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LOCAL TETRAHYDROBIOPTERIN AUGMENTS REFLEX CUTANEOUS VASODILATION IN AGED HUMAN SKIN

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by

Anna E. Stanhewicz

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The thesis of Anna E. Stanhewicz was reviewed and approved* by the following:

W. Larry Kenney
Professor of Physiology and Kinesiology
Thesis Advisor

Lacy A. Holowatz
Assistant Professor of Kinesiology

James A. Pawelczyk
Associate Professor of Physiology and Kinesiology

Donna H. Korzick
Associate Professor of Physiology and Kinesiology
Head of the Intercollege Graduate Degree Program in Physiology

*Signatures are on file in the Graduate School

ABSTRACT

Reflex cutaneous vasodilation (VD) is attenuated in aged skin, potentially resulting in decreased heat loss during heat exposure. Constitutive NO-synthase (NOS) is necessary for full expression of reflex cutaneous VD. Tetrahydrobiopterin (BH_4) acts as an essential cofactor for NOS activity by preventing NOS uncoupling and reducing oxidant stress. Reduced BH_4 bioavailability is associated with primary aging. We hypothesized that acute local BH_4 administration would augment NO-dependent VD in aged skin during hyperthermia. Three intradermal microdialysis fibers were placed in the ventral forearm skin of 11 young (Y, 22 ±1 years) and 11 older (O, 73±2 years) human subjects for local infusion of (1) control: Ringers solution, (2) BH_4 administration: 10mM BH_4, and (3) NOS inhibition: 20mM N^G-nitro-L-arginine methyl ester (L-NAME), respectively. Red cell flux was measured at each site by laser-Doppler flowmetry (LDF) as reflex VD was induced using a water perfused suit. After a 1ºC rise in oral temperature mean body temperature was clamped and L-NAME was perfused at each site. Cutaneous vascular conductance was calculated (CVC = LDF/MAP). VD was attenuated at the control site in O (O: 0.6 ± 0.1 vs. Y: 1.0 ± 0.2 CVC; $p<0.05$). BH_4 administration increased VD in O (1.2 ± 0.2 CVC; $p<0.05$) but not Y (1.0 ± 0.2 CVC). NOS inhibition similarly attenuated VD at the BH_4 site in both groups (O: 0.7 ± 0.1 vs. Y: 0.5 ± 0.1 CVC; $p<0.05$). BH_4 administration increased absolute maximal CVC in O (BH_4: 2.0 ± 0.2 vs. control: 1.4 ± 0.2 CVC; $p<0.5$). Acute local BH_4 administration increases NO-dependant reflex cutaneous VD in aged skin during whole body heat stress, suggesting that reduced BH_4 contributes to attenuated VD in older humans.

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Chapter 1

INTRODUCTION

Background and Significance

The human cutaneous circulation can accommodate extremely high blood flows. During passive thermal stress in a supine position, blood flow to the skin increases up to 8 liters per minute (or ~60% of cardiac output) allowing for convective heat transfer from the body core to the surface where heat dissipation can occur (Rowell, 1993). Under normothermic conditions the cutaneous vasculature is under tonic adrenergic control. With an increase in body temperature, skin blood flow is first increased through withdrawal of tonic adrenergic tone and then further increased through active reflex cutaneous vasodilation (VD) (Roddie et al., 1957). Reflex cutaneous VD, in conjunction with sweat evaporation, allows for the dissipation of heat from the skin to the environment, minimizing heat storage and the consequent rise in core temperature during environmental heat stress.

Reflex cutaneous VD is attenuated with primary aging (Kenney, 1988; Kenney et al., 1997). On average, healthy humans aged $\geq$ 65 years have a 25 – 50% reduction in skin blood flow (SkBF) compared with young adults. This attenuation is apparent even when subjects are matched for fitness level (Kenney, 1988), hydration status (Kenney et al., 1990), and acclimation status (Armstrong and Kenney, 1993) suggesting that this is a primary aging phenomenon. Age-related impairments occur at multiple points along the sympathetic efferent arm controlling heat-induced reflex VD and include an attenuated

neural drive (Grassi et al., 2003), reduced contribution of cholinergic co-transmitter(s) (Holowatz et al., 2003), and impaired downstream signaling in both endothelial and vascular smooth muscle cells (Minson et al., 2002; Holowatz et al., 2003; Holowatz et al., 2006b)

Reflex cutaneous VD is mediated by sympathetic co-transmission of acetylcholine and unknown co-transmitter(s) (Kellogg et al., 1995). These neurotransmitters initiate the downstream nitric oxide (NO) production required for full expression of the reflex VD response (Kellogg et al., 1998). In healthy young subjects approximately 30 - 40% of the full reflex VD response is mediated by NO signaling with the other approximately 60 – 70% relying on cofactors such as prostaglandins, histamine, vasoactive intestinal peptide and substance P (Bennett et al., 2003; Wong et al., 2004; McCord et al., 2006). With aging, the cofactor-mediated contribution to the overall reflex VD response is attenuated and consequently, older individuals rely predominantly on NO for reflex VD in response to increased core temperature (Holowatz et al., 2003).

NO is synthesized through the activity of two constitutively expressed nitric oxide synthases (NOS; endothelial-NOS and neuronal-NOS). The NOS enzyme is a dimer that relies on the presence of essential cofactors and available substrate to couple the oxidation of L-arginine to the reduction of molecular oxygen to produce NO and L-citrulline (Liu and Gross, 1996). When either substrate or cofactor availability is limited, or under conditions of elevated oxidant stress, the NOS dimer can destabilize and uncouple (Munzel et al., 2005). This uncoupling disrupts the normal flow of electrons through the dimerized complex and results in the production of superoxide radicals rather than NO (Vasquez-Vivar et al., 1998; Xia et al., 1998).

One age-associated impairment in downstream VD signaling is upregulated arginase activity. Arginase catalyzes the conversion of L-arginine to L-ornithine in the last step of the urea cycle, limiting substrate availability for NO synthesis by NOS and reciprocally regulating NO production (Berkowitz et al., 2003). With aging, upregulated arginase activity results in decreased NO production and attenuated endothelial function (Berkowitz et al., 2003). Local L-arginine supplementation or arginase inhibition augments NO-dependent reflex VD in aged skin, demonstrating that increasing available substrate restores VD function in aged cutaneous vessels (Holowatz et al., 2006b).

Another age-associated impairment in cutaneous VD signaling is elevated oxidant stress due to a combined increase in production and decreased clearance of reactive oxygen species (ROS) (Donato et al., 2007). Local ascorbate supplementation augments NO-dependent reflex VD in aged skin, pointing to a role for oxidant stress in the age associated attenuation in the reflex VD response to heat stress (Holowatz et al., 2006a).

In addition to upregulated arginase activity and increased oxidant stress, there are other potential mechanisms associated with impaired downstream signaling and attenuated NO-dependent reflex VD in aged skin. Tetrahydrobiopterin (BH_4) serves as an essential cofactor for pteridine-requiring monoxygenases (Raman et al., 1998). Consequently, BH_4 bioavailability is required for enzymatic systems in vascular control and is important to both VD and vasoconstriction in the healthy vasculature (Kaufman, 1993). Specifically, BH_4 plays a critical role in NOS dimerization and NO production (Raman et al., 1998). Reduced BH_4 bioavailability may contribute to several pathologies leading to vascular dysfunction (Higashi et al., 2006; Porkert et al., 2008; Lang et al., 2009; Schmidt et al., 2010).

BH_4 is a naturally occurring cofactor that is synthesized *de novo* from guanosine triphosphate (GTP) in a series of three reactions initiated and regulated by the activity of GTP cyclohydrolase 1 (GTPCH-1) (Takikawa et al., 1986; Thony et al., 2000). BH_4 can also be resynthesized from its oxidized form, dihydrobiopterin (BH_2), in the so called salvage or "biopterin recycling" pathway (Thony et al., 2000). In the salvage pathway, BH_2 is reduced back to BH_4 through the activity of dihydrofolate reductase (DHFR) (Crabtree et al., 2009; Crabtree and Channon, 2011). Taken together, *de novo* synthesis and biopterin recycling maintain BH_4 bioavailability.

BH_4 serves as an essential cofactor for NOS and therefore plays a critical role in NO-dependent VD. BH_4 binds to the oxygenase domain of the NOS dimer, stabilizing the iron-moiety and maintaining the functional conformation of the monooxygenase (Raman et al., 1998). When BH_4 bioavailability is limited, uncoupling of the NOS dimer can occur, reducing NO synthesis and promoting oxidative stress (Vasquez-Vivar et al., 1998). In primary aging, high oxidant stress may deplete bioavailable BH_4 by (1) direct oxidation of existing BH_4 to dihydrobiopterin (BH_2) and/or (2) reducing BH_4 synthesis *in vivo* (Vasquez-Vivar, 2009). This attenuation may contribute to the vascular dysfunction associated with aging.

Animal models of vascular disease have responded to exogenous BH_4 treatment with improved markers for vascular health (Schmidt et al., 2010). Similarly, *in vivo* studies examining human populations that exhibit vascular dysfunction have shown that administration of exogenous BH_4 improves endothelial function (Higashi et al., 2006; Cosentino et al., 2008; Porkert et al., 2008). The proposed mechanisms for these improvements in endothelial function are the NOS-coupling and antioxidant properties of

the BH_4 molecule. However, these mechanisms have yet to be fully described and few *in vivo* studies have addressed the functional role of BH_4 in attenuated NO-dependent reflex cutaneous VD in aged humans.

The primary focus of the present study was to determine if local BH_4 administration increases NO-dependent VD in aged human skin. The cutaneous circulation provides a unique, easily accessible vascular bed in which to study the mechanisms of microvacular function (Holowatz et al., 2008). BH_4 administration by microdialysis improves vasoconstrictor function in aged skin in response to cold stress (Lang et al., 2009). We hypothesized that acute, local BH_4 administration by microdialysis would increase NO-dependent VD in aged skin during whole body heating.

Specific Aim and Hypothesis

Specific Aim: The purpose of the present study was to determine if administration of BH_4, a cofactor for NOS, increases NO-dependent reflex cutaneous VD in aged skin.

Hypothesis: Acute local administration of BH_4 will increase NO-dependent reflex cutaneous VD in aged skin during whole body heat stress.

