The Pennsylvania State University

The Graduate School

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REFLEX CUTANEOUS VASODILATION: INFLUENCES OF PRIMARY HUMAN AGING AND HYPERTENSIVE VASCULAR PATHOLOGY

A Thesis in

Kinesiology

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

May 2007

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ABSTRACT

Reflex cutaneous vasodilation is attenuated with primary human aging and hypertensive vascular pathology, rendering these populations more vulnerable to complications from heat-related illness. Full expression of reflex cutaneous vasodilation is dependent on functional nitric oxide (NO), which is reduced with primary human aging and hypertensive vascular pathology. NO bioavailability may be decreased by (1) upregulated arginase (Arg) activity, which reciprocally regulates the NO-synthase (NOS) substrate L-arginine (L-arg) and/or (2) increased oxidant stress. Furthermore, there may be a mechanistic link between upregulated Arg activity and increased oxidant stress through NOS uncoupling. Therefore, the purpose of this series of experiments was to investigate the underlying mechanisms mediating impaired reflex cutaneous vasodilation in aged and hypertensive cutaneous vasculature.

In the first study we investigated age-related alterations in exogenous acetylcholine-induced vasodilation. While acetylcholine is capable of modulating reflex-mediated vasodilation, the precise mechanisms through which acetylcholine induces vasodilation and whether those downstream mechanisms change with aging are unclear. We tested the hypotheses that both NO- and prostanoid-mediated pathways contribute to exogenous acetylcholine-induced vasodilation and that both are attenuated with advanced age. Twelve young (Y: 23±1 yr) and 10 older (O: 69±1 yr) subjects underwent infusions of 137.5 μM acetycholine at 4 intradermal microdialysis (MD) sites: control (Co, Ringer's), NO synthase inhibited (NOS-I, 10 mM L-NAME), cyclooxygenase inhibited (COX-I, 10 mM ketorolac) and NOS-I+COX-I. For all studies red blood cell flux was monitored using laser-Doppler flowmetry and cutaneous vascular conductance (CVC) was calculated (laser-Doppler flux/MAP) and normalized to maximal CVC (%CVC_{max}) (28mM sodium nitroprusside + local heating to 43°C). Baseline %CVC_{max} was increased in the older at COX-I sites (COX-I 16±1, NOS-I+COX-I 16±2 vs. Co 10±1 %CVC_{max}; $p<0.001$) but not in the young, suggesting an age-related shift toward COX-derived vasoconstrictors contributing to basal cutaneous vasomotor tone. There was no

difference in peak %CVC_{max} during acetylcholine infusion between age groups and the response was unchanged by NOS-I (O: NOSI 35±5 vs. Co 38±5 %CVC_{max}; p=0.84) (Y: NOSI 41±4 vs. Co 39±4 %CVC_{max}; p=0.67). COX-I and NOS-I+COX-I attenuated the peak CVC response to Ach in both groups (COX-I Y: 22±2 vs. O: 29±3 %CVC_{max} vs. Co; p<0.001 both groups; NOS-I+COX-I Y: 29±2 vs. O: 32±3 %CVC_{max} vs. Co; p<0.001 both groups). We concluded that acetylcholine directly induces cutaneous vasodilation through prostanoid and non-NO-, non-prostanoid-dependent pathways. Further, older subjects have a diminished prostanoid contribution to acetylcholine-induced vasodilation.

In the second study we hypothesized that increased Arg activity contributes to attenuated vasodilation in aged skin by limiting L-arg for NOS-mediated NO synthesis. Five MD fibers were placed in forearm skin of 10 young (Y: 23±1 years) and 9 older (O: 68±1 years) human subjects serving as Co, NOS-I, Arg-inhibited (Arg-I: 5.0mM (S)-(2-boronoethyl)-L-cysteine + 5.0mM N-hydroxy-nor-L-arginine), L-arg supplemented (L-arg: 10.0mM L-arginine) and combined Arg-inhibited + L-arg sites. For all whole body heating studies reflex vasodilation was induced by using a water-perfused suit to increase oral temperature (T_{or}) 0.8-1.0°C. Cutaneous vasodilation during heating was attenuated in the older subjects (Y: 42±1 vs. O: 30±1 %CVC_{max}, p<0.001) at Co sites. NOS inhibition decreased vasodilation in both age groups compared to Co (Y: 22±2, O: 18±2 %CVC_{max}, p<0.001). Arg inhibition, L-arg supplementation, and Arg inhibition + L-arg supplementation augmented vasodilation in the older subjects (Arg-inhibited: 46±4, L-arg: 44±4, Arg-inhibited + L-arg: 46±5 %CVC_{max}, p<0.001 vs. Co) but not in young subjects (Arg-inhibited: 46±4, L-arg: 38±4, Arg-inhibited + L-arg: 44±4 %CVC_{max}, p>0.05 vs. Co). Increasing L-arg for NO synthesis by either Arg inhibition or direct L-arg supplementation abolishes the age-related deficit in reflex cutaneous vasodilation.

The third study tested the hypothesis that age-related increases in oxidant stress attenuates reflex cutaneous vasodilation, therefore acute antioxidant administration alone and combined with Arg inhibition to increase L-arginine availability would increase reflex cutaneous vasodilation in aged skin. Eleven young (Y, 22±1 years) and 10 older

(O, 68±1 years) men and women were instrumented with four intradermal MD fibers. MD sites served as Co, NOS-I, L-ascorbate supplemented (Asc: 10mM L-ascorbate), and Asc + Arg-inhibited (AA+Arg-I: 10mM L-ascorbate + 5mM BEC + 5mM nor-NOHA). Cutaneous vasodilation during heating was attenuated in the older subjects (Y: 37±3 vs. O: 28±3 $\%CVC_{max}$, $p<0.05$). NOS-I significantly decreased vasodilation in both groups compared to the Co site (Y: 20±4, O: 15±2 $\%CVC_{max}$, $p<0.05$ vs. Co within group). Asc and Asc + Arg-I did increase vasodilation beyond Co in the older subjects (Asc: 35±4 $\%CVC_{max}$, Asc + Arg-I: 41±3 $\%CVC_{max}$; $p<0.001$) but did not in the young subjects (Asc: 36±3 $\%CVC_{max}$; Asc + Arg-I: 40±5 $\%CVC_{max}$, $p>0.05$). Combined Asc + Arg-I resulted in a greater increase in vasodilation than did Asc alone in the older subjects ($p=0.001$). Acute Asc supplementation increased reflex cutaneous vasodilation in aged skin. When combined with Arg inhibition to increase L-arg availability Asc supplementation resulted in a further increase in vasodilation above Asc alone, effectively restoring cutaneous vascular conductance to the level of young subjects.

In the fourth study we tested the hypothesized that NO-dependent vasodilation would be attenuated in essential hypertensive human skin due to upregulated Arg activity and acute Arg inhibition or L-arg supplementation would augment reflex cutaneous vasodilation. Five MD fibres were placed in skin of 8 unmedicated subjects with essential hypertension (HTN: MAP: 112±1 mmHg) and 9 age-matched normotensive (AMN) (MAP: 87±1 mmHg) men and women to serve as: Co, NOS-I, Arg-I, L-arg supplemented, and combined Arg-I + L-arg. The $\Delta\%CVC_{max}$ between the Co and NOS-I site was calculated as the difference between Co and NOS-I sites. Maximal CVC was attenuated in the HTN subjects by ~25% compared to AMN subjects ($p<0.001$). With a 1.0°C increase in body core temperature (T_{or}) $\%CVC_{max}$ was not different between the groups (HTN: 43±3 vs. AMN: 45±3$\%CVC_{max}$, $p>0.05$). NOS-I significantly decreased $\%CVC_{max}$ in both groups but $\%CVC_{max}$ was greater in the HTN group (HTN: 32±4 vs. AMN: 23±3$\%CVC_{max}$, $p<0.05$). Arg-I alone augmented $\%CVC_{max}$ only in the HTN group (HTN: 65±5 vs. AMN: 48±3 $\%CVC_{max}$, $p<0.05$). L-arg alone did not effect $\%CVC_{max}$ in either group (HTN: 49±5 vs. AMN: 49±3 $\%CVC_{max}$, $p>0.05$). Combined

Arg-I + L-arg augmented %CVC_{max} in both subject groups compared to their respective control sites (HTN: 60±7 vs. AMN: 61±3 %CVC_{max}, both $p<0.05$ vs. respective control sites). The Δ %CVC_{max} between the control and NOS-I sites was attenuated at $\Delta T_{or}>0.5°C$ in the HTN group ($p<0.001$ vs. AMN). Vasodilation is attenuated with HTN due to decreased NO-dependent vasodilation and can be augmented with Arg inhibition but not L-arg supplementation, suggesting that Arg is upregulated with HTN.

In the fifth study we tested the hypothesis that increased oxidant stress contributed to attenuated NO-dependent reflex cutaneous vasodilation in hypertensive human subjects, further antioxidant supplementation, alone and combined with Arg inhibition would augment NO-dependent cutaneous vasodilation. Nine unmedicated subjects with essential hypertension (HTN: MAP=112±1mmHg) and 9 age-matched normotensive (AMN: MAP=81±10) men and women were instrumented with 4 intradermal microdialysis (MD) fibers: Co, NOS-I, Asc supplemented, and Asc + Arg-I. Oral temperature (T_{or}) was increased by 0.8°C via a water-perfused suit and L-NAME was then perfused through all MD sites to quantify the change in vasodilation due to NO. Cutaneous vasodilation was attenuated in essential hypertensive skin (HTN: 35±3 vs. AMN: 42±4 %CVC_{max}, $p<0.05$). Asc and Asc+A-I augmented cutaneous vasodilation in HTN (Asc: 57±5, Asc + A-I: 53±6 %CVC_{max}, $p<0.05$ vs. Co), but not AMN. %CVC_{max} after NOS-I in the Asc and Asc + A-I was increased in HTN (Asc: 41±4, Asc + A-I: 40±4 vs. Co: 29±4 %CVC_{max}; both $p<0.05$) and was further decreased in HTN (Asc: -19±4, Asc + A-I: -17±4 %CVC_{max}, $p<0.05$ vs. Co) but not AMN. Antioxidant supplementation alone or combined with Arg inhibition augments attenuated reflex cutaneous vasodilation in HTN skin through NO- and non-NO-dependent mechanisms.

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LIST OF ABBREVIATIONS

Acetylcholine	Ach
Analysis of Variance	ANOVA
Ascorbate	Asc
Arginase	Arg
Arginase-Inhibited	Arg-I
Body Core Temperature	T_c
Cutaneous Vascular Conductance	CVC
Cutaneous Vascular Conductance % maximum	$\%CVC_{max}$
Cyclooxygenase	COX
Cyclooxygenase-Inhibited	COX-I
Forearm Blood Flow	FBF
Forearm Vascular Conductance	FVC
L-arginine	L-arg
Laser-Doppler Flux	LDF
Mean Arterial Pressure	MAP
Intradermal Microdialysis	MD
Nitric Oxide	NO
Nitric Oxide Synthase	NOS
Nitric Oxide Synthase-Inhibited	NOS-I
Oral Temperature	T_{or}
Reactive Oxygen Species	ROS
Skin Blood Flow	SkBF
Skin Temperature	T_{sk}

ACKNOWLEDGEMENTS

There are many people that deserve a special thank you for their role in the studies presented in this dissertation and in my scientific training. I have been extremely fortunate to have been able to work with a spectacular team of creative professionals who are always willing to lend a helping hand. They have supported and encouraged me throughout the highs and lows of this endeavor, mentored me, offered critical feedback, and above all have always been there as a sounding board. I would not have been able to complete the studies that comprise this dissertation without their hard work, and I would not be the scientist and person I am today without them.

To my academic advisor and mentor, Dr. Larry Kenney, you have truly exemplified what it is to be a mentor. You have given me the opportunity and the means to develop into a successful scientist. You taught me the importance of generating creative hypothesis-driven scientific ideas and how to clearly articulate these ideas in grant proposals, manuscripts and presentations. I thank you for not accepting anything less than "flawless science, perfectly packaged". I hope that one day I can give the quality mentoring that you have given me to my own students.

To Dr. Christopher Minson, thank you for being incredibly patient with me as a young masters student and for igniting my initial spark of excitement about human physiology. You led me to Larry, and you continue to encourage me. You taught me how much fun the laboratory environment can be.

To Drs. David Proctor and James Pawelczyk, thank you for always encouraging me, making me think outside the box, and for sharing in my excitement. Thank you for always having an open door and for being willing to listen and offer your unique perspectives about science and about life.

To Dr. Kelly Dowhower-Karpa, thank you for your professional, clinical, and personal support. You are a personal role model and I am encouraged that you are a dedicated scientist, clinician, mother and wife.

To Dr. Caitlin Thompson-Torgerson, I am deeply indebted to you for all the help you have given to me throughout the years, but above all I am grateful for your friendship. I have had a great deal of fun and learned so much about science from our endless "skinomics" discussions in the lab.

To Dr. David DeGroot, thank you for your helping hands, and for your friendship. I have been extremely fortunate to spend my time in graduate school with you. From the early days of studying for physiology 571 exams to editing each others dissertations, you are a true friend. A true friend will help you move (twice), and will drive you to the emergency room when you learn knives and margaritas don't mix.

To Jane Pierzga, thank you for all you do to keep the lab running. I've looked to you for personal support and I always knew you would do whatever it took to get the endless projects, IRB and GCRC paperwork, and purchasing completed with style.

To Lindsay Baker and Jim Lang, thank you both for all of your help with the studies, and for putting up with me, especially during grant writing periods.

I would like to thank my family for valuing education and for your endless emotional support. It has been difficult being so far away from you, especially during an eventful several years, but I appreciate your understanding and encouragement.

Finally, to my husband, my best friend, mi amor, Simon, I cannot offer you enough thanks. You have made many sacrifices so that I could pursue my love of physiology. Our move all the way across the country was the beginning of a great adventure together. You have always been my source of endless support and I could not have done this without you. You have witnessed and shared in my tears of sadness, frustration and joy throughout this adventure. I want you to know how much I appreciate you and that I feel truly blessed to have you in my life.

El amor más fuerte y más puro no es el que sube desde la impresión, sino el que desciende desde la admiración.
-Santa Catalina de Siena

Chapter 1

INTRODUCTION

Background and Significance

The cutaneous circulation has a tremendous capacity to reflexly increase blood flow for thermoregulatory purposes. Under resting thermoneutral conditions, whole body skin blood flow is approximately 200-500ml/min. With rising body temperature an integrated cardiovascular response ensues through activation of the sympathetic nervous system, resulting in a robust increase in skin blood flow that can reach 7-8 liters/min in young healthy subjects (Rowell, 1974). However, primary human aging and hypertensive vascular pathology result in an attenuated skin blood flow response during thermal stress mediated by impairments in peripheral cutaneous vasodilatory signaling (Kenney & Kamon, 1984; Kenney *et al.*, 1984; Kenney *et al.*, 1997; Holowatz *et al.*, 2003). Together with altered central cardiovascular responses during hyperthermic stress (Minson *et al.*, 1998), the attenuated skin blood flow response renders these populations more vulnerable to cardiovascular and heat-related complications during exposure to high environmental heat (Semenza *et al.*, 1999; McGeehin & Mirabelli, 2001).

