DIETARY NITRATE SUPPLEMENTATION AUGMENTS NITRIC OXIDE SYNTHASE-DEPENDENT AND INDEPENDENT REFLEX CUTANEOUS VASODILATION IN HEALTHY HUMANS

by

ERICA L. LEVITT

B.S., Kansas State University, 2012

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Kinesiology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2013

Approved by:

Major Professor
Brett J. Wong, Ph.D.

Copyright

Abstract

Beetroot juice (BRJ) has been shown to increase NO-dependent dilation through both NOS-dependent and NOS-independent pathways. We hypothesized BRJ supplementation would augment reflex cutaneous active vasodilation. Subjects were equipped with two microdialysis fibers on the forearm and randomly assigned as control (Ringer's) or NOS inhibition (20mM L-NAME). Whole-body heating was achieved via water-perfused suits to raise core temperature (Tc; ingestible telemetric pill) 0.8°C. Maximal cutaneous vasodilation was reached by administering 54mM SNP and local heating to 43°C. Skin blood flow was measured via laser-Doppler flowmetry and mean arterial pressure determined; cutaneous vascular conductance (CVC) was calculated and expressed as $\%CVC_{max}$. Subjects underwent heat stress pre- and post-nitrate supplementation (3 days of BRJ: 5mM, 0.45g nitrates per day). BRJ increased the plateau CVC at control (pre: 57 ± 3 vs. post: 80 ± 5 $\%CVC_{max}$) and L-NAME (pre: 36 ± 3 vs. post: 52 ± 6 $\%CVC_{max}$; $p<0.05$ for all conditions) sites. The %NO contribution increased from pre- to post-BRJ (pre: 44 ± 5 $\%CVC_{max}$ vs. post: 64 ± 6 $\%CVC_{max}$; $p<0.05$). These data suggest that BRJ augments the NOS-dependent and NOS-independent component of reflex cutaneous vasodilation.

Table of Contents

List of Tables

List of Figures

Acknowledgements

I would like to acknowledge the many people who made my research and the writing of this thesis possible.

I am especially grateful to everyone who participated as a subject in my research. Many of you are close friends, and I appreciate your willingness to pull yourself away from your own studies to spend time in the lab with me.

I would like to thank Jeremy Keen and Jena Eder for all their help with data collection, and for their investment of time on my behalf both in and outside the lab. Jeremy's continual patience and support have been an enormous source of encouragement for me throughout this process.

I would also like to acknowledge my committee members, Dr. David Poole and Dr. Tim Musch. I would like to thank them for their time spent with me for this project and their passion for research. I could not have had more inspiring role models. Thank you for all the challenges you both presented me throughout my undergraduate and graduate studies. These are the moments from my education here I will remember the most, and have used to develop myself as a stronger person.

Finally, it has been a great privilege to work with my advisor, Dr. Brett Wong. Words cannot fully express my appreciation for his guidance and patience as I conducted my research over the past several months. Dr. Wong has been tremendously supportive and more than generous with his time and advice. I have benefited from our many open discussions, and while setting the highest of standards he has provided the encouragement I have needed to strive to meet them.

Dedication

To my mother, father, and my sister: Your support and guidance is more than anyone could ever ask for. No words can do justice the gratitude I have for you and all the opportunities I have been blessed with in my lifetime. Thank you for always encouraging me to chase after my dreams, and for your involvement in this dream come true.

Chapter 1 - Introduction

In humans, the onset of heat stress initiates a rise in core temperature and is associated with an increase in cutaneous vasodilation and sweating (Fox et al. 1958). It is well known that the cutaneous circulation is regulated by two branches of the sympathetic nervous system (Kellogg et al. 1995; Kellogg et al. 1998; Rowell et al. 1974). These include the adrenergic vasoconstrictor nerves and the cholinergic active vasodilator nerves (Kellogg et al. 2006). With an increase in core body temperature, the sympathetic cholinergic vasodilator nerves reflexively mediate increases in skin blood flow and can direct 60% of cardiac output, or ~8 l/min of blood flow to the skin (Edholm et al. 1957; Kellogg et al. 1995; Kellogg et al. 2006; Rowell et al. 1974).

The mechanisms for control of the cutaneous active vasodilator system in human skin remain unresolved. The current, accepted mechanism to explain cutaneous active vasodilation in response to body heating is the co-transmission theory (Kellogg et al. 1995). This theory suggests the release of acetylcholine and an unknown substance(s) are responsible for the sweat response and cutaneous vasodilation, respectively. Kellogg et al. provided evidence for acetylcholine as the primary mechanism for the sweat response to passive heat stress, inasmuch as a muscarinic receptor antagonist eliminated the sweat response, which resulted in minimal attenuation of the cutaneous vasodilator response (Kellogg et al. 1995). However, it remains to be elucidated as to if any one vasodilator is involved, or rather if it is a combination of several potential vasodilators that contribute to the responses observed in reflex vasodilation. A multitude of potential vasodilators and vasodilator pathways have been proposed as possible substances to support the co-transmission theory. These vasodilators include: neurokin-1

receptors (Wong et al. 2005), vasoactive intestinal peptide (VIP) (Bennett et al. 2003), VIP receptors (VPAC; Kellogg et al. 2010), histamine 1 (H1) receptors (Wong et al. 2004), transient receptor potential vanilloid type-1 (TRPV-1) channels (Wong & Fieger, 2012), prostaglandins (McCord et al. 2006), and nitric oxide (NO) (Kellogg et al. 1998; Wilkins et al. 2003). In addition to these co-transmitters for active vasodilation, Figure 1 demonstrates other potential mechanisms also contribute to the skin blood flow response in healthy humans during reflex cutaneous vasodilation. Pathways and production of nitric oxide in heat stress conditions will be further examined in the present investigation.

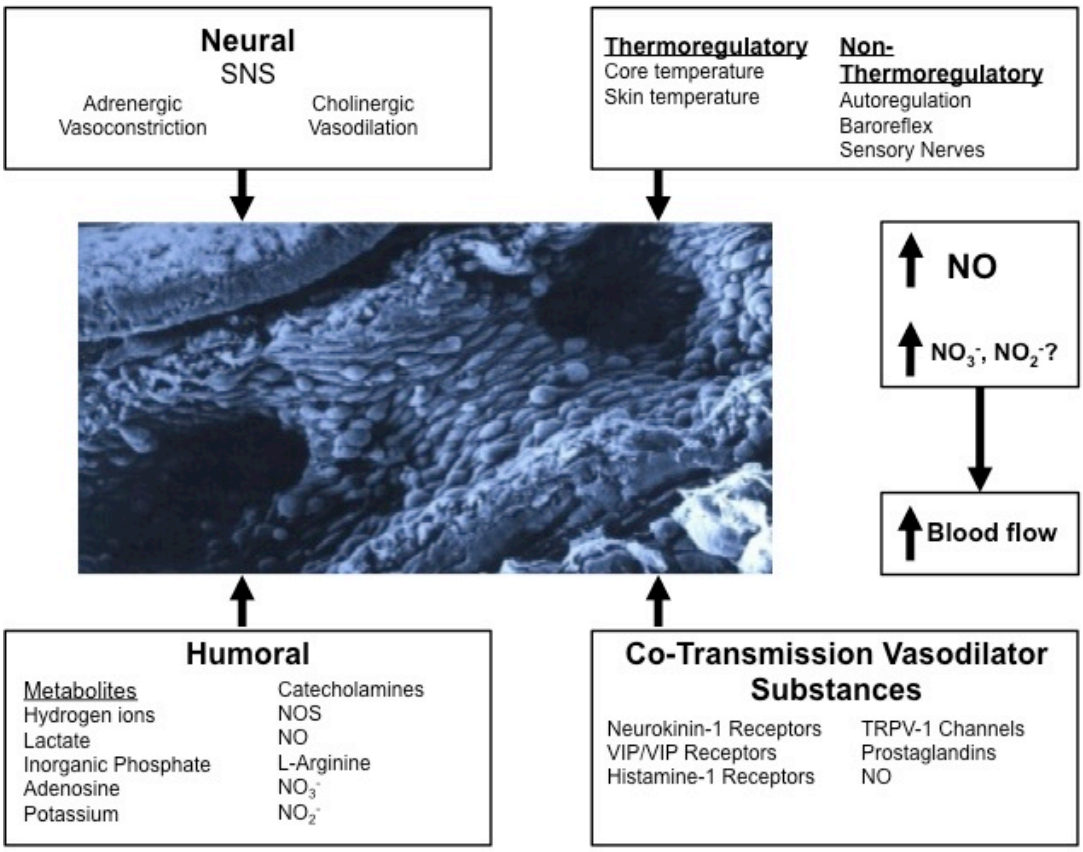

Figure 1. Potential mechanisms for control of skin blood flow during reflex cutaneous vasodilation. This figure demonstrates a collection of factors influencing cutaneous blood flow with specific attention to NO through the NO-dependent pathway (i.e., L-arginine) and the NO-independent pathway (i.e., nitrate reduction to nitrite, and subsequently to NO).