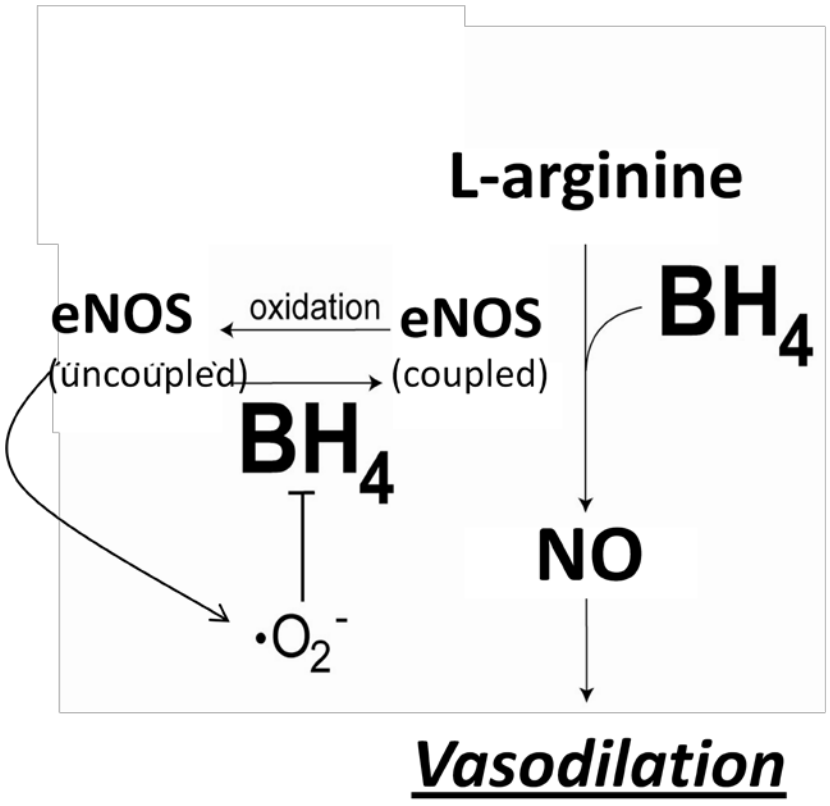

Figure 1.1 Schematic representation of the role of tetrahydrobiopterin in endothelial nitric oxide synathase –mediated nitric oxide production. eNOS, endothelial nitric oxide synthase; BH_4, tetrahydrobiopterin; NO, nitric oxide; $\cdot O_2$, superoxide.

Chapter 2

LOCAL TETRAHYDROBIOPTERIN ADMINISTRATION AUGMENTS REFLEX CUTANEOUS VASODILATION IN AGED HUMANS

Introduction

Skin blood flow (SkBF) is controlled by dual sympathetic innervation comprising an adrenergic vasoconstrictor system and a cholinergic active vasodilator system (Grant, 1938). With increasing body temperature, SkBF first increases through passive withdrawal of tonic adrenergic constrictor tone and then increases further through the active vasodilator system (Roddie et al., 1957). Active vasodilation (VD) is mediated by sympathetic release of acetylcholine and unknown cotransmitter(s) (Kellogg et al., 1995), which induce nitric oxide (NO) production. In the vascular endothelium, NO is synthesized by the constitutively expressed nitric oxide synthases (NOS; eNOS and nNOS) in response to these neurotransmitters (Kellogg et al., 2009). NO is required for full expression of reflex VD and activation of these pathways is responsible for ~30 – 40% of the total VD response (Kellogg et al., 1998; Shastry et al., 1998). The other ~ 60 – 70% of reflex VD relies on cofactors such as prostaglandins, histamine, vasoactive intestinal peptide and substance P (Bennett et al., 2003; Wong et al., 2004; McCord et al., 2006). With aging, the cofactor-mediated contribution to the overall reflex VD response is attenuated and consequently, older individuals rely predominantly on NO for VD in the heat (Holowatz et al., 2003).

Primary aging is associated with an attenuated vasodilator response to increases in core temperature (Kenney et al., 1997) due to a decreased co-transmitter contribution as well as an attenuated NO-dependent vasodilator response (Holowatz et al., 2003). The decrease in NO bioavailability in aged human skin results from both a decrease in NO production through mechanisms such as up-regulated vascular arginase (Berkowitz et al., 2003) and increased NO degradation through increased oxidant stress (Donato et al., 2007). Furthermore, NOS is a dimeric enzyme, requiring coupling of the oxygenase and reductase domains for NO production (Raman et al., 1998; Andrew and Mayer, 1999). In conditions where substrate (L-arginine) is limited or oxidant stress is increased, the NOS dimer can destabilize and uncouple, producing superoxide rather than functional NO (Vasquez-Vivar et al., 1998; Munzel et al., 2005).

In addition to upregulated arginase activity and increased oxidative stress there are other mechanisms potentially associated with the decrease in NO bioavailability that likely contribute to attenuated reflex cutaneous VD in aged skin. One such mechanism is a decreased availability of tetrahydrobiopterin (BH_4) associated with primary aging (Delp et al., 2008; Sindler et al., 2009). Mechanistically, BH_4 stabilizes the functional conformation of the NOS dimer and reduces oxidant stress both within and around the NOS molecule (Raman et al., 1998; Vasquez-Vivar et al., 1998). Without BH_4, or when BH_4 is limited, the NOS dimer uncouples, disrupting the flow of electrons necessary for the production of NO (Cosentino and Luscher, 1999). With this disruption comes an increased production of superoxide which further diminishes BH_4 bioavailability and increases oxidant stress (Vasquez-Vivar et al., 1998). *In vivo* studies examining vascular function in human populations that exhibit vascular dysfunction have shown that

administration of exogenous BH_4 increases measures of NO-dependent VD (Cosentino et al., 2008; Porkert et al., 2008). However, the functional role of BH_4 in NOS uncoupling and its potential contribution to attenuated NO-dependent reflex cutaneous VD in aged humans is unclear.

The purpose of this study was to determine if exogenous administration of BH_4 would increase NO-dependent reflex cutaneous VD in aged humans.

Methods

Subjects

Experimental protocols were approved by the Institutional Review Board at The Pennsylvania State University. Written and verbal consent were obtained voluntarily from all subjects prior to participation according to the declaration of Helsinki. Studies were performed on eleven young (23±3 yr, 7 men and 4 women) and eleven older (73±2 yr, 5 men and 6 women) healthy subjects. Each subject underwent a complete medical screening including resting ECG, physical examination, lipid profile, blood chemistry (Quest Diagnostics Nichol Institute, Chantilly, VA) and VO_2 max test to test for the prevalence of underlying cardiovascular disease. Subjects were screened for neurological, cardiovascular and dermatological diseases. All subjects were normally active, nonhypertensive, nondiabetic, healthy nonsmokers who were not taking prescription medications with primary or secondary vascular effects. Subjects who were taking 81mg aspirin daily as a preventative measure were required to cease treatment for a two week washout period before the study visit. All premenopausal women were studied during the early follicular phase of their menstrual cycle.

Instrumentation

All protocols were performed in a thermoneutral laboratory with the subject in a semisupine position and the experimental arm supported at heart level. Upon arrival to the laboratory, subjects were instrumented with three intradermal microdialysis (MD) fibers (10-mm, 30-kDa cutoff membrane, MD 2000, Bioanalytical Systems, West Lafayette, IN, USA) placed in the skin of the ventral left forearm using sterile technique.

Prior to MD fiber placement, ice packs were applied to the MD sites for 5 minutes. Ice provides anesthetic relief from the discomfort of needle insertion as well as minimizes the attenuation in peak cutaneous vascular conductance (CVC) values resulting from MD fiber placement(Hodges et al., 2009). For each fiber, a 25-guage needle was inserted horizontally into the dermis such that the entry and exit points were ~2.5cm apart. MD fibers were then threaded through the lumen of the guide needle and the needle was removed such that the membrane of the MD fiber remained under the skin. The microdialysis fibers were taped in place and randomly assigned to receive 1) lactated Ringer solution to serve as control, 2) 10mM BH_4 (Sigma, St. Louis, MO) for BH_4 administration, and 3) 20mM N^G-nitro-L-arginine (L-NAME; Calbiochem) to inhibit NOS-mediated production of NO. All pharmacological agents were mixed just prior to usage, dissolved in lactated Ringer solution, sterilized with syringe microfilters (Acrodisc, Pall, Ann Arbor, MI) and wrapped in foil to prevent degradation of the drug due to light exposure. During the insertion trauma resolution period (60 - 90 min), solutions were perfused through the microdialysis fibers at a rate of 2μl/min (Bee Hive controller and Baby Bee microinfusion pumps, Bioanalytical systems). Pilot studies were conducted to determine the optimal dose of BH_4, defined as a dose that would maximally augment reflex vasodilator function but did not alter baseline CVC. 20mM L-NAME concentrations were utilized to completely inhibit NOS activity.

Skin temperature was controlled using a water-perfused suit that covered the entire body except the head, hands, feet and experimental arm. Copper-constantan thermocouples were placed on the surface of the skin at six different sites; calf, thing, abdomen, chest, shoulder and back to continually monitor skin temperature. Each

subject's heart rate was monitored throughout the protocol and arterial blood pressure was measured every 5 minutes (Cardiocap, GE Healthcare) and verified by brachial auscultation regularly throughout the protocol. Oral temperature (T_{or}) was measured as an index of changes in body core temperature using a thermister placed in the sublingual sulcus throughout baseline and whole body heating. Local skin temperature over each microdialysis site was maintained at 33ºC throughout baseline and whole body heating (Moor Instruments SHO2) to eliminate locally mediated responses to skin warming or cooling.

To obtain an index of skin blood flow, cutaneous red blood cell flux was continually measured directly over each experimental site with an integrated laser-Doppler flowmetry (LDF) probe placed in a local heater (MoorLAB, Temperature Monitor SH02; Moor Instruments, Devon, UK). CVC was calculated as red blood cell flux divided by the mean arterial pressure (MAP) and expressed in absolute CVC and percent of maximum VD (%CVCmax; 28mM SNP and local heat to 43). MAP was calculated as diastolic pressure plus one third pulse pressure.