Neural Control of Skin Blood Flow

The cutaneous circulation is controlled by dual sympathetic innervation consisting of an adrenergic vasoconstrictor system and an active vasodilator system (Grant & Holling, 1938). With rising body core temperature (T_c) skin blood flow is increased initially by withdrawal of adrenergic vasoconstrictor tone. Upon reaching a specific body core temperature threshold, skin blood flow further increases through activation of the active vasodilator system (Roddie *et al.*, 1957a). Cutaneous active vasodilation is mediated by the co-release of acetylcholine and an unknown neurotransmitter from

sympathetic cholinergic active vasodilator nerves which mediate sweating and cutaneous vasodilation, respectively (Kellogg *et al.*, 1995). Vasoactive intestinal peptide (VIP) (Bennett *et al.*, 2003; Wilkins *et al.*, 2004a), substance P (Wong & Minson, 2006), and histamine 1 receptor activation (Wong *et al.*, 2004) all contribute to reflex vasodilation through NO-dependent mechanisms, which are required for full expression of active vasodilation (Kellogg *et al.*, 1998a; Shastry *et al.*, 1998). Additionally, acetylcholine is capable of modulating the initial rise in skin blood flow through NO-dependent mechanisms (Shibasaki *et al.*, 2002). In total, NO contributes approximately 30-40% to reflex vasodilation in young healthy skin (Kellogg *et al.*, 1998a; Shastry *et al.*, 1998), however there is the potential for a great deal of redundancy in the mechanisms that mediate reflex cutaneous vasodilation especially with respect to NO.

Primary Human Aging and Skin Blood Flow

Human aging in the absence of overt pathology is associated with an attenuated rise in skin blood flow during heat stress (Kenney, 1988; Kenney *et al.*, 1997; Holowatz *et al.*, 2003). While central cardiovascular responses to heat stress are also impaired in the aged (Minson *et al.*, 1998), peripheral alterations in neural and vascular vasodilatory signaling are clearly evident. Aged subjects respond to systemic hyperthermia with a lower skin blood flow for a given rise in T_c, as a result of decreased sensitivity of the active vasodilator system (Kenney *et al.*, 1997). Aged humans display a reduced functional contribution of sympathetic co-transmitter-mediated vasodilation, and instead rely primarily on attenuated NO-dependent vasodilation to increase skin blood flow during hyperthermia (Minson *et al.*, 2002; Holowatz *et al.*, 2003). These data suggest that the age-related deficit in active vasodilation could be due to (1) a reduction in the efferent sympathetic neural stimulus, (2) decreased vascular responsiveness to sympathetic stimulus, and/or (3) alteration in downstream second messenger vascular signaling, most notably NO bioavailability.

Acetylcholine-Induced Vasodilation

In young healthy skin acetylcholine is capable of modulating the initial rise in skin blood flow during hyperthermia (Shibasaki *et al.*, 2002). Muscarinic receptor antagonism decreases the initial rise by ~30% (Kellogg *et al.*, 1995) and acetylcholinesterase inhibition augments skin blood flow with small increases in core temperature through NO-dependent mechanisms (Shibasaki *et al.*, 2002). Because this initial rise is blunted in healthy older subjects (Holowatz *et al.*, 2003), one putative mechanism mediating this attenuation is decreased vascular responsiveness to acetylcholine and/or impairments in downstream signaling mediating cutaneous vasodilation. Studies using iontophoresis techniques to deliver actylcholine non-invasively to the cutaneous vasculature suggest that both NO and cyclooxygenase-(COX) derived vasodilators (prostanoids) contribute to acetylcholine-mediated vasodilation (Berghoff *et al.*, 2002). However, the electrical current used to deliver acetylcholine to the skin with iontophoresis complicates the interpretation of these data because the mode of electrical current and delivery vehicle used with this technique stimulate vasodilation by prostanoid and NO-dependent mechanisms (Khan *et al.*, 1997; Abou-Elenin *et al.*, 2002; Khan *et al.*, 2004). Therefore, the precise identity and potential age-related alterations in the second messenger systems involved in, and the contribution of each of these systems to, acetylcholine-mediated vasodilation in human skin are unclear.

Hypertension and Skin Blood Flow

Chronically elevated systemic vascular resistance causes impairments in vasodilatory responses resulting from reduced NO-dependent vasodilatation and structural maladaptations including vascular smooth muscle hypertrophy (Taddei *et al.*, 1998; Taddei & Salvetti, 2002). Impaired NO bioavailability associated with hypertension appears to be not only a consequence of the disease but is also likely involved in its etiology (Warnholtz *et al.*, 2004). Furthermore, the vascular

maladaptations associated with essential hypertension have been likened to accelerated vascular aging (Brunner *et al.*, 2005), suggesting common mechanisms underlying endothelial dysfunction with both vascular aging and essential hypertensive pathology.

Essential hypertensive humans exhibit attenuated cutaneous vasodilatory responses during local (Carberry *et al.*, 1992) and systemic thermal stress (Kenney *et al.*, 1984). Our laboratory has previously shown that skin blood flow in hypertensive subjects is reduced during exercise in the heat. Because the incidence of essential hypertension increases with advancing age (AHA, 2006), hypertension-associated decreased systemic NO bioavailability coupled with healthy age-related deficits in NO- and non-NO-dependent mechanisms (Holowatz *et al.*, 2003) may combine to significantly attenuate reflex cutaneous vasodilation. However, the precise contribution of NO to reflex cutaneous vasodilation and the involvement of mechanisms limiting NO bioavailability in hypertensive human skin remain unclear.

Putative Mechanisms Affecting NO Bioavailability with Aging and Hypertension

NO bioavailability in the vasculature is dictated by the balance of NO production and degradation. Several putative mechanisms exist through which primary aging and hypertension may independently decrease NO bioavailability in the cutaneous vasculature, most notably (1) augmented arginase activity and (2) increased oxidant stress (Figure 1-1). Arginase catalyzes the conversion of L-arginine to L-ornithine and urea in the final reaction of the urea cycle, and is capable of limiting NO synthesis through nitric oxide synthase (NOS) by competing for the common substrate L-arginine (Hecker *et al.*, 1995; Wu & Morris, 1998). Arginase is upregulated in animal models of vascular aging (Berkowitz *et al.*, 2003; White *et al.*, 2006) and hypertension (Johnson *et al.*, 2004; Zhang *et al.*, 2004a; Demougeot *et al.*, 2005; Demougeot *et al.*, 2006), and in these models inhibition of arginase restores endothelium-dependent vasodilation to specific agonists through NO-dependent mechanisms.

An additional mechanism contributing to decreased NO bioavailability is through an increase in oxidant stress. With both aging and hypertension, oxidant stress increases

through increased production of reactive oxygen species (ROS) through NAD(P)H oxidases, xanthine oxidases, mitochondrial sources, uncoupled endothelial NOS (eNOS), and decreased antioxidant defense mechanisms (Warnholtz *et al.*, 2004). One of the most deleterious ROS, superoxide, readily reacts with newly synthesized NO forming peroxynitrite four times faster than it can be degraded by superoxide dismutase (Beckman, 1996). Superoxide may also serve as a direct pro-constrictor stimulus (Bailey *et al.*, 2005). Additionally, there may be a functional link between augmented arginase activity and increased superoxide production through uncoupled endothelial NOS (eNOS). eNOS uncoupling results from peroxynitrite-mediated oxidization of the essential NOS cofactor tetrahydrobiopterin (BH_4) and/or L-arginine deficiency (Heinzel *et al.*, 1992; Toth *et al.*, 2002; Kuzkaya *et al.*, 2003, Landmesser, 2003 #106).

In aged and hypertensive human forearm muscle vasculature, acute infusions of the potent antioxidant ascorbate restore NO-dependent vasodilation (Taddei *et al.*, 1998; Taddei *et al.*, 2001). Ascorbate acts by directly scavenging ROS and by stabilizing the essential NOS cofactor BH_4 (Toth *et al.*, 2002). However, to date, there have been no systematic investigations into the influence of oxidant stress on attenuated cutaneous vasodilation in these populations.

Summary

Five separate studies comprising this dissertation were performed to investigate the mechanisms underlying attenuated reflex cutaneous vasodilation in aged and hypertensive skin. Because the precise identity of the neurotransmitter(s) mediating reflex vasodilation remains elusive, but the putative neurotransmitter(s) candidates mediate cutaneous vasodilation in part through NO-dependent mechanisms, these studies have focused on mechanisms affecting NO bioavailability in the cutaneous circulation with aging and hypertension. The first study investigated age-related alteration to the downstream second messenger systems involved in exogenous acetylcholine-induced vasodilation. The subsequent four studies investigated the roles of arginase, L-arginine

availability, and oxidative stress in attenuated reflex cutaneous vasodilation in aged and essential hypertensive humans.

Specific Aims and Hypotheses

Specific Aim 1: The purpose of the study, "Mechanisms of acetylcholine-mediated vasodilatation in young and aged human skin," was to characterize acetylcholine-induced vasodilation in the skin of young and older subjects. Specifically, by infusing exogenous acetylcholine directly into the cutaneous vasculature via intradermal microdialysis along with specific antagonists, the aim was to delineate the NO- and COX-dependent contributions to acetylcholine-mediated vasodilation.

> Hypothesis 1: Acetylcholine-induced vasodilation in aged skin would be attenuated due to decreased NO- and COX-dependent vasodilation.

Specific Aim 2: The purpose of the study, "L-arginine supplementation or arginase inhibition augment reflex cutaneous vasodilatation in aged human skin," was to determine the role of arginase in attenuated reflex cutaneous vasodilation in aged humans.

> Hypothesis 2: Arginase inhibition alone and with concurrent L-arginine supplementation would augment cutaneous vasodilation during passive whole body heat stress by increasing L-arginine availability for NO synthesis.

Specific Aim 3: The purpose of the study, "Acute ascorbate supplementation alone or combined with arginase inhibition augment reflex cutaneous vasodilation in aged human skin," was to determine the effects of acute ascorbate administration, alone and combined with arginase inhibition, in attenuated reflex cutaneous vasodilation in aged skin.

> Hypothesis 3a: Ascorbate supplementation alone would augment reflex cutaneous vasodilation in aged skin.

Hypothesis 3b: Increasing L-arginine availability for NO synthesis through NOS by inhibiting arginase in combination with ascorbate supplementation would enhance reflex vasodilation over ascorbate supplementation alone.

Specific Aim 4: The purpose of the study, "Upregulation of arginase activity contributes to attenuated reflex cutaneous vasodilatation in hypertensive humans," was to determine the role of arginase in reflex cutaneous vasodilation in humans with essential hypertension. Because arginase is upregulated in the skin with primary human aging (65-85 years) (Holowatz *et al.*, 2006b), we sought to control this by investigating the role of arginase in reflex vasodilatation in age- and sex-matched controls.

Hypothesis 4a: Reflex vasodilation in hypertensive subjects would be attenuated due to a reduced NO contribution.

Hypothesis 4b: Acute arginase inhibition alone or with concurrent L-arginine supplementation would significantly augment reflex vasodilation in hypertensive skin while only modestly increasing reflex vasodilatation in the age-matched control group.

Specific Aim 5: The purpose of the study, "Ascorbate increases NO- and non-NO-dependent reflex cutaneous vasodilation in hypertensive humans," was to determine the relative role of oxidant stress and augmented arginase activity on attenuated NO-dependent vasodilation in human hypertensive cutaneous vasculature. We sought to control for age-related increases in oxidant stress and arginase activity (Holowatz *et al.*, 2006a, b) by matching humans with essential hypertension to age- and sex-matched controls.

Hypothesis 5a: Acute antioxidant (ascorbate) supplementation in the skin would augment NO-dependent reflex cutaneous vasodilation

Hypothesis 5b: Arginase inhibition combined with ascorbate supplementation would further enhance NO-dependent reflex vasodilation over ascorbate supplementation alone.

Figure **1-1**

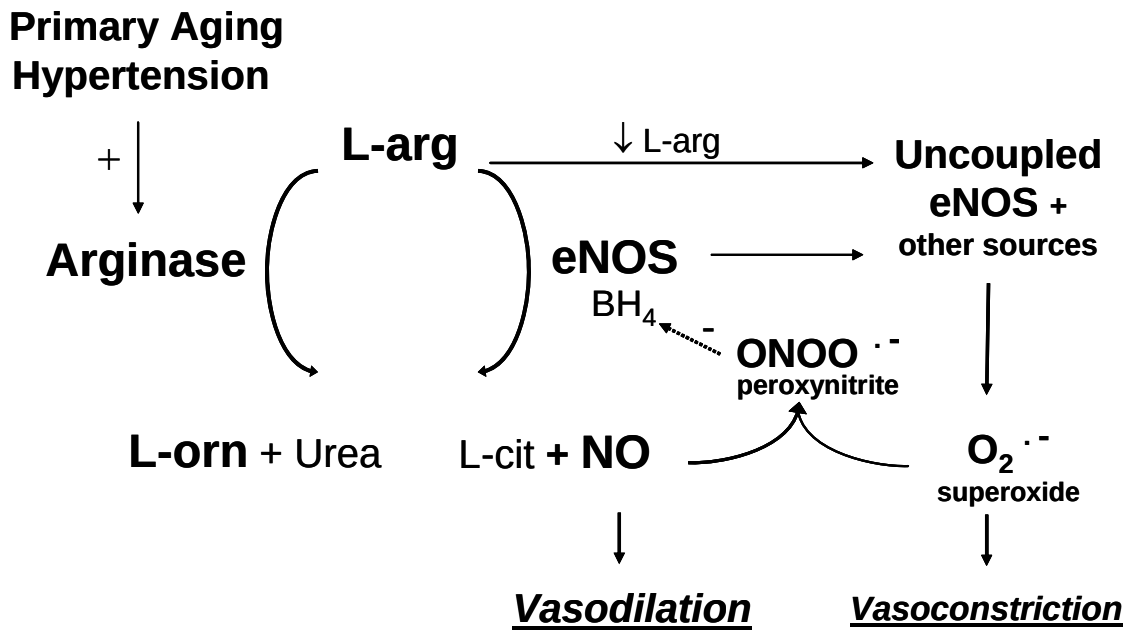

Figure **1-1**: Schematic depiction of the ways in which primary human aging and essential hypertension may affect cutaneous microvascular signaling and lead to decreased NO bioavailability and impaired reflex cutaneous vasodilation.
L-orn: L-ornithine; L-cit: L-citrulline; BH_4: tetrahydrobiopterin

Chapter 2

REVIEW OF THE LITERATURE

The role of the cardiovascular system during exposure to environmental heat is to mediate the transport of heat to the body surface for elimination via heat exchange mechanisms. Thus in humans, increasing blood flow to the cutaneous circulation is one of the primary effector responses critical to the maintenance of body core temperature (T_c) during hyperthermia. Under resting thermoneutral conditions the cutaneous circulation is over perfused relative to its metabolic demands (200-500ml/min), but can increase to 8L/min with maximal systemic heat stress (Rowell, 1974). With primary human aging and hypertensive vascular pathology there is an attenuated rise in skin blood flow during heat stress, which has negative thermoregulatory and cardiovascular functional consequences for these vulnerable populations. This review will focus on the neural regulation of the cutaneous circulation as it pertains to vasodilatory mechanisms and evidence for the putative mechanisms mediating attenuated reflex cutaneous vasodilation in primary aged and essential hypertensive humans.

