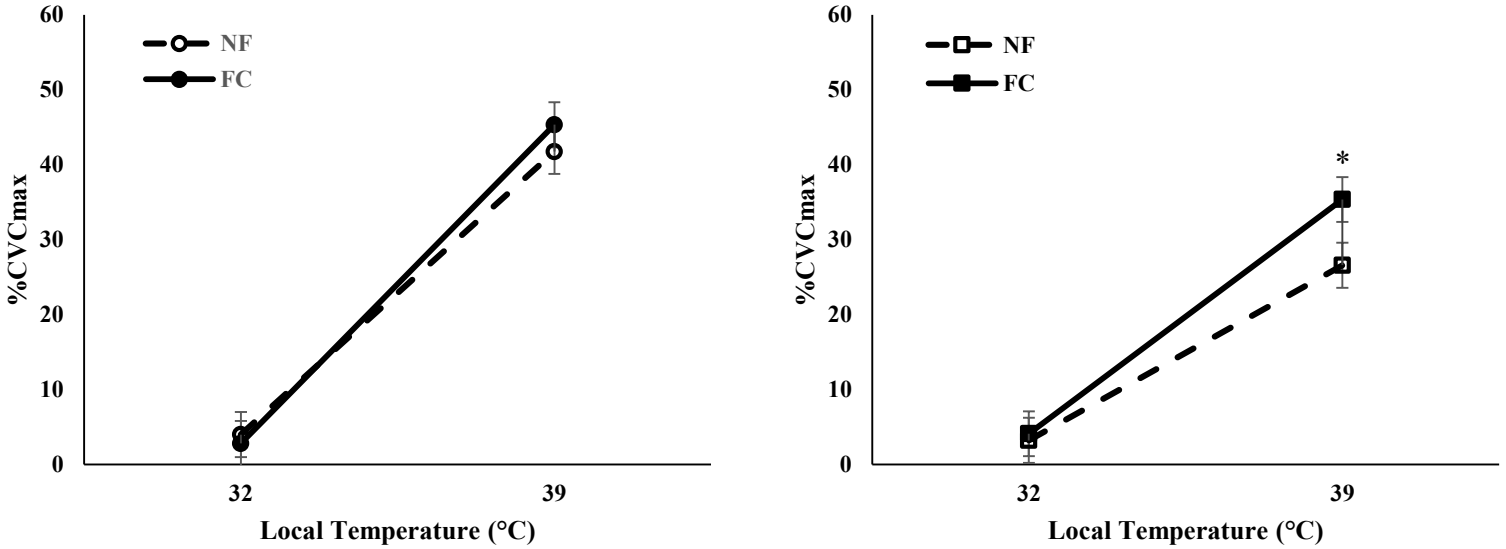
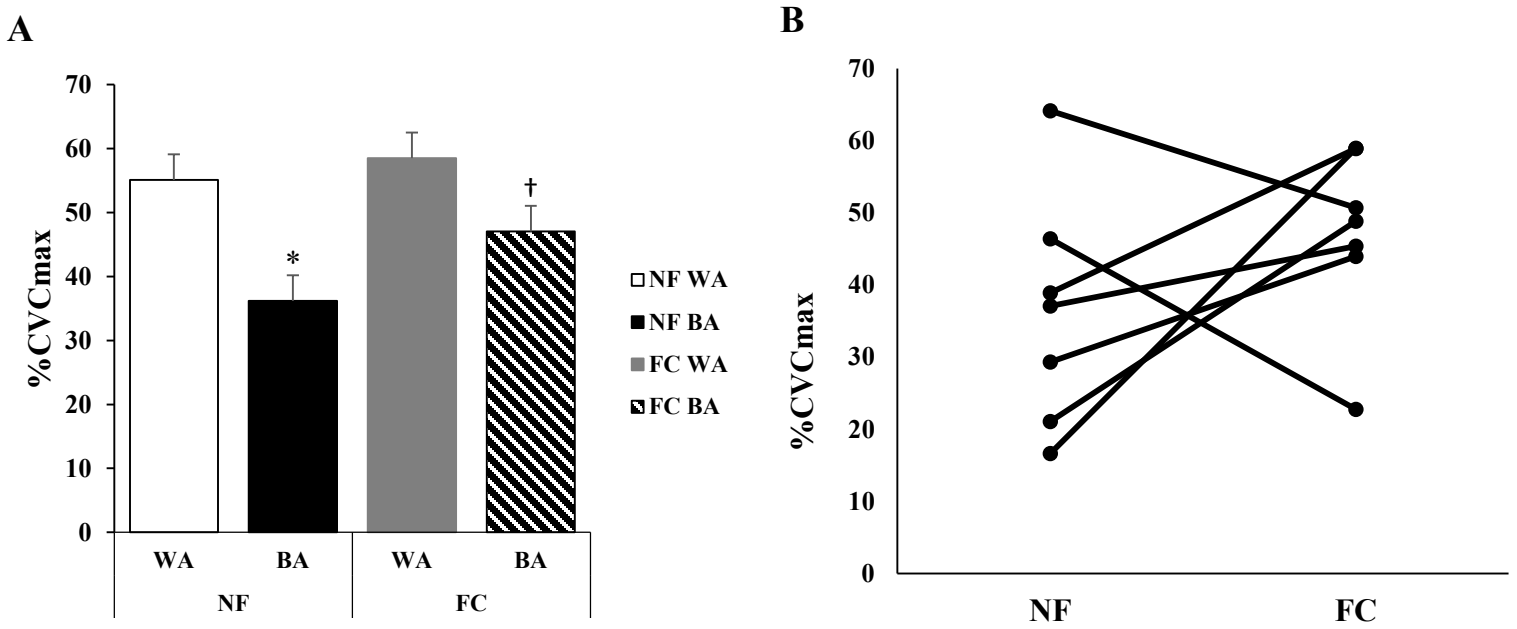


Figure 5.5 A summary of %CVCmax for the plateau phase at 39 °C heating of skin between young black Americans and white Americans at the Ringer's site

A, the plateau %CVCmax at 39 °C heating between groups; B, individual %CVCmax at 39 °C heating between non-flavanol (NF) and flavanol-rich cocoa (FC) beverages in black American group. Open bar, white American (WA) group with NF; black-colored bar, black American (BA) group with NF; grey-colored bar, WA group with FC, pattern-filled bar, BA group with FC. *$P < 0.01$, significant difference vs. white American group; †$P < 0.01$, significant difference vs. NF. Values are means ± SE.



Chapter 6: Study Three

The Effects of Acute Flavanol Intake on Methacholine-induced Cutaneous Vasodilation in Healthy, Young Black Americans

6.1 ABSTRACT

Black American adults have increased morbidity and mortality stemming from cardiovascular disease and related risk factors relative to white American adults. Furthermore, it is known that the underlying impairments manifest early in young adults prior to any overt signs of risk in this population. We have recently demonstrated that nitric oxide dependent cutaneous microvascular function is attenuated in young black Americans relative to age-matched white Americans. Thus, we hypothesized that young black Americans would have an attenuated vasodilatory response to methacholine (MCh)-induced cutaneous vasodilation when compare to age-match white Americans as indexed by the dose of MCh required to elicit 50% of the maximal vasodilation (EC_{50}). We also tested the hypothesis that acute consumption of flavanol-rich cocoa (FC) would produce a leftward shift in the dose-response curve of MCh-induced cutaneous vasodilation in young black Americans, while there would be no effects of acute consumption of FC in young white Americans.

Fourteen young adults (7 black Americans and 7 white Americans) participated in this double-blind crossover study. Data were collected on two different days, separated by a minimum of one week. A single intradermal microdialysis membrane was inserted in the forearm and skin blood flow was indexed via a laser-Doppler probe housed within a local heater over the membrane site. After the placement of the laser-Doppler probe and local heater, the local heater was clamped at 32 ºC, and there was a 60-90 min waiting period to while the hyperemia associated with the the needle insertion subsided. After a 60-90 min waiting period, subjects were randomly assigned to consume either a non-flavanol containing (NF) beverage or FC beverage. It was followed a 75-min waiting period as close to a 2-hour post-ingestion. Following a 75-min waiting period, the microdialysis site received with increasing concentration of MCh (1×10^{-6} M; 7 doses at 10-fold increments)

disserved in lactated Ringer's solution, with each dose being administered for 6 min at perfusion rate of 2 μl/min. After the last dose of MCh, local skin temperature was raised and clamped at 43 ºC, and 28 mM of sodium nitroprusside (SNP) was perfused for approximately 30 min to induce maximal vasodilation. Cutaneous vascular conductance (CVC) was calculated as cutaneous blood flux/mean arterial pressure and normalized as a percentage of maximal CVC (%CVCmax). A four-parameter logistic curve fit was constructed to compare does-reseponse curves of MCh-induced vasodilation between groups or test beverages with an extra sum of squares F-test.