Experimental Protocol

After MD fiber placement and the insertion trauma resolution period, baseline data was collected (20 min). Throughout this baseline period, mean skin temperature was held at 34ºC by perfusing thermoneutral water through the suit. After baseline, 52ºC water was perfused through the suit to clamp mean skin temperature at 48 ºC. At a 1ºC rise in T_{or}, mean body temperature was clamped. After 5 minutes of steady laser-Doppler flux values, 20mM L-NAME was perfused through each MD fiber at a rate of 4µl/min to

competitively inhibit NOS within each site. L-NAME perfusion was discontinued after laser-Doppler flux values decreased to a steady plateau (~40 minutes). At that time whole body heating was terminated, the water perfusing the suit was returned to 34ºC, and subjects were returned to thermoneutral conditions. After completion of the whole body heating protocol, 28mM sodium nitroprusside (SNP; Nitropress, Abbott Laboratories, Chicago, IL) was perfused through each MD site at a rate of 4µl/min along with simultaneous local heating of the skin to 43ºC at each site to obtain maximal CVC values.

Data Acquisition and Analysis

CVC data from the control, BH_4 and NOS inhibited sites were acquired at 40 Hz, digitized, and stored on a personal computer until further data analysis (Windaq, Dataq Instruments, Akron, OH). CVC values were averaged over a 5 minute period of stable laser-Doppler flux at baseline and for every 0.1ºC rise in T_{or} during the whole body heating protocol. Maximal CVC values were averaged over a stable plateau in laser-Doppler flux during perfusion of 28mM SNP and local heating to 43ºC. ΔCVC during NOS-inhibition represents the difference in plateau CVC values at each site before and after L-NAME perfusion.

Paired students t-tests were used to detect significant differences between the young and older groups for physical characteristics. A two-way repeated-measures mixed model analysis of variance (ANOVA) was used to detect differences for the ΔCVC and differences in maximal CVC. A three-way repeated-measures mixed model ANOVA was conducted to detect age and MD local drug treatment differences over the rise in T_{or} (SAS, version 9.1.3, Cary, North Carolina, USA). Post hoc comparisons with Bonferroni

corrections were performed when necessary to determine where differences between groups and drug treatments occurred. The level of significance was set at $\alpha=0.05$ for main effects. Values are presented as mean ± SE.

Results

Subject characteristics are presented in Table 2.1. Age groups were well matched for body-mass index, fasting blood glucose, systolic blood pressure (SBP), diastolic blood pressure (DBP) and high density lipoproteins (HDL). The older subject group had significantly higher low density lipoproteins (LDL) than younger subjects.

Figure 2.1 shows absolute CVC values as a function of increasing core temperature (ΔT_{or}) during whole body heating and following NOS inhibition (L-NAME, 20mM) in control, NOS-inhibited and BH_4 treated sites in young and older subjects. Compared to young, older subjects exhibit an attenuated vasodilation at the control site beginning at a ΔT_{or} of 0.3ºC (Fig. 2.1A, $p<0.05$). With NOS inhibition (L-NAME) young and older subjects had similar vasodilatory responses throughout heating (Fig. 2.1B). Local administration of BH_4 augmented the vasodilator response in older subjects such that the older VD response matched that of the young (Fig. 2.1C).

Figure 2.2 shows %CVCmax as a function of increasing core temperature (ΔT_{or}) during whole body heating and following NOS inhibition (L-NAME, 20mM) in control, NOS-inhibited and BH_4 treated sites in young and older subjects. Compared to young, older subjects exhibit an attenuated vasodilation at the control site beginning at a ΔT_{or} of 0.3ºC (Fig. 2.3A, $p<0.05$). With NOS inhibition (L-NAME), young and older subjects had similar vasodilatory responses throughout heating (Fig. 2.2B). Local administration of BH_4 augmented the vasodilator response in older subjects such that the older VD response matched that of the young (Fig. 2.3C).

Figure 2.3 shows the mean reduction in CVC (panel A) and %CVCmax (panel B) at control and BH_4 administered sites in young and older subjects with NOS inhibition

(L-NAME, 20mM) when mean body temperature was clamped. The decrease in the dilation response with NOS-inhibition represents NOS-dependent dilation. NOS-dependent dilation was reduced at the older control site compared to the young control site (CVC; O: 0.2±0.04 vs. Y: 0.4±0.8, $p<0.05$) (%CVCmax; O: 12.7% ± 2.2 vs. Y: 24.6% ± 4.3, $p<0.05$). Compared to the older control site, local BH_4 administration increased NOS-dependent dilation in the older subject group (CVC; BH_4: 0.7 ± 0.1 vs. control: 0.2 ± 0.04, $p<0.05$) (%CVCmax; BH_4: 32.2% ± 3.4 vs. control: 12.7% ± 2.2, $p<0.05$). Compared to the young control site, local BH_4 administration did not change NOS-dependent dilation in the young subjects (CVC; BH_4: 0.5±0.1 vs. control: 0.4±0.8) (%CVCmax; BH_4: 24.5 ± 4.4% vs. control: 21.6 ± 4.4%).

Table 2.2 presents maximal CVC values measured at each microdialysis site during combined SNP infusion and 43°C local heating. Older subjects had a lower maximal CVC compared to young subjects at the control site (O: 1.4 ± 0.2 vs. Y: 1.9 ± 0.2; $p\leq0.001$). Local BH_4 administration increased maximal CVC in the older group (BH_4: 2.0 ± 0.2 vs. control: 1.4 ± 0.2, $p\leq0.001$) but not the younger group (BH_4: 1.8 ± 0.2 vs. control: 1.9 ± 0.2).

Discussion

The principal finding of this study was that local BH_4 administration increased reflex cutaneous VD in aged human skin through increased NO-dependent VD. BH_4 was effective at increasing skin blood flow in the aged subject group but had no effect in the young subject group. Furthermore, BH_4 increased maximal CVC in the older subject group but had no effect in the young subject group.

In healthy young subjects ~ 30 - 40% of the full reflex VD response is mediated by NO signaling with the other ~ 60 – 70% relying on cofactors such as prostaglandins, histamine, vasoactive intestinal peptide and substance P (Bennett et al., 2003; Wong et al., 2004; McCord et al., 2006). With aging, the cofactor-mediated contribution to the overall reflex VD response is attenuated and consequently, older individuals rely predominantly on NO for VD in the heat (Holowatz et al., 2003).

NO is synthesized in the cutaneous vasculature by the constitutively expressed NOS isoforms, endothelial-NOS (eNOS) and neuronal-NOS (nNOS) in response to local and whole body heat stress (Kellogg et al., 2009). Kellogg et.al. have reported that NO-dependent reflex VD is mediated exclusively by nNOS in young, healthy subjects (Kellogg et al., 2008b). These same authors report that NO-dependent dilation in response to local heat is mediated exclusively by eNOS in young, healthy subjects (Kellogg et al., 2008a). Stewart et al. have reported nNOS mediated NO-dependent dilation in response to local heat in young subjects with postural orthostatic tachycardia syndrome (Stewart et al., 2007). These conflicting results may suggest redundancy in NO-dependent dilator pathways, and further investigation is warranted.

BH_4 acts as an essential cofactor for all constitutively expressed NOS isoforms by stabilizing the iron moiety of the oxygenase domain and maintaining the functional conformation of the NOS dimer (Raman et al., 1998). In the absence of BH_4, or when BH_4 is limited, the NOS dimer destabilizes and may uncouple disturbing the flow of electrons required for NO synthesis and resulting in the production of superoxide rather than NO (Cosentino and Luscher, 1999; Moens and Kass, 2007). Thus, NOS is highly dependent on adequate BH_4 availability for NO synthesis.

BH_4 bioavailability is decreased with primary aging (Delp et al., 2008; Sindler et al., 2009) potentially leading to NOS uncoupling. This uncoupling may be responsible in part for attenuated endothelium derived NO (Higashi et al., 2006). Our results support the proposed mechanism by which BH_4 administration increases reflex cutaneous VD in aged human skin through an augmentation in NOS coupling and increased NO synthesis through NOS. *In vivo* studies conducted on both animal models of vascular pathology and human populations that exhibit endothelial dysfunction have shown that increasing BH_4 availability improves endothelial function and increases NO-dependent VD in conduit vessels (Cosentino et al., 2008; Porkert et al., 2008). By administering BH_4 locally we were able to augment NO-dependent dilation and restore reflex cutaneous VD in the older subject group, suggesting that a similar effect can be seen in aging.

An unexpected finding of this study was the increased maximal CVC in the older BH_4 treated site. This increase in maximal CVC suggests that local BH_4 administration augments NO signaling and increases subsequent relaxation in aged vascular smooth muscle. Maximal cutaneous blood flow decreases as a function of age (Martin et al., 1995; Minson et al., 2002). Aged adults rely predominantly on NO-dependent dilation for

full expression of reflex VD in the heat, and interventions that increase substrate availability for NOS (Holowatz et al., 2006b) or reduce oxidant stress (Holowatz et al., 2006a) in the skin are capable of increasing NO-dependent VD and augmenting reflex VD in the aged cutaneous vasculature. However, none of these interventions have reported increased maximal CVC, suggesting that they do not improve vascular smooth muscle reactivity. Our data suggest that acute, local BH_4 administration increases vascular smooth muscle reactivity and provides evidence for a novel role of BH_4 in cutaneous VD.

BH_4 may affect smooth muscle reactivity to NO by acting as a potent antioxidant. BH_4 is capable of reducing peroxynitrate at a rate constant several fold higher than ascorbate (Gao et al., 2009). This may explain why increased maximal CVC was observed here, but has not been observed with localized ascorbate treatment. Additionally, oxidation of the soluble guanylyl cyclase (sGC) of the vascular smooth muscle cell results in a loss of enzyme activity and inactivates NO signaling (Lucas et al., 2000). Cumulative, BH_4 may affect smooth muscle reactivity to NO by maintenance of redox balance in the aged cutaneous vasculature.

Acute, local BH_4 administration increases reflex VD in aged skin. This augmentation is due to an increase in NO-dependent dilation, potentially through increased NOS coupling and NO production, and increased vascular smooth muscle reactivity. Although other interventions have successfully augmented NO-dependent VD, BH_4 administration is novel in its ability to increase vascular smooth muscle reactivity. This dual role of BH_4 in improved vascular function is novel, and further exploration is

required to fully elucidate its mechanistic role in smooth muscle reactivity and NO-dependent vasodilation.