Nitric oxide has been shown to contribute ~35-45% to cutaneous active vasodilation (Kellogg et al. 1998; Shastry et al. 1998). The NO component to cutaneous active vasodilation was demonstrated through blockade of nitric oxide synthase (NOS), resulting in an attenuated, but not abolished skin blood flow response to whole body heating (Kellogg et al. 1998), suggesting full expression of NO was required to achieve reflex cutaneous vasodilation. However, the precise mechanism underlying the release of NO during passive heat stress was unknown (Shastry et al. 1998). Wilkins et al. (2003) subsequently demonstrated that NO has a direct vasodilator role to reflex cutaneous vasodilation and may also work synergistically with the unknown transmitter(s) (Wilkins et al. 2003). Recent evidence from Kellogg et al. (2008) determined the specific NOS isoform responsible for active vasodilation to be neuronal nitric oxide synthase (nNOS), activated by centrally mediated reflex responses to passive heat stress.

The classical pathway used for NO generation through the conversion of L-arginine (with cofactors Ca^{2+} and BH_4) to L-citrulline and NO has been shown to be NOS-dependent, and thus requires oxygen for the conversion to NO (Moncada et al. 1993). Conversely, the nitrate-nitrite-NO pathway has been shown to be a NOS-independent pathway for NO generation, when the NOS-dependent pathways and its corresponding enzymes are compromised, such as in hypoxic conditions (i.e. low PO_2) or a state of acidosis (Benjamin et al. 1994, Lundberg et al. 1994, Lundberg et al. 2008). Nitrate and nitrite derive from two primary sources: endogenously via the L-arginine classical pathway for NO production or through exogenous sources in the diet such as vegetable consumption (i.e. leafy greens such as spinach and beetroot) (Lundberg et al. 2004; Lundberg et al. 2008). The conversion from nitrate to nitrite, and subsequently to NO requires the enterosalivary circulation and the upper gastrointestinal tract (Govoni et al. 2004; Lundberg et al. 2004). Nitrate is rapidly reduced to nitrite by anaerobic facultative commensal bacteria and

other reductase enzymes in the mouth and saliva (Govoni et al 2008; Lundberg et al. 2004). Nitrate can further be reduced to nitrite in the acidic state of the stomach and small intestine, where nitrite can be actively taken up by the salivary circulation, enter the systemic circulation, or subsequently be converted to NO (Lundberg et al. 2004).

Recent literature provides evidence of cardiovascular health benefits elicited from consumption of nitrate sources in both healthy and disease populations. Consumption of dietary nitrates has been shown to increase exercising skeletal muscle blood flow (Ferguson et al. 2012), reduce the oxygen cost of exercise (Bailey et al. 2009; Larsen et al. 2007), improve exercise tolerance in peripheral artery disease patients (Kenjale et al. 2011), and to lower blood pressure (Webb et al. 2008; Larsen et al. 2006). It has been suggested that many of these cardiovascular benefits are due to an increase in bioavailable NO originating from the reduction of nitrate to nitrite and subsequently to NO.

There are multiple factors and mechanisms responsible for control of skin blood flow in reflex cutaneous vasodilation in healthy humans. As demonstrated in Figure 1, there are additional neural and humoral stimuli, including the previously stated co-transmitters and potential vasodilator(s) that regulate cutaneous blood flow. NO is required for full expression of reflex cutaneous vasodilation, thus the focus of the present investigation was to identify a role for NO production through both the NOS-dependent and NOS-independent pathways via dietary nitrates under passive heat stress conditions.

Reflex cutaneous vasodilation has been shown to be attenuated in healthy aged individuals as well as in disease populations, such as hypertension and hypercholesterolemia (Holowatz et al. 2003; Holowatz et al. 2007). The reduced thermoregulatory response in these individuals has been attributed, in part, to a decrease in bioavailable NO. Inasmuch as dietary

nitrates have previously been shown to yield various cardiovascular health benefits and increase bioavailable NO, it is possible that dietary nitrate supplementation may improve cutaneous blood flow and thermoregulation in healthy populations. The effect of dietary nitrate supplementation on cutaneous blood flow in response to passive heat stress is currently unknown. Therefore, the purpose of the present investigation was to determine the effects of dietary nitrate supplementation via beetroot juice on the skin blood flow response to whole body heating. We hypothesized that three days of nitrate supplementation via beetroot juice would augment the NO component of cutaneous reflex vasodilation in healthy humans.

Chapter 2 - Methods

Subjects and Ethical Approval

Seven subjects (7 men; age 22 ± 1 yr; height 179 ± 8 cm; body mass 80 ± 14 kg; body mass index 25 ± 4 kg/m^2) volunteered to participate in this study. The Institutional Review Board at Kansas State University approved all protocols of the study, and written and verbal informed consent was obtained from each subject prior to participation. All studies conformed to guidelines as set forth by the Declaration of Helsinki. All subjects participated in a standard health history screening to verify the absence of cardiovascular disease, non-obese, and nonsmoking status. All tests were performed with the subjects in the supine position and the experimental arm at heart level.

Dietary Nitrate Supplementation and Intervention

All subjects reported to the laboratory for pre-and post-supplement experimental sessions (see below). Following the pre-supplement experimental session, all subjects were instructed to consume 70 ml/day of 0.45 g concentrated nitrate-enriched beetroot juice shot (Beet It, James White Drinks, Ipswich, UK) as one 70 ml shot per day for three days (Bailey et al. 2009; Larsen et al. 2007). The third 70 ml shot was consumed in the laboratory approximately two hours prior to the start of the post-supplement whole body heating protocol. Consumption of nitrates approximately 1.5-2 hours prior to the start of the protocol has previously been demonstrated to elicit peak circulating plasma nitrite levels (Webb et al. 2008; Vanhatalo et al. 2010). All subjects were asked to refrain from exercise, caffeine, and alcohol 12 hours prior to pre-testing and during the three days of beetroot juice supplementation. The subjects were also asked to refrain from eating foods high in nitrate content, such as leafy greens (e.g., spinach, > 300 g/day), and to refrain from using antibacterial mouthwash products before testing and during the

supplementation period (Govoni et al. 2008). A venous blood sample was taken prior to each experimental protocol to determine plasma nitrate and nitrite concentration for both pre- and post-supplement whole body heating trials. Blood samples were centrifuged and stored in a nitrite-free EDTA vacutainer and stored at -80°C. Subjects were instructed to fast for 9-12 hours prior to blood sampling.

Instrumentation

During both pre- and post-supplement testing, the subjects were equipped with two microdialysis fibers on the ventral side of the left forearm. Ice was used to numb the skin before fiber placement (Hodges et al. 2009). Microdialysis fibers were placed by using a 23-gauge needle inserted into the intradermal layer of the skin. The fibers were then threaded through the lumen of the needle; the needle was removed from the skin leaving the semi-permeable membrane of the fiber in place under the skin. Microdialysis fibers were used to perfuse drugs to local areas of the skin on the forearm. The membranes of the microdialysis fibers were 10 mm in length with a 55-kDa molecular mass cutoff (CMA 31 Linear Probe; CMA Microdialysis; Kista, Sweden). Placement of the microdialysis fibers results in a mild trauma response that was allowed to subside (~45-90 minutes) before starting the whole body heating protocol.

Laser Doppler flowmetry was used as an index of skin blood flow by measuring red blood cell flux. Local heating units were placed directly over the center of each microdialysis site (PF5020 local heating units and PeriFlux 5020 Temperature Unit; Perimed; Jarfalla, Sweden). An integrated laser-Doppler probe (Probe 413; Perimed; Jarfalla, Sweden) with seven emitting and receiving probes were placed in the center of each local heating unit, to measure red blood cell flux directly over each microdialysis site.

Subjects wore a water-perfused suit to control whole body temperature (Allen Vanguard, Ottawa, Ontario, Canada) that covered the entire body except for the hands, feet, head, face, and the experimental forearm. The water-perfused suit was covered with a water impermeable rain suit to limit evaporative heat loss during whole body heating.

Subject Monitoring

Core body temperature was measured via telemetric ingestible pill (CorTemp Data Recorder, CorTemp Temperature Sensor, Wireless Sensing Systems and Design, Palmetto, FL, USA). Subjects were instructed to ingest the pill approximately 2-3 hours prior to arriving at the laboratory the day of each experimental test. Temperatures were recorded approximately every minute during baseline, the period of whole body heating, and during the cooling period. Whole body heating persisted until subjects' core temperature was raised 0.8-1.0°C.

Subjects' heart rates were monitored with an electrocardiogram throughout the duration of the protocol. Blood pressure was measured beat-by-beat via photoplethysmography (NexfinHD; BMEYE, Amsterdam, The Netherlands) with a cuff placed on a finger on the right, non-experimental arm. Beat-by-beat blood pressure was verified via automated brachial auscultation on the right, non-experimental arm every 5 minutes (S/5 Light Monitor; Datex-Ohmeda, GE Healthcare; Madison, WI, USA).