The dose response curves on %CVCmax between the white American group and the black American group in each dietary intervention were similar (all $P > 0.05$). In the white Americans group, the dose response curves on %CVCmax were similar ($P = 0.869$) between NF and FC beverages. The log EC_{50} of dose-response curves on %CVCmax for NF and FC were not significantly different (NF: -3.64 ± 0.63 vs. FC: -4.06 ± 0.19 log EC_{50}; $P = 0.720$). In the black Americans group, the dose response curves on %CVCmax were similar between NF and FC intake ($P = 0.322$). The log EC_{50} for non-flavanol and flavanol were not significantly different (NF: -4.12 ± 0.19 vs. FC: -4.09 ± 0.19 log EC_{50}; $P = 0.516$).

Contrary to our hypotheses, the log EC_{50} of dose-response curves on %CVCmax was similar between the white American group and the black American group. Furthermore, the acute consumption of FC did not alter the dose-response curves of MCh-induced cutaneous vasodilation in both groups. This data suggests that 1) cutaneous microvascular endothelial function in response to incremental doses of MCh might remain intact in both black American and white American adults, and 2) acute consumption of FC has no effects on MCh-induced cutaneous vasodilation in microvasculature.

6.2 INTRODUCTION

Black American adults are at increased morbidity and mortality of cardiovascular disease (CVD) and its risk factors including myocardial infarction [123, 124], stroke [125], hypertension and type 2 diabetes [3, 126] when compare to white American adults. One potential mechanism of higher morbidity and mortality of CVD in black Americans is endothelial dysfunction. Endothelial dysfunction in conduit vessels is commonly observed in young black Americans without any overt sign of CVD. Several studies have provided evidence that endothelial dysfunction is linked with increased CVD risks in young black Americans and other populations. Bassett et al. (1992) investigated vasodilatory capacity in normotensive young black and white Americans. They reported that vasodilatory capacity of the forearm resistance vessels is lower in young black American men when compared to young white American men [28]. Similarly, Campia et al. (2002) demonstrated that black American adults have blunted endothelium-dependent and -independent vasodilation of the brachial artery indexed by flow-mediated dilation and nitroglycerin-mediated dilation relative to white American adults [127]. Taken together, these findings indicate an early onset of endothelial dysfunction in young black Americans prior to any overt signs of risk compared with young white Americans.

Although most studies of endothelial function in human vessels have focused on macrovessels by using plethysmography, there is an emerging evidence that endothelial dysfunction presents in microcirculation prior to the macrocirculation. Recently, it has been recognized that the microcirculation is an ideal location to predict CVD risk. There is compelling evidence that endothelial dysfunction in microvasculature is an early predictive marker of CVD events [128, 129] including hypertension and diabetes [130]. To support these previous findings we have recently demonstrated that nitric oxide (NO)-dependent

vasodilation in cutaneous microvasculature is significantly attenuated in young black Americans relative to age-matched white Americans, indicating the present of microvascular endothelial dysfunction in young black Americans.

Diet is one of the modifiable lifestyle factors that has a pivotal role in the prevention of CVD and its risk factors [84, 85]. Flavonoids are present in more than 5,000 plants in nature that are classified based on their chemical structure. They are usually subdivided into several subgroups according to which functional groups are attached to the 'C-ring' of this 3-ring carbon backbone [131]. The main subgroups of flavonoids include flavanols (cocoa, tea, fruits, and wine), flavonols (broccoli, onion, tea, and tomato), flavones (herbs), isoflavones (soybean), flavanones (juices), and anthocyanidins (berries and wine) [87]. Flavonoids are digested mainly in the small intestine and the liver. Flavonoids metabolites, which are chemically and structurally distinct from flavonoids, reach the target tissues through blood circulation [131, 132] and each subgroup of flavonoids leads to different physiological effects on cardiovascular health [133, 134].

Flavanols, a subgroup of flavonoids, have recently gained emerging interest because it has been shown that flavanols have beneficial effects on cardiovascular health. Research has shown that flavanols independently have beneficial effects on cardiovascular health [85, 92]. The exact underlying mechanisms of decreased CVD and its risk factors with flavanols have not been fully elucidated; however, recent evidence suggest that flavanols increases NO bioavailability in the endothelium and improve endothelial function [89]. Furthermore, we have recently found that cerebral vasomotor reactivity in response to hypercapnia is attenuated in college-aged black Americans relative to age-matched white Americans, suggesting an impaired endothelial function in cerebral microcirculation [25], and such attenuated cerebral vasodilatory capacity in young black Americans improved with acute flavanol intake [26].

Thus, we hypothesized that 1) young black Americans might have a increased 50 % of the maximal response (EC_{50}) of dose-response curve on methacholine (MCh)-induced cutaneous vasodilation when compare to age-match white Americans, and 2) acute consumption of flavanol-rich cocoa (FC) would produce a leftward shift in the dose-response curve of MCh-induced cutaneous vasodilation in young black Americans, while there would be no effects of acute consumption of FC in young white Americans.

6.3 METHODS

Ethical Approval and Subjects

Healthy, young adults aged 18-30 were recruited for this study. The groups were compromised of 7 black American and 7 white American subjects. All subjects completed a questionnaire on medical history, medications, and lifestyle behaviors prior to participation including some optional questions about their ethnic background as well as that of their birth parents. Ethnicity of each subject was self-defined as black American or white American, and the subject was only accepted if both parents are black American or white American, respectively. Before participation, all experimental procedures were explained to subjects and they were given an opportunity to ask questions. All subjects were not taking any medications known to alter cardiovascular function. Subjects were normally active with no history of cardiovascular, metabolic, or neurological disease. Current smokers or individuals who quit smoking within the prior two years were excluded [50]. All female subjects were only studied during the early follicular phase (within 1-3 days of the start of menstruation) to minimize the effects of female sex hormones [51, 52].

Instrumentation and Measurements

This study was completed during two different visits conducted at the same time of day, separated by the minimum of one week. Each subject was instrumented with a single intradermal cutaneous microdialysis membrane. The membrane was dedicated for the local infusion of lactated Ringer's solution. On each experimental day, subjects reported to the Environmental and Autonomic Physiology Laboratory (BEL 846) inside Belmont Hall at The University of Texas at Austin, having refrained from strenuous exercise and alcohol beverages for 24 hr and from caffeine and food for 12 hr. Subjects were asked to follow a low-flavanol diet in the three consecutive days prior to each testing day to control dietary

intake of flavanols. On the first visit, subjects read and signed the Consent to Participate form and filled out the Research Health Questionnaire. In this time, subjects were encouraged to ask any questions regarding the experimental protocol. Then, height and weight were obtained in both days to provide subject's baseline characteristics (Seca 763, Seca, Chino, CA).

Subjects were then asked to lie quietly down on the patient's bed in the semi-supine position for the remainder of data collection. Five electrodes were attached to the subject for the assessment of cardiac rhythms and heart rate (HR) measured via an electrocardiogram (HP Patient Monitor, Agilent, Santa Clara, CA) interfaced with a cardiotachometer (CWE, Ardmore, PA). A blood pressure cuff was put on the upper arm for the assessment of arterial blood pressure measured from auscultation of the brachial artery via electrosphygmomanometry (Tango+; SunTech, Raleigh, NC).

Subjects were instrumented with a single intradermal microdialysis membrane (CMA 31 Linear Microdialysis Probe, 55 kDa cut-off membrane; CMA Microdialysis AB, Holliston, MA) in the dermal space of the dorsal forearm. After the placement of the microdialysis membrane, the site was perfused lactated Ringer's solution at rate of 2 μl/min via an infusion pump (Pump 11, Harvard Appartus, Natick, MA) [54-56]. Then, the local skin blood flow was continuously measured via a laser-Doppler flowmetry (LDF) probe (VP7 A/T with moorVMS-LDF2; Moor Instruments, Wilmington, DE) housed within a 3-cm diameter local heater (PeriFlux System 5000; Perimed, Sweden), which was placed above the microdialysis membrane. After placement of the laser-Doppler and local heater, the local heater was clamped at 32 ºC, and there was a 60-90 min waiting period to subside hyperemia driven by the needle insertion.