Limitations:

Older subjects had a significantly higher LDL than younger subjects. This could suggest a possible effect of cholesterol. However, the older subject group had LDL and total cholesterol values below 120 mg/dl and 200 mg/dl respectively. These values classify the older subject group as non-hypercholesterolemic according to the guidelines set by the American Heart Association and do not indicate increased cardiovascular disease risk in this group.

One alternate explanation for our results is that the augmented reflex cutaneous VD in older subjects is due to the antioxidant properties of BH_4 independent of its role in NOS coupling/uncoupling status. ROS oxidize NO to peroxynitrite (Taddei et al., 2001). Reducing oxidant stress through local supplementation with ascorbate can augment the VD response in aged skin (Holowatz et al., 2006a). However, ascorbate has been shown to mediate its effects on VD through its protection and stabilization of the BH_4 molecule (Huang et al., 2000).

In summary, attenuated NO-dependent reflex VD in aged skin is likely due, in part, to an age-related decrease in BH_4. Local BH_4 administration increased reflex cutaneous VD by increasing NO-dependent VD and smooth muscle reactivity. This treatment, however, did not alter the VD responses in the young subject group. These data suggest that age associated reductions in BH_4 bioavailability may contribute to the attenuated reflex cutaneous VD response to heat stress seen in older humans.

Tables and Figures

Table 2.1 Subject Characteristics

Variable	Young	Older
Sex, M/F	7,4	5,6
Age, years	23 ± 1	73 ± 2 *
BMI, kg/m^2	25 ± 3	26 ± 1
Fasting blood glucose, mg/dl	89 ± 2	95 ± 4
LDL, mg/dl	72 ± 5	105 ± 9 *
HDL, mg/dl	53 ± 5	61 ± 4
SBP, mmHg	118 ± 4	126 ± 3
DBP, mmHg	77 ± 3	76 ± 2

Values are mean ± SEM for 11 young and 11 older subjects. HDL: high density lipoprotein, LDL: low density lipoprotein, BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure. * $p < 0.05$ significant difference compared to young subject group.

Table 2.2 Absolute Maximal CVC (SNP perfusion, 28mM + local heat, 43°C) Measured at Each Intradermal Microdialysis Site

Site	Maximal CVC
Control young	1.9 ± 0.2 *
Control older	1.4 ± 0.2
NOS-inhibited young	2.3 ± 0.3
NOS inhibited older	1.6 ± 0.2
BH_4 young	1.8 ± 0.2
BH_4 older	2.0 ± 0.2 *

Control: lactated ringers; NOS-inhibited: L-NAME; BH_4: tetrahydrobiopterin administration. *p <0.05 compared to older control.

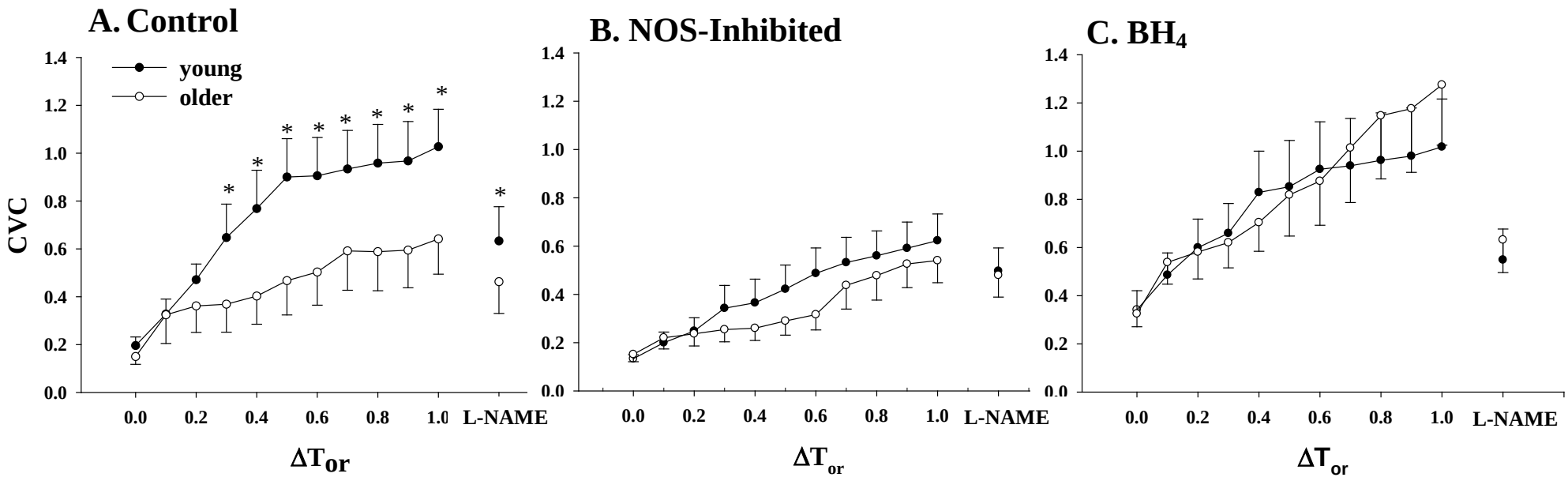

Figure 2.1 Group mean ± SEM of CVC response in young and older subjects during whole body heating (ΔT_{or}) and following NOS inhibition (L-NAME, 20mM) in control, NOS-inhibited and BH_4 treated sites. A: Control site. Older subjects have an attenuated vasodilatory response to increased core temperature compared to young beginning at a 0.3°C rise. B. NOS-Inhibited site. With NOS inhibition (L-NAME) young and older subjects have similar vasodilatory responses. C: BH_4 administered site. BH_4 administration augmented the vasodilation response in older subjects. * $p < 0.05$ significant difference between young and older within sites.

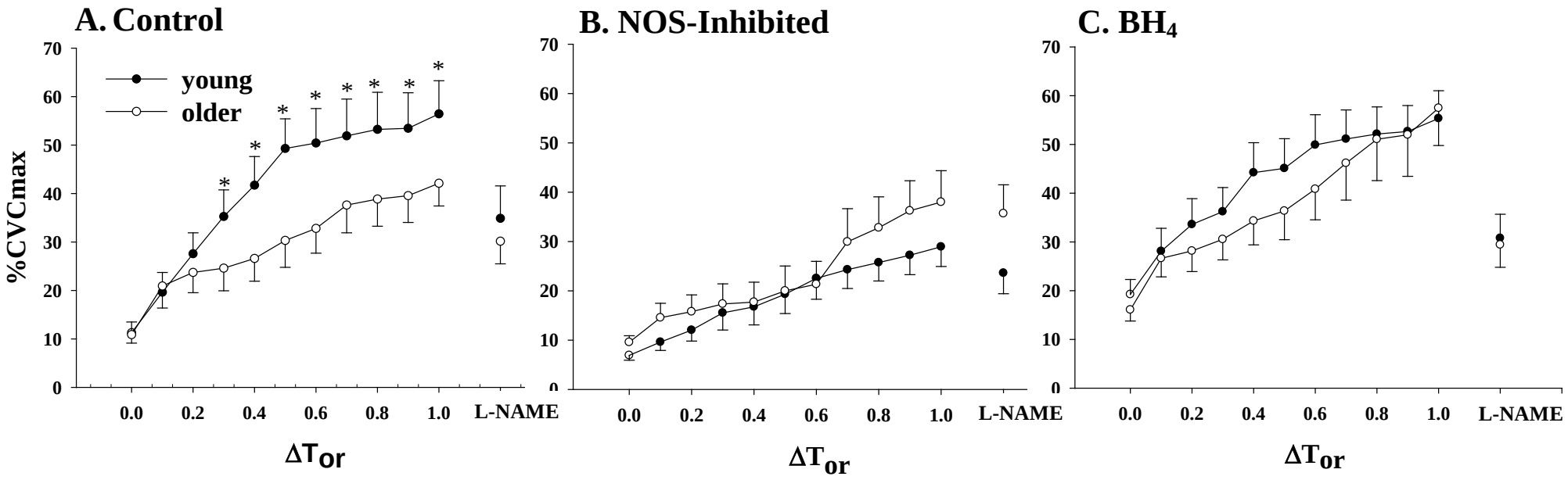

Figure 2.2 Group mean ± SEM of %CVCmax response in young and older subjects during whole body heating (ΔT_{or}) and following NOS inhibition (L-NAME, 20mM) in control, NOS-inhibited and BH_4 treated sites. A: Control site. Older subjects have an attenuated vasodilatory response to increased core temperature compared to young beginning at a 0.3°C rise. B. NOS-Inhibited site. With NOS inhibition (L-NAME) young and older subjects have similar vasodilatory responses. C: BH_4 administered site. BH_4 administration augmented the vasodilation response in older subjects. * $p < 0.05$ significant difference between young and older within sites.