Protocols

Baseline skin blood flow, blood pressure, and core temperature data were collected for 10-15 minutes following the trauma response following microdialysis placement. During this time thermoneutral water (32-33°C) was pumped through the water-perfused suit. Drug infusion through each microdialysis site was randomized and each site received one of two treatments: 1) lactated Ringer's solution that served as a control; 2) 20 mM of the L-arginine analog N^G-*nitro-*

L-arginine methyl ester (L-NAME; Tocris Bioscience; Minneapolis, MN, USA) dissolved in lactated Ringer's solution to inhibit nitric oxide synthase (Kellogg et al. 1999). See Figure 2 for a representative schematic of drug infusions in the whole body heating protocol. All drugs were perfused for approximately 60 minutes prior to starting whole body heat stress. Drugs were perfused via microdialysis at a rate of 2µl/min with a microinfusion pump (Bee Hive controller and Baby Bee Syringe Pumps, Bioanalytical Systems, West Lafayette, IN).

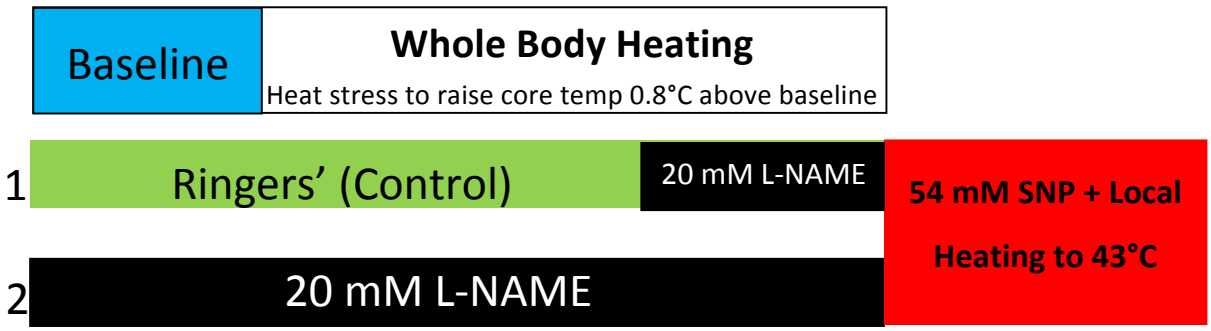

Figure 2. Schematic illustration of the experimental protocol. Following microdialysis placement and trauma resolution, Ringer's and L-NAME were infused prior to and during whole body heating. L-NAME was infused into the control site once core temperature was raised 0.8°C above baseline. Whole body heating via 50°C was used to raise core temperature 0.8°C above baseline. At the end of the whole body heat stress protocol, all sites were simultaneously infused with 54 mM SNP and locally heated to 43°C to elicit maximal vasodilation.

Whole body heat stress was initiated by pumping 50°C water through the water-perfused suit. Whole body heating continued until subjects' core temperature was raised 0.8-1.0°C above baseline. Core temperature was maintained at this level until a 5-10 minute plateau in skin blood flow for both sites was reached. Once a plateau in skin blood flow was achieved, 20 mM L-NAME was infused into the control site until skin blood flow decreased and reached a plateau. Once a new plateau was achieved at the control site in response to L-NAME (post-L-NAME drop), subjects were cooled by pumping thermoneutral water through the suit and the plastic rain

suit was removed. Maximal skin blood flow was elicited via infusion of 54mM sodium nitroprusside (SNP) at a rate of 4µl/min and by increasing the temperature of the local heaters to 43°C. This dose of SNP with local heating has been previously shown to elicit maximal cutaneous vasodilation in human skin (Kellogg et al. 1999).

Data Acquisition and Analysis

Data were digitized and stored at 50 Hz on a personal computer and analyzed offline using signal-processing software (Windaq; Dataq Instruments, Akron, OH, USA). Skin blood flow data was converted to cutaneous vascular conductance (CVC), calculated as red blood cell flux divided by mean arterial pressure (RBF/MAP). CVC data was expressed as a percentage of maximal vasodilation (%CVC_{max}) via SNP infusion and local heating to 43°C. CVC data were averaged over a stable 5 minute period for baseline, whole body heating plateau, and maximal skin blood flow.

Percent nitric oxide synthase component (%NOS) dependent vasodilation was calculated using the following equation: %NOS = [(Control WBH plateau before L-NAME – Control post-L-NAME drop) / Control WBH plateau before L-NAME] * 100

Data was analyzed using one-way and two-way repeated-measures ANOVA and paired t-tests as appropriate. All statistical analyses were performed using SigmaStat 3.5 (Systat Software; Point Richmond, CA, USA). All values are presented as mean ± SEM and p-values < 0.05 were considered statistically significant.

Chapter 3 - Results

Pre- and post-BRJ systolic, diastolic, and mean arterial blood pressure data are shown in Figures 3-5, respectively, at baseline, during whole body heating, and at the end of the protocol (i.e., during SNP infusion and local heating to 43°C; thermoneutral conditions). Systolic blood pressure was not significantly different from pre-BRJ to post-BRJ for any phase of the protocol, as depicted in Figure 3.

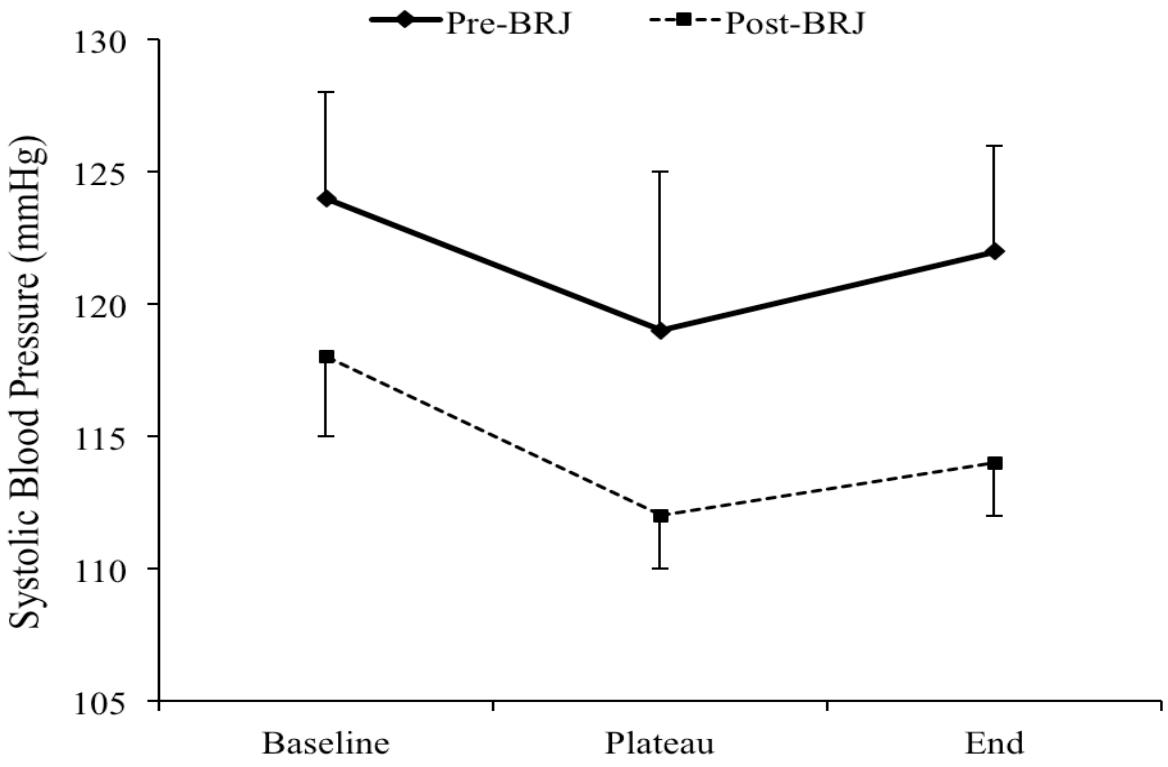

Figure 3. Values are mean ± SEM. There was no significant difference in systolic blood pressure pre- to post-BRJ in any phase of the whole body heating protocol. Plateau defined as the plateau during whole body heating at a 0.8°C increase in core temperature.

Diastolic blood pressure (Figure 4) was significantly reduced at baseline from pre-BRJ to post-BRJ (79 ± 3 mmHg vs. 66 ± 3 mmHg; $p < 0.05$) and at the end of the protocol (pre: 74 ± 2 mmHg vs. post: 63 ± 3 mmHg; $p < 0.05$). There was no effect of BRJ on diastolic blood pressure during whole body heating (pre: 68 ± 2 mmHg vs. post: 66 ± 2 mmHg).