Thermoregulatory Reflex Control of Cutaneous Vasodilation

Skin blood flow (SkBF) is neurally controlled by two distinct branches of the sympathetic nervous system (Lewis & Pickering, 1931; Grant & Holling, 1938). Under thermoneutral and cool conditions, alterations in cutaneous vascular tone are mediated by sympathetic adrenergic vasoconstriction. With a rise in skin and body core temperature (T_{sk} and $T_{c,}$ respectively), SkBF initially passively increases by a withdrawal of sympathetic vasoconstrictor adrenergic tone, leading to a two-fold increase in SkBF. Thereafter, once T_c has reached a critical threshold (ΔT_c =+0.3-0.4°C), sympathetic

cholinergic nerves release neurotransmitter(s) to mediate active vasodilation as well as to initiate sweating (Roddie *et al.*, 1957a).

Cutaneous active vasodilation is purportedly mediated by the co-transmission of acetycholine and an unknown neurotransmitter(s), where acetylcholine primarily mediates the sweating response and the unknown neurotransmitter(s) co-released with acetylcholine from cholinergic nerves mediates cutaneous vasodilation. In support of this hypothesis, muscarinic receptor antagonism abolishes sweating but only modestly delays the initial rise in SkBF with rising Tc, while pre-synaptic blockade of cholinergic nerves with botulinum toxin abolishes both sweating and active vasodilation (Kellogg *et al.*, 1995). An alternative hypothesis to cholinergic co-transmission is that two separate nerves mediate sweating and active vasodilation, respectively, and both nerves types are sensitive to botulinum toxin (Johnson, 2006). The specific identity of the unknown neurotransmitter(s) mediating active vasodilation and whether one or two nerves mediates the response remains unclear and is a topic of debate, but putative cholinergic neurotransmitter candidates include the vasoactive neuropeptides: vasoactive intestinal peptide (VIP), substance P, and calcitonin gene-related peptide (CGRP). Evidence from the rabbit ear model of active vasodilation suggests that the cholinergic neurotransmitter(s) mediates cutaneous vascular smooth muscle cell relaxation through cyclic adenylate cylcase (cAMP)-dependent mechanisms (Farrell & Bishop, 1997; Wilkins *et al.*, 2003). Immunoreactivity to these peptides has been demonstrated in human cutaneous nerves (Schulze *et al.*, 1997). Furthermore, VIP (Bennett *et al.*, 2003), histamine receptor 1 (H1) (Wong *et al.*, 2004), and neurokinin 1 (NK1) (Wong & Minson, 2006) receptor activation have all been shown to contribute to active vasodilation.

Functional nitric oxide (NO) is required for full expression of active cutaneous vasodilation (Kellogg *et al.*, 1998a, Shastry *et al.*, 1998) through activation of soluble cyclic guanylyl cyclase (cGMP)-dependent mechanisms. Inhibition of NO-synthase (NOS) reduces active vasodilation by approximately 30-40% in young healthy skin (Kellogg *et al.*, 1998a; Shastry *et al.*, 1998). NO synthesis during active vasodilation may be stimulated from a variety of sources including direct stimulation from the

putative neurotransmitter(s) involved in active vasodilation. Acetylcholine (Shibasaki *et al.*, 2002), VIP (Wilkins *et al.*, 2004a), and substance P (Wong & Minson, 2006) in part mediate cutaneous vasodilation through NO-dependent mechanisms. Additionally, the contribution of each of these putative neurotransmitters to the different phases of the SkBF response during rising T_c creates the potential for a great deal of redundancy in active vasodilation, especially with respect to NO mechanisms. For example, acetylcholine is capable of modulating the initial rise in active vasodilation (T_c <0.5°C) through NO-dependent mechanisms but not in the established plateau phase (T_c >0.6°C), based on the observations that prolonged active vasodilation is insensitive to muscarinic receptor antagonism (Roddie *et al.*, 1957a; Shastry *et al.*, 2000), but the initial rise in skin blood flow can be augmented by acetylcholinesterase inhibition. Activation of H1 receptors also contributes to the rise in SkBF through NO pathways, which has been linked to VIP signaling (Wong *et al.*, 2004), but VIP also has an NO-dependent component which is independent of H1 receptor activation (Wilkins *et al.*, 2004a). One putative source of VIP-induced histamine and NO is the degranulation of cutaneous mast cells (Figure 2-1). However, the precise source and potential interplay between VIP, histamine and NO signaling and their contribution to the initial vs. late phases of active vasodilation remain unclear.

Along with a potential direct contribution of NO to active vasodilation from a variety of putative neurotransmitter signals, NO is also capable of mediating vasodilation synergistically with the sympathetic co-transmitter(s). The resultant cutaneous vasodilation in the presence of NO and sympathetic co-transmitter(s) is greater than the sum of the contributions of each of these individual vasodilator pathways (Wilkins *et al.*, 2003). The precise mechanism of this synergistic interaction may occur prejunctionally, where NO enhances the release of sympathetic co-transmitter(s) (Grider *et al.*, 1992), or downstream involving the respective second messenger systems (Farrell & Bishop, 1997). This later hypothesis is attractive considering the potential interactions between neurotransmitter(s) activated cAMP and NO activated cGMP-dependent mechanisms through the phosphodiesterases.

In addition to NO, cyclooxygenase (COX)-dependent second messenger systems also contribute to active vasodilation (McCord *et al.*, 2006). Whether COX-derived vasodilators are independent of or interact with the NO pathway remains unknown, but combined inhibition of COX and NOS attenuates active vasodilation in an additive fashion, suggesting independent mechanisms. One potential stimulus for COX is through acetylcholine mechanisms. Studies utilizing iontophoresis or exogenous infusions to locally deliver acetylcholine to the cutaneous vasculature suggest that acetylcholine-mediated vasodilation in human skin is both NO- and prostanoid-dependent (Noon *et al.*, 1998; Kellogg *et al.*, 2005). In this construct, prostanoid production through acetylcholine may contribute to the early phase of active vasodilation and further prostanoid production in the latter phases of active vasodilation may be the result of shear stress mechanisms (McCord *et al.*, 2006). An alternative stimulus for COX-derived vasodilators is through endothelial NK1 receptor activation of the inositol triphosphate (IP_3) pathway, resulting in increased intracellular calcium and activation of both NOS and COX pathways (Wong & Minson, 2006). Collectively, the putative neurotransmitters(s) likely mediate the synthesis of both COX-derived vasodilators and NO which independently contribute to active vasodilation.

Reflex Cutaneous Vasodilation and Primary Human Aging

Human aging in the absence of overt pathology is associated with attenuated cutaneous vasodilation during thermal stress (Kenney & Hodgson, 1987; Kenney, 1988; Kenney *et al.*, 1990; Kenney *et al.*, 1997). This impaired vasodilatory response is apparent even when subjects are matched for fitness level (VO_{2max}) (Kenney, 1988), acclimation (Armstrong & Kenney, 1993), and hydration status (Kenney *et al.*, 1990), suggesting that this is a primary aging phenomenon. On average, healthy aged humans display a 25-50% reduction in SkBF during body heating coupled with a blunted increase in cardiac output and less redistribution of blood flow from the renal and splanchnic vascular bed (Minson *et al.*, 1998). For a given rise in T_c there is a decrease in the slope of the SkBF:T_c relation, suggesting that aging affects the peripheral mechanisms

mediating the rise in SkBF (Kenney *et al.*, 1997). Additionally, the origins of the age-related reduction in SkBF are due to decreased sensitivity of the active vasodilator system and not age-related alteration in adrenergic mechanisms (Kenney *et al.*, 1997).

Considering that NO is required for full expression of active vasodilation (Kellogg *et al.*, 1998a; Shastry *et al.*, 1998) and the documented system wide impairments in NO bioavailability with primary aging (Adeagbo *et al.*, 2003; Suschek *et al.*, 2003; Chicoine *et al.*, 2004; Touyz & Schiffrin, 2004; Zhang *et al.*, 2004b), reduced NO-dependent cutaneous vasodilation may contribute to attenuated reflex vasodilation. In an earlier study, we hypothesized that NO-dependent vasodilation would be attenuated and this reduction would account for the overall reduced active vasodilator response. Contrary to our hypothesis, we found that healthy aged cutaneous vasculature relies primarily on impaired NO-dependent mechanisms to increase SkBF during hyperthermia, and the non-NO-dependent (functional co-transmitter) contribution to reflex vasodilation was significantly attenuated (Holowatz *et al.*, 2003). Aged humans require a much greater increase in T_c ($\Delta T_c \geq 0.9°C$) to stimulate significant contributions of non-NO-dependent pathways to active vasodilation compared to young subjects ($\Delta T_c \geq 0.6°C$) (Figure 2-2). Although, NO-dependent vasodilation in aged cutaneous vessels was reduced overall (Minson *et al.*, 2002) and the NO contribution to reflex vasodilation was reduced with mild increases in T_c (ΔT_c 0.3-0.6°C) compared to young subjects, NO played a significant role in reflex vasodilation in aged skin especially with moderate increase in T_c (ΔT_c 0.6°C-0.9°C).

Because acetylcholine is capable of modulating the initial rise in SkBF in the early phase of active vasodilation, and aged subjects display reduced SkBF in this T_c range, one potential mechanism to explain impaired skin blood flow is decreased sensitivity of aged cutaneous vessels to acetylcholine mediated by alterations in acetylcholine second messenger signaling. To date, there have been no systematic investigations into the underlying mechanisms mediating age-related alteration in acetylcholine-mediated cutaneous vasodilation.

Acetylcholine-Induced Vasodilation

The precise mechanism and potential age-related alterations of acetylcholine-induced vasodilation in human cutaneous vasculature remains unclear. Intraarterial infusion of acetylcholine in the human forearm muscle vasculature produces profound vasodilation that is inhibited with co-infusion of competitive NO-synthase inhibitors, and restored with L-arginine infusion. Thus in the human forearm circulation, acetylcholine-induced vasodilation is almost exclusively mediated by NO mechanisms (Vallance *et al.*, 1989b, a). Age- and pathology-related impairments in forearm vasodilation to acetylcholine are well established (Taddei *et al.*, 2001), suggesting globalized pathology-associated reductions in NO bioavailability in the vasculature. However, these studies measured whole forearm blood flow with venous occlusion plethysmography (VOP) which represents predominantly muscle but also bone and SkBF. Because SkBF is impossible to quantify using VOP when underlying muscle blood flow is changing, it has been assumed that acetylcholine also induces cutaneous vasodilation though NO-dependent mechanisms. Additionally, it is uncertain to what extent delivery of pharmacological agents intraarterially reaches and affects the cutaneous vasculature (Dietz *et al.*, 1994; Shastry *et al.*, 1998). In contrast to forearm muscle vasculature, *in vitro* subcutaneous vessel experiments suggest that acetylcholine-mediated vasodilation is primarily COX-, and non-NO-non-COX-dependent, with NO playing a minimal role.

In order to circumvent the problems associated with assessing acetylcholine-induced vasodilation *in vivo* in the cutaneous vasculature with intraarterial drug delivery and VOP, iontophoresis has been utilized to locally deliver acetylcholine directly to the cutaneous vasculature. However, the iontophoresis technique has several limitations that make the interpretation of these data difficult. In the absence of any vasoactive drug, the electrical current used to deliver charged molecules to the cutaneous vasculature induces vasodilation (Grossmann *et al.*, 1995; Durand *et al.*, 2002a; Durand *et al.*, 2002c). This current-induced increase in SkBF is sensitive to COX-inhibition and sensory nerve blockade (Morris & Shore, 1996; Noon *et al.*, 1998). Additionally, the quantity of acetylcholine delivered to the cutaneous vasculature is dependent on the resistance of the

vehicle used to deliver the drug (Asberg *et al.*, 1999; Abou-Elenin *et al.*, 2002). Vasodilation to iontophoresis of acetylcholine in aged skin is attenuated (Algotsson *et al.*, 1995), but the precise signaling mechanisms underlying this reduction have not been systematically explored.

Reflex Cutaneous Vasodilation and Essential Hypertension

Persistent elevations in peripheral vascular resistance have widespread deleterious effects on the vasculature, including vascular smooth muscle hypertrophy and decreased endothelium-dependent vasodilation associated with reduced bioavailability of NO (Folkow, 1982; Sihm *et al.*, 1995; Taddei *et al.*, 1997, 1998; Rizzoni *et al.*, 2000; Taddei & Salvetti, 2002). In the cutaneous circulation essential hypertension is associated with an attenuated reflex vasodilation and a subsequent reduction in core-to-skin heat transfer during whole body heat stress (Kenney & Kamon, 1984; Kenney *et al.*, 1984; Kenney, 1985). Functionally, epidemiological evidence suggests that a significant fraction of individuals treated for heat related illness have pre-existing hypertensive conditions (Tucker *et al.*, 1985).

Only a few well-controlled studies have examined skin blood flow responses during hyperthermia in human hypertensive populations. These investigations have been primarily descriptive in nature and have focused on structural microvascular changes rather than underlying alterations in vasodilatory mechanisms (Antonios *et al.*, 1999a; Antonios *et al.*, 1999b; Kellogg *et al.*, 1999; Maver & Strucl, 2000; Serne *et al.*, 2001). These studies have demonstrated that maximal vasodilation in response to the local application of heat (Carberry *et al.*, 1992) and reflex cutaneous vasodilation during systemic hyperthermia are attenuated in hypertensive human subjects (Kenney *et al.*, 1984). Using both laser-Doppler flowmetry and venous occlusion plethysmography, Carberry *et al.* found that essential hypertensive subjects had significantly lower forearm vascular conductance both at rest and during maximal local heating of forearm skin (Carberry *et al.*, 1992). This reduction in maximal skin blood flow was attributed to structural changes in the cutaneous microcirculation likely reflecting significant vascular

smooth muscle hypertrophy. Similarly, Kenney *et al.* has previously shown that unmedicated essential hypertensive subjects exhibit diminished forearm blood flow during dynamic leg exercise-induced hyperthermia in comparison to age-matched normotensive control subjects (Kenney *et al.*, 1984). Any increases in forearm blood flow using this paradigm are caused solely by an increase in SkBF (Johnson *et al.*, 1976). Normotensive control subjects had a four-fold increase in forearm blood flow, while hypertensive subjects showed little increase in forearm blood flow with dynamic leg exercise, demonstrating an attenuated skin blood flow response in the hypertensive subject group (Figure 2-3) (Kenney *et al.*, 1984).

One potential explanation for the reduced resting and attenuated skin blood flow responses observed in hypertensive subjects during thermoneutral and hyperthermic conditions, respectively, is augmented basal cutaneous vasoconstrictor tone. In this construct, enhanced adrenergic vasoconstriction would limit the degree to which active cutaneous vasodilation could increase blood flow. Examination of the contribution of the adrenergic vasoconstrictor system and the active vasodilator system in hypertensive subjects revealed little or no change in active cutaneous vasodilatory function among hypertensives compared to normotensive controls (Kellogg *et al.*, 1998b). However, there are several methodological concerns with this study that make it difficult to generalize these findings. First, the hypertensive subjects were medicated in this study and abstained from their pharmacotherapy for only one week prior to experimentation. This may not have been a sufficient period of time abstaining from antihypertensive pharmacotherapy to observe significant differences in cutaneous vasodilatory function due to vascular remodeling. Secondly, Kellogg et al. only reported the laser-Doppler flux data normalized to a percentage of maximum and they did not report absolute maximal cutaneous vascular conductance. Considering the lower maximal skin blood flow in hypertensive subjects (Carberry *et al.*, 1992), this normalization scheme may be masking hypertensive differences in reflex vasodilation.