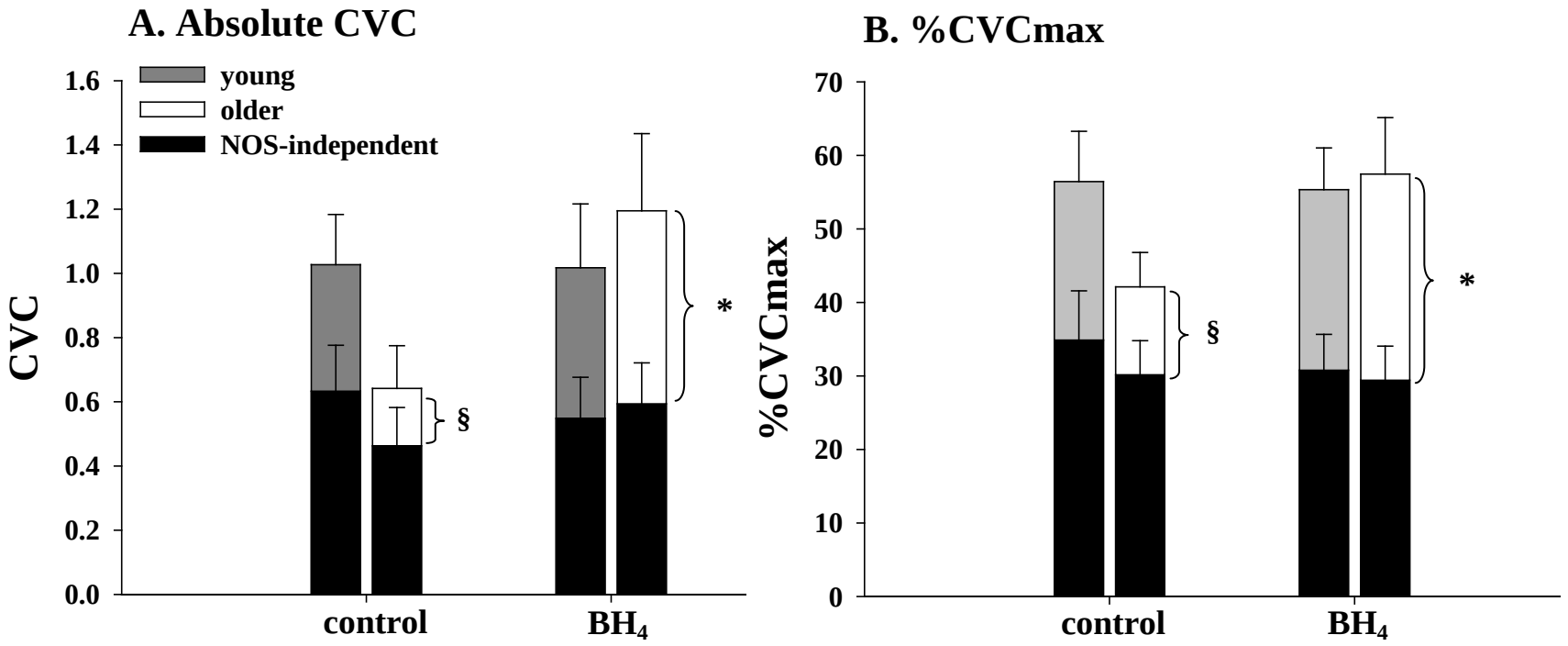

Figure 2.3 Group mean ± SEM of vasodilatory response in young and older subjects at 1°C rise in oral temperature in control and BH_4 administered sites. Panel A presents absolute CVC. Panel B presents %CVCmax. The complete bars represent mean peak response at 1°C rise in oral temperature. The black bars represent mean plateau response after NOS-inhibition (L-NAME, 20mM) at clamped 1°C rise in oral temperature. The mean decrease from peak response to L-NAME plateau represents NOS-dependent dilation at 1°C rise in oral temperature. Older subjects have an attenuated NOS-dependent dilation compared to young subjects at the control site. BH_4 administration increases NOS-dependent dilation in older subjects. § $p < 0.05$ significant difference compared to young control. *$p < 0.05$ significant difference compared to older control.

CHAPTER 3

ADDITIONAL DATA FROM CH. 2: LOCAL TETRAHYDROBIOPTERIN ADMINISTRATION COMBINED WITH ARGINASE INHIBITION

Introduction

In chapter 2 we showed that local tetrahydrobiopterin (BH_4) administration increases NO-dependent reflex vasodilation (VD) in aged human skin. In addition to cofactor availability, NO production and consequent VD depends on adequate substrate (L-arginine) availability. One potential mechanism limiting L-arginine for NO synthesis and full reflex cutaneous vasodilation in aged skin is through upregulation of vascular arginase. Local inhibition of arginase increases NO-dependent VD in aged skin (Holowatz et al., 2006b). BH_4 administration and arginase inhibition target different mechanisms that effect NO production, however both increase NO-dependent VD in aged skin.

Arginase catalyzes the conversion of L-arginine to L-ornithine in the last step of the urea cycle and is capable of limiting NO production by competing with NOS for the common substrate L-arginine (Berkowitz et al., 2003). When substrate is absent or limited the NOS dimer uncouples and produces superoxide (O_2^-) rather than functional NO (Munzel et al., 2005). Overall, increased arginase activity results in decreased NO production and increased O_2^- production by NOS.

Arginase is upregulated with primary aging and contributes to the decline in vascular function (Berkowitz et al., 2003; White et al., 2006; Santhanam et al., 2007).

Arginase inhibition restores NOS coupling in animal models of vascular aging (Kim, Bugaj et al. 2009) and increases NO-dependent reflex vasodilation in aged human skin (Holowatz et al., 2006b; Kim et al., 2009).

We hypothesized that acute arginase inhibition combined with administration of the essential NOS cofactor BH_4 would augment reflex VD in an additive manner by increasing NOS coupling through the essential cofactor BH_4 and increasing available L-arginine for NO synthesis by inhibiting arginase activity. To completely inhibit arginase we used a combination of (S)-(2-boronoethyl)-L-cysteine-HCl (BEC) a slow binding competitive inhibitor of arginase I and II, and N^{ω}-hydroxy-*nor*-L-arginine (nor-NOHA) a high-affinity inhibitor of arginase.

After initial data collection we found that arginase inhibition combined with BH_4 administration attenuated VD in the young subject group. One potential explanation for this finding was a negative interaction between the arginase inhibitors and the BH_4 that was leading to an impairment of VD signaling in the healthy cutaneous vasculature. We hypothesized that one of the arginase inhibitors was interacting with the BH_4 to decrease VD in the young subject group. Specifically, we thought that the boronic acid complex of the BEC may be oxidizing the BH_4. Therefore, additional data was collected from a subset of the young subject group (n=4).

Methods

Data presented in figure 3.1 were collected during the full study reported in chapter 2. Therefore all methodological procedures were the same as chapter 2. The only variation from these methods was the addition of a fourth microdialysis fiber, placed in the ventral forearm during the placement of the other three fibers. This additional fiber was perfused with a combination of 5mM (S)-(2-boronoethyl)-L-cysteine-HCl (BEC; Calbiochem, San Diego, CA), 5.0mM N^{ω}-hydroxy-*nor*-L-arginine (nor-NOHA; Calbiochem) and 10mM BH_4 (Sigma, St. Louis, MO) for simultaneous arginase inhibition and BH_4 administration. Following the insertion trauma resolution period, the whole body heating protocol was completed. Data are represented as a percent of maximal CVC (%CVCmax).

In order to examine possible interactions between the arginase inhibitors and BH_4, five intradermal microdialysis (MD) fibers were placed in the ventral forearm skin of 4 young (23±1 yr, 2 men, 2 women), healthy subjects who had completed the study presented in chapter 2. Each fiber was randomly assigned to receive either 1) lactated Ringer solution to serve as control, 2) 5mM BEC, 3) 10mM BH_4 + 5mM BEC, 4) 5mM nor-NOHA or 5) 10mM BH_4 + 5mM nor-NOHA. Following the insertion trauma resolution period, the whole body heating protocol was completed. All data were collected and analyzed according to the procedures described in chapter 2 and are presented as %CVCmax.

Results

Figure 3.1 shows the VD response to whole body heating (ΔT_{or}) and following NOS inhibition (L-NAME, 20mM) in control and arginase-inhibited + BH_4 administered sites in young and older subjects. Arginase inhibition combined with BH_4 administration attenuated VD in young subjects compared to their control site beginning at a 0.5°C rise. Arginase inhibition combined with BH_4 administration did not alter NO-dependent VD or absolute maximal CVC in young or older subject groups.

Figure 3.2 shows the VD response to whole body heating (ΔT_{or}) and following NOS inhibition (L-NAME, 20mM) in the control, BEC and BEC + BH_4 sites (panel A) and control, nor-NOHA and nor-NOHA + BH_4 sites (panel B) in a subset (n=4) of young subjects who had previously completed the study described in chapter 2. Neither arginase inhibitor alone or combined with BH_4 administration attenuated the VD response to whole body heating compared to the control site.

Discussion

The primary finding from this additional pilot study was that arginase inhibition using a cocktail of 5mM BEC + 5mM nor-NOHA combined with BH_4 administration attenuates reflex cutaneous VD in young, healthy human skin. Contrary to our original hypothesis, this combination of arginase inhibition and BH_4 administration did not augment VD in the older subject group. In a subset of young subjects, BH_4 combined with BEC alone or BH_4 combined with nor-NOHA alone did not attenuate the VD response to whole body heating.

One potential explanation for the observed attenuation in VD is the oxidation of BH_4 by one of the arginase inhibitors thereby converting BH_4 to dihydrobiopterin (BH_2), a competitive inhibitor of BH_4. BH_2 acts as an inhibitor of NOS by competing with BH_4 for the binding site but lacking the coupling and antioxidant properties of BH_4 (Moens et al., 2011). BH_2 can be reduced back to BH_4 by dihydrofolate reductase (DHFR), but in conditions where BH_4 is readily oxidized, the rate of oxidation can outstrip the activity of DHFR and the BH_4/BH_2 ratio decreases (Vasquez-Vivar et al., 2002). This ratio has been implicated in endothelial dysfunction (Crabtree et al., 2008; Takeda et al., 2009) and could be a possible mechanism by which VD was attenuated in the young subject group.

Another potential mechanism by which the arginase inhibitor combined with BH_4 could attenuate VD in young subjects is inhibition of one or more cotansmitters that mediate ~ 60 – 70% of the total reflex VD response. Prostaglandins, histamine, vasoactive intestinal peptide and the substance P have all been identified as contributors to the full expression of reflex cutaneous VD (Kellogg et al., 1995; Bennett et al., 2003;

Wong et al., 2004; McCord et al., 2006). Our project was not designed to examine specific cotransmitter(s) contributions; however the observation that the reduction in VD with NOS inhibition was not different between the control and combination sites suggests that perhaps the cotransmitter(s) contribution rather than NO synthesis by NOS was affected.

In summary, we found that arginase inhibition (5mM BEC + 5mM nor-NOHA) combined with BH_4 administration attenuated reflex cutaneous VD in young subjects. Further investigation is needed to examine the BH_4:BH_2 ratio and fully describe the mechanisms behind these data.