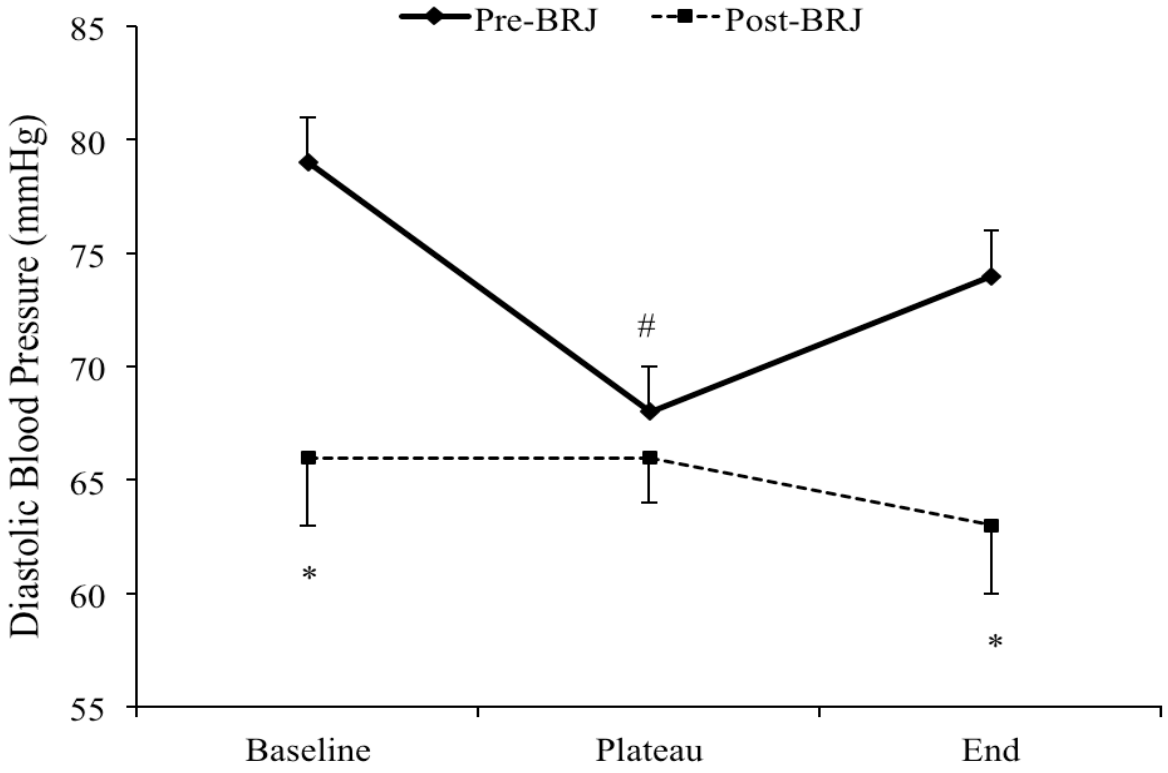

Figure 4. Values are mean ± SEM. Diastolic blood pressure was significantly reduced pre- to post-BRJ. Diastolic blood pressure at baseline and at the end of the protocol was significantly reduced pre vs. post-BRJ. Diastolic blood pressure during the plateau of whole body heating was significantly reduced compared to baseline and the end of the protocol for pre-BRJ only. Plateau defined as the plateau during whole body heating at a 0.8°C increase in core temperature. End of protocol defined as return to thermoneutral conditions during SNP infusion and local heating to 43°C.
*, $p < 0.05$ vs. pre-BRJ
#, $p < 0.05$ vs. baseline, end of protocol

Figure 5 displays mean arterial pressure data for each phase of the protocol. Mean arterial pressure at baseline was significantly reduced from pre-BRJ (97 ± 4 mmHg) to post-BRJ (82 ± 4 mmHg; $p < 0.05$) and mean arterial pressure at the end of the protocol was significantly reduced from pre-BRJ (93 ± 1 mmHg) to post-BRJ (79 ± 4 mmHg; $p < 0.05$). There was no effect of BRJ supplementation on mean arterial pressure during whole body heating (pre: 85 ± 3 mmHg vs. post: 80 ± 1 mmHg).

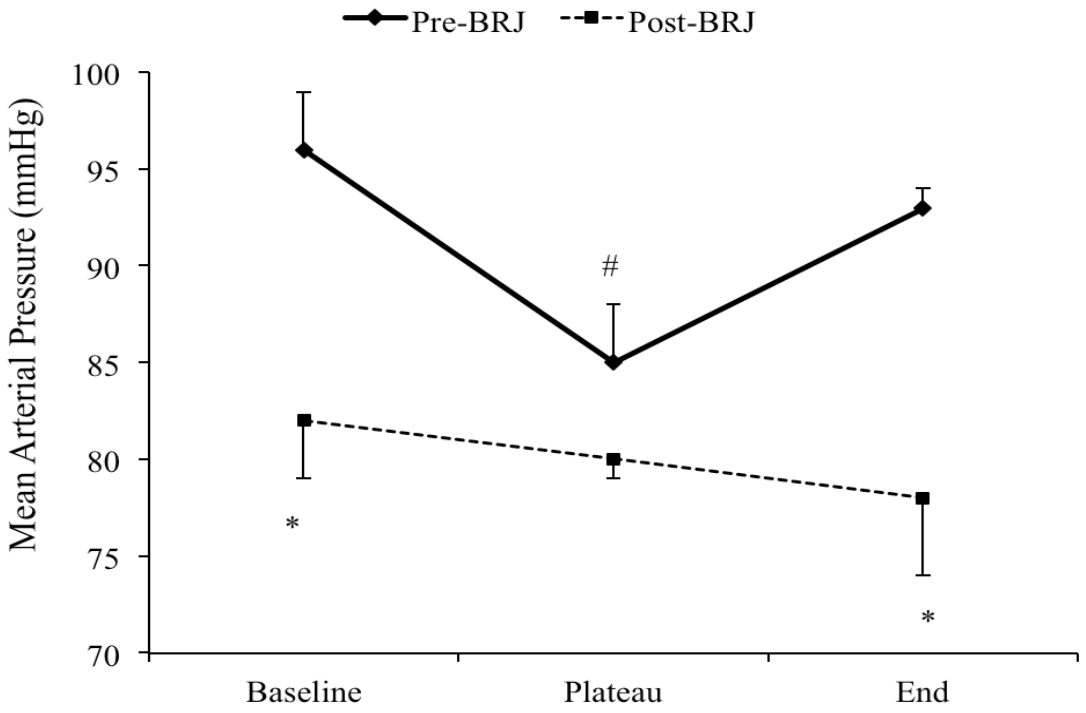

Figure 5. Values are mean ± SEM. Mean arterial pressure was significantly attenuated pre- to post-BRJ for three days. Mean arterial pressure at baseline and at the end of the protocol was significantly reduced pre- to post-BRJ. Mean arterial pressure during the plateau of whole body heating was significantly reduced compared to baseline and the end of the protocol for pre-BRJ only. Plateau defined as the plateau during whole body heating at a 0.8°C increase in core temperature. End of protocol defined as return to thermoneutral conditions during SNP infusion and local heating to 43°C.
*, $p < 0.05$ vs. pre-BRJ
#, $p < 0.05$ vs. baseline, end of protocol

Figure 6 shows the group mean whole-body heating plateau $\%CVC_{max}$ data. The increase in $\%CVC_{max}$ at the plateau during whole body heating was augmented pre- to post-BRJ for both the control (pre-BRJ: 57 ± 3 $\%CVC_{max}$ vs. post-BRJ: 80 ± 5 $\%CVC_{max}$; $p < 0.05$) and L-NAME treatment site (pre-BRJ: 36 ± 3 $\%CVC_{max}$ vs. 52 ± 6 $\%CVC_{max}$; $p < 0.05$). The increase in CVC at L-NAME sites was attenuated compared to control sites both pre- and post-BRJ ($p < 0.05$ for all conditions).