To date there have been no systematic investigations into the mechanisms underlying hypertension-associated reductions in reflex cutaneous vasodilation. Because of the functional importance of NO in reflex vasodilation and documented globalized

endothelial dysfunction that occurs with hypertension (Taddei *et al.*, 1997, 1998; Taddei & Salvetti, 2002; Brunner *et al.*, 2005), decreased NO bioavailability may contribute to attenuated reflex cutaneous vasodilation. Therefore, mechanisms affecting NO bioavailablity in hypertensive cutaneous vasculature are ideal targets for investigation.

Putative Mechanisms Affecting NO Bioavailability with Primary Aging and Essential Hypertension

NO bioavailability in the vasculature is dictated by the balance between NO production and degradation. Several putative mechanisms exist through which primary human aging and/or essential hypertension decrease the bioavailability of NO, most notably (1) augmented arginase activity and (2) increased oxidant stress. Vascular arginase metabolizes L-arginine, the substrate for nitric oxide synthase (NOS), to L-ornithine and urea thereby decreasing substrate availability for NO synthesis (Berkowitz *et al.*, 2003; Johnson *et al.*, 2004). Reactive oxygen species (ROS) convert newly synthesized NO into peroxinitrite before NO can initiate downstream vasodilation. ROS activity increases with both aging and hypertension by (1) increased enzymatic ROS production and (2) decreased antioxidant defense mechanisms. Augmented arginase activity may also potentiate ROS production through uncoupled NOS, where NOS itself produces superoxide as a result of decreased L-arginine and/or essential cofactor availability. Furthermore, there is a mechanistic link between augmented vascular arginase activity and the pathogenesis of deleterious vascular remodeling with essential hypertension through an increase in the polyamine and proline precursor L-ornithine which contributes to vascular smooth muscle cell proliferation (Wu & Morris, 1998; Durante *et al.*, 2001).

Arginase

Arginase has recently been recognized as an important enzyme capable of regulating the bioavailability of NO. Arginase catalyzes the conversion of L-arginine to L-ornithine and urea in the final step the urea cycle. L-ornithine is the precursor for polyamine and proline synthesis which are important in collagen formation and tissue growth (Wu & Morris, 1998; Durante *et al.*, 2001). There are two isoforms of arginase with two separate genes encoding each isoform. Arginase I is most abundantly expressed in the liver and arginase II is expressed extrahepatically; both isoforms have been localized in the vasculature in endothelial and smooth muscle cells (Bachetti *et al.*, 2004). Arginase is capable of modulating NO production by competing for L-arginine, the common substrate for both endothelial NOS (eNOS) and arginase. Increased arginase activity may help to explain the L-arginine paradox, where although the intracellular concentration of L-arginine far exceeds the K_m for NOS (~2-20μmol/l), the addition of exogenous L-arginine increases NO-dependent vasodilation (Wu & Morris, 1998). Arginase has a higher K_m for L-arginine (~2-20mmol/l), but the maximum activity of arginase is 1000 times that of NOS (Wu & Morris, 1998).

In a recent series of investigations, Berkowitz et. al. (Berkowitz *et al.*, 2003) immunolocalized arginase in the endothelium and vascular smooth muscle of aged rodent conduit arteries. Aged mice exhibited an increase in endothelial expression and activity of arginase which contributed to impaired endothelial function through NO-dependent mechanisms. NO-dependent vasodilation was enhanced with both acute and chronic arginase inhibition in aged vessels compared to young control vessels. Interestingly, L-arginine supplementation did not alter NO-dependent vasodilation in the absence of arginase inhibition, suggesting that the intracellular L-arginine pool accessible to eNOS is reciprocally regulated by arginase. Although both isoforms of arginase are expressed in the vasculature, the available arginase inhibitors are not isoform specific. Antisense oligonucleotide approaches to knockout arginase I in aged rat aortic rings revealed that arginase I contributes to age-related endothelial dysfunction in rat conduit vasculature (White *et al.*, 2006). However, endothelial tissue culture evidence suggests that the

arginase isoforms are localized in different subcellular locations with arginase I in the cytosol and arginase II in the mitochondria (Topal *et al.*, 2006). Either arginase isoform may be activated and upregulated with endothelial dysfunction.

Isolated vessel studies using hypertensive and atherosclerotic animal models also suggest endothelial arginase is upregulated and contributes to the etiology of endothelial dysfunction associated with vascular disease (John & Schmieder, 2003; Ming *et al.*, 2004). Spontaneously hypertensive rats (SHR) exhibit increased expression of arginase I and II in the vasculature as well as increased arginase activity (Demougeot *et al.*, 2005). Chronic arginase inhibition prevents the rise in blood pressure and improves endothelium-dependent vasodilation in response to acetylcholine by increasing NO bioavailability. Other studies examining the effect of acute arginase inhibition in the vasculature have produced similar results. Arginase inhibition in resistance arterioles of Dahl salt sensitive hypertensive rats complete restorated endothelium-dependent vasodilation (Johnson *et al.*, 2004; Demougeot *et al.*, 2005) and partial restoration of endothelium-dependent vasodilation in hypertensive porcine conduit coronary vessels (Zhang *et al.*, 2004a). However, in procine coronary arteries, arginase inhibition did not completely restore NO-dependent vasodilation compared to control vessels, suggesting that other alterations in NO bioavailability may also occur with hypertension. Cumulatively, these finding suggest that upregulated arginase contributes to impaired NO-dependent vasodilation and endothelial dysfunction with hypertension and vascular aging.

To date, relatively few *in vivo* studies have examined the role of vascular arginase in endothelial dysfunction in humans. *In vitro* vascular studies suggest that augmented vascular arginase activity may contribute to the etiology of pulmonary arterial hypertension (PAH). PAH subjects exhibit increased arginase activity in blood serum and increased arginase II expression in cultured pulmonary endothelial cells (Xu *et al.*, 2004). In human skin, arginase activity was first identified in 1951 (Van Scott, 1951). Moreover, both arginase isoenzymes are present in the skin and their activity is increased in certain inflammatory dermatological conditions (Kampfer *et al.*, 2003; Wessagowit *et*

al., 2004). This finding is not surprising considering that the product of the arginase catalyzed reaction, L-ornithine, is the precursor for polyamine and collagen synthesis.

Oxidant Stress

Reactive oxygen species (ROS) play a pivotal role in the pathogenesis of hypertension and are also associated with age-related impairments of endothelial function. It is well established that with healthy aging and cardiovascular disease, including hypertension, the concentrations of ROS are elevated due to increased production by NAD(P)H oxidases, NOS uncoupling, and increased mitochondrial ROS production (Freeman & Crapo, 1982). Furthermore, there is a reduction in the activities of antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), which modulate ROS degradation (Kohen, 1999; Khodr & Khalil, 2001). Elevations in superoxide anion concomitantly with hypertension produces a deleterious cascade that leads to decreased NO, further increases in harmful ROS production through uncoupled eNOS, and enhanced vasoconstriction.

Superoxide is readily degraded by reacting with NO and produces peroxynitrite. Superoxide clearance via this mechanism occurs four times faster than metabolism via superoxide dismutase (Lassegue & Griendling, 2004). Peroxynitrite can then oxidize critical cofactors for NOS including tetrahydobiopterin (BH_4), leading to uncoupling of NOS where NOS itself donates electrons to molecular oxygen forming superoxide instead of NO (Toth *et al.*, 2002; Kuzkaya *et al.*, 2003; Landmesser *et al.*, 2003). NOS uncoupling occurs when cofactors are low and under circumstances in which there is limited L-arginine availability (Kuzkaya *et al.*, 2003; Landmesser *et al.*, 2003). Therefore, a mechanistic link exists between augmented arginase activity, limited L-arginine availability, and increased superoxide production through uncoupled NOS. Superoxide can directly produce vasoconstriction through a variety of mechanisms including stimulation of cyclooxygenase pathways, activation of the thromboxane A_2 receptors, and stimulation of the Rho/Rho-kinase pathway (Jin *et al.*, 2004; Bailey *et al.*, 2005; Vanhoutte *et al.*, 2005). Thus, increased superoxide with hypertension leads to

attenuated vasodilation through impaired vasodilation from NO and augmented vasoconstrictor tone.

In human essential hypertension, increased ROS production, particularly superoxide, is observed throughout the development and progression of the disease. The administration of the water soluble antioxidant ascorbic acid augments endothelium-dependent vasodilation by increasing NO bioavailability in hypertensive subjects (Taddei *et al.*, 1998; Frei, 1999; Taddei *et al.*, 2001; Cross *et al.*, 2003; Hirooka *et al.*, 2003). Interestingly, healthy aging is also associated with increased oxidant damage and reduced NO-dependent vasodilation, but ROS do not significantly contribute to attenuated NO-dependent vasodilation until after the age of 60 (Taddei *et al.*, 2001). As such, the administration of intraarterial ascorbic acid, which is capable of stabilizing BH_4 by preventing oxidation by peroxynitrite (Toth *et al.*, 2002), or oral BH_4 supplementation has been shown to improve endothelium-dependent vasodilation in both essential hypertensive and healthy aged human subjects (Taddei *et al.*, 1998; Frei, 1999; Cross *et al.*, 2003; Eskurza *et al.*, 2005).

The Skin as a Model Circulation for the Assessment of Vascular Function

In addition to the functional thermoregulatory importance of the cutaneous circulation, it is an easily accessible, representative vascular bed to assess endothelial function (Sax *et al.*, 1987; RG *et al.*, 2003; Abularrage *et al.*, 2005). NO is a key signaling molecule in cutaneous vasculature that directly mediates vasodilation in response to thermal stimuli (Shastry *et al.*, 2000; Charkoudian *et al.*, 2002; Minson *et al.*, 2002; Holowatz *et al.*, 2003; Minson & Wong, 2004; Wong *et al.*, 2004; Houghton *et al.*, 2005; McCord *et al.*, 2006; Wong & Minson, 2006). Primary human aging- and hypertension-induced deficits in vascular function, including attenuated vasoreactivity, are evident in the cutaneous circulation and mirror systemic changes of generalized endothelial dysfunction (Sax *et al.*, 1987; RG *et al.*, 2003). However, the precise mechanisms underlying reduced cutaneous vasoreactivity have not been systematically explored. Therefore, the cutaneous circulation is an ideal vascular bed for the *in vivo*

examination of the mechanisms that mediate primary human aging- and hypertension--induced vascular dysfunction.

> "Microvascular dysfunction is a systemic process that occurs in a similar fashion in multiple tissue beds throughout the body (Sax *et al.*, 1987; RG *et al.*, 2003). Identification of impaired microvascular blood flow and vasoreactivity by noninvasive means can lead to early identification of patients at risk for peripheral vascular and coronary artery disease. Furthermore, it can provide a qualitative assessment of the effects of a given treatment." (Abularrage *et al.*, 2005)

Figure 2-1

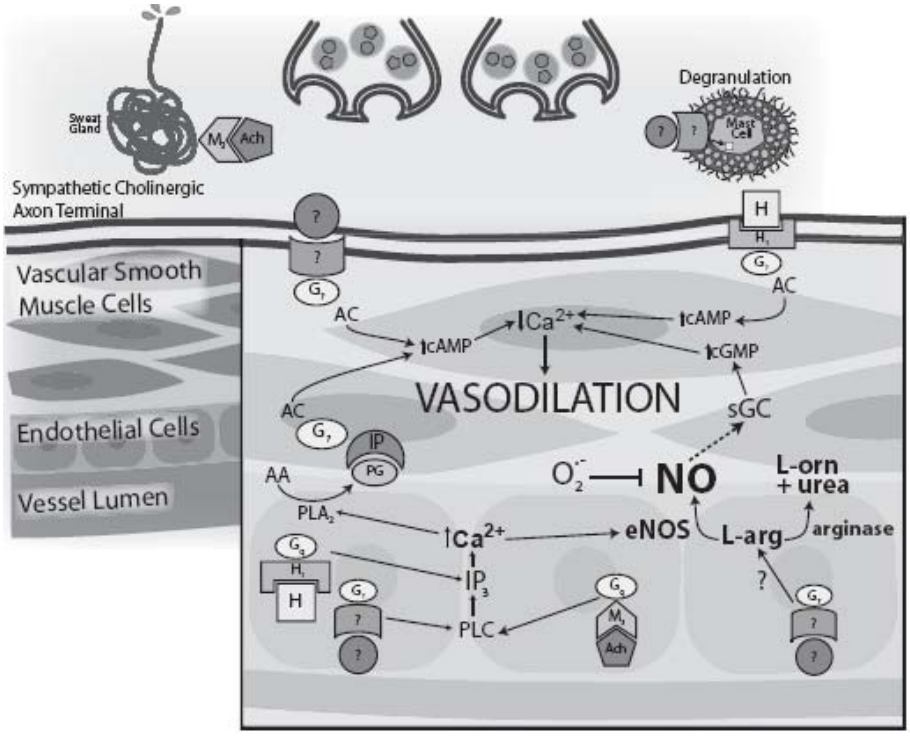

Figure **2-1**: Schematic representation of the putative mechanism of active vasodilation with relevant age-related alterations. Acetylcholine mediates the sweating response through muscarinic receptor (M_2) activation on the sweat glands and may modulate the initial rise in active vasodilation through NO and COX-dependent prostanoid synthesis. In this schematic the unknown neurotransmitter (?) and receptor (?) mediate vasodilation through adenylate cyclase (AC) mechanisms and may also increase NO synthesis through inositol triphosphate (IP_3)-mediated increases in intracellular calcium (Ca^{2+}). Histamine (H) also contributes to active vasodilation through NO-dependent and NO-independent mechanisms. Putative neurotransmitters involved in active vasodilation include vasoactive intestinal peptide (VIP), substance P, and calcitonin gene related peptide (CGRP) which may induce histamine release through the degranulation of cutaneous mast cells. With aging there is a reduction in both the functional neurotransmitter and NO contributions. NO-dependent vasodilation is decreased by an age-related upregulation of arginase activity and increased oxidant stress.

PG, prostaglandins; PLA2, phospholipase A2; AA, arachadonic acid; IP, prostaglandin receptor; G, G-protein coupled receptor; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; L-orn, L-ornithine; L-arg, L-arginine; $O_2^{\cdot-}$, superoxide; sGC, soluble guanylyl cyclase.