Experimental Protocol

Following a 60-90 min waiting period, subjects consumed either a FC beverage or a nutrient-matched placebo beverage with the exception that it contained no flavanols (NF), randomly. Energy, minerals, and other nutrient components of NF and FC beverage are presented in Table 6.1. A third party person prepared (randomized order), appropriately documented, and administered the test beverage keeping it double-blind. The beverage was mixed in 300 ml of water, and subjects were asked to finish consuming the beverage within 5 min. Upon completion of the beverage, a timer was immediately started to ensure that the second measures of interest were taken as close to a 2-hour post-ingestion. Following a 75-min waiting period, the microdialysis site received increasing concentrations of MCh (1 x 10-6 M; 7 doses at 10-fold increments) dissolved in lactated Ringer's solution, with each dose being administered for 6 min at perfusion rate of 2 μl/min. This approach has been commonly used for dose-response studies to construct dose- response curve and to estimate the effective concentration of EC_{50} [135-137]. After the last dose of MCh, local temperature was raised to 43 ºC at a rate of 0.5 every 5 seconds and clamped at 43ºC combined with infusion of 28 mM of sodium nitroprusside (SNP) for approximately 30 min through the microdialysis membrane to induce maximal vasodilation. At the end of local skin heating at 43 ºC, the heating element was turned off and removed from the skin to minimalize the possibility of 'desensitization' in the skin with prolonged heating [93, 94].

Data Analysis

The LDF (in flux units) and skin temperature data were integrated with a data acquisition system (MP150, BIOPAC Systems, Goleta, white Americans), and recorded on a laboratory computer for off-line analysis (Acknowledge, BIOPAC, Goleta, white

Americans). The red blood cell flux recorded via LDF in the final minute of each stage (baseline, each dose, and SNP at 43 ºC) was averaged. In the last minute of each stage, blood pressure was also obtained via auscultation at the brachial artery to calculate mean arterial pressure (MAP), which was used to calculate cutaneous vascular conductance (CVC) at each stage. CVC was then presented as a percentage of maximal CVC (%CVCmax), which was obtained during 43 ºC heating plus 28 mM SNP infusion. A four-parameter logistic curve fit in non-linear regression model was constructed to compare does-reseponse curves of MCh-induced vasodilation between groups or test beverages with an extra sum of squares F-test.

Statistical Analysis

A paired *t*-test showed no significant differences in physical characteristics and hemodynamic variables obtained under fasting conditions during rest on each of the experimental days, so these data were averaged. Analysis of physical characteristics and hemodynamic variables between racial groups was performed using independent t-tests. A three-way mixed model was conducted to estimate the effect of group, MCh dose, and test beverage on the %CVCmax. Both the main effects of group, MCh dose, and test beverage and the interaction between group, MCh dose on each test beverage were assessed (Stata 13; StataCorp LP, College Station, TX). Dose-response curves for %CVCmax was constructed using a nonlinear fitting technique for identification of log EC_{50} for each subject (Prism, Graphpad Software). The mean log EC_{50} of MCh dose-response curves between race or test beverages was then compared with an extra sum of squares F-test. The post-hoc comparison with Tukey's HSD were performed when a significant interaction was identified. All data are presented as means ± SE unless otherwise stated. The level of significance was set at $P < 0.05$.

6.4 RESULTS

Subject characteristics

As shown in Table 6.2, subjects were well matched for age, height, weight, and body mass index (all $P > 0.05$). Resting systolic blood pressure (SBP), diastolic blood pressure (DBP), MAP, and HR were not different between groups. (all $P > 0.05$) (Table 6.3).

The Effects of Acute Flavanol Intake on Cutaneous Microvascular Function

No statistically significant differences in absolute CVC and %CVCmax in response to MCh were observed between male and female for either the white American or the black American groups; thus, the data from both sexes in each group were combined.

Baseline %CVCmax at 32 °C. Table 6.4 shows the baseline %CVCmax between post ~10 min and post ~2 hr beverage intake in both groups. In the white American group, the baseline %CVCmax was similar between at the beginning of dietary intervention protocol (NF: 12 ± 2 vs. FC: 10 ± 2 %CVCmax; $P = 0.870$) and approximately 2 hr after beverage given (NF: 11 ± 2 vs. FC: 11 ± 2 %CVCmax; $P = 0.922$). In the black American group, the baseline %CVCmax was similar between at the beginning of dietary intervention protocol (NF: 7 ± 2 vs. FC: 8 ± 2 %CVCmax; $P = 0.682$) and approximately 2 hr after beverage given (NF: 7 ± 2 vs. FC: 8 ± 2 %CVCmax; $P = 0.639$). No racial difference in the baseline %CVCmax was observed either at the beginning of dietary intervention protocol between NF and FC beverages (NF: $P = 0.101$; FC: $P = 0.433$ respectively) or approximately 2 hr after beverage given (NF: $P = 0.137$; FC: $P = 0.277$), respectively. No significant three-way interaction (group, time, and beverage) was observed ($P = 0.745$). There were no interactions between group and time, time and beverage, or beverage and group (all $P > 0.05$). There was no main effect of group ($P = 0.115$), time ($P = 0.636$), or

beverage (P = 0.989). This data indicates that the baseline %CVCmax either at the beginning of dietary intervention or 2 hr after beverage given was the same between NF and FC; thus, we used %CVCmax at approximately 2 hr after beverage given as the baseline %CVCmax on each dose-response curve.

MCh-induced and SNP-induced absolute maximal CVC. Figure 6.1 shows that absolute maximal CVC at the highest dose (1M) of MCh and 43 °C heating + 28 mM SNP infusion. In white Americans, absolute maximal CVC was similar either maximal dose of MCh (NF: 3.0 ± 0.3 vs. FC: 3.2 ± 0.3 flux·mm Hg^{-1}; P = 0.319) or 43°C heating + 28 mM SNP infusion (NF: 3.3 ± 0.3 vs. FC: 3.5 ± 0.3 flux·mm Hg^{-1}; P = 0.475). In black Americans, absolute maximal CVC was similar either maximal dose of Mch (NF: 2.8 ± 0.3 vs. FC: 2.7 ± 0.3 flux·mm Hg^{-1}; P = 0.661) or 43°C heating + 28 mM SNP infusion (NF: 2.9 ± 0.3 vs. FC: 3.1 ± 0.3 flux·mm Hg^{-1}; P = 0.589). No racial difference in absolute maximal CVC was observed either at maximal dose of Mch between NF and FC beverages (NF: P = 0.711; FC: P = 0.193 respectively) or at 43 °C heating + 28 mM SNP infusion between NF and FC beverages (NF: P = 0.397; FC P = 0.337 respectively). No three-way interaction was observed (P = 0.529). There were no interactions between group and time, time and beverage, or beverage and group (all $P > 0.05$). There was no main effect of group, time. Or beverage (all $P > 0.05$). This data indicates that the maximal cutaneous vasodilation in microvasculature occurred with maximal dose of MCh alone when compare to SNP infusion, and was not different between black Americans and white Americans or NF and FC beverages.

Dose response curves on %CVCmax. Figure 6.2 shows endothelium-dependent vasodilation in response to incremental dose of MCh. MCh dose-response curves for NF and FC intake were compared to evaluate endothelium-dependent vasodilation in the black American and white American groups, respectively. In the white American group, the

dose-response curves on %CVCmax were similar between NF and FC beverages (P = 0.869). The log EC_{50} was not significantly different between NF and FC beverages (NF: -3.64 ± 0.63 vs. FC: -4.06 ± 0.19 log EC_{50}; P = 0.720). HillSlope was similar between NF and FC beverages (P = 0.720). Top and bottom were also not different between NF and FC beverages (P = 0.104 and P = 0.687, respectively). In black American group, the dose response curves on %CVCmax were similar between NF and FC beverages (P = 0.322). The log EC_{50} was not significantly different between NF and FC beverages (NF: -4.12 ± 0.19 vs. FC: -4.09 ± 0.19 log EC_{50}; P = 0.516). HillSlope was similar between NF and FC beverages (P = 0.130). Top and bottom were also not different between NF and FC beverages (P = 0.659 and P = 0.652, respectively). The dose-response curves on %CVCmax between white Americans and black Americans in each test beverage were similar. (all $P > 0.05$).

6.5 DISCUSSION

The primary aim of this study was to clarify the effects of acute flavanol intake on cutaneous microvascular function between young black American and white American individuals. In the present investigation, we hypothesized that 1) young black Americans might have an increased EC_{50} of dose-response curve on MCh-induced cutaneous vasodilation when compare to age-match white Americans, and 2) acute consumption of FC beverage would produce a leftward shift in the dose-response curve of MCh-induced cutaneous vasodilation in young black Americans, while there would be no effects of acute flavanol intake in young white Americans. Contrary to our hypotheses, the EC_{50} of dose-response curve on MCh-induced cutaneous vasodilation was similar between racial groups. Furthermore, the acute consumption of FC beverage did not alter the dose-response curve on MCh-induced cutaneous vasodilation in both races.