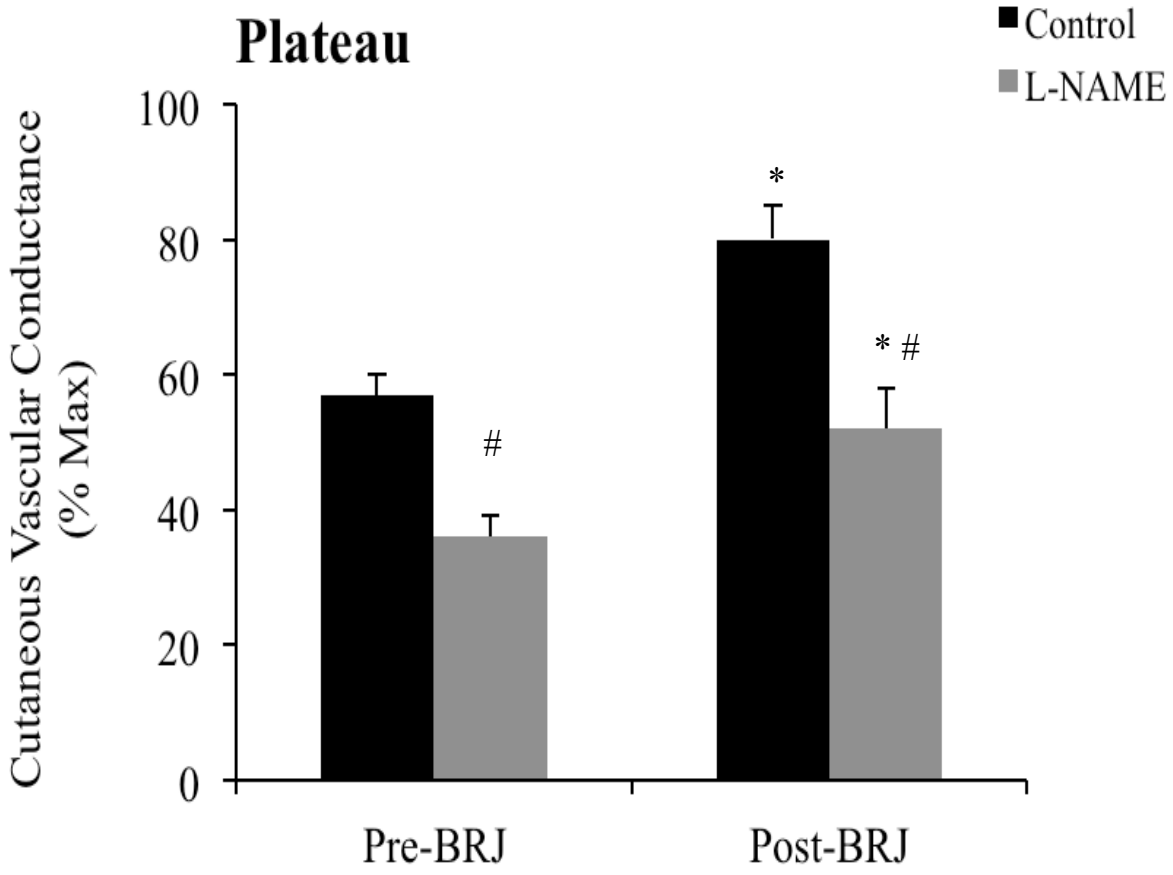

Figure 6. Values are mean ± SEM. There was a significant increase in the whole body heating plateau $\%CVC_{max}$ from pre- to post-BRJ for both the control and L-NAME sites. Plateau defined as the plateau during whole body heating at a 0.8°C increase in core temperature. L-NAME sites were attenuated compared with control both pre- and post-BRJ.
*, $p < 0.05$ vs. pre-BRJ
#, $p < 0.05$ vs. control

Figure 7 presents the mean group L-NAME drop $\%CVC_{max}$ data (i.e., decrease in CVC in response to L-NAME infusion at the plateau of heat stress at control sites). The decrease in $\%CVC_{max}$ was significantly greater pre- to post-BRJ (pre: 39 ± 3 $\%CVC_{max}$ vs. post: 25 ± 4 $\%CVC_{max}$).

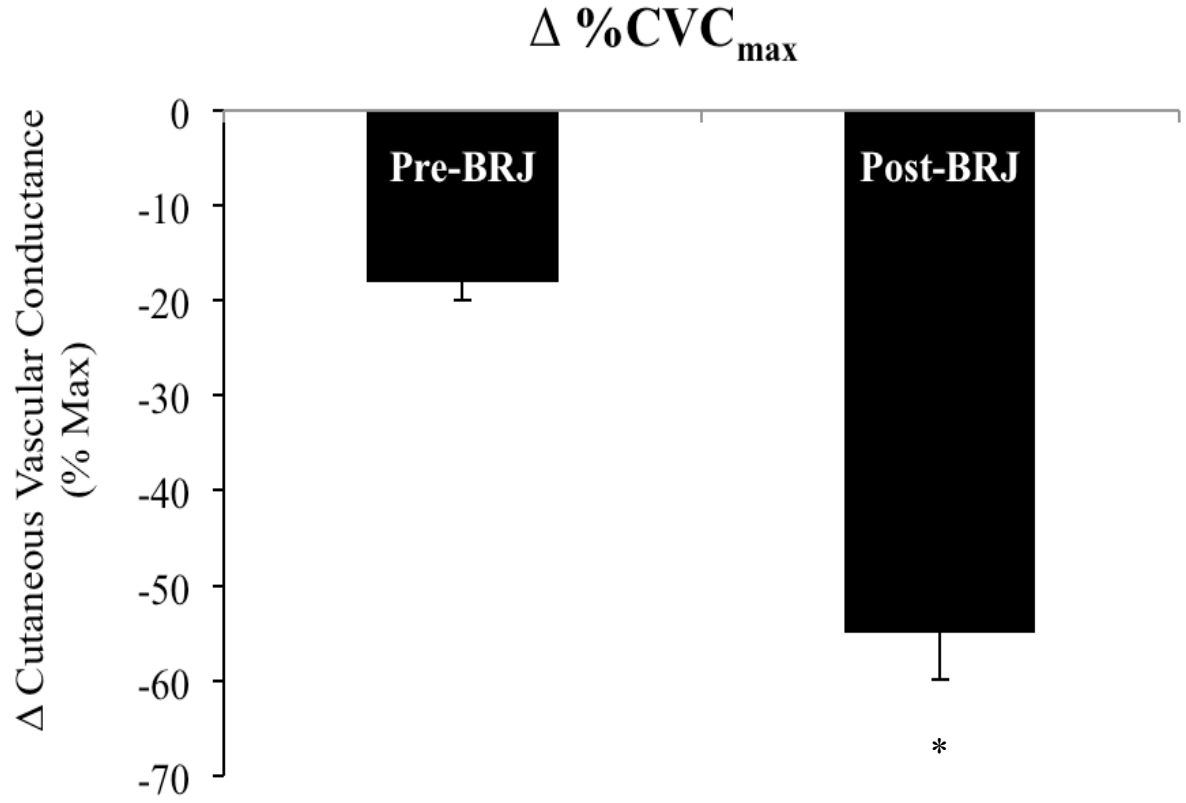

Figure 7. Values are mean ± SEM. The decrease in $\%CVC_{max}$ during the L-NAME infusion at control sites was further reduce from pre- to post-BRJ. The reduction in CVC from the plateau in heat stress to the plateau following L-NAME infusion in the control site (i.e., post-L-NAME drop) was significantly reduced from pre- to post-BRJ.
*, $p < 0.05$ vs. pre-BRJ

Figure 8 depicts mean group data of the contribution of NO to reflex cutaneous vasodilation. Beetroot juice augmented the NO component of reflex cutaneous vasodilation (pre-BRJ: 44 ± 5 %CVC_{max} vs. post-BRJ: 64 ± 6 %CVC_{max}; $p < 0.05$).