Figure 2-2

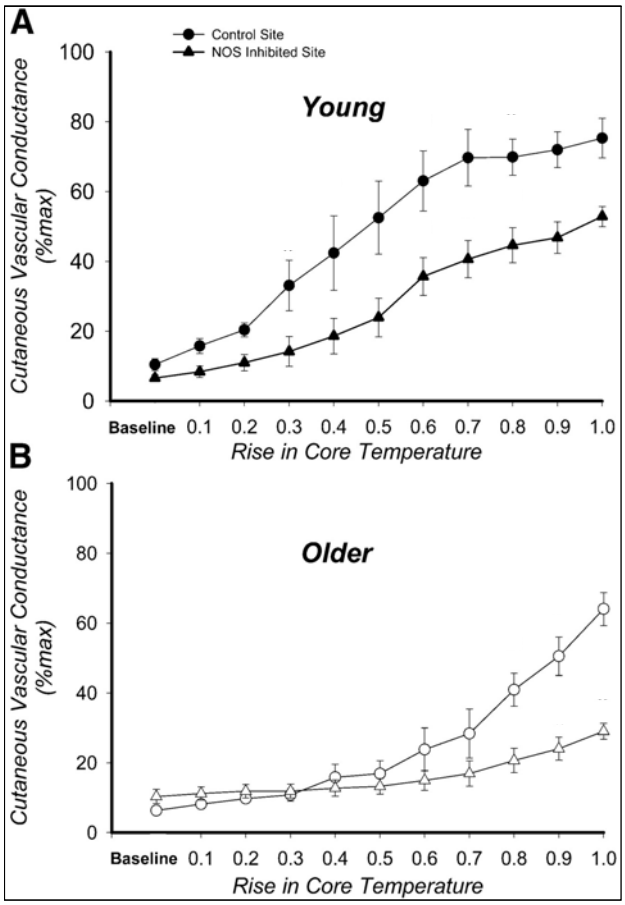

Figure **2-2**: Cutaneous vascular conductance (% of maximal) mean ± SE responses of A: young men (18-26 yr) and B: older men (65-81 yr) during passive whole body heating in a control (Ringer infusion only) and nitric oxide synthase (NOS)-inhibited (L-NAME infusion) microdialysis sites. (Holowatz *et al.*, 2003)

Figure 2-3

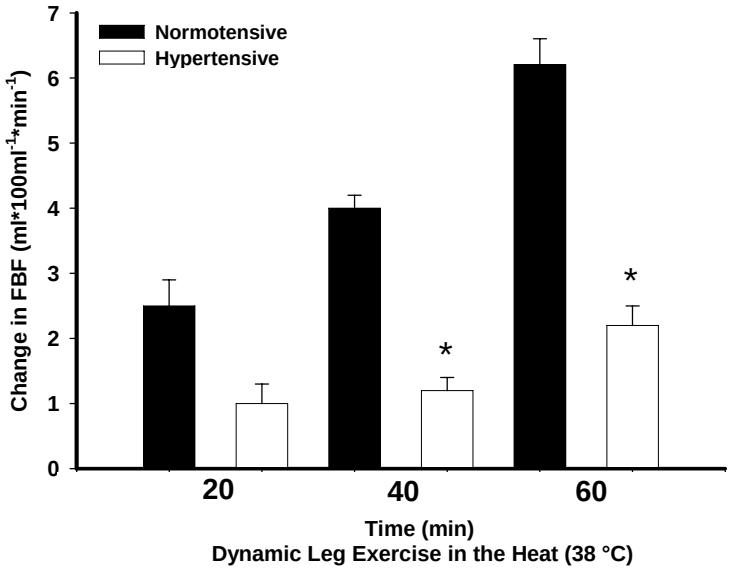

Figure **2-3**: Changes in forearm blood flow (FBF) during dynamic leg exercise in the heat (38 °C) in hypertensive and normotensive human subjects. Hypertensive subjects had an attenuated rise in FBF during hyperthermia. *$p<0.05$
Adapted from Kenney *et al.*, 1984.

Chapter 3

MECHANISMS OF ACETYLCHOLINE-MEDIATED VASODILATATION IN YOUNG AND AGED HUMAN SKIN

Introduction

Skin blood flow is controlled by two branches of the sympathetic nervous system, a noradrenergic vasoconstrictor system and a cholinergic active vasodilator system (Grant & Holling, 1938). As core body temperature begins to rise, the initial increase in skin blood flow is mediated by a release of vasoconstrictor tone; upon reaching a specific threshold sweating and reflex active vasodilatation are initiated, stimulating the co-release of acetylcholine and an unknown vasodilator from sympathetic cholinergic nerves (Roddie *et al.*, 1957a; Kellogg *et al.*, 1995). Nitric oxide (NO) appears to contribute approximately thirty percent to the total reflex vasodilatory response (Kellogg *et al.*, 1998a; Shastry *et al.*, 1998). Vasoactive intestinal peptide has been identified as a potential cutaneous cholinergic vasodilator substance and is known to have a NO-dependent component {Bennett, 2003 #6; Wilkins, 2004 #7.

With advanced age there is an attenuated reflex vasodilatory response {Kenney, 1988 #10; Kenney, 1997 #9; Martin, 1995 #8}. The initial rise in skin blood flow during hyperthermia is blunted in healthy older subjects, such that a greater rise in core temperature is required to observe significant reflex vasodilatation (Wilkins *et al.*, 2003). Shibasaki and colleagues (2002) demonstrated that acetylcholine may directly contribute to the initial rise in skin blood during hyperthermia in young healthy subjects. When acetylcholinesterases were inhibited by neostigmine administration, an augmented initial vasodilatory response was observed; however, this augmentation was abolished by the co-administration of neostigmine and a nitric oxide synthase inhibitor (Shibasaki *et al.*, 2002). Furthermore, muscarinic receptor antagonism with atropine has no effect on skin blood flow when it is administered after skin blood flow has reached a plateau during established hyperthermia (Shastry *et al.*, 2000). These results suggest that that

acetylcholine mediates a portion of the initiation of reflex cutaneous vasodilatation in young subjects, most likely through NO-dependent mechanisms.

The precise mechanisms of acetylcholine-mediated vasodilatation in the cutaneous vasculature of young subjects remain unclear. It is generally hypothesized that acetylcholine produces endothelium-dependent vasodilatation through NO-dependent, prostanoid-dependent, and non-NO-, non-prostanoid-dependent pathways. Furthermore, acetylcholine can produce vasoconstriction by acting directly on the vascular smooth muscle (Collier & Vallance, 1990). Our understanding of the mechanisms of acetylcholine-mediated vasodilatation is limited by the techniques used to deliver acetylcholine to the cutaneous vasculature. Most studies investigating these mechanisms have used iontophoresis to deliver acetylcholine to the skin, where the anodal current from this technique can cause cutaneous vasodilatation through prostanoid-dependent mechanisms (Grossmann *et al.*, 1995; Khan *et al.*, 1997; Noon *et al.*, 1998; Durand *et al.*, 2002a; Durand *et al.*, 2002c). Moreover, iontophoretically-initiated cutaneous vasodilatation varies as a function of the resistance of the vehicle used to dissolve and deliver acetylcholine (Asberg *et al.*, 1999; Droog & Sjoberg, 2003; Khan *et al.*, 2004).

Acetylcholine-mediated vasodilatation in the cutaneous vasculature is also attenuated with advanced age (Algotsson *et al.*, 1995). Studies in human forearm and cutaneous microvasculature demonstrate that there is a reduction in prostanoid-dependent vasodilatation with healthy aging due to both an increase in thromboxane vasoconstrictor activity and a decrease in prostacyclin-mediated vasodilator activity (Taddei *et al.*, 1997; Buus *et al.*, 2000; Heymes *et al.*, 2000). Furthermore, we have previously shown a decrease in NO-mediated vasodilatation in aged skin (Minson *et al.*, 2002). These data suggest that downstream pathways in acetylcholine-mediated vasodilatation may be attenuated in aged skin. Therefore the purpose of the present study was to characterize acetylcholine-mediated vasodilatation in the skin of young and older subjects. Specifically, by infusing exogenous acetylcholine directly into the cutaneous vasculature via intradermal microdialysis along with specific antagonists, we sought to delineate the NO contribution and the prostanoid-dependent contribution to acetylcholine-mediated vasodilatation. Finally, we hypothesized that older subjects would have attenuated

acetylcholine mediated-vasodilatation due to decreased NO- and prostanoid-dependent vasodilatation.

Methods

Subjects

Studies were performed on 12 young (23±1 yr) and 10 older (69±1 yr) men and women. Experimental protocols were approved by the Institutional Review Board at The Pennsylvania State University and conformed to the guidelines set forth by the Declaration of Helsinki. Verbal and written consent was voluntarily obtained from all subjects before their participation. Each subject underwent a complete medical screening including blood chemistry, lipid profile evaluation (Quest Diagnostics Nichol Institute, Chantily, VA), physical exam, and an assessment of VO_{2peak} (Parvomedics, Salt Lake City, UT). All subjects were screened for the presence of cardiovascular, dermatological, and neurological disease. Subjects were normally active, normotensive, healthy nonsmokers who were not currently taking medications, including aspirin therapy, hormone replacement therapy, or oral contraceptives. All young female subjects were studied in the early follicular phase of their menstrual cycle. All subjects were asked to abstain from all non-steroidal anti-inflammatory products for at least a week prior to participation in the experiment.

Instrumentation

Upon arrival to the laboratory, subjects were seated in a reclined position and instrumented with four intradermal microdialysis fibres (MD 2000, Bioanalytical Systems, IN) (10-mm, 20-kDa cutoff membrane) in the skin on the ventral side of the forearm. Microdialysis sites were spaced at least 4.0 cm apart to insure no cross-reactivity of pharmacological agents being delivered to the skin. Placement of the

microdialysis fibres was accomplished at each site by first inserting a 25-gauge needle through the unanaesthetized skin using sterile technique. The entry and exit points were ~2.5 cm apart. The microdialysis fibre was then threaded through the lumen of the needle, and the needle was withdrawn, leaving the fibre in place. The microdialysis fibres were then taped in place and attached to the outlet port of 4 individual stopcocks. Lactated Ringers solution was perfused through the microdialysis fibres at a rate of 2.0µl/min (Harvard microinfusion pump, South Natick, MA).

Measurements

To obtain an index of skin blood flow, cutaneous red blood cell flux (RBC) was measured with a laser-Doppler flowmeter probe placed in a local heater (Moor Instruments, Temperature Monitor SH02, MoorLAB, UK) over each microdialysis fibre. All laser-Doppler probes were calibrated using Brownian standard solution before each experiment. Cutaneous vascular conductance was calculated as RBC flux divided by mean arterial pressure. Blood pressure was measured via brachial auscultation every ten minutes during the protocol. Mean arterial pressure was calculated as diastolic blood pressure plus ⅓ pulse pressure.

Protocol

After placement of the microdialysis fibres, RBC flux over each microdialysis site was monitored to ensure that the initial hyperemia caused by the insertion trauma had resolved before the protocol started. Microdialysis fibres were randomly assigned to receive 10 mM N^G-nitro-L-arginine methyl ester (L-NAME; Calbiochem, San Diego, CA) to inhibit NO production by nitric oxide synthase (NOS), or 10 mM ketorolac (Sigma Chemical Inc., St Louis, MO) to non-specifically inhibit cyclooxygenase (COX), thereby inhibiting all prostanoid and thromboxane products through COX. Both NOS and COX were inhibited in a third microdialysis site with 10 mM L-NAME + 10 mM

ketorolac at final dilution. Our laboratory previously showed that this dose of L-NAME is sufficient to maximally inhibit NO production in both subject groups (Minson *et al.*, 2002). Pilot work was performed by infusing separated microdialysis fibres with 2.5, 5, 10, 15, 20, and 25mM doses of ketorolac and then infusing increasing doses of acetylcholine (68.7, 137.5, 550uM). We observed a dose dependent rise in skin blood flow with increasing does of acetylcholine. Further, doses of ketorolac greater than 10mM did not further reduce the skin blood flow response to acetylcholine; therefore 10mM ketorolac was used to maximally inhibit production of vasoactive substances from cylooxygenase. All pharmacological agents were dissolved in lactated Ringers solution. A fourth microdialysis fibre was perfused with lactated Ringers solution and served as a control site. These infusions were maintained throughout the protocol.

The microdialysis fibres were perfused with assigned pharmacological agents at a rate of 2.0µl/min for at least 75 minutes before acetylcholine infusion to ensure adequate NOS and COX inhibition. After 10 minutes of baseline measurements of RBC flux were obtained, each microdialysis fibre was perfused with 137.5 µM acetylcholine (Sigma Chemical Inc., St Louis, MO) for one minute at a rate of 2.0µl/min. Extensive pilot studies were performed to determine the appropriate dose of acetylcholine that corresponded with the magnitude of cutaneous vasodilatation that is observed at the initiation of reflex vasodilatation. Precise infusion volumes were obtained using Liquid Switch stopcocks (CMA Microdialysis, Solma, Sweden). Infusions at the different microdialysis sites were separated by at least 2 minutes. Following a return to baseline RBC flux and at least twenty minutes after the first acetylcholine infusion, a second infusion of the same dose was repeated at all sites. RBC flux was again allowed to return to baseline values. Blood pressure was measured at baseline, peak, and upon returning to baseline during each acetylcholine infusions. After completion of both acetylcholine infusions, 28 mM sodium nitroprusside (SNP; Nitropress, Abbot Laboratories, Chicago, IL, USA) was perfused through all microdialysis fibres to achieve maximal CVC at all sites. Local heating of the skin to 43°C was conducted after SNP infusion to ensure maximal CVC had been obtained.

Data Acquisition and Analysis

Data were acquired using Labview software and National Instruments data acquisition system (Austin, TX). The data were digitized, recorded and stored on a personal computer for further analysis. The CVC data were averaged over 30 second periods and are presented as a percentage of maximal CVC (%CVC_{max}).

Statistical Analyses

Student's t-tests were used to determine significant differences between the young and the old groups for physical characteristics. A two-way repeated measures analysis of variance was conducted within each group to detect potential differences between infusions at the different drug treatment sites. A three-way repeated measures analysis of variance was conducted to detect differences between subject groups at the drug treatment sites over time (SAS statistical software, version 8.01). Planned comparison tests including Tukey post-hoc tests were performed when appropriate to determine where differences between groups and drug treatment occurred. The level of significance was set at α=0.05. Values are means ± standard error.

Results

The physical characteristics of the subjects are presented in Table 3-1. There was no difference between the groups for body mass index but the older subject group had a significantly lower VO_{2peak} ($p<0.001$). There was no difference between the groups for total cholesterol ($p=0.12$), however there was a significant age difference in both high density and low density lipoproteins ($p=0.01$, $p=0.02$ respectively). There was a significant difference for mean arterial pressure between the groups ($p=0.002$), however MAP did not change over the duration of the protocol.

No significant physiological differences in the $\%CVC_{max}$ responses to acetylcholine were observed between the sexes for either the older or the younger subject group, therefore the data from both sexes in each group were combined. When drug treatment sites were compared within age group over time there were no difference in $\%CVC_{max}$ between infusion 1 and infusion 2 ($p=1.0$) for both subject groups. Therefore data from both infusions were combined.

Group mean responses in all drug treatment sites over time to 137.5 μM acetylcholine infusion are shown in Figure 3-1 A for the young subject group and Figure 3-1 B for the older subject group. There were no differences between the responses in the control and NOS inhibited sites in either subject group. In the young subjects, COX inhibition caused a significant attenuation of the response in comparison to the control site ($p<0.001$). Additionally, with NOS + COX inhibition the response was attenuated compared to control ($p<0.001$) but it was increased compared to COX inhibition alone ($p<0.001$). In the older subjects COX inhibition and NOS + COX inhibition attenuated the $\%CVC_{max}$ response to acetylcholine ($p<0.001$), but there was no significant difference between these two drug treatment sites ($p=0.86$).

Group mean data for baseline $\%CVC_{max}$ in all of the drug treatment sites in young and older subjects are shown in Figure 3-2. There was no difference between groups for baseline values at the control site or the NOS inhibited site, (O: 10±1 vs Y: 12±1 $\%CVC_{max}$, $p=0.18$) and (O: 10±1 vs. Y: 11±1 $\%CVC_{max}$, $p=0.34$) respectively. There was a significant increase in baseline $\%CVC_{max}$ above the control site in the older subject group in the COX inhibited site that was not seen in the younger subject group (O: 16±1 vs Y: 10±1 $\%CVC_{max}$, $p<0.001$; within group vs. control O: $p<.001$, Y: $p=0.25$). NOS + COX inhibition similarly resulted in an increase in baseline $\%CVC_{max}$ above control values in only the older subject group (O: 16±2, vs. Y: 14±1 $\%CVC_{max}$, $p<.001$; within group vs. control O: $p<.001$, Y: $p=0.19$).