It is well demonstrated that black Americans have reduced endothelial function in response to a variety of stimuli. Forearm blood flow (FBF) responses to exercise [28], ischemia [63], and mental stress [64] were significantly lower in healthy black Americans when compare to healthy white Americans. Cardillo et al. (1999) have also reported that FBF was attenuated in young black Americans following separate infusions of isoproterenol [138]. However, several cautions must be taken in interpreting our results in light of these previous studies. It has been reported that there is some heterogeneity in arteries in endothelial and smooth muscle cell in regards to shape and function [139, 140]. Galle et al. (1993) have also demonstrated that different sized vessels exhibit heterogeneous vasodilatory responses [141]. Importantly, it is notable that laser Doppler-induced indices of cutaneous microcirculation do not correlate with conduit vessel response to flow-mediated dilation either in control subjects or in a coronary artery disease patients [142]. Furthermore, the use of iontophoresis technique has received considerable attention.

The iontophoresis technique used in Cardillo's study requires infusing vasoactive substances into intradermal space by using electrical charges. It has been indicated that non-specific effects of the electrical charges and individual differences in skin electrical resistance could influence the responses to intradermal drug infusion by iontophoresis. These factors can confound the results and make it difficult to ensure if vasoactive substances are actually delivered into intradermal space by this technique [143, 144], indicating the potential limitation for the use of iontophoresis technique. In the current study, we determined the effects of acute flavanol intake on MCh-induced cutaneous vasodilation in microvasculature by combining intradermal microdialysis. Thus, there is the possibility that different size and location of arteries, and measurements may lead to different blood flow responses in this study.

We have previously found that acute flavanol intake improves cutaneous microvascular function in response to thermally induced hyperemia in young black Americans. However, the acute consumption of FC beverage did not alter the dose-response curve of MCh-induced cutaneous vasodilation in both races in the current study. The reason for this remain unclear; however, there is the possibility that other vasoactive substances might be activated and confounded the results on MCh-induced vasodilation. Although NO released from endothelial cells acts as a potent vasodilator and is activated in response to a variety of stimuli including acetylcholine (Ach), MCh, also results in the release of other vasoactive substances including prostaglandins and endothelium-derived hyperpolarizing factors (EDHFs) [53, 145]. Thus, there is the possibility that upregulation of EDHFs and/or prostaglandins may compensate for decreased NO-dependent vasodilation and lead to a maintained endothelial function in young black Americans in the current study. Additional studies are needed to explore the effects of EDHFs and/or prostaglandins in response to MCh-induced hyperemia in young black Americans.

Another possibility is that MCh-induced vasodilation in microvasculature may be mediated by different mechanisms, which is distinct from the release of NO. Cyclic guanosine monophosphate (cGMP) is synthesized from guanosine triphosphate (GTP) by soluble guanylate cyclase (sGC), and NO binds to sGC so vasodilation; thus, cGMP has a major role in the regulation of vascular smooth muscle tone [146, 147]. It has shown that NO is not involved in the NO-cGMP pathway on ACh-mediated vasodilation [148, 149]. In addition, studies with endothelial NO synthase (eNOS) knockout mice have shown that ACh-induced vasodilation was only slightly attenuated in isolated first-order gracilis muscle arterioles [150, 151]. Notably, a recent study has demonstrated that the inhibition of the activation of sGC on responses to NO does not alter ACh-induced vasodilation in the pulmonary and systemic vascular beds of the rat [152]. These findings suggest that NO has no significant role in ACh-induced vasodilation. We intradermally administrated MCh instead of ACh to eliminate the confounding effects of acetlycholinesterase [153]. Thus, mechanistic studies are required to confirm this possibility.

In the current study, we observed that acute consumption of FC did not alter the dose-response curve on MCh-induced cutaneous vasodilation in both races. In line with the possibility that mentioned above, it is likely that there would be no effects of MCh-induced vasodilation in microvasculature even if acute consumption of FC would improve NO-dependent vasodilation. Taken together, our results suggest that NO does not play a significant role in MCh-induced vasodilation so that acute flavanol intake does not show any changes in NO-dependent vasodilatory response to MCh-induced hyperemia in young black Americans.

Limitations

A limitation of the current study is that we did not include blood analysis such as flavanol metabolites, and nitrate/nitrite to quantify these metabolites in the circulation. However, we allowed a 2-hour window in the present study because studies of flavanol ingestion have previously demonstrated that plasma concentration of flavanols peak after 2 hours from the ingestion [92, 119-122]. Furthermore, we believe that the flavanol content in FC beverage (792 mg) used in the present study was high enough to elicit the benefits of acute flavanol intake in microvascular function when compare to previous studies [26, 90]. Another limitation of the current study is that we measured MCh-induce vasodilation by infusing lactated Ringer's solution with single intradermal microdialysis membrane. This may limits the conclusion that can be drawn from our findings. For future studies, using multiple microdialysis sites for NOS inhibitor and/or sGC inhibitor will provide a more reliable and mechanistic approach to clarify the involvement of NO on MCh-induced vasodilation.

Conclusion

In conclusion, the results of the current study show that the EC_{50} of dose-response curve on MCh-induced vasodilation is not different between young black American and white Americans. Furthermore, our finding indicates that the acute consumption of FC does not alter the dose-response curve on MCh-induced cutaneous vasodilation in both races. Additional mechanistic studies are required to confirm our findings.

6.6 TABLES AND FIGURES

Table 6.1 Product nutrition facts

Per serving	Flavanol	Non-flavanol
Calories	224	229
Fat, g (calculated)	3	0
Sat fat, g	1.5	0
Trans fat, g	0	0
Cholesterol, mg	16.5	18
Sodium, mg	546	555
Carbohydrates, g	37.5	39
Dietary fiber, g	7.5	7.5
Sugar, g	25.5	30
Protein, g	21	21
Vitamin A, IU	15	12
Vitamin C, mg	3	4.5
Calcium, mg	643.5	729
Iron, mg	1.5	0
Magnesium, mg	169.5	61.5
Potassium, mg	1170	1014
Flavanols 1-10, mg	**370.8**	0.0
Monomers	**97.2**	0.0
Catechin, mg	**26.7**	0.0
Epicatechin, mg	**70.5**	0.0
2-10 mers	**273.6**	0.0
Total flavanols, mg*	**729.0**	0.0

* Total flavanols include all flavanols that are greater than 10 polymers.

Table 6.2 Subject characteristics

N, no. of subjects; M, male; F, female; BMI, body mass index. Values are means ± SD.

	White American (N=7)	Black American (N=7)	*P*-value
Age (years)	22 ± 4	22 ± 4	0.89
Sex (M/F)	4/3	4/3	1.00
Height (cm)	171 ± 11	169 ± 10	0.82
Weight (kg)	70 ± 14	69 ± 13	0.92
BMI (kg/m^2)	24 ± 2	24 ± 2	0.87

Table 6.3 Baseline hemodynamic variables

NF, beverage without flavanols, FC, flavanol-rich cocoa beverage; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure. All data were collected in the last minute prior to MCh infusion. Baseline hemodynamics were not different between NF and FC in both groups (all $P > 0.05$). Values are means ± SD.

	White American		Black American	
	NF	FC	NF	FC
HR (beats/min)	63 ± 11	59 ± 8	69 ± 11	65 ± 7
SBP (mmHg)	114 ± 9	114 ± 6	119 ±10	120 ± 5
DBP (mmHg)	66 ± 7	67 ± 7	70 ± 10	71 ± 7
MAP (mmHg)	82 ± 7	82 ± 4	87 ± 9	87 ± 5

Table 6.4 Baseline cutaneous vascular conductance as a percentage of maximal vasodilation in response to local heating (%CVCmax) at ~10 min and ~2 hour after each test beverage given in both groups

NF, beverage without flavanols; FC, flavanol-rich cocoa beverage. At 32 °C, the baseline %CVCmax either at the beginning of dietary intervention or 2 hours after beverage given was the same between NF and FC in both groups (all $P > 0.05$). Values are means ± SE.

		Post ~10 min	Post ~120 min
White American	NF	12 ± 2	10 ± 2
	FC	11 ±2	11 ± 2
Black American	NF	7 ± 2	8 ± 2
	FC	7 ± 2	8 ± 2

Figure 6.1 A summary of absolute maximal cutaneous vascular conductance between the highest dose (1M) of MCh on 42 °C heating and 28 mM SNP infusion on 43 °C heating in both group

NF, beverage without flavanols; FC, flavanol-rich cocoa beverage. No racial difference in absolute maximal CVC was observed either at maximal dose of Mch between NF and FC beverages or at 43°C heating + 28 mM SNP infusion between NF and FC beverages. Values are means ± SE.