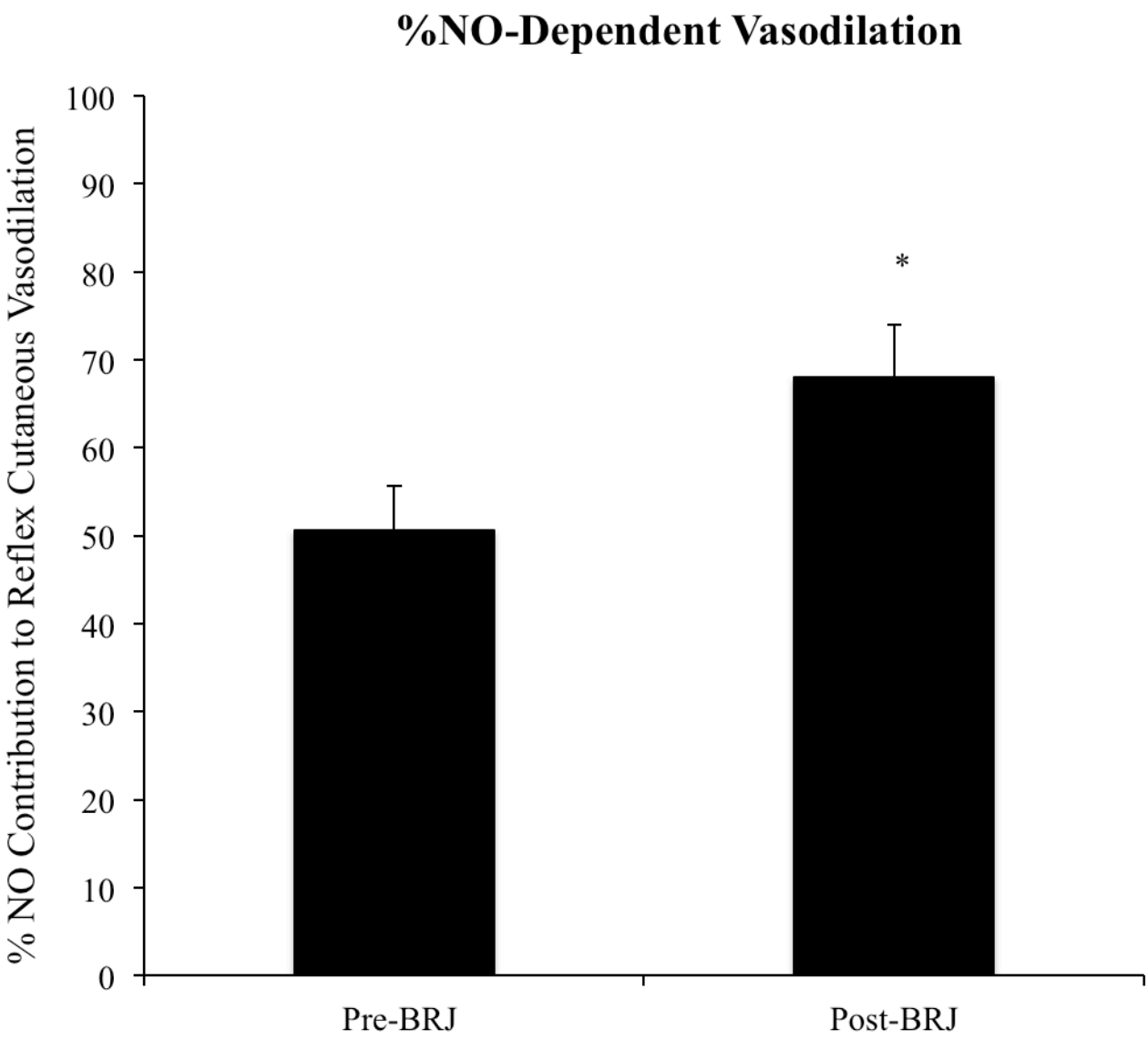

Figure 8. Values are group mean ± SEM. These data represent the %NOS-dependent contribution to vasodilation in whole body heating following three days of BRJ. There was a significant increase in %NOS-dependent vasodilation following three days of BRJ.
*, $p < 0.05$ vs. pre-BRJ

Absolute maximal CVC data are shown in Table 1. Neither BRJ nor microdialysis treatment (control vs. L-NAME) had any effect on maximal CVC values.

Absolute Maximal CVC Values

Treatment Site		Maximal CVC Values (mV/mmHg)
Pre-BRJ	Control	1.72 ± 0.14
Pre-BRJ	L-NAME	1.80 ± 0.20
Post-BRJ	Control	1.58 ± 0.13
Post-BRJ	L-NAME	1.74 ± 0.22

Table 1. Values are group mean ± SEM. There was no significant difference in absolute maximal CVC between the control and L-NAME sites pre- or post-BRJ.

Chapter 4 - Discussion

To our knowledge, this is the first investigation of the effects of dietary nitrate supplementation via beetroot juice on reflex cutaneous vasodilation in healthy humans. The primary finding of this investigation suggests that nitrate supplementation augments reflex cutaneous vasodilation significantly through both NOS-dependent and NOS-independent pathways, and thus may improve thermoregulation in healthy subjects. We observed a significant augmentation of the plateau phase of reflex cutaneous vasodilation at both the control and L-NAME sites following three days of nitrate supplementation. The increase in CVC following nitrate supplementation in both treatment sites suggests a significant improvement in NO production through NOS-independent mechanisms (Figure 5). We also observed a greater decrease in CVC in response to L-NAME infusion during the plateau at the control site from pre- to post-BRJ. This greater reduction in the post-L-NAME drop from pre- to post-BRJ further suggests there is a significant improvement in the NOS-dependent component to reflex cutaneous vasodilation (Figure 6). These data together suggest the NO component to cutaneous active vasodilation may be augmented through both NOS-dependent and NOS-independent mechanisms.

Despite the use of an exogenous NOS inhibitor, we observed a significant increase in CVC at L-NAME treatment sites during the plateau post-nitrate supplementation, which is suggestive of augmented NOS-independent vasodilation via exogenous bioavailable NO derived from beetroot juice. The conversion from nitrate to nitrite, and subsequently NO, was augmented in passive heat stress conditions, with increased core and arterial blood temperature and possibly a reduced pH (i.e., acidosis) of the blood. Nitrite reduction to NO has previously been demonstrated by Cosby et al. (2003) to elicit vasodilation and increase forearm blood flow in

humans via deoxyhemoglobin, suggesting hypoxia and acidosis are necessary to elicit reductions from nitrite to NO via the NOS-independent pathway (Cosby et al. 2003). Whether changes in skin, blood, or core temperature have a similar effect on the reduction of nitrate to nitrite to NO as do changes in pH and PO_2 remains to be elucidated.

We further observed significant reductions in both diastolic and mean arterial blood pressure following three days of nitrate supplementation. Diastolic blood pressure and mean arterial blood pressure were attenuated pre- to post-nitrate supplementation at baseline and at the end of the protocol during thermoneutral conditions in healthy subjects. Reductions in both diastolic and mean arterial pressure are consistent with previous studies following nitrate supplementation. Webb et al. demonstrated that ingestion of nitrates (beetroot juice, 500 ml) lowered both systolic and diastolic blood pressures and were associated with peak concentrations of plasma nitrite (Webb et al. 2008). Larsen and colleagues (2006) have also observed reductions in diastolic and mean blood pressure in young healthy subjects after three days of sodium nitrate supplementation (dose of 0.1 mmol per kg of body weight). Taken together, these results are consistent with our findings, and further suggest that dietary nitrates were reduced to NO.

In addition to thermoregulatory control of skin blood flow, there is also non-thermoregulatory control of the cutaneous vasculature via the baroreflex (Kellogg et al 1990). Non-thermoregulatory control of skin blood flow via the baroreflex detects reductions in mean arterial pressure and responds by increasing sympathetic nerve activity. Cui et al. demonstrated increased frequency of skin sympathetic nerve activity in whole body heating, suggesting that activation of skin sympathetic nerve activity is centrally mediated either by vasodilator or sudomotor control, or both (Cui et al. 2006). Our finding that there was no difference in mean arterial pressure between pre- and post-nitrate supplementation during hyperthermia suggests

baroreflex control of mean arterial pressure may be heightened in response to the decrease in resting mean arterial pressure following nitrate supplementation. During whole body heating post-BRJ, we observed a slight decrease in pulse pressure. Although this decrease in pulse pressure was not statistically significant compared to pre-BRJ, a decrease in pulse pressure has been shown to elicit a pressor response (Ead et al. 1952; Sagawa 1983). It is possible that as pulse pressure decreases during heat stress post-BRJ a mild pressor response is elicited by the baroreflex maintain mean arterial pressure. It is possible in heat stress conditions that mean arterial pressure may be closely regulated by skin sympathetic nerve activity in the presence of additional bioavailable NO via nitrate supplementation.

As previously shown, consumption of dietary nitrates elicits many potential cardiovascular health benefits. The findings from the present investigation are consistent with previous literature by demonstrating significant reductions in blood pressure in healthy individuals. The primary findings of the study also suggest improved microvascular function in young, healthy individuals due to an increase in NOS-dependent and independent vasodilation following nitrate supplementation. It is well known that NO is required for full expression of cutaneous active vasodilation, and in aging, for example, the NO component to reflex cutaneous vasodilation is attenuated (Holowatz et al. 2003; Kellogg et al. 1998, Shastry et al. 1998). It is possible that nitrate supplementation may augment NOS-dependent vasodilation in healthy, aged individuals, thus restoring the NO component to reflex cutaneous active vasodilation. Whether nitrate supplementation can augment reflex cutaneous vasodilation in healthy aging or in patient populations, such as hypertension or hypercholesterolemia, is unknown but our data suggest nitrate supplementation may be a safe and effective means by which to improve thermoregulation in these populations.

Limitations

There are at least two limitations to address in the present investigation. The first experimental limitation was the use of L-NAME as a non-selective NOS isoform inhibitor. Specific eNOS and nNOS isoform inhibitors would have allowed for quantification of each respective NOS isoform contribution to the response. By use of specific NOS isoform inhibitors we would have been able to determine any differences from pre- to post-BRJ and if one specific NOS isoform was upregulated versus another after consumption of beetroot juice. Second, we did not quantify serum levels of plasma nitrate and nitrite from pre- to post-BRJ. Measuring changes in plasma nitrate and nitrite levels would have allowed us to determine if changes in blood pressure were correlated to peak nitrite levels pre- to post-supplement intervention as previously discussed and presented in current mechanistic studies with nitrate supplementation (Webb et al. 2008). Although indirect, our data, demonstrating a reduction in both diastolic and mean arterial blood pressure, would appear to confirm that three days of nitrate supplementation was adequate in increasing plasma nitrate and nitrite concentrations.