Group mean data for peak $\%CVC_{max}$ responses to infusion of 137.5 μM acetylcholine in both subject groups are shown in Figure **3-3**. There was no difference between the groups for the peak responses at the control site (O: 38±5 vs. Y: 39±4 $\%CVC_{max}$, $p=0.74$). Furthermore, there were no significant differences between the

control and the NOS inhibited sites within or between groups (O: 35±5 vs. Y: 41±4 $\%CVC_{max}$, p=.16; within group vs. control O: p=.84, Y: p=.67). COX inhibition significantly attenuated the peak responses to acetylcholine in both subject groups compared to the control site (O: 29±3 vs. Y: 22±2 $\%CVC_{max}$, p<.001). However, the older subject group exhibited attenuation, but to a lesser degree, in peak $\%CVC_{max}$ when COX was inhibited when compared to the young subject group (p<0.001). Inhibiting both NOS + COX decreased the peak responses in both subject groups vs control (O: 32±3, Y: 29±2 $\%CVC_{max}$, p<.001). Peak responses during NOS+COX inhibition were increased compared to COX inhibition alone (p<0.001) in the young subject group but not in the older subject group (p=0.68).

Discussion

The major findings of the present study were that in young and older subjects, cutaneous vasodilatation to exogenous acetylcholine is mediated by prostanoid-dependent and non-NO-, non-prostanoid-dependent pathways. NO does not directly contribute to acetylcholine-mediated cutaneous vasodilatation in either age group. The older subjects exhibited an increase in baseline $\%CVC_{max}$ in sites where COX was inhibited, suggesting that there is an increase in COX pathway vasoconstrictor products that contribute to basal cutaneous vasomotor tone. Finally, there is an attenuated prostanoid-dependent contribution in the vasodilatory response to exogenous acetylcholine as skin ages.

Mechanisms of acetylcholine-mediated vasodilatation in young skin

Our findings implicate prostanoid-dependent as well as non-NO-, non-prostanoid dependent contributions to exogenous acetylcholine-mediated vasodilatation but indicate no direct role for NO. This *in vivo* finding supports *in vitro* data from Buus et al. (2000), who concluded that while the L-arginine/NO pathway is present in human isolated subcutaneous vessels, the NO pathway contributes only minimally to acetylcholine-

mediated vasodilatation (Buus *et al.*, 2000). Studies using iontophoresis application of acetylcholine have also demonstrated a limited role for NO in the cutaneous response to acetylcholine (Khan *et al.*, 1997; Noon *et al.*, 1998). As validated by the data presented here, those authors attributed much of the observed cutaneous vasodilatation to prostanoid-dependent pathways.

In contrast to our finding for a limited direct role for NO in acetylcholine-mediated vasodilatation, Boutsiouki et al. (2004) recently recovered NO byproducts in microdialysis dialysate in response to acetylcholine infusion. Dialysate recovery of NO metabolites was subsequently abolished using 5mM L-NAME, but NOS inhibition only partially attenuated the vasodilator response by ~30% (Boutsiouki *et al.*, 2004), suggesting the involvement of other vasodilator pathways. Moreover, direct measurements of cutaneous NO *in vivo* during 160mM acetylcholine infusion have demonstrated limited measurable increases (Zhao *et al.*, 2004) . Our data do not support a direct role for NO; however it is possible that a small NO-dependent vasodilatation may be observed with higher doses of acetylcholine such as those that were used in the Boutsiouki study. One possible mechanistic explanation for a limited direct role for NO in acetylcholine-mediated vasodilatation is that NO may be quenched by superoxide anions produced from the COX pathway before acting on the vascular smooth muscle (Marcelin-Jimenez & Escalante, 2001; Bratz & Kanagy, 2004). Additionally, animal studies have shown that the NOS and the COX pathways interact in a complex manner capable of modulating enzymatic cross talk (Bratz & Kanagy, 2004), such that inhibiting one pathway may augment the vasodilator contributions of other pathways. In this construct, it may be possible to recover NO metabolites from microdialysis dialysate but observe little or no direct contribution of NO and a significant COX contribution to acetylcholine-mediated vasodilatation.

We found that a significant portion of acetylcholine-mediated vasodilatation could not be abolished with either COX or NOS inhibition; by process of elimination, this suggests a non-NO-, non-prostanoid-dependent pathway contributes to acetylcholine-mediated vasodilatation in the skin in humans. The remaining vasodilatation in response to acetylcholine infusion could be attributed to endothelial derived hyperpolarization

factors (EDHF), which are most likely products of the arachadonic acid pathway. Although the identity of EDHF is unknown, metabolites of the cytochrome p450 enzymes and epoyeicosatrienic acids are good candidates as potenital EDHFs (Hecker *et al.*, 1994; Mombouli & Vanhoutte, 1997; Hatoum *et al.*, 2004). We found a significant portion of vasodilatation could not be blocked in the presence of COX inhibitors or with simultaneous NOS and COX inhibition (Figures 3-1 and 3-3), suggesting that EDHF-type substances may be more abundant in the skin when COX is inhibited due to an increase in the availability and metabolism of arachadonic acid. One unexpected finding in the present study was that the cutaneous vascular response to acetylcholine was actually higher when both NOS and COX were inhibited compared to COX inhibition alone. The most likely explanation for this response is an upregulation of the EDHF pathway(s) when all other vasodilatory pathways are inhibited, which further suggests the possibility of cross talk and redundant mechanisms at play in these pathways. Additional research is needed with specific inhibitors of arachadonic acid metabolism to determine possible EDHFs at play in this response. However, it is likely that there are many EDHFs with varied distribution within the branches of the cutaneous vasculature as well as redundancy in dilator mechanisms between NO, prostanoids, and EDHFs (Osanai *et al.*, 2000). Exogenous infusion of acetylcholine into the skin through microdialysis may prove to be a useful tool in future *in vivo* research on the identity of EDHFs in the cutaneous vasculature.

Age-related changes in acetylcholine-mediated vasodilatation

Our findings indicate that aged human skin exhibits altered contributions of COX products to both tonic cutaneous blood flow and exogenous acetylcholine-meditated vasodilatation. In the older subjects, baseline $\%CVC_{max}$ was significantly lower in comparison to the younger subjects in the control site but was elevated in sites where COX was inhibited. One likely explanation for this response is that COX isoenzymes can produce both vasoconstrictor (PGE_2, thromboxane A_2) and well as vasodilator (PGI_2) substances, and with advanced age there is a shift in the balance between COX

vasoconstrictor and vasodilator products to favor vasoconstriction (Taddei *et al.*, 1997; Matz *et al.*, 2000a; Matz *et al.*, 2000b). Specifically, there may be alterations in the expression of the COX isoenzymes (Heymes *et al.*, 2000). Data from the forearm circulation, where both NO- and prostanoid-dependent pathways contribute to acetylcholine-mediated vasodilation, suggests increases in COX derived endothelial vasoconstrictor products in subjects with pathology induced endothelial dysfunction (Taddei *et al.*, 1997). This age-related shift toward increased vasoconstriction through alteration in COX products as well as COX isoenzyme expression may help to explain the baseline responses in the older subject group in the present study. Our data suggest that there is an increase in vasoconstrictor COX products that contribute to tonic cutaneous vascular tone in aged skin. Furthermore, peak $\%CVC_{max}$ responses to infusions of acetylcholine in COX inhibited sites were augmented in older subjects compared to the young subjects, suggesting the non-specific COX inhibition antagonized an up-regulated COX-mediated vasoconstriction. Taken together, these data suggest that with advanced age there is a shift toward increased vasoconstrictor products of COX and a decrease in COX-mediated vasodilatation in the cutaneous vasculature.

We originally hypothesized we would find an attenuated vasodilatory response to exogenous acetylcholine in the older subjects. Healthy aging, in the absence of overt pathology, is associated with mild endothelial dysfunction (Singh *et al.*, 2002), and a decreased vasodilatory response to acetylcholine. Surprisingly, we did not find significant differences in the responses to acetylcholine between the subject groups at the control site. Although, we did not observe a significant difference in terms of $\%CVC_{max}$, aged skin exhibits reduced maximal CVC (Martin *et al.*, 1995) which may be masking the expected reduction in endothelium-dependent vasodilatation with aging. That is, our choice to scale CVC as a percent of maximal conductance in order to compare between experimental sites may be concealing larger potential differences between subject groups. We did, however observe alteration in the downstream pathways mediating acetylcholine-mediated vasodilatation in aged skin including an attenuated vasodilator prostanoid contribution.

Acetylcholine contributions to attenuated reflex vasodilatation with aging

With advanced age there is attenuated reflex vasodilatation, including a substantial reduction in the initial rise in skin blood flow. We had previously attributed this attenuation in older subjects to a decreased ability of aged skin to respond to acetylcholine (Holowatz *et al.*, 2003). However, in the present study we found that healthy older subjects did not exhibit a significant reduction in cutaneous vasodilatation to this dose of exogenous acetylcholine. Shibasaki et al. (2002) have implicated acetylcholine mediating vasodilatation through NO-dependent mechanisms in the initial rise in skin blood flow during hyperthermia. These authors observed an augmented initial rise in skin blood flow during acetylcholinesterase inhibition but an attenuated response with concurrent NO synthase inhibition, suggesting that acetylcholine contributes to the initial rise in skin blood flow during hyperthermia through NO-dependent mechanisms (Shibasaki *et al.*, 2002). However, recent evidence has established a role for an histamine 1 (H1) receptor-mediated component to the initial rise in reflex vasodilatation which is partially dependent on NO (Wong *et al.*, 2004). Therefore, it is possible that acetylcholine does not work directly through NO-dependent mechanisms during reflex cutaneous vasodilatation, and instead, what Shibasaki et al. attributed to NO-dependent vasodilatation through acetylcholine may have been an H1 NO-dependent contribution. In other words, concurrent acethylcholinesterase and NO synthase inhibition would inhibit any NO-dependent vasodilatation whether it was mediated from acetylcholine or histamine-dependent mechanisms. In light of the evidence from Wong et al. (2004) and the data from our current study, it is possible that acetylcholine mediates vasodilatation during the initial rise in skin blood flow during hyperthermia through non-NO-dependent mechanisms. An alternative hypothesis in terms of attenuated reflex cutaneous vasodilatation with aging is that older subjects may have a reduced H1 receptor-mediated NO-contribution to the initial rise in skin blood flow. The contribution of H1 receptor-mediated reflex vasodilatation in aged skin as well as the downstream acetylcholine contributions to the initial rise in skin blood flow during hyperthermia needs further investigation.

Limitations

Acetylcholine produces vasodilatation through both endothelium-dependent mechanisms as well as through neurogenic mechanisms, such as the axon reflex (Berghoff *et al.*, 2002), although the precise mechanisms of the axon reflex and a potential role for acetylcholine remain unclear. Many studies examining acetylcholine-mediated vasodilatation have used iontophoresis as a way to deliver acetylcholine to the skin, where anodal current from this technique alone has been shown to cause an axon reflex that is sensitive to systemic COX inhibition using acetylsalicylic acid (Morris & Shore, 1996; Berghoff *et al.*, 2002; Durand *et al.*, 2002b; Durand *et al.*, 2002c). Moreover, other studies examining the acetylcholine-induced axon reflex have used direct subcutaneous injections of acetylcholine (Douglas & Ritchie, 1960). In this instance, the trauma from the injection can alone stimulate neurogenic axon reflexes. Boutsiouki et al. (2004) recently found that acetylcholine delivered through a microdialysis fibre produced localized vasodilatation in response to low doses of acetylcholine and a more widespread flare response with an accompanying itch sensation at doses above 6.25 mM (Boutsiouki *et al.*, 2004). Our dose of acetylcholine was very small in comparison to the study by Boutsiouki et al., and we did not observe a flare response nor did any of our subjects report feeling an itching sensation with acetylcholine infusion. Our data are limited in that they do not allow us to differentiate between an axon reflex and endothelium-dependent vasodilatation. However, we attempted to minimize the potential for neurogenic vasodilatation by using the skin specific technique of microdialysis instead of iontophoresis and by delivering a low dose of acetylcholine.

Methodological steps were taken in the present study to use sufficient doses of L-NAME and ketorolac to maximally inhibit the cutaneous vasodilatory response to stimulators of the NO and COX pathways. Inhibition of the NO synthase pathway has been demonstrated using 10mM L-NAME which maximally inhibits NO production in response to local heating of the skin of both young and aged subject groups (Minson *et al.*, 2002). With regard to the dose of ketorolac used in this study, increasing the concentration of the COX antagonist did not further decrease the skin blood flow

response to acetylcholine. Additionally, our dose of ketorolac per volume of tissue was higher that what is commonly given during arterial infusion studies (Dinenno & Joyner, 2004; Schrage *et al.*, 2004). Even though we are confident that we would not have seen a different skin blood flow response to acetylcholine with higher doses of antagonists in either pathway investigated, we cannot be certain that we maximally inhibited both the NO and the COX pathways. Therefore, we cannot rule out a larger role for NO or COX products in the cutaneous vascular response to acetylcholine.

Summary

In summary, we found that NO did not directly contribute to acetylcholine-mediated vasodilatation in either age group. Furthermore, prostanoid-dependent and non-NO-, non-prostanoid-dependent pathways contribute to cutaneous acetylcholine-mediated vasodilatation. Our data suggest that older subjects exhibit alterations in COX vasoactive products to favor vasoconstriction. This shift may contribute to basal cutaneous vascular tone and to the attenuated vasodilator prostanoid contribution to acetylcholine-mediated vasodilatation.