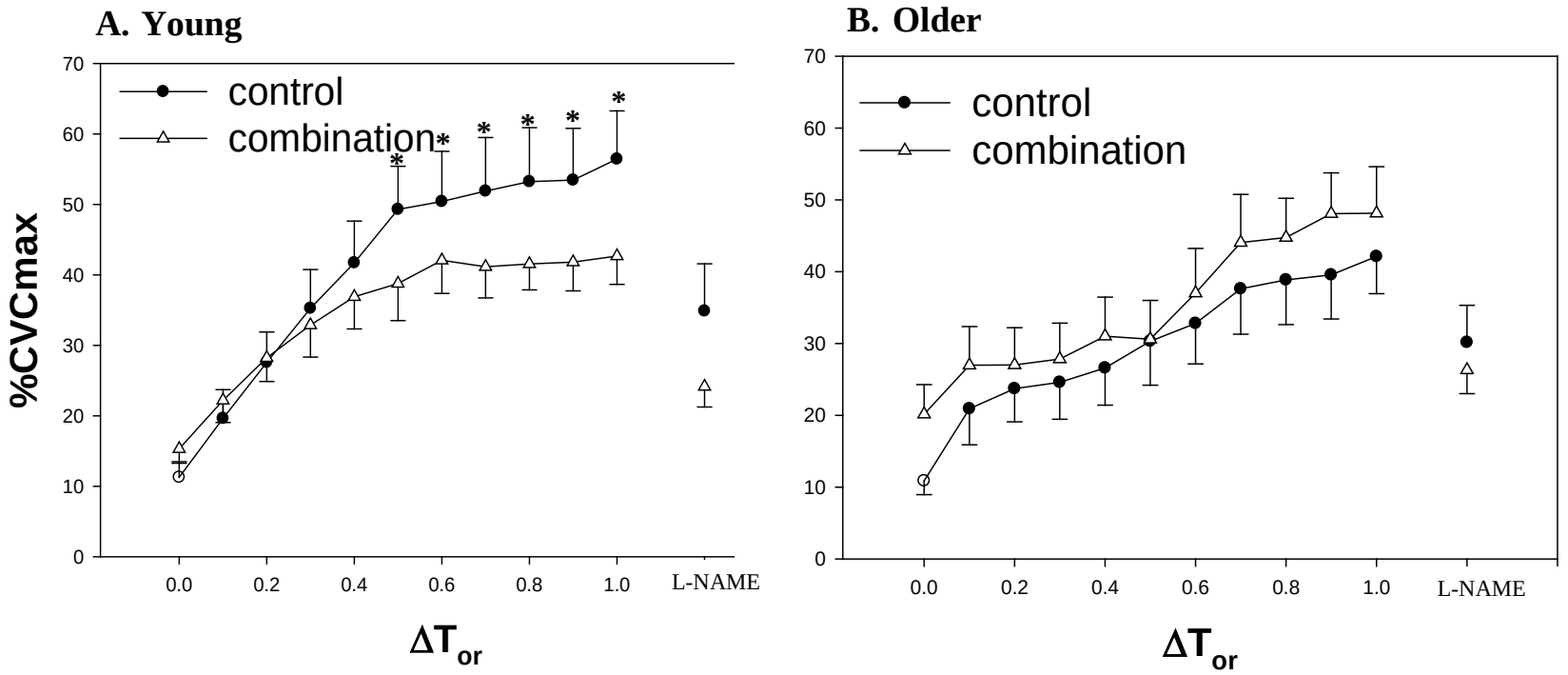

Figure 3.1 Group mean ± SEM of vasodilatory (%CVC max) response in young and older subjects during whole body heating (ΔT_{or}) and following NOS inhibition (L-NAME, 20mM) in control and combined arginase inhibited and BH_4 administered sites. Arginase inhibition combined with BH_4 administration attenuated %CVCmax response in young subjects beginning at 0.5°C rise in T_{or} (panel A). Arginase inhibition combined with BH_4 administration did not augment %CVCmax response in older subjects (panel B). * $p < 0.05$ significant difference between combination and control within subject groups.

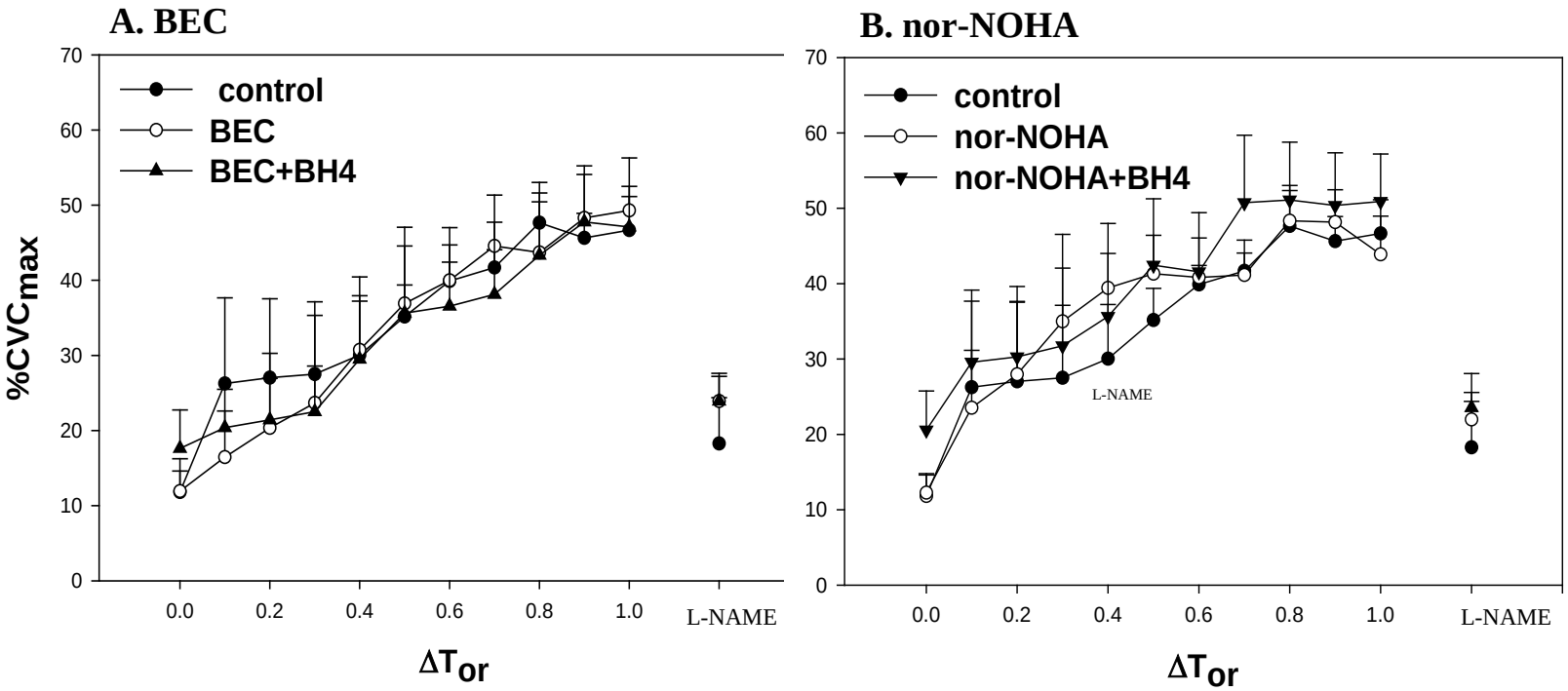

Figure 3.2 Group mean ± SEM of vasodilatory (%CVC max) response in young subjects (n=4) during whole body heating (ΔT_{or}) and following NOS inhibition (L-NAME, 20mM) in control, BEC and BEC + BH_4 administered sites (panel A) and control, nor-NOHA and nor-NOHA + BH_4 administered sites (panel B). Neither arginase inhibitor alone or combined with BH_4 administration attenuated the VD response to whole body heating compared to control.

Chapter 4

CONCLUSIONS AND FUTURE DIRECTIONS

The primary findings of this study were that local tetrahydrobiopterin (BH_4) administration 1) increased NO-dependent reflex cutaneous vasodilation (VD) and 2) increased maximal CVC in aged skin. In summary, these data suggest that acute, local BH_4 administration increases the reflex cutaneous VD response to passive whole body heating seen in older subjects.

Implications

Reflex cutaneous vasodilation and vasoconstriction are both impaired with primary aging (Grassi et al., 2003; Degroot and Kenney, 2007). BH_4 administration increases NO-dependent dilation and maximal CVC in aged skin. Acute, local BH_4 administration also augments cutaneous vasoconstriction (VC) induced by whole body cooling in older subjects through the proposed role of BH_4 as an essential cofactor for tyrosine hydroxylase (Lang et al., 2009). Taken together, these data suggest that BH_4 is essential for neurovascular control mechanisms mediating the control of skin blood flow during both hypothermia and hyperthermia.

Primary aging alters the central cardiovascular responses underlying human thermoregulatory control. During direct passive heating, older men exhibit an age-related reduction in SkBF associated with attenuated redistribution of blood flow away from the splanchnic and renal circulations (Minson et al., 1998). This reduced ability to

redistribute blood from the visceral circulation suggests a diminished vasoconstrictor stimulus and/or sensitivity in these vascular beds and fits into the model of decreasing vascular function with age. Increasing BH_4 bioavailability systemically may improve the age-related decline in central cardiovascular responses to heat stress through improved VD function in the cutaneous circulation and improved VC function in the visceral vascular beds.

Future Research Directions

1. Our results suggest that locally increasing BH_4 concentrations augments vasodilator function. In addition, local BH_4 administration augments cutaneous vasoconstriction induced by whole body cooling in older subjects through its proposed role as an essential cofactor for tyrosine hydroxylase and subsequent nor-epinephrine synthesis (Lang et al., 2009). Taken together, these data suggest that BH_4 may play a dual role in the control of skin blood flow. Therefore, strategies for easily increasing bioavailable BH_4 may be an important area of study. Oral supplementation with pharmacological preparations of BH_4 reduces blood phenylalanine concentrations in patients with BH_4 responsive phenylkatonuria by increasing the availability of BH_4, an essential cofactor for phenylalanine hydroxylase (Guttler et al., 1984). Additionally, oral supplementation with BH_4 improves vascular function in animal and human models of vascular dysfunction (Cosentino et al., 2008; Porkert et al., 2008). Taken together, these data suggest that oral supplementation strategies may be an

effective intervention strategy for improved microvascular function in the aged. Oral BH_4 supplementation may expand the range of blood flow to the aged cutaneous vasculature.