Conclusions

To our knowledge, this is the first study to identify a role for dietary nitrate supplementation via beetroot juice on the skin blood flow response to reflex cutaneous vasodilation. Our data suggest dietary nitrate supplementation augmented reflex cutaneous vasodilation in young, healthy subjects via: 1) NOS-independent vasodilation, as evidenced by an increase in CVC from pre-to post-BRJ in the plateau phase for both control and L-NAME treatment sites; and 2) NOS-dependent vasodilation, as evidenced by a larger reduction in CVC during the post-L-NAME drop phase of the whole body heating protocol. These data suggest that dietary nitrate supplementation via beetroot juice is a safe and effective method for improving

thermoregulation in young, healthy humans, as evidenced by increasing reflex cutaneous vasodilation via both NOS-dependent and NOS-independent pathways.

References

Bailey SJ, Winyard P, Vanhatalo A, Blackwell JR, DiMenna FJ, Wilkerson DP, Tarr J, Benjamin N, Jones AM (2009). Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *J Appl Physiol* 107: 1144-1155.

Benjamin N, O'Driscoll F, Duncan C, Smith L, Golden M (1994). Stomach NO synthesis. *Nature* 368: 502.

Bennett LAT, Johnson JM, Stephens DP, Saad AR, Kellogg DL Jr (2003). Evidence for a role for vasoactive intestinal peptide in active vasodilatation in the cutaneous vasculature of humans. *J Physiol* 552: 223-232.

Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X, Huang KT, Shields H, Kim-Shapiro DB, Schechter AN, O Cannon III R, Gladwin MT (2003). Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nature Medicine* 9: 1498-1505.

Cui J, Sathishkumar M, Wilson TE, Shibasaki M, Davis SL, Crandall CG (2006). Spectral characteristics of skin sympathetic nerve activity in heat-stressed humans. *Am J Physiol Heart Circ Physiol* 290: H1601-H1609.

Ead HW, Green JH, Neil E (1952). A comparison of the effects of pulsatile and non-pulsatile blood flow through the carotid sinus on the reflexogenic activity of the sinus baroreceptors in the cat. *J Physiol* 118: 509-519.

Edholm OG, Fox RH, MacPherson RK (1957). Vasomotor control of the cutaneous blood vessels in the human forearm. *J Physiol* 139: 455-465.

Ferguson SK, Hirai DM, Copp SW, Holdsworth CT, Allen JD, Jones AM, Musch TI, Poole DC (2013). Impact of dietary nitrate supplementation via beetroot juice on exercising muscle vascular control in rats. *J Physiol* 591: 547-557.

Fox RH, Hilton SM (1958). Bradykinin formation in human skin as a factor in heat vasodilatation. *J Physiol* 142: 219-232.

Govoni M, Jansson EA, Weitzberg E, Lundberg JO (2008). The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide* 19: 333-337.

Hodges GJ, Chiu C, Kosiba WA, Zhao K, Johnson JM (2009). The effect of microdialysis needle trauma on cutaneous vascular responses in humans. *J Appl Physiol* 106: 1112-1118.

Holowatz LA, Houghton BL, Wong BJ, Wilkins BW, Harding AW, Kenney WL, Minson CT (2003). Nitric oxide and attenuated reflex cutaneous vasodilation in aged skin. *Am J Physiol Heart Circ Physiol* 284: H1662-H1667.

Holowatz LA, Thompson-Torgerson CS, Kenney WL (2008). The human cutaneous circulation as a model of generalized microvascular function. *J Appl Physiol* 105: 370-372.

Johnson JM, Kellogg DL Jr (2010). Thermoregulatory and thermal control in the human cutaneous circulation. *Front Biosci (Schol Ed)* 2: 825-853

Kellogg DL Jr (2006). In vivo mechanisms of cutaneous vasodilation and vasoconstriction in humans during thermoregulatory challenges. *J Appl Physiol* 100: 1709-1718.

Kellogg DL Jr, Crandall CG, Liu Y, Charkoudian N, Johnson JM (1998). Nitric oxide and cutaneous active vasodilation during heat stress in humans. *J Appl Physiol* 85: 824-829.

Kellogg DL Jr, Johnson JM, Kosiba WA (1990). Baroreflex control of the cutaneous active vasodilator system in humans. *Circ Res* 66: 1420-1426.

Kellogg DL Jr, Liu Y, Kosiba IF, O'Donnell D (1999). Role of nitric oxide in the vascular effects of local warming of the skin in humans. *J Appl Physiol* 86: 1185-1190.

Kellogg DL Jr, Pergola PE, Piest KL, Kosiba WA, Crandall CG, Grossmann M, Johnson JM (1995). Cutaneous active vasodilation in humans is mediated by cholinergic nerve cotransmission. *Circulation Research* 77: 1222-1228.

Kellogg DL Jr, Zhao JL, Wu Y (2008). Neuronal nitric oxide synthase control mechanisms in the cutaneous vasculature of humans in vivo. *J Physiol* 586: 847-857.

Kellogg DL Jr, Zhao JL, Wu Y, Johnson JM (2010). VIP/PACAP receptor mediation of cutaneous active vasodilation during heat stress in humans. *J Appl Physiol* 109:95-100.

Kenjale AA, Ham KL, Stabler T, Robbins JL, Johnson JL, VanBruggen M, Privette G, Yim E, Kraus WE, Allen JD (2011). Dietary nitrate supplementation enhances exercise performance in peripheral arterial disease. *J Appl Physiol* 110: 1582-1591.

Larsen FJ, Weitzberg E, Lundberg JO, Ekblom B (2007). Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiol* 191: 59-66.

Larsen FJ, Ekblom B, Sahlin K, Lundberg JO, Weitzberg E (2006). Effects of dietary nitrate on blood pressure in healthy volunteers. *N Engl J Med* 355: 2792-2793.

Lundberg JO, Weitzberg E, Alving K (1994). Intragastric nitric oxide production in humans: Measurements in expelled air. *Gut* 35: 1543-1546.

Lundberg JO, Govoni M (2004). Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic Biol Med* 37: 395-400.

Lundberg JO, Weitzberg E, Gladwin MT (2008). The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov* 7: 156-167.

McCord GR, Cracowski J, Minson CT (2006). Prostanoids contribute to cutaneous active

vasodilation in humans. *Am J Physiol Regul Integr Comp Physiol* 291: R596-R602.

Moncada S, Higgs A (1993). The L-arginine-nitric oxide pathway. *N Engl J Med* 329: 2002-2012.

Rowell LB (1974). Human cardiovascular adjustments to exercise and thermal stress. *Physiol Rev* 54: 75-159.

Sagawa K (1983). Baroreflex control of systemic arterial pressure and vascular bed. In *Handbook of Physiology*, Chapter 14, pages 453-496.

Shastry S, Dietz NM, Halliwill JR, Reed AS, Joyner MJ (1998). Effects of nitric oxide synthase inhibition on cutaneous vasodilation during body heating in humans. *J Appl Physiol* 85: 830-834.

Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Pavey, TG, Wilkerson DP, Benjamin N, Winyard PG, Jones AM (2010). Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. *Am J Physiol Regul Integr Comp Physiol* 299: R1121-R1131.

Webb AJ, Patel N, Loukogeorgakis S, Okorie M, Aboud Z, Misra S, Rashid R, Miall P, Deanfield J, Benjamin N, MacAllister R, Hobbs AJ, Ahluwalia A (2008). Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension* 51: 784-790.

Wilkins BW, Holowatz LA, Wong BJ, Minson CT (2003). Nitric oxide is not permissive for cutaneous active vasodilatation in humans. *J Physiol* 548: 963-969.

Wong BJ, Fieger SM (2012). Transient receptor potential vanilloid type 1 channels contribute to reflex cutaneous vasodilation in humans. *J Appl Physiol* 112: 2037-2042.

Wong BJ, Tublitz NJ, Minson CT (2005). Neurokinin-1 receptor desensitization to consecutive microdialysis infusions of substance P in human skin. *J Physiol* 568: 1047-1056.

Wong BJ, Wilkins BW, Minson CT (2004). H1 but not H2 histamine receptor activation contributes to the rise in skin blood flow during whole body heating in humans. *J Physiol* 560: 941-948.