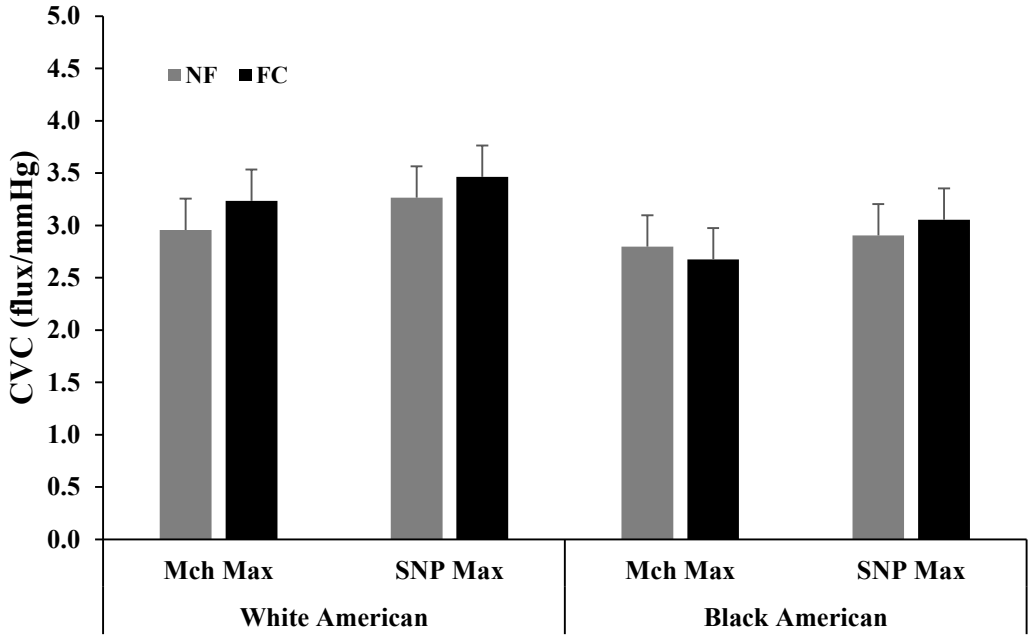
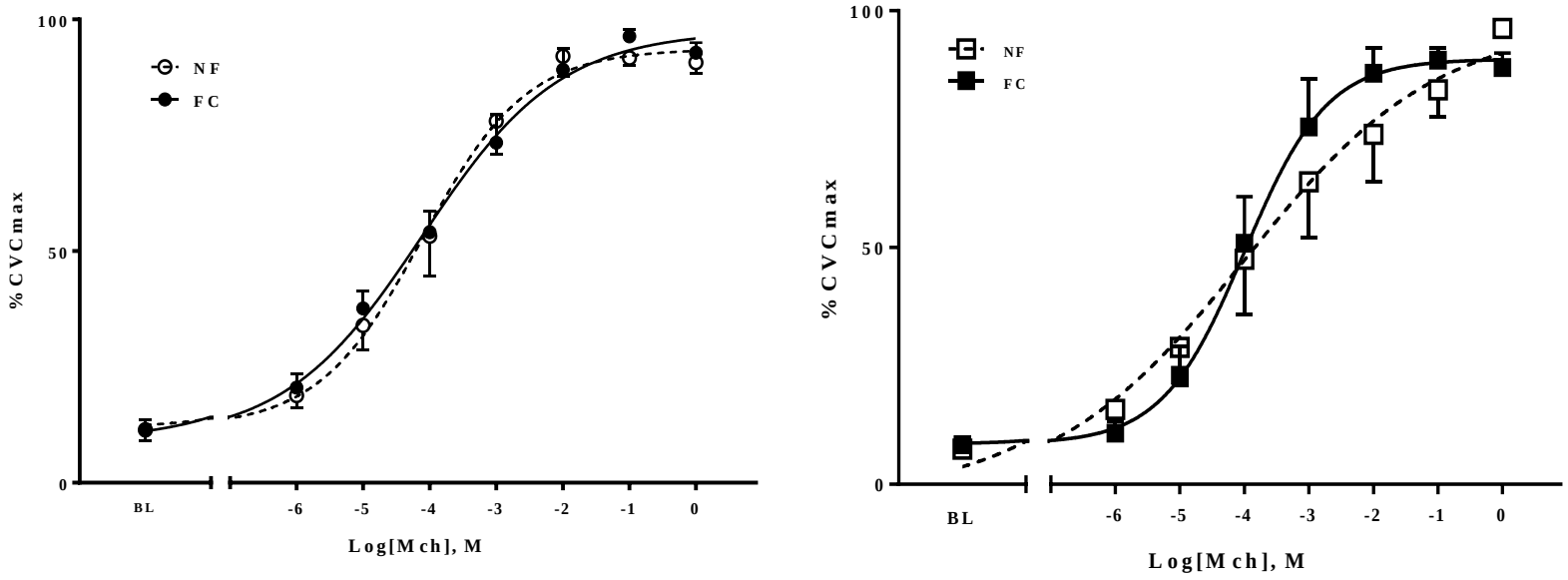


Figure 6.2 A summary of cutaneous vasodilation in response to incremental doses of methacholine (MCh) in both groups

Left panel, whit American group; right panel, black American group. NF, beverage without flavanols; FC, flavanol-rich cocoa beverage. Log EC_{50}, Hillslope, top, and bottom were not different between NF and FC beverages in both groups (all $P > 0.05$). Values are means ± SE.



Chapter 7: Review of Literature

This literature review summarizes the relevant research that shows microvascular function is impaired in healthy, young black Americans relative to their white counterparts. The topics in this review of literature include: 1) cardiovascular disease (CVD) in black Americans; 2) microvascular dysfunction as it relates to CVD; 3) the evidence of microvascular dysfunction in black Americans; 4) potential mechanisms of attenuated microvascular function in black Americans; 5) the benefits of flavanols on cardiovascular health; 6) potential mechanisms of improved microvascular function with flavanols; and 7) the use of local heating protocol with microdialysis as a means to assess microvascular function.

7.1 Overview of Cardiovascular Disease in Black Americans

Black Americans are the largest minority group in the United States. An analysis of the U.S. Census in 2010 show that black American population constitutes 13% of the total population [154]. In the United States, overall mortality rates rapidly declined during this century; however, the mortality rates in black American population are 50% higher than white counterparts [155]. In addition, the life expectancy at birth for black American men and women is 6.9 and 5.2 years lower than white Americans, respectively [156]. CVD is a class of disease, which includes hypertension, heart disease, stroke, and peripheral artery disease. Although overall mortality from CVD has continued to decrease over recent decades, CVD is still the leading cause of deaths in the United States. It is currently estimated that 83 million adults suffer from CVD. In 2008, the estimated annual direct and indirect costs associated with the treatment of CVD was already about 300 billion dollars [1].

Research has shown that black Americans have, traditionally, experienced higher rates of mortality and morbidity from CVD than white Americans for every decennial year back to 1900 [3, 4, 78]. Black American men and women (44% and 49%, respectively) have higher prevalence of CVD than white American men and women (37% and 32%, respectively) [79]. Furthermore, CVD prevalence is higher in young black Americans than age-matched white Americans (35-44 years) and this racial disparity between young black Americans and white Americans may not be explained by clinical and socioeconomic factors [24]. Specifically, black American adults have increased CVD risk factors including myocardial infarction [123, 124], stroke [125], hypertension and type 2 diabetes [3, 126] when compare to white American adults.

Given the higher mortality and prevalence of CVD in black Americans relative to white Americans, black Americans are at greater burden than their white counterpart in the

United States even though overall mortality rates and prevalence of CVD have improved during recent decades. Therefore, CVD is one of the crucial determinants of racial differences in mortality between black Americans and white Americans, and it is imperative to investigate underlying mechanisms of increased mortality rates of CVD and its risk factors in young black Americans.

7.2 Pathophysiology of Microvascular Endothelial Dysfunction

CVD includes a variety of diseases in the heart and vasculature [1]. It is well established that atherosclerosis is a common pathological stage for the development of CVD, which is initiated by the impairment of the endothelium in vasculature [157-159]. The microvasculature consists of small arterioles, capillaries, and venules, and its main functions include the delivery of oxygen and nutrients to the cell, elimination of carbon dioxide and other metabolic byproducts from the cell, controlling blood pressure, and regulation of body temperature. A large proportion of the endothelium lies on the internal surface of microvessels and produces vasoactive molecules to keep vascular tone and homeostasis [77, 160].

The endothelium is a single intimal layer of arterial walls, and it was initially considered a physical barrier to protect and separate blood and its contents from underlying tissue in the past. However, the endothelium is now believed to serve as not only a physical barrier but also the place where endothelial cells maintain vascular homeostasis by the interplay with various vasoactive molecules [161]. The major functions of the endothelium are to regulate vascular homeostasis and maintain the balance between: 1) vasodilation vs. vasoconstriction; 2) inhibition vs. stimulation of smooth muscle cell proliferation & leukocyte migration; and 3) thrombogenesis vs. fibrinolysis. Therefore, endothelial dysfunction can interrupt one or all of these functions, resulting in the development of CVD [129].