2. Primary aging is associated with increased oxidant stress due to increased production and decreased clearance of reactive oxygen species (Donato et al., 2007). In this highly oxidative environment BH_4 is rapidly oxidized to BH_2 which is unable to act as a cofactor for NOS activity and is instead a competitive inhibitor with BH_4. This diminished BH_4:BH_2 ratio is implicated in NOS uncoupling and has been suggested as a plausible explanation for endothelial dysfunction (Vasquez-Vivar et al., 2002; Takeda et al., 2009). This mechanism has yet to be examined in human skin. Biochemical analysis of skin punch biopsy samples may be used to quantify the decrease in BH_4:BH_2 ratios observed with aging. Additionally, pharmacological interventions that alter BH_4:BH_2 ratio through intradermal microdialysis may be used examine the role of diminished BH_4:BH_2 ratio on reflex cutaneous vasodilation in aged skin. Taken together, these techniques may elucidate the mechanisms underlying reduced BH_4:BH_2 ratio in the vasculature and examine their effects on the attenuated vasodilator response in aged human skin.
3. The role of BH_4 in smooth muscle reactivity to NO requires further exploration. One potential explanation for this improvement in reactivity may by stabilization of the soluble guanylyl cyclase (sGC) dimer through its heme domain (Lucas et al., 2000). sGC mediates downstream NO signaling for relaxation within the vascular smooth muscle cell (Collier and Vallance, 1989) and requires both α- and

β-subunits as well as the presence of a prosthetic heme group for activation of the sGC by NO (Craven and DeRubertis, 1978, 1983). Oxidation of the heme group results in the loss of enzyme activity and may explain, in part, attenuated maximal blood flow in aged skin. However, further study is required to address these mechanism(s). *Ex vivo* analysis of skin punch biopsy samples may elucidate age related changes in sGC receptor concentration and conformation while *in vitro* cell culture work may provide insight into the role of acute and chronic BH_4 treatment on sGC receptor conformation and reactivity to exogenous NO.

Appendix

Informed Consent

INFORMED CONSENT FORM FOR CLINICAL RESEARCH STUDY
The Pennsylvania State University

Title of Project: ***Mechanisms of the NO-contribution to reflex cutaneous vasodilation with age Part II***

Principal Investigator: **Lacy Holowatz, Ph.D.**
Address: 113 Noll Laboratory
University Park, PA 16802
Phone: 814-867-1781 Email: lma191@psu.edu

Co-Investigator: **W. Larry Kenney, Ph.D.**
Address: 102 Noll Laboratory
Phone: 814-863-1672

Research Assistant: **Susan Slimak, RN**
Phone: 814-863-8556, email: sks31@psu.edu
Jane Pierzga, M.S., Research Assistant
Phone: 814-865-1236

This is to certify that I, _____ have been given the following information with respect to my participation as a volunteer in a program of investigation.

Note: This study had 2 parts. We have completed Part 1. Part 2 of the study is similar to that of Part 1. However, some of the substances used in Part 2 are different.

1. <u>Purpose of the study:</u> When you are exposed to the heat, nerves in your skin make natural chemicals that cause the skin's blood vessels to get bigger. This increases the amount of blood flowing through those vessels. This increased flow helps to cool your body. As you age, you cannot increase the blood flow in your skin as well as when you are younger. So, aging can make you more prone to illness in extreme heat. This study looks at whether the change in skin blood flow with age is due to the actions of those natural chemicals. To do this, we use "microdialysis" (MD). This technique involves placing very thin plastic tubing between the layers of your skin. The largest part of the tubing is about 6x the diameter of a human hair. We pump fluid like that found in your body's tissues through the tubing. The tubing acts like very small blood vessels in your skin by allowing some substances to pass between the fluid in the tubing and the fluid in your skin. During the experiment, we will add substances to the fluid in the tubing. The substances can only reach a 2.5 cm^2 (0.4 $inch^2$), nickel-sized area of skin at each tube. These substances are like some natural chemicals found in your body. The substances are:

1. BH_4 – *((6R)-5,6,7,8-Tetrahydrobiopterin dihydrochloride*) – a natural substance found in your cells. BH_4 shuts down waste products that produce reactive oxygen molecules in cells. It may also help blood vessels to get bigger.

2. L-NAME (*N^G-nitro-L-arginine methyl ester*) – like a natural protein found in your cells. It stops chemical reactions that involve that protein.
3. BEC (*(s)-(2-boronoethyl)-L-cysteine-HCl*) – like a natural protein found in your cells. It stops chemical reactions that involve that protein.
4. nor-NOHA (*N-hydroxy-nor-L-arginine*) – like a natural protein found in your cells. It stops chemical reactions that involve that protein.
5. SNP (*sodium nitroprusside*) causes your blood vessels to get as large as they can.

Also, We measure the flow in vessels near the tubing by shining a weak laser light onto your skin.

2. Procedures: ***You will participate on the circled days. Please read the descriptions of the circled days.***

Then write your initials by the circled days. You may request personnel of the same gender to perform procedures.

_____ initial Screening Day 1: You do not eat or drink after midnight during the night before your exam. You report to the Noll Lab for your appointment. When you arrive, the staff draws 15 ml (1 Tbsp) of blood from a vein in your arm. If you take thyroid medicine, we draw 3.5 ml (0.2 Tbsp) more blood for a thyroid test. We send the blood sample to a lab to see if the proteins, blood cells, electrolytes, etc. are within normal levels. After the blood draw, we give you juice and a snack bar. You have an examination by the medical staff that includes blood pressure, check-up, height, weight, and 12-lead ECG**.** We send the blood sample to a lab that tests it for wellness markers. The lab destroys the sample after testing it. If you are a woman of childbearing-age, you submit a urine sample for a pregnancy test. We may measure the thickness of folds of skin at several places on your body to determine your percent body fat.

_____ initial Screening Day 2: You report to the Noll Lab for a medical history and graded exercise test (GXT). Bring clothes in which you can exercise. You may use clothing we provide. We measure your blood pressure and the electrical activity of your heart. During the test, you wear a nose clip and breathe into a tube to measure the oxygen and carbon dioxide you breathe out. The researcher adjusts the harness that holds the tube so that you are comfortable. During the test, you rate how hard you are working by using a numbered scale matched to short phrases (rating of perceived exertion or RPE scale). For the GXT, you exercise on a treadmill (or bike) to measure your fitness level. You will walk if you are in the older group or run if you are in the young group. The treadmill's grade (or bike's resistance) increases a little every 2 minutes. The exercise becomes harder. The test is most accurate if you do your best to exercise as long as you can. However, you can stop whenever you want to stop. The test is 10-20 minutes long.

_____ initial "Role of BH_4" During the experiment, men wear shorts. Women wear shorts and a sports bra. We can provide this clothing. When you arrive at the laboratory, we measure your blood pressure and heart rate then you wash your forearm and pat it dry. We tape 6 wires to your skin that measure skin temperatures. Also, we tape three ECG leads to your chest to measure heart rate. Then you don a suit that has tubing lining the inside and lie down. Water that is 33°C (91.4°F) flows through the suit's tubing. Then we prepare the MD sites on your arm.

Microdialysis (MD): We place a tight band around your upper arm so your veins are easily seen. We make pairs of pen-marks on your arm 2.5 cm (1 inch) apart and away from veins. The MD

tubing will enter and exit your skin at the marks. We remove the tight band. We clean your arm with an orange-colored Betadine fluid and alcohol. We place an ice bag on your arm for 5 minutes to numb your skin. Then we insert a thin needle into your skin at each entry mark. The needle's tip travels between the layers of skin for 2.5 cm (1 inch) and leaves your skin at the matching exit mark. The tubing is threaded through the needle. Next, we withdraw the needle leaving the tubing in your skin. You will have up to 5 sets of tubing in your skin. Any redness of your skin subsides in about 60 minutes. We tape a pencil-sized probe over each site where there is thin tubing in your skin. Then we start the plain fluid (Lactated Ringer's solution) flowing through the tubing in your skin. When the redness on your arm is gone, the study begins.

During the experiment, we measure:
Skin Blood Flow: We place pencil-sized probes over the tubing in your skin. The probes use a weak laser light to measure blood flowing in the small vessels at those sites.
Skin Temperature: We tape 6 wires to your skin: (calf, thigh, abdomen, chest, back, and upper arm)
ECG and Heart Rate: We place 3 sticky disks on your chest to measure your heart's rate and electrical activity.
Blood Pressure: We may use any of the following methods. One method uses a cuff that inflates on your upper arm while the researcher listens with a stethoscope at the inside of your elbow. The other method uses a machine that automatically inflates a cuff on your arm and takes a reading. We may use more than one method to make sure that we get a good reading.
Forearm Blood Flow: We place a blood pressure cuff around your wrist and upper arm. We place a strain gauge that looks like a rubber band around your forearm between the cuffs. During the experiment, we perform a measurement every 10 minutes for about 3 minutes. For the measurement, the wrist cuff inflates to stop blood flow to your hand. The upper arm cuff inflates allowing blood flow into your arm while blocking blood flowing out. This causes a slight increase in the size of your forearm that can be seen by the gauge. During each measurement, the wrist cuff remains inflated while the upper arm cuff switches 6 times between inflation and deflation. Then both cuffs are deflated.
Body Temperature: We place a wire under your tongue to measure your body's temperature.

When the experiment begins, you will rest for 20 minutes. Then we add the test-substances to the plain fluid running through the tubing.
Probe 1. Lactated Ringer's only
Probe 2. Lactated Ringer's + L-NAME
Probe 3. Lactated Ringer's + BEC + nor-NOHA
Probe 4. Lactated Ringer's + BH_4
Probe 5. Lactated Ringer's + BEC + nor-NOHA + BH_4

After 60 minutes, we perform another 20-minute baseline and set of measurements. We collect the fluid exiting the MD tubing in you skin. Later, we test this fluid for a substance that shows us to what extent the cells have reactive oxygen present. If any of the fluid remains after testing, we destroy the fluid 2 years after we publish the study. Then we increase the temperature of the water flowing through the suit to 48°C (118.4°F). The heating continues until your body's temperature rises 1.0°C (1.8°F) or until you wish to stop. We collect the fluid exiting the MD tubing during the heating phase, too. Your skin blood flow at the MD sites rises and then stays at the higher level. When the skin blood flow is stable at the new level, we add L-NAME to the fluid running through probes 1, 3, 4, and 5. We will turn down the temperature of the water flowing through the suit to 45°C (113°F). Heating ends when the skin blood flow at probes 1, 3, 4

and 5 is stable at a new level or you wish to stop. When heating ends, cooler water 22°C (71.6°F) flows through the suit's tubing to cool you quickly. Then 33°C (91.4°F) flows through the suit's tubing to keep your comfortable. Also, we stop the flow of test substances through the MD tubing. Lastly, Lactated Ringer's + SNP will flow through all tubing and we heat the laser probes' holders to 43°C (109.4°F) for 30 – 45 minutes. This creates the greatest amount of blood flow possible. Then the study ends. The places where the tubing enters and exits your skin will be cleaned with alcohol, and the tubing will be pulled from your skin. A sterile bandage will be placed over the sites where the tubing was in your skin. We place a bag of ice on your arm for 10 minutes to reduce any bruising that may occur. We measure your blood pressure and heart rate before you leave.