Appendix A - Individual Responses

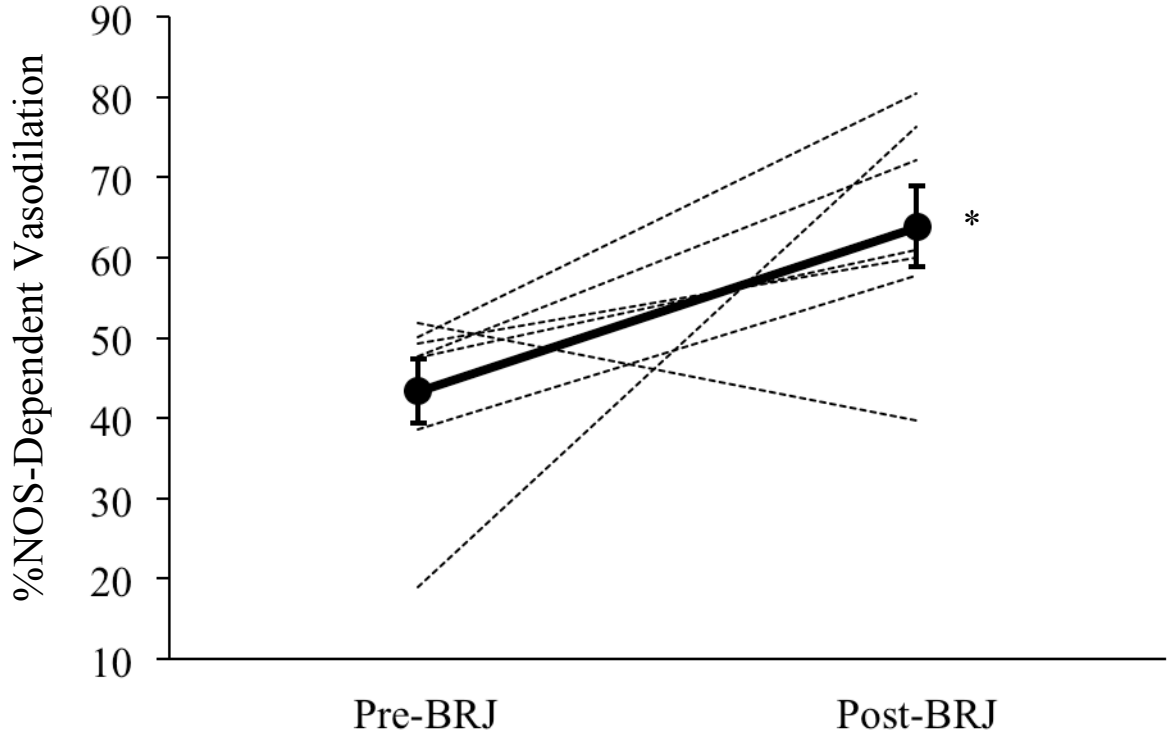

Figure A.1. These data represent the individual responses of all subjects (n = 7) of the NO-component to reflex cutaneous vasodilation from pre- to post-BRJ. Group mean data (solid line) represents the increase in %NO-dependent vasodilation in all subjects from pre- to post-BRJ.

Appendix B – Control Site Skin Blood Flow Tracing

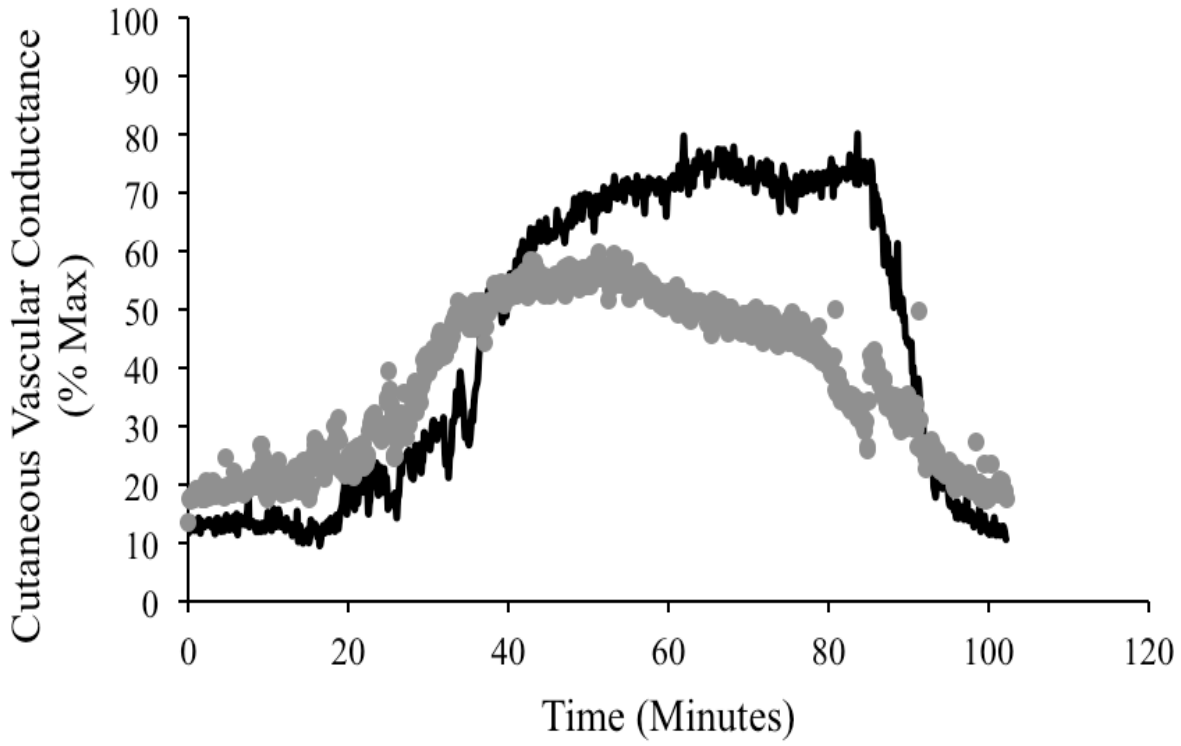

Figure B.2. A representative tracing of the skin blood flow response to reflex cutaneous vasodilation in one subject in the control site from pre-BRJ (gray line) to post-BRJ (black line). This figure suggests improved NOS-dependent and NOS-independent vasodilation. Improved NOS-dependent vasodilation was demonstrated by a further reduction in CVC during the post-L-NAME drop, post-BRJ. Improved NOS-independent vasodilation was suggested by an increase in CVC in the whole body heating plateau of the protocol post-BRJ.

Appendix C – L-NAME Site Skin Blood Flow Tracing

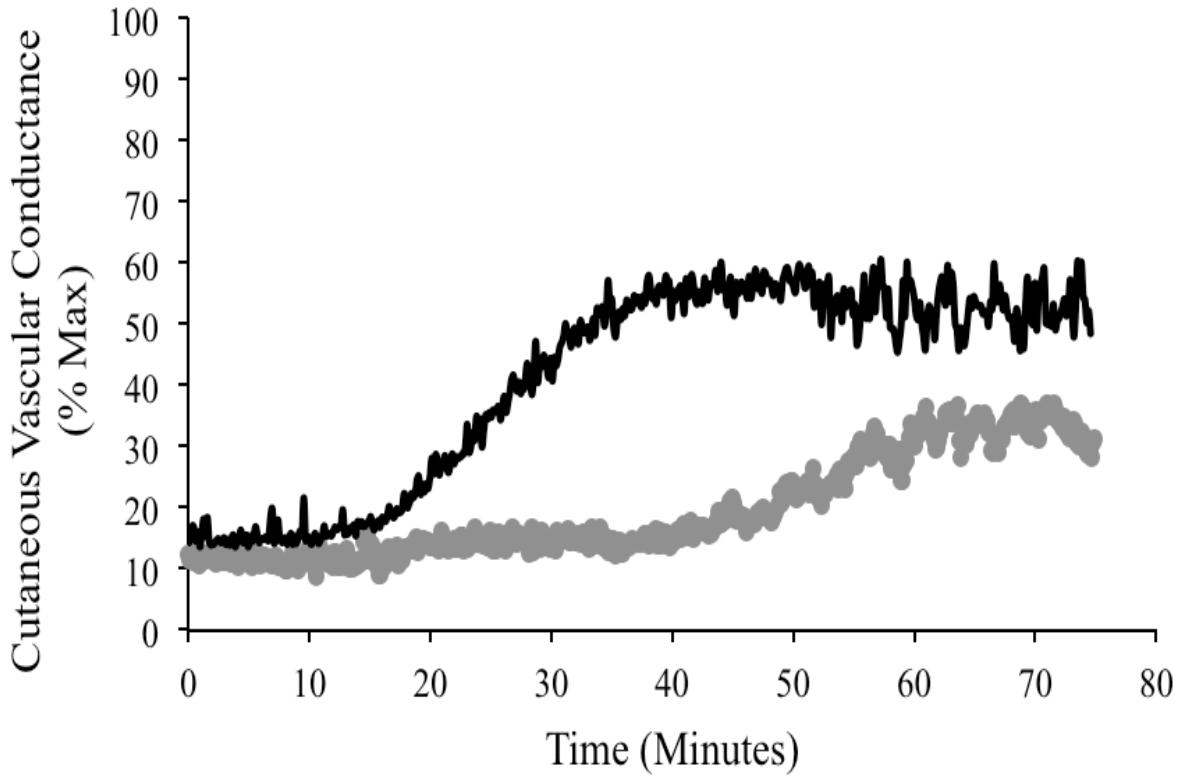

Figure C. 3. A representative tracing of the skin blood flow response to reflex cutaneous vasodilation in one subject in the L-NAME treatment site from pre-BRJ (gray line) to post-BRJ (black line). This figure suggests an improved NOS-independent vasodilation as demonstrated by an increase in CVC in the plateau phase of whole body heating post-BRJ. This increase in CVC in a NOS-inhibited treatment site suggests an increase in NOS-independent vasodilation post-BRJ.