NO is a potent vasodilator formed by the reaction of NO synthase (NOS), which divides the substrate L-arginine into NO and L-citrulline. Three constitutive isoforms of NOS can produce NO, including endothelial NOS (eNOS), neuronal NOS, and inducible NOS [129, 162]. Several cofactors are also related to NO production, including tetrahydrobiopterin (BH_4), nicotinamide adenine dinucleotide phosphate (NADPH)

oxidase, heme, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) [163, 164].

In the endothelium, once NO is produced and released from the endothelial cells, it diffuses to the vascular smooth muscle cells using its highly lipophilic and diffusible properties. At the smooth muscle cells NO binds to and thus activates guanylate cyclase, which triggers a cascade of intracellular events ultimately resulting in vasodilation. Signaling molecules, such as adenosine, bradykinin, endothelial growth factor, and serotonin, also participate in cell signaling which contributes to endothelium-dependent vasodilation [165, 166]. In addition, NO-independent vasodilatory substances exist and responsible for vascular homeostasis. Kellogg et al. (2005) administered ketorolac, an inhibitor of prostaglandin production, directly into the interstitial space within local areas of the skin by using intradermal microdialysis. They found that prostaglandins are a potent vasodilator and released from endothelial cells [145]. Several recent investigations also suggest that endothelium-derived hyperpolarization factors probably affect NO production via an independent pathway [167, 168].

The endothelium releases not only vasodilator substances such as NO and prostaglandins, but also secretes vasoconstrictor substances. For example, Kinlay et al. (2001) reported that endothelin-1 accounts for 39% of the coronary tone in normal arteries and acts as a vasoconstrictor in atherosclerotic coronary arteries [169]. Vasoconstrictor prostanoids generated at the endothelium and the conversion of angiotensin I to angiotensin II at the surface of the endothelium also modulate vascular tone, acting as vasoconstrictors [170].

Therefore, vascular tone is ultimately the function of a coordinated balance between endothelium-derived vasoconstrictors and vasodilators, and it is critical to prevent endothelial dysfunction and to keep overall vascular health. In a normal condition, the

endothelial cells regulate vascular homeostasis by balancing between vasoconstriction and vasodilation and thereby protecting the initiation of the atherosclerotic process. However, acute and/or chronic disruptions in the balance between vasoconstriction and vasodilation can lead to endothelial dysfunction.

7.3 MICROVASCULAR DYSFUNCTION IN BLACK AMERICANS

As stated above, black Americans have higher rates of mortality and morbidity from CVD including stroke, coronary artery disease, hypertension, and metabolic syndrome compared to white Americans [3, 4]. The exact mechanisms which increases the risk of cardiovascular diseases in black Americans are complicated and multifactorial; however, recent studies show that endothelial dysfunction, related to decreased NO bioavailability in this population, is a major contributor for the increased risk of cardiovascular diseases [23, 27].

Post-occlusive reactive or thermal hyperemia on the blood vessels has been widely used as a measure of endothelial function because an increased shear stress on the endothelial cells elicit an increase in NO production and vasodilation [165, 171]. For this reason, many investigators have used venous occlusion plethysmography to test endothelial function in macro- and micro-vasculatures. Previous studies have reported that the vascular dilatory responses to hyperemia in resistance vessels are attenuated in young black Americans relative to young white Americans. For example, Bassett et al. investigated vasodilatory capacity in normotensive young black and white Americans. They found that vasodilatory capacity of the forearm resistance vessels was lower in young black Americans men when compared to young white American men [28].

In addition, forearm vasodilatory response to isoproterenol (β-adrenergic agonist) was blunted in normotensive young black American men relative to white American men [172]. NO mediated forearm blood flow responses to the administration of methacholine and sodium nitroprusside (SNP) were attenuated in healthy young black American men compared with white American men [30]. Similarly, Campia et al. noted that black Americans have blunted endothelium-dependent and -independent vasodilation of the brachial artery indexed by flow-mediated dilation and nitroglycerin-mediated dilation

relative to white Americans [127]. Therefore, these results might indicate early onset of endothelial dysfunction in healthy young black Americans prior to any overt signs of risk compared with young white Americans.

Although most studies on endothelial function in human vessels have focused on macrovessels by using plethysmography, there is an increased interest in how endothelial function contributes to alterations in the microcirculation. Researchers recently have recognized that impairment of microvascular function is involved in cardiovascular and metabolic diseases. Racial differences of macrovascular function have been investigated; however, the role of racial differences in microvascular function are not fully understood. Therefore, this study is important to elucidate the role of microcirculation in black Americans.

7.4 Potential Mechanisms of Microvascular Dysfunction

Structurally, the active eNOS enzyme is a dimer. On the C-terminal, a reductase domain binds NADPH, FMN, and FAD. On the other side, the N-terminal oxygenase domain contains a heme group and the binding sites for BH_4, oxygen, and L-arginine. The eNOS dimer is normally inactivated and associated with caveolae which are a type of lipid raft in the endothelial cell membrane. Once intracellular Ca^{2+} concentration increases with stimulation such as shear stress, calmodulin is activated and then released from caveolin-1 and, in turn, binds to eNOS and results in the production of NO [173, 174]. When the tight regulation of eNOS is disturbed, the eNOS dimer is uncoupled and the oxygenase domain begin to generate superoxide anion (O_2^-) instead of NO thus decreased NO bioavailability. The eNOS uncoupling may arise from: 1) upregulation of NADPH oxidase and/or xanthine oxidase (XO); 2) BH_4 deficiency; 3) A limited availability of L-arginine; and 4) upregulation of asymmetric dimethylarginine (ADMA).

Upregulation of NADPH oxidase and XO. NADPH oxidase is an important source of reactive oxygen species (ROS) [175]. NADPH oxidase on the endothelial cell membrane transfers electrons from NADP to molecular oxygen and produce O_2^-. XO is another important source of ROS in the vasculature. This enzyme donates electrons to produce O_2^- and hydrogen peroxide (H_2O_2). It has been found that XO inhibition reduces O_2^- production and improves NO-dependent vasodilation [176]. Taken together, it has been suggested that the upregulation of NADPH oxidase and/or XO are related to eNOS uncoupling.

BH_4 deficiency. BH_4 is the essential cofactor for the generation of NO by eNOS. The lack of BH_4 in the endothelial cells can cause eNOS uncoupling [177]. For example, the infusion of BH_4 into the human arterial circulation improves endothelium-dependent vasodilation in smokers [178], suggesting that a decrease in BH_4 concentration may cause eNOS uncoupling [68, 179]. In addition, one possible mechanism of eNOS uncoupling

related to BH_4 has suggested that the formation of peroxynitrite ($ONOO^-$) due to elevated oxidation may oxidize BH_4 [67]. Excessively produced O_2^- by NADPH oxidase and/or XO can bind with NO and form $ONOO^-$. The oxidization of BH_4 by $ONOO^-$ is converted to BH_3 or BH_2 which are inactive forms of BH_4, leading to eNOS uncoupling [68-70]. Furthermore, it has been assumed that $ONOO^-$ might oxidize the zinc-thiolate cluster of eNOS and destabilize eNOS dimer so eNOS is uncoupled [71]. Therefore, oxidative stress may decrease BH_4 availability and result in increasing eNOS uncoupling.

Limited availability of L-arginine. eNOS produces endothelium-dependent NO from L-arginine. The availability of L-arginine may be one contributor to eNOS uncoupling. Studies have shown that a low concentration of L-arginine can limit the production of NO in both animals and human cells [32, 71]. This data suggests that a decrease in the amount of L-arginine may reduce NO bioavailability due to eNOS uncoupling. L-arginine in human cells not only binds with eNOS, but also with arginase [34, 35, 38-40]. Therefore, the concentration of arginase can also cause eNOS uncoupling due to competition for the share the substrate L-arginine.

Upregulation of ADMA. ADMA found in blood plasma is also closely related to uncoupled eNOS. ADMA, an endogenous competitive antagonist of eNOS, interferes with L-arginine in the production of NO [180, 181]. Vallance et al. (1992) initially assumed the hypothesis that ADMA was related to NOS. They administrated L-NG-monomethyl-L-arginine (L-NMMA) and found that the high concentrations of plasma ADMA was negatively correlated to NOS activity in chronic renal failure patients, suggesting that ADMA is one of the important endogenous NO inhibitor [182]. Data from other studies have supported the idea that ADMA may play a role in the inhibition of eNOS [183, 184]. In addition, increased oxidative stress might promote the expression of ADMA by

inactivating dimethylarginine dimethylaminohydrolase, which also contributes to eNOS uncoupling [185].

Taken together, endothelial function might be related to NO bioavailability, which is closely dependent on eNOS coupling. The eNOS may be regulated via NADPH and XO, BH_4, L-arginine, and arginase. Therefore, endothelial dysfunction due to a decrease in NO bioavailability might occur when any of these factors are disrupted.