Table **3-1**: Subject Characteristics. Values are means±SE; HDL, high density lipoprotein; LDL, low density lipoprotein; MAP mean arterial pressure. *Significant difference from younger subjects ($p<0.05$)

Variable	Young	Older
Sex (M, F)	7, 5	5, 5
Age, years	23±1	69±1*
BMI, kg/m^2	23±1	23±1
$VO_{2\ peak,}$ ml/kg/min	37±2	23±1*
Total Cholesterol, mg/dl	160±7	184±11
HDL, mg/dl	36±5	53±4*
LDL, mg/dl	75±7	109±10*
Resting MAP, mmHg	86±2	95±2*

Figure 3-4

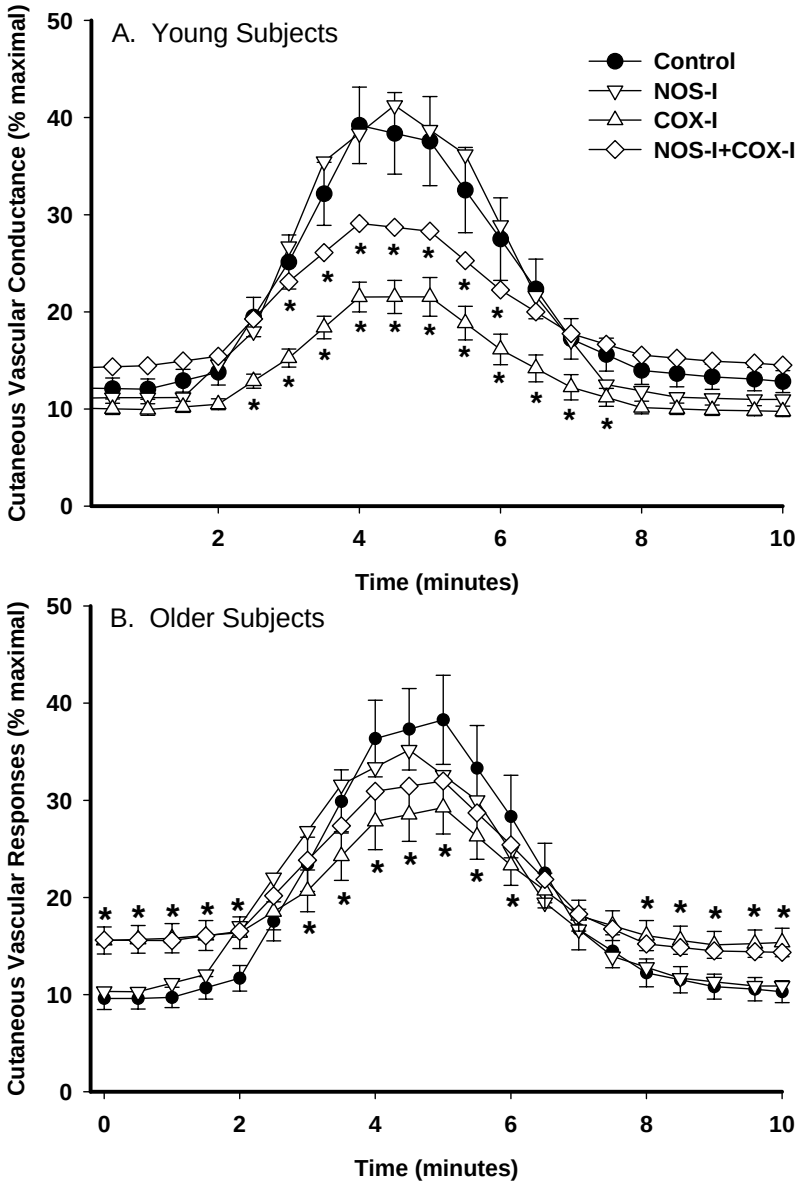

Figure **3-4**: Group mean responses in drug treatment sites to infusion of 137.5µM acetylcholine in **A:** young subjects (n=12), and **B:** older subject (n=10). ● Control site lactated Ringer infusion, ▽ nitric oxide synthase inhibited (NOS-I) 10mM L-NAME, △cylooxygenase inhibited (COX-I) 10mM ketorolac, ◊ nitric oxide synthase and cyclooxygenase inhibited (NOS-I+COX-I) 10mM L-NAME + 10mM ketorolac. Standard error bars omitted or the NOS-I and NOS-I+COX-I sites for clarity. *Significant difference from the control site ($p<0.05$)

Figure 3-2

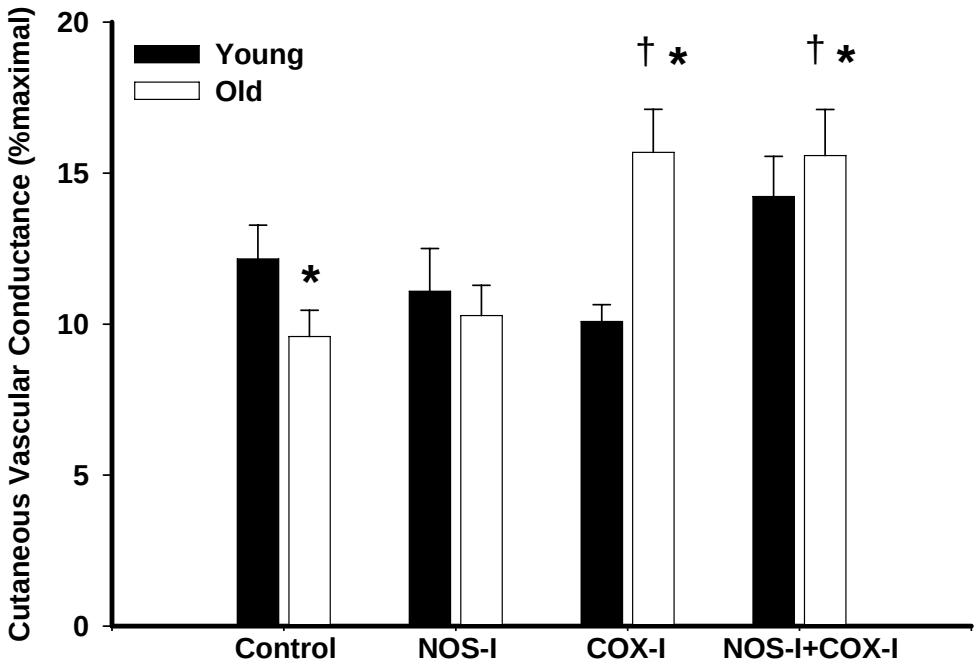

Figure **3-2**: Baseline $\%CVC_{max}$ in all drug treatment sites before infusion of 137.5µM acetylcholine, solid bars represent the young subject group (n=12) and the open bars represent the older subject group (n=10). Treatment with ketorolac alone (COX-I) and ketorolac + L-NAME (COX-I + NOS-I) significantly increased baseline in the older subjects. * $p<0.05$ significant difference between groups, †$p<.005$ significant vs. control site older subject group, ‡ $p<.005$ significant vs. control site young subject group.

Figure 3-3

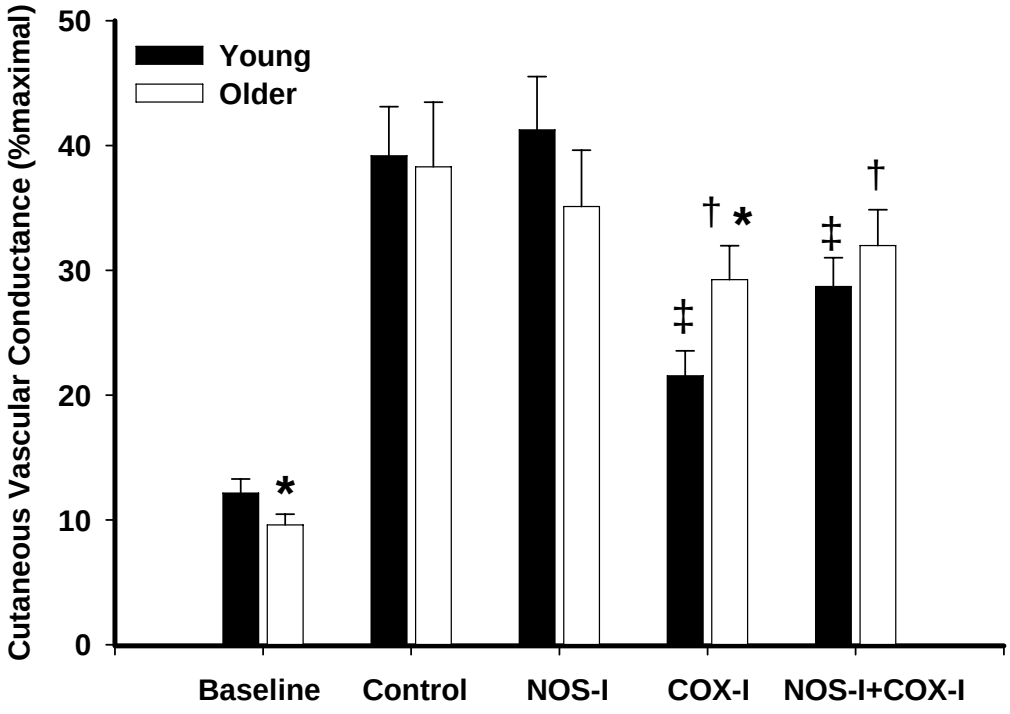

Figure **3-3**: Peak %CVC_{max} responses in all drug treatment sites to infusion of 137.5µM acetylcholine, solid bars represent the young subject group (n=12) and the open bars represent the older subject group (n=10). Peak %CVC_{max} was attenuated in both subject groups in sites treated with ketorolac (COX-I), but not in sites treated with L-NAME (NOS-I) alone,* $p<0.05$ significant difference between groups †$p<.005$ significant vs. control site older subject group, ‡ $p<.005$ significant vs. control site young subject group.

Chapter 4

L-ARGININE SUPPLEMENTATION OR ARGINASE INHIBITION AUGMENTS REFLEX CUTANEOUS VASDILATATION IN AGED HUMAN SKIN

Introduction

Skin blood flow is neurally controlled by two distinct branches of the sympathetic nervous system, an adrenergic vasoconstrictor system and an active vasodilator system (Grant & Holling, 1938). Under normothermic conditions, the cutaneous vasculature is under tonic adrenergic control. However, with rising body core temperature, skin blood flow initially increases through a withdrawal of tonic vasoconstriction, and upon reaching a specific threshold skin blood flow further increases by the active vasodilator system (Roddie *et al.*, 1957a). Cutaneous active vasodilatation is mediated by sympathetic cotransmission of acetylcholine and an unknown neurotransmitter (Kellogg *et al.*, 1995). Vasoactive intestinal peptide (VIP) and histamine 1 (H1) receptor activation contribute to reflex cutaneous vasodilatation (Bennett *et al.*, 2003; Wilkins *et al.*, 2004b; Wong *et al.*, 2004). Moreover, these vasodilator pathways mediate downstream nitric oxide (NO) - dependent vasodilatation, which is required for full expression of the reflex vasodilatory response (Shastry *et al.*, 1998).

Aged humans exhibit attenuated cutaneous vasodilatory responses during hyperthermia (Kenney *et al.*, 1997), resulting from a diminished neurogenic cotransmitter contribution and an increased reliance on impaired NO-dependent vasodilatation (Holowatz *et al.*, 2003). Attenuated NO-dependent vasodilatation is associated with decreased NO bioavailability. In aged skin decreased NO bioavailability is likely multifaceted, involving several potential signaling pathways, including dysregulated utilization of the NO substrate, L-arginine (L-arg).

One potential mechanism that has been implicated in reduced L-arg availability for NOS is augmented vascular arginase (Arg) activity (Hecker *et al.*, 1995; Berkowitz *et*

al., 2003). Arg is constitutively expressed in two isoforms (Arg I and Arg II), which catalyze the conversion of L-arg to L-ornithine and urea during the final step of the urea cycle. Arg I is most likely the predominant isoform in the vasculature and is capable of reciprocally regulating endothelial NOS by competing for the common substrate L-arg (Berkowitz *et al.*, 2003; White *et al.*, 2006) (Figure 4-1).

In aged and hypertensive animal models of microvasculature dysfunction, augmented Arg activity contributes to impaired NO bioavailability (Berkowitz *et al.*, 2003; John & Schmieder, 2003; White *et al.*, 2006), and both acute and chronic inhibition of Arg restore endothelial NO-dependent vasodilatation. Furthermore, augmented Arg activity may serve as a potential explanation for the "L-arginine paradox", which states that despite the high intracellular concentrations of L-arg that far exceed the Michaelis constant for endothelial NOS ($2\text{-}10^{-6}$ M) (Grody *et al.*, 1987; Griffith & Stuehr, 1995), NO-dependent vasodilatation *in vivo* can be further increased by the administration of exogenous L-arg. Taken together, these findings suggest that increased Arg activity may limit the available pool of L-arg for NOS and, therefore, the bioavailability of NO. However, the role of Arg in regulating intracellular stores of L-arg and subsequent NO-dependent vasodilatation in humans remains unclear.

Therefore, the purpose of this study was to determine the role of Arg in reflex cutaneous vasodilatation in aged humans. We hypothesized that Arg inhibition alone and with concurrent L-arg supplementation would augment cutaneous vasodilatation during passive whole body heat stress by increasing L-arg availability for NO synthesis.

Methods

Subjects

Experimental protocols were approved by the Institutional Review Board at The Pennsylvania State University and conformed to the guidelines set forth by the Declaration of Helsinki. Verbal and written consent was voluntarily obtained from all

subjects prior to participation. Studies were performed on 10 young (18-27 years) and 9 older (65-72 years) men and women. Each subject underwent a complete medical screening, including blood chemistry, lipid profile evaluation (Quest Diagnostics Nichol Institute, Chantily, VA, USA), physical examination, and an assessment of maximal oxygen uptake (VO_{2max}) (SensorMedics Corperation, Yorba Linda, CA, USA). All subjects were screened for the presence of cardiovascular, dermatological, and neurological disease. Subjects were normally active, normotensive, non-diabetic, healthy non-smokers who were currently not taking medications, including aspirin therapy, hormone replacement therapy or oral contraceptives. All young female subjects were studied on days 2-7 of the early follicular phase of their menstrual cycle.

Instrumentation and Measurements

All protocols were performed in a thermoneutral laboratory with the subject in the supine position with the experimental arm at heart level. Upon arrival to the laboratory between the hours of 0700-0900, subjects were instrumented with five intradermal microdialysis fibres (MD 2000, Bioanalytical Systems, IN, USA) (10 mm, 20 kDa cutoff membrane) in the skin on the right ventral forearm. Microdialysis sites were at least 4.0 cm apart to insure no cross-reactivity of pharmacological agents being delivered to the skin. Microdialysis fibres were placed at each site by first inserting a 25 gauge needle through unanaesthetized skin using sterile technique. The entry and exit points were ~2.5 cm apart. The microdialysis fibre was then threaded through the needle, and the needle was withdrawn, leaving the fibre in place. The microdialysis fibres were taped in place and perfused with lactated Ringer's solution during the insertion trauma resolution period at a rate of 2.0μL min^{-1} (Bee Hive controller and Baby Bee microinfusion pumps, Bioanalytical Systems, IN, USA) for 60-90 minutes.

To obtain an index of skin blood flow, cutaneous red blood cell (RBC) flux was measured with an integrated laser-Doppler flowmeter probe placed in a local heater (MoorLAB, Temperature Monitor SH02, Moor Instruments, Devon, UK) on the skin directly above each microdialysis membrane. All laser-Doppler probes were calibrated

using Brownian standard solution. Cutaneous vascular conductance (CVC) was calculated as RBC flux divided by mean arterial pressure.

To control whole body temperature, subjects wore a water-perfused suit that covered the entire body except head, hands, and experimental arm and a water-impermeable rain suit to minimize evaporative heat loss. The subject's electrocardiogram was monitored throughout the protocol, and blood pressure was measured via brachial auscultation every 5 minutes. Oral temperature (T_{or}) was continuously monitored during baseline and throughout whole body heating with a thermister placed in the sublingual sulcas as an index of body core temperature. The subjects were instructed to keep the thermister in the same location in the sublingual sulcus and not to open their mouths or speak during the protocol. Mean skin temperature was calculated as the unweighted average of six copper-constantan thermocouples placed on the chest, middle back, abdomen, upper arm, thigh, and calf. During the insertion trauma resolution and baseline periods, thermoneutral water (34°C) was perfused through the suit to clamp whole body temperature. During whole body heating, 50°C water was perfused through the suit to raise subject's T_{or} 1.0°C above baseline body temperature.

Experimental Protocol

A schematic representation of the protocol is illustrated in Figure 4-2. RBC flux over each microdialysis site was monitored during the insertion trauma resolution period. Following this period, microdialysis sites were randomly assigned to receive 1) 10.0mM N^G-nitro-L-arginine (L-NAME) to inhibit NO production by NOS, 2) the combination of 5.0mM (s)-(2-boronoethyl)-L-cysteine-HCl (BEC) and 5.0mM N omega-hydroxy-nor-L-arginine (nor-NOHA) to inhibit Arg (Calbiochem, San Diego, CA, USA), 3) 10.0mM L-arginine (Sigma) to supplement the substrate for NOS and Arg, or 4) 5.0mM BEC + 5.0mM nor-NOHA + 10.0mM L-arg to inhibit Arg and supplement the substrate for NOS and Arg. All pharmacological agents were dissolved in lactated Ringers solution. A fifth microdialysis site was perfused with only lactated Ringers to serve as control.