3. Discomforts and risks:

Graded Exercise Test (GXT): You will likely have tiredness, sweating, and breathlessness. You will also have increased heart rate and muscle fatigue. You may also have lightheadedness, fainting, nausea, or muscle cramp, but these occur less frequently. More severe reactions include irregular heartbeat, chest pain, heart attack (< 0.05%), and death (< 0.02%). Severe reactions are rare. We monitor you closely during the test.

Treadmill (or bike): A bike may be used if you are unable to use the treadmill. It is possible for you to stumble or fall on the treadmill (or bike) leading to cuts, scrapes, dislocations, broken bones, head injury, abnormal heart rhythms, or even death. We will tell you the safe use of the treadmill (or bike) and watch you closely during the test. We make all changes in speed slowly, and assist you on and off the treadmill (or bike).

Microdialysis: The risks are less than that for a blood draw because microdialysis uses only a small, localized area of skin. In contrast, a blood draw involves not only skin, but also large blood vessels and blood. You will probably experience some pain and bruising like that from a blood draw. However, we use ice to numb your arm during the insertion of the tubing. Also, the small needle reduces pain during placement of the tubing. You will probably not have pain after the tubing is in place. You may feel a little pain when the tubing is removed from your skin. You may become lightheaded or may faint. Sometimes the tubing can break during removal from your skin. Then we remove the tubing by pulling on the other end of it. This produces no additional risk for you. The tubing could break so that a small piece is left under your skin. This has not occurred in any of our studies. If this happened, we would treat any tubing remaining in your skin like a splinter. The thin layer of skin over the tubing may have to be cut to allow removal. Mild pressure with sterile gauze stops any slight bleeding that may occur. Infection is possible. Sterile techniques and supplies like those used in a hospital keep the risk minimal. We apply a sterile bandage after the experiment. We tell you how to take care of the site.

Fluid flowing through the tubing: The substances flowing through the tubing only go to a 2.5 cm^2 (0.4 $inch^2$) area of skin at each tubing site. The amount that enters the skin is very small. However, there is a chance of your having a bad reaction to the substances. This reaction could produce redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or collapse. If a bad reaction should occur, medical help will be summoned right away.

Lactated Ringer's Solution: This fluid is similar to the natural fluids in your skin. This fluid contains salt, potassium, lactate, and chloride. The acid content is like that of your body's fluids. A bad reaction to this fluid is highly unlikely.

L-NAME, BH_4, BEC, nor-NOHA, and SNP: Only minute amounts of these substances enter the nickel-sized area of skin around the MD tubing. We and other researchers have used these substances (L-NAME, BEC, nor-NOHA, BH_4, SNP) in human skin. There have been no reports of bad reactions.

Laser Doppler Flowmetry: Weak lasers can hurt your eye if you stare into the light for a long time. We do not turn on the laser until the probes are taped to a surface. The tape may irritate your skin.

Skin Temperature: The wires taped to your skin are not harmful, but the tape may irritate.

Body Temperature: We place a plastic-coated wire placed under your tongue. There is a small chance that it could irritate your mouth. Also, you could become tired from holding the wire in place. We can tape the wire to your face to help you. The tape could irritate your skin.

Blood Pressure (Manual or CardioCap 5): We measure your blood pressure using the method common in a doctor's office and/or with a CardioCap 5 machine. Both methods use a cuff that inflates on your upper arm. As the cuff slowly deflates, we listen with a stethoscope at the bend in your elbow or the CardioCap 5 takes a reading. During the short time we inflate the cuff, your arm may feel numb or tingly.

Forearm Blood Flow: Your arm and wrist may feel numb when the cuffs are inflated, and the cuffs may cause temporary bruising.

ECG: This machine measures the electrical activity of your heart. You will have 3-12 wires from the machine taped to spots on your chest. There have been no adverse effects. The tape may irritate your skin.

Betadine: Hospitals and researchers use this orange-colored fluid to clean and sterilize the skin. You could have a bad reaction to Betadine if you are allergic to iodine. You will inform us if you have these allergies so that we will use alcohol instead. A bad reaction could cause redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or collapse.

Latex: Some gloves and medical materials are made of latex rubber. You will inform us if you are allergic to latex and decline to participate in the study.

Blood Draw: Blood draws often cause mild pain, bruising, swelling, or bleeding. There is also a slight chance of infection or a small clot. You may become lightheaded or may faint. To keep the chance of infection minimal, the staff uses the same techniques used in hospitals.

Ratings of Perceived Exertion (RPE) Scale: During the GXT, we use this scale to assess how hard you feel you are working. The numbers on the scale refer to descriptions of effort. The only correct answers are the one that truly describe what you are feeling.

Local Heating: We measure the temperature of your skin under the holders. The skin will feel very warm but will not hurt. The heating will make the skin of your arm under the holders red like when you take a hot bath. The redness will not last more than several hours. Some people may be more sensitive to the heating than others. If your arm feels too hot, you will tell us, and we will reduce or stop the heating.

Whole Body Heating: You will feel very warm and will sweat. Although you will be lying down and bad reactions are unlikely, body heating can possibly cause tiredness, cramps, quick shallow breathing, an unsteady breathing pattern, lightheadedness, heart trouble, chest pain, or feeling sick to your stomach. We watch you closely, and remind you to keep us aware of how you feel. The heating part of the experiment ends, and we cool you right away if we observe these or other related signs.

Skin Fold Measurements: Your percent body fat is measured using a tool that looks like tongs. The tongs gently measure the thickness of skin folds at several places on your body.

4. a. Benefits to me: You will receive a medical screening that could inform you about your health. You could gain some knowledge about how your body works.

b. Potential benefits to society: This study can find some of the changes that impair the body's resistance to heat stress with age. These results could suggest ways to prevent or treat the changes that make older people prone to heat illness. This could help to prevent heat illness and death in older people. Also, as more people grow older, their health concerns have a greater impact on society. Also, the project helps to provide important experience, education, and degree-work for students of Penn State.

5. Alternative procedures that could be utilized: The procedures used in this study are used in many other research labs around the world. The procedures are the best ways to explore the questions and accomplish the goals of this research.

6. Time duration of the procedures and study: You will need to visit the Noll Lab on for the following:

_____ initial Day 1 is for the first part of the screening that should last no longer than 1/2 hour.

_____ initial Day 2 is for the 2nd part of the screening that should last no more than 1 hour.

_____ initial Day 3, "Role of BH_4": 5 hours.

7. Statement of confidentiality: Volunteers are coded by an identification number for statistical analyses. All records are kept in a secure location. All records associated with your participation in the study will be subject to the usual confidentiality standards applicable to medical records (e.g., such as records maintained by physicians, hospitals, etc.), and in the event of any publication resulting from the research no personally identifiable information will be disclosed. The Office of Human Research Protections in the U.S. Department of Health and Human Services, the U.S. Food and Drug Administration (FDA), The Penn State University Office for Research Protections (ORP) and The Penn State University Institutional Review Board may review records related to this project.

8. Right to ask questions: Please contact Lacy Holowatz (W: 814-867-1781, H: 814-880-9217), Susan Slimak (W: 814-863-8556, H: 814-237-4618), or Jane Pierzga (W: 814-865-1236,

H: 814-692-4720) with questions, complaints, or concerns about this research. You can also call this number if you feel this study has harmed you. If you have any questions, concerns, or problems about your rights as a research participant or would like to offer input, please contact Penn State University's Office for Research Protections (ORP) at (814) 865-1775. The ORP cannot answer questions about research procedures. Questions about research procedures can be answered by the research team.

9. Compensation: You will receive a lab T-shirt.
You will receive $10.00 for each of the 5 MD probes inserted in your arm (maximum $50.00). You will receive $20.00 more for completing the study.
Total = $70.00
You are paid an amount of money equal to the part of the experiment that you complete. For instance, if you complete only half of the experiment you will be paid for each probe that was inserted plus $10.00. This is because $10.00 is one half of $20.00. You may be asked to repeat the experiment. If you agree to repeat the experiment, you will be paid for the repeated experiment as stated above.
Total payments within one calendar year that exceed $600 will require the University to annually report these payments to the IRS. This may require you to claim the compensation that you receive for participation in this study as taxable income.

10. Injury Clause: In the unlikely event you become injured as a result of your participation in this study, medical care is available. Please call Lacy Holowatz (W: 814-863-2948, 814-880-9217) or Jane Pierzga (W: 814-865-1236, H: 814-692-4720). It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

11. Voluntary participation: Your being in this study is voluntary. You may withdraw from this study at any time by telling the researcher. If you decide to withdraw, you will not have a penalty or loss of benefits you would receive otherwise. You may decline to answer certain questions. You may decide not to comply with certain procedures. However, your being in the study may be contingent upon answering these questions or complying with the procedures. The researcher may end your role in the study without your consent if the researcher deems that your health or behavior adversely affects the study or increases risks to you beyond those approved by the Institutional Review Board and agreed upon by you in this document. You have been given an opportunity to ask any questions you may have, and all such questions or inquiries have been answered to your satisfaction.

12. In the event that abnormal test results are obtained, you will be apprised of the results immediately and advised to contact a health care provider for follow-up.

If your blood pressure is above normal, we will advise you to inform a health care provider. High blood pressure is a condition that can develop over many years, and you have may had this condition for a long time. You will need health care for this condition. You may wish to talk with a medical provider of your choice before being in our study. We will give the results of our measurements to you or, upon your request, send them to your doctor.

You must be 18 years of age or older to take part in this research study. If you agree to take part in this research study and the information outlined above, please sign your name and indicate the date below.

You will be given a copy of this signed and dated consent form for your records.

_____

Volunteer **Date**

I, the undersigned, have defined and explained the studies involved to the above volunteer.

_____

Investigator **Date**

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