7.5 THE BENEFITS OF FLAVANOLS ON CARDIOVASCULAR HEALTH

Overtime, there has been an emerging interest for flavonoids due to their potential beneficial effects on cardiovascular health. Epidemiological studies have explored whether the dietary intake of flavonoids is related to CVD risk; however, the data from many epidemiological studies have shown mixed results [84, 186-190].

The effects of flavonoids on cardiovascular health. Over 4,000 flavonoid have been classified based on their chemical structure. The basic flavonoid skeleton consists of 15 carbon atoms with two aromatic rings (Ring A and Ring B) that are connected by 3-ring carbon backbone (Ring C) [191, 192]. They are usually subdivided into several subgroups according to which functional groups are attached to the 'C-ring' of this 3-ring carbon backbone [131]. The main subgroups of flavonoids include flavanols (cocoa, tea, fruits, and wine), flavanones (juices), flavonols (broccoli, onion, tea, and tomato), anthocyanidins (berries and wine), flavones (herbs), and isoflavones (soybean). It has estimated that average daily total flavonoid intake of U.S. adults is 189.7 mg/day mainly from flavanols (83.5%), flavanones (7.6%), flavonols (6.8%), anthocyanidins (1.6%), flavones (0.8%), and isoflavones (0.6%) [86]. Recently, flavanols, a subgroup of flavonoids, have gained increasing attention due to their reported benefits on cardiovascular health. Flavanols are abundant in cocoa-containing products, green tea, red wine, and soy products [87]. The estimated mean flavanol intake of U.S. adult is in the range of 50-100 mg/day [88]. Flavonoids are digested mainly in the small intestine and the liver. Flavonoids metabolites, which are chemically and structurally distinct from flavonoids, reach the target tissues through the circulation [131, 132] and each subgroup of flavonoids leads to different physiological effects on cardiovascular health [133, 134].

It have been indicated that flavonoids are inversely associated with CVD. For example, Hertog et al. (1993) reported that individuals who consumed higher amount of

flavonoids in their diet had a 68% lower cardiovascular risk when compared with individuals who consumed less flavonoids in their diet [193]. Sesso et al. (1999) also observed a significant reduction of cardiovascular risk in individuals who drank more than one cup of flavonoids containing tea per day than individuals who did not drink flavonoids containing beverage [194]. Interestingly, it has been suggested that flavonoid intake in the form of tea might have benefits for individuals with established CVD. In this prospective cohort study, cardiovascular risk reduced in individuals who consumed moderate and heavy tea beverage (31% and 39% respectively) among 1,900 individuals with acute myocardial infarction [195].

On the contrary, other studies have demonstrated that there is no relationship between flavonoid intake and cardiovascular risk. For example, the results from the Scottish Heart Healthy Study indicates there is no relationship between coffee or tea consumption and coronary heart disease [196]. Similarly, Rimm et al. (1996) found that flavonoid intake did not significantly associate with coronary heart disease [197]. In addition, a 14-year follow-up study demonstrated that tea consumption to which milk was added was not related to ischemic heart disease in a cohort of 1,900 Welsh men [198]. These complicated results from epidemiological studies, regarding the effects of flavonoid on cardiovascular health, might be related to subtle differences in the chemical structures of several subgroups in flavonoids [132]. Therefore, it might be an important implication for dietary recommendations if the positive vascular effects of flavonoids are partly due to a limited number of flavonoid subgroups.

The benefits of flavanol-rich cocoa on cardiovascular health. Flavanols are distinguished from other subgroups of flavonoids by the presence of a hydroxyl group at position 3 of ring C [87]. Flavanols are mainly present in a mixture of monomeric (−) epicatechin and (+) catechin and oligomeric and polymeric proanthocyanidin flavanols

[199]. Furthermore, a small amount of gallocatechin and epigallocatechin have also been indentified [200]. Cocoa, a well-known source of flavanols, has especially received a great deal of attention because of their independent beneficial effects on cardiovascular health. The observation of Kuna Indians of Panama by Hollenberg et al. initiated this attention. They have conducted a series of studies and found that individuals living in the island of San Blas, which is located just off the coast of Panama, have a significantly lower incidence of CVD when compared to individuals who moved to the mainland from San Blas. These investigators have reported the possibility that the difference in incidence of CVD between these Kuna Indians is dependent on the consumption of cocoa containing beverages that are mostly consumed by Kuna Indians living on the island. [201-206]. Numerous epidemiological and prospective follow-up studies have supported this hypothesis, which confirms an inverse correlation between the consumption of flavanol-rich cocoa and cardiovascular risk [16, 18, 207, 208].

The exact underlying mechanisms involved in reducing CVD risk factors with the supplementation of flavanol-rich cocoa are not been fully understood; however, recent evidence suggest that flavanol intake might modulate cardiovascular risk via the improvement of endothelial function, the inhibition of oxidative low-density lipoprotein production, and/or the decrease in blood pressure. A number of meta-analyses has shown that cocoa intake lowers both systolic and diastolic blood pressure which leads to a decrease in cardiovascular risk, presumably due to an improvement of endothelial function with an increased NO bioavailability [89, 209, 210]. In support of this hypothesis, it has been reported that acute and long-term supplementation of flavanols improve endothelial function in healthy individuals [91, 96, 97] and more effectively than in individuals with endothelial dysfunction [90, 92].

Although it is not conclusively explored which specific active compounds of cocoa are responsible for the improvement of CVD risks, studies have suggested that epicatechin and its metabolites are major contributors. [90, 92, 211]. In addition, it has been proven that the acute effects of epicatechin on vasculature are similar between the consumption of chemically isolated pure epicatechin from cocoa and the consumption of epicatechin from flavanol-rich cocoa. [121], indicating that the specific flavanols in cocoa are capable for improving vascular health.

Taken together, it is still controversial if the consumption of flavanol-rich cocoa could provide beneficial effects on CVD and its related risk factors; however, a majority of studies suggest that the specific subgroups of flavanols have a significant role in improving vascular function and prevent CVD. Therefore, additional investigation will provide more knowledge of the relationship between flavanols and cardiovascular health.

7.6 Potential Mechanisms of Improved Vascular Health with Flavanols

The underlying mechanisms by which flavanol intake improves endothelial function in young black Americans has not been fully explored. However, given that endothelial dysfunction due partly to decrease in NO bioavailability is commonly observed in black Americans [23, 66, 101] and that flavanols are exert positive effects on endothelial function in part by an increase in NO bioavailability [121, 212], it is plausible to suggest that the flavanol intake may improve endothelial function in young black Americans, via an improvement in NO mediated vasodilation. Hypothesized mechanistic pathways include an increase in antioxidant capacity, an increase in eNOS expression/activity, a decrease in arginase activity, and/or an increased in L-arginine availability.

Flavanols exert their beneficial actions in part through their antioxidant like properties via the neutralization of ROS and the inhibition of $ONOO^-$ production. The aromatic rings (Ring A and Ring B) delocalize electrons and scavenge ROS. These aromatic rings directly neutralize free radicals including O_2^- and chelate metals such as Fe^{2+} and Cu^+, and thus decrease in ROS [18]. It has been reported that consumption of flavanol-rich cocoa improves serum antioxidant capacity, indicating that flavanols preserve endothelial function from oxidative stress and endogenous ROS [213], It has been demonstrated that reduced NO bioavailability in young black Americans (22 years) is related to the upregulation of O_2^- (65), which leads to a reduction in NO bioavailability as they react with NO to form $ONOO^-$ (68, 69). Similarly, Manson et al. (2005) have shown that NO production is improved by a NADPH oxidase inhibitor in young black Americans (102). Additionally, studies have demonstrated that epicatechins reduce the production of free radicals including O_2- through of NADPH oxidase, which in turn, improves NO bioavailability (103, 104). These findings suggest flavanols may affectively reduce

oxidative stress and endogenous ROS and improve NO bioavailability in young black Americans.

Beyond antioxidant capacity of flavanol-rich cococa, another potential mechanism of improved NO bioavailability associated with flavanol consumption in young black Americans is an increase in eNOS coupling by indirect pathways, including upregulation of eNOS activity, inhibition of arginase activity, and/or improvement of L-arginine availability in the endothelial cells. In vitro studies, endothelial cells incubated with flavonoid-rich red wine upregulated eNOS mRNA and protein expression, producing more bioactive NO (up to 3 times) when compared to control cells with no treatment (105). Plant extracts rich in flavonoids also upregulated eNOS activity in cultured endothelial cells (106, 107) and rat aorta (108). In terms of arginase activity, it has been suggested that flavanol-rich cocoa reduces arginase activity in human endothelial cells, leading to increase in local concentrations of the substrate, L-arginine. A series of studies by Schnorr et al. demonstrated that arginase II mRNA expression in human umbilical vein endothelial cells (HUVECs) is reduced in two hours when they incubated with epicatechines. They also observed that arginase II activity was reduced in rats with high flavanol intake for 28 days and in human erythrocytes with high flavanol intake for 24 hours, respectively, indicating that flavanol-rich cocoa may reduce arginase activity in human endothelial cells and leads to increase in local concentrations of the substrate, L-arginine. [109]. These findings suggest the possibility that flavanols may improve eNOS coupling by indirect pathways, including upregulation of eNOS activity, inhibition of arginase activity, and/or improvement of L-arginine availability in the endothelial cells.

7.7 LOCAL HEATING OF SKIN TO EVALUATE MICROVASCULAR FUNCTION

In the human cutaneous vasculature, both neurogenic reflexes and local control factors regulate skin blood flow (SkBF). The skin is comprised of glabrous and non-glabrous (hairy) portions and non-glabrous skin is predominantly present on most of the body surface [214]. In the non-glabrous skin, a change in skin temperature elicits two sympathetic pathways that are responsible for the control of the cutaneous vasoreflexes: an adrenergic vasoconstriction system and an active vasodilation system. For example, the direct local cooling of the skin reduces SkBF mediated by reflex activation of the sympathetic nervous system and requires norepinephrine release from vasoconstrictor nerve terminals. On the other hand, local heating of skin to 42°C causes maximal cutaneous vasodilation [113, 215] independent of changes in core temperature and activation of thermoregulatory reflexes [216].

Local heating of skin elicits cutaneous blood flow reactivity in two distinct phases that is marked by an initial peak and is followed by a plateau [43, 62, 112]. The local axon reflex mechanism primarily mediated by sensory nerves which induce a rapid initial peak in SkBF the first 10 min which is minimally dependent on NO. This is followed by a plateau in SkBF after about 20~30 min that is approximately 50–75 % dependent on NO [62, 77, 113, 166] and involves several pathways including transient receptor potential vanilloid type-1 (TRPV1) channels [114], adenosine receptors [115], and reactive oxygen species [116], while the remaining 30% of vasodilation on the plateau depends on endothelium-derived hyperpolarizing factors (EDHFs) that stimulate calcium-activated potassium channels on endothelial and smooth muscle cells [110].

It has been suggested that the human cutaneous circulation serves as a surrogate vascular bed for the assessment of global vascular health in vivo. For example, impaired cutaneous microvascular reactivity is observed before any clinical signs of microvascular

dysfunction during the early stages of diseases [42], suggesting the correlation between skin vascular reactivity and a systemic microvascular dysfunction. For this reason, SkBF response to local heating has been widely used as a means to examine microvascular dysfunction because of its ease of accessibility and non-invasive characteristics [44-46].

Additionally, the combination of laser-Doppler flowmetry (LDF) and intradermal microdialysis has been successfully used in skin reactivity tests to measure microvascular function and determine the underlying mechanisms of microvascular dysfunction in both healthy and diseased populations. Vasoactive substances delivered via intradermal microdialysis allow the investigation of how cutaneous vascular reactivity is affected in those with a higher risk of CVD that is related to endothelial dysfunction. For example, endothelial dysfunction is closely associated with NO bioavailability, which has been shown via the combination of LDF and intradermal microdialysis in populations with elevated CVD risk factors including smoking [47], hypercholesterolemia, and essential hypertension [49]. Therefore, the combination of a typical local heating protocol and microdialysis might provide better knowledge to understand the underlying mechanisms of microvascular dysfunction in black Americans.

Chapter 8: Conclusion

8.1 SUMMARY

The overall goal of this dissertation was to investigate potential mechanisms of microvascular dysfunction in young black Americans and to test the hypothesis that microvascular function might be improved by dietary flavanol supplementation in young black Americans. In a series of studies within this dissertation we combined laser-Doppler flowmetry and microdialysis technique to measure microvascular function and determine the underlying mechanisms of microvascular dysfunction.

In study 1, we explored the potential mechanisms of impaired cutaneous microvascular function in young black Americans. We hypothesized that microvascular function in response to local heating of skin would be attenuated in young black Americans relative to age-matched white Americans due largely to the lack of nitric oxide (NO) bioavailability. We also hypothesized that such attenuated cutaneous microvascular function in young black Americans would be improved with an intradermal infusion of L-arginine and arginase inhibitors, respectively. Our results suggest that limited NO bioavailability due partly to a relative deficit of L-arginine in the endothelium is one of the mechanisms by which microvascular dysfunction occurs in young black Americans when compared to age-matched white Americans. This conclusion is in accordance with previous findings that a low concentration of L-arginine could limit the production of NO in both animals [32] and human cells [33] , indicating that a decrease in the amount of L-arginine may reduce NO bioavailability. Furthermore, our results suggest that increased competition from arginase is not a likely mechanism of microvascular dysfunction in young black Americans as we did not observe any differences in %CVCmax, an index of microvascular function, between the lactated Ringer's infused and arginase-inhibited microdialyis sites in both racial groups. This conclusion is consistent with those of Holowatz et al. (2006) who

previously reported that intradermal administration of arginase inhibitors did not alter cutaneous vasodilation in young subjects [34], indicating that upregulation of arginase activity is not a limiting factor of NO bioavailability in young individuals. Taken together, considering attenuated microvascular function due partly to limited NO bioavailability in young black Americans and the observed restoration of cutaneous microvascular function with the administration of L-arginine, a relative deficit of L-arginine in the endothelium is one of the mechanisms by which microvascular dysfunction occurs in young black Americans.

Study 2 was designed to explore the effects of acute flavanol intake on thermally induced vasodilation in young black Americans. We hypothesized that acute flavanol intake would improve microvascular function in response to local heating of skin in young black Americans relative to young white Americans. We further hypothesized that such improvement with flavanol intake would be related to an increase in NO bioavailability. Our results suggest that acute consumption of flavanol-rich cocoa improved NO-dependent microvascular endothelial function in young black Americans relative to age-matched white Americans. The underlying mechanisms by which flavanols improve NO bioavailability in young black Americans are needed further mechanistic investigation to confirm our results; however, decreased oxidative stress and/or increased endothelial NO synthase (eNOS) coupling associated with flavanol intake have been proposed as major contributors [103, 104]. Taken together, our results suggest that acute flavanol intake may improve NO bioavailability and microvascsular function in young black Americans through reducing oxidative stress level and/or increasing eNOS coupling.

Study 3 was designed to explore the effects of acute flavanol intake in methacholine (MCh)-induced vasodilation in young black Americans. We hypothesized that young black Americans would require a higher dose of Mch to achieve 50% of maximal dilation (EC_{50})

when compare to age-match white Americans. We further hypothesized that acute flavanol intake would produce a leftward shift in the dose-response curve of MCh-induced cutaneous vasodilation in young black Americans, while there would be no effects of acute flavanol intake in young white Americans. Contrary to our hypotheses, we found that the EC_{50} of dose-response curve on MCh-induced cutaneous vasodilation was similar between racial groups. Furthermore, the acute consumption of flavanol-rich cocoa did not alter the dose-response curve on MCh-induced cutaneous vasodilation in both races. This study was not mechanistic by design so we are not able to explain the exact mechanisms of our findings. However, it is likely that MCh-induced hyperemia may activate other vasoactive substances including prostaglandins and endothelium-derived hyperpolarizing factors (EDHFs), which compensate for decreased NO-dependent vasodilation [53, 145] and contribute to maintain microvascular endothelial function in young black Americans. It is also plausible that MCh-induce vasodilation in microvasculature may be mediated by different mechanisms, which are distinct from the release of NO (134, 135). In line with this possibility, it is likely that there would be no effects of MCh-induced vasodilation in microvasculature even if acute flavanol intake would improve NO-dependent vasodilation. Taken together, our results suggest that NO does not play a significant role in MCh-induced vasodilation so that acute flavanol intake does not show any changes in NO-dependent vasodilation in young black Americans; however, additional mechanistic studies in larger group are required to confirm our findings.

8.2 PERSPECTIVES

Despite remarkable evidence that endothelial dysfunction is a major contributor to develop cardiovascular disease (CVD) and its risk in black Americans, the exact mechanisms of endothelial dysfunction in microvasculature have not fully elucidated. Our

findings that limited availability of L-arginine in the endothelium is a potential mechanism of attenuated NO-dependent microvascular function in young black Americans, at least in part, provide clinical relevance of the heightened risk of CVD in this population. Similarly, the benefits of flavanol-rich cocoa in vascular health have been explored at length, while there is uncertainty whether acute consumption of flavanol-rich cocoa improve microvascular function in young black Americans. Our findings that acute consumption of flavanol-rich cocoa improved NO-dependent microvascular function is young black Americans provide a novel potential therapeutic target to delay onset of microvascular endothelial dysfunction and prevent the development of CVD in this population. Future mechanistic studies are needed to determine the underlying mechanisms of improved NO-dependent microvascular function with acute flavanol intake in young black Americans.

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