Our laboratory previously showed that a 10.0mM dose of L-NAME was sufficient to maximally inhibit NOS in both subject groups (Minson *et al.*, 2002). Extensive pilot work was conducted to ensure that the concentrations of arginase inhibitors maximally inhibited the arginase pathway. Briefly, varying concentrations (0.1mM, 1.0mM, 2.5mM, 5.0mM 10.0mM) of each BEC + nor-NOHA were delivered to different skin microdialysis sites during a standardized local heating protocol described elsewhere (Minson *et al.*, 2001). Similar pilot testing was conduced using (2.5mM, 5.0mM, 10.0mM, and 20.0mM) L-arg. Increasing concentrations above 2.5mM BEC + 2.5mM nor-NOHA and 5.0mM of L-arg did not further increase the NO-dependent plateau phase of the local heating response.

All microdialysis sites were perfused with assigned pharmacological agents continuously for at least 60 minutes prior to the start of the baseline and during the baseline and heating periods at a rate of 2.0μL min^{-1}. Baseline data were collected for 20 minutes prior to the start of whole body heating. After the baseline data collection period, whole body heating was conducted to raise T_{or} by 1.0°C. At the end of the heating protocol, each microdialysis site was perfused with 28.0mM sodium nitroprusside (SNP; Nitropress, Abbot Laboratories, Chicago, IL, USA) at a rate of 4.0μL min^{-1} to achieve maximal CVC. Local heating of the skin to 43°C was conducted simultaneously with SNP infusion to ensure maximal CVC had been obtained.

Data Acquisition and Analysis

Data were acquired using Labview software and National Instruments data acquisition system (Austin, TX, USA). The data were collected at 40 Hz, digitized, recorded and stored on a personal computer for further analysis. CVC data were averaged over 3-minute periods for baseline and every 0.1°C rise in T_{or} and are presented as a percent of maximal CVC (%CVC_{max}). Two reviewers blinded to the age of the subjects and to the pharmacological treatment of the microdialysis sites visually identified the absolute T_{or} and delta T_{or} (ΔT_{or}) at which the threshold for reflex cutaneous vasodilatation was initiated in each microdialysis site.

Student's t-tests were used to determine significant differences between the young and older groups for physical characteristics and baseline T_{or}. A two-way repeated measures analysis of variance (ANOVA) was conducted to detect age and pharmacological treatment effects on the threshold T_{or} (absolute T_{or} and ΔT_{or}) for reflex cutaneous vasodilatation. A three-way repeated measures ANOVA was conducted to detect differences between subject groups at the pharmacological treatment sites over the rise in T_{or} (SAS, version 8.01). Planned comparison tests, including Tukey *post hoc* tests, were performed when appropriate to determine where differences between groups and drug treatments occurred. The level of significance was set at α=0.05. Values are presented as means ± SEM.

Results

The physical characteristics of the subjects are presented in Table 4-1. There were no differences between the groups for body mass index or mean arterial pressure, but the older group had a significantly lower VO_{2max} ($p=0.002$). Total cholesterol and low density lipoprotein levels were significantly higher in the older subject group ($p=0.001$ for both), but there was no difference in high density lipoproteins between the age groups.

Table 4-2 shows the threshold values for reflex cutaneous vasodilatation in both age groups. There was no difference between groups in baseline T_{or} ($p=0.12$). The threshold for reflex vasodilatation at the control site and the NOS inhibited site was significantly lower in the young subject group vs. the aged subject group ($p<0.05$). There was no difference between the control site, the Arg-inhibited, the L-arg supplemented, or the Arg-inhibited + L-arg supplemented within each age group ($p>0.05$).

Figure 4-3 illustrates the $\%CVC_{max}$ responses across the rise in body core temperature in both age groups. In the young subject group the differences between the control site and the NOS inhibited site started at ΔT_{or}=0.4°C; this difference was observed in the aged subject group at $\Delta T_{or} \geq 0.6$°C ($p<0.001$ between groups). There was no difference in the $\%CVC_{max}$ responses in the Arg-inhibited compared to the control site

in the young subject group (Figure 4-3 A). However, in the older subject group, Arg inhibition significantly increased $\%CVC_{max}$ above the level of the control site at $\Delta T_{or} \geq 0.7°C$. Similarly, CVC in the L-arg supplemented (Figure 4-3 B) and the Arg-inhibited + L-arg supplemented (Figure 3C) site was not a significantly different from the control site in young subjects but was significantly augmented compared to the control site in the older subject group starting at $\Delta T_{or}=0.7°C$. Moreover, Arg inhibition, L-arg supplementation, and Arg inhibition + L-arg supplementation in the older subject groups increased cutaneous vasodilatation such that there was no difference between these microdialysis sites compared the young subject group's control site ($P>0.05$).

Figure 4-4 summarizes the $\%CVC_{max}$ responses in each microdialysis treatment site with a 1.0°C rise in body core temperature. Baseline $\%CVC_{max}$ is included for visual comparison. Older subjects had significantly attenuated responses in the control and the NOS-inhibited sites compared to young subjects ($P>0.05$). There were no difference between young and older subjects in the Arg-inhibited, L-arg supplemented, or Arg-inhibited + L-arg supplemented treated sites. Arg inhibition, L-arg supplementation, and Arg inhibition + L-arg supplementation significantly increased $\%CVC_{max}$ above the level of the control site in the older subjects but not in young subjects.

Discussion

The principle finding of the present study was that acute Arg inhibition, L-arg supplementation, or both in the cutaneous vasculature selectively augments reflex cutaneous vasodilatation in aged human subjects. In young subjects, these treatments did not significantly alter the cutaneous vasodilatory response during whole body heat stress. Furthermore, the effects of combined Arg inhibition and L-arg supplementation in aged skin were not additive. These results suggest that Arg activity may be increased in aged human skin and may limit the intracellular availability of L-arg for NOS. Cumulatively, these data demonstrate that the age-related deficit in reflex cutaneous vasodilatation can be restored by either 1) inhibiting Arg to replenish the available pool of L-arg for NOS,

or by 2) directly supplementing superphysiological concentrations of L-arg to effectively saturate the Arg and NOS pathways (Figure 4-1).

Our results indicate that NO bioavailability is compromised in aged human skin and that pharmacological interventions specifically targeting the L-arg/NO pathway can increase cutaneous blood flow during hyperthermia. In young human subjects, NO is required for full expression and contributes approximately 40-50% to the total reflex cutaneous vasodilatory response (Shastry *et al.*, 1998); additionally both VIP and H1 receptor activation mediate vasodilatation through NO-dependent mechanisms (Wilkins *et al.*, 2004b; Wong *et al.*, 2004). Furthermore, NO is capable of mediating cutaneous vasodilatation synergistically with sympathetic cotransmitters, resulting in a combined vasodilatation that is greater than the sum of the individual contributions (Wilkins *et al.*, 2003). In the context of human ageing, we have previously shown that older subjects have an impaired cotransmitter contribution to cutaneous vasodilatation with significant increases in body core temperature (Holowatz *et al.*, 2003). Instead, the aged rely on an impaired NO-dependent mechanism to increase blood flow to the skin during thermal stress. Our current findings suggest that in aged human skin, the intracellular stores of L-arg available for NOS and NO-dependent vasodilatation are insufficient to support the full expression of the increase in skin blood flow during hyperthermia. Alternatively, the interventions employed in the present investigation affecting the L-arg/NO pathway may have capitalized on the synergistic relationship between NO and the cotransmitter(s) mediating reflex cutaneous vasodilatation; by increasing the amount of bioavailable NO in the cutaneous vasculature, the total vasodilatory response of both NO and non-NO mediated vasodilatation may have been augmented.

Our data show that the cutaneous vasodilatation in the combined treatment site was not significantly different compared to the arginase inhibited or L-arginine supplemented treatments alone, indicating that the effects of the individual treatments were not additive. One potential explanation for this finding is that the individual drug treatments were sufficient to replenish intracellular L-arg for NO synthesis through NOS, such that the L-arg/NOS pathway was operating near V_{max}. Another possibility is that we maximized the capacity of the cutaneous vessels to vasodilate at this level of

hyperthermic stress (1.0°C) approaching a "ceiling effect" with our individual drug treatments and that increasing the degree of hyperthermic stress may have unmasked further vasodilatation in the combined treatment site.

There are several putative mechanisms affecting the L-arg/NO pathway and subsequent NO-dependent vasodilatation that may be impaired in aged skin. The most plausible mechanisms directly alter the intracellular availability of L-arg for NOS and include augmented Arg activity, the subcellular distribution of L-arg in relation to NOS, and age-related increases in endogenous NOS inhibitors. Additionally, these mechanisms have also been suggested as possible explanations for the L-arg paradox where the intracellular concentration of L-arg far exceed the K_m for NOS but the addition of exogenous L-arg augments NO-dependent vasodilatation.

Our *in vivo* findings implicate a role for augmented Arg activity limiting the availability of L-arg for NOS and subsequent NO-dependent cutaneous vasodilatation in humans. These results are in agreement with *in vitro* isolated vessel investigations where Arg I is capable of reciprocal regulation of endothelial NOS and its activity is upregulated in aged vessels (Berkowitz *et al.*, 2003; White *et al.*, 2006). These authors found that pre-treatment with Arg inhibitors directly restored NO signalling and L-arg responsiveness in aged vessels. Similarly, our data also demonstrates that the age-associated decline in cutaneous vasodilatory function was restored by Arg inhibition. However, in contrast to the study by Berkowitz et al., we were able to induce augmented cutaneous vasodilatation with L-arg supplementation alone in the absence of concurrent Arg inhibition. These divergent findings may be due to species, tissue, and vascular tree differences and methodological differences including the dose of L-arg delivered to the vasculature. Alternatively, another explanation involves the intracellular compartmentalization of L-arg available for NO synthesis in relation to NOS localization. In young endothelial cells, intracellular L-arg is sequestered in several pools including 1) a pool associated with the cationic amino acid transporter (CAT) that is freely exchangeable with the extracellular space and associated with caveolar NOS and 2) a pool in the cytosolic fraction where Arg I is localized that is non-freely exchangeable (McDonald *et al.*, 1997; Flam *et al.*, 2001). Localized cutaneous L-arg supplementation

most likely affected the caveolar associated pool of L-arg whereas Arg inhibition likely increased the availability of L-arg in the cytosolic pool.

Another putative explanation for the L-arg paradox is the accumulation of endogenous NOS inhibitors. Asymetric dimethylarginine (ADMA) is the most abundant of the endogenous NOS inhibitors in humans and both hyperlipidemia and age-related impairments in microvascular function and subsequent NO bioavailability have been linked to increases in AMDA (Miyazaki *et al.*, 1999; Kielstein *et al.*, 2003). The mechanisms mediating increased AMDA are two fold, which include an increase in its formation and a decrease in degradation via oxidative stress (Fliser, 2005). *In vivo* human studies have demonstrated that AMDA-mediated NOS inhibition in the forearm circulation is reversible by the administration of L-arg (Kielstein *et al.*, 2005). In the context of the current study, an increase in endogenous NOS inhibitors in the aged subjects could help explain why cutaneous vasodilatation was significantly augmented with L-arg supplementation in the absence of Arg inhibition.

Our findings show that direct L-arg supplementation to the cutaneous vasculature through intradermal microdialysis significantly improves cutaneous vasodilatory function during hyperthermia in aged humans. Other investigations examining the effects of L-arg supplementation on vasodilatory responsiveness have reported mixed results depending on the dose, duration and route of administration. Consistent with our data, L-arg infusion into forearm and coronary vascular beds have more consistently demonstrated an increase in NO bioavailability and improved endothelium-dependent vasodilatation (Chauhan *et al.*, 1996; Pernow *et al.*, 2003; Perticone *et al.*, 2005). In contrast, recent evidence from a clinical trail examining the effects of oral L-arg supplementation (9 mg day^{-1}) on non-invasive measures of resting vascular function (pulse pressure, arterial compliance, pulse wave velocity and arterial elastance) in patients following acute myocardial infarction failed to demonstrate a significant effect of L-arg treatment (Schulman *et al.*, 2006). These non-specific tests of arterial stiffness during resting conditions may have not been sufficient to observe significant differences between treatment and placebo groups. Both, *in vitro* and *in vivo* human data in the forearm circulation suggest that potent vasodilatory stimuli enhance L-arg transport through CAT-

1, significantly increasing NO production (Parnell *et al.*, 2004). In the present study we stimulated the cutaneous microvasculature to induce pronounced vasodilatation through whole body hyperthermia; in this construct it is feasible that a sufficient vasodilatory stimulus to the vasculature is necessary to observe increased vasodilatation with L-arg supplementation.

Limitations

Our aim in the present study was to investigate the role of arginase and L-arg availability in the regulation of cutaneous blood flow during systemic hyperthermia. We chose to use five separate microdialysis treatment sites in each subject during passive whole body heat stress and compare between sites. Furthermore, our chosen pharmacological treatments specifically target the Arg pathway and are therefore instrumental in studying the interplay between Arg and NO (Cox *et al.*, 1999; Tenu *et al.*, 1999). However, we did not choose to inhibit NOS subsequent to the established plateau in skin blood flow to quantify the NO contribution within each microdialysis treatment site due to the longer duration of whole body heating and associated increased cardiovascular risk and discomfort for the aged subjects. This additional data would have helped to clarify the pharmacological action of our chosen Arg inhibitors and the synergistic role between NO and cotransmitter-mediated vasodilatation during reflex cutaneous vasodilatation.

Our findings that the aged subjects had attenuated cutaneous vasodilatation in the control site and rely on impaired NO-dependent vasodilatation are consistent with our previous investigations. However, in both the aged and young subjects we observed systematically attenuated levels of cutaneous vasodilatation (~30%CVC_{max}) with increasing core body temperature in comparison to our previous study (Holowatz *et al.*, 2003). In our previous studies we induced maximal vasodilatation by infusion of 28mM SNP, whereas in the present study we induced maximal vasodilatation by both infusion of this dose of SNP and by simultaneously locally heating the skin to 43°C. The most likely explanation for this discrepancy between studies is that we obtained a truer level of

maximal vasodilatation with our current protocol, accounting for the consistent difference in cutaneous vascular conductance across age groups. It is likely that our higher maximal CVC values attained in this study account for the lower normalized CVC values.

Summary

In conclusion, this study demonstrated that both Arg inhibition and direct L-arg supplementation through intradermal microdialysis in the cutaneous vasculature selectively augments reflex cutaneous vasodilatation in aged humans. Moreover, these data suggest that NO bioavailability is decreased in aged skin due to limited L-arg availability by an age-related upregulation of vascular Arg activity. The age-related impairment in cutaneous vasodilatation during whole body heat stress can be restored by replenishing the available pool of L-arg for NO synthesis through NOS.

Table **4-1**: Subject Characteristics. Values are means±SEM; HDL, high density lipoprotein; LDL, low density lipoprotein; MAP mean arterial pressure. *Significant difference vs. young subject group ($p<0.05$)

Variable	Young	Older
Sex (M, F)	5, 5	4, 5
Age, years	23±1	69±1*
BMI, kg/m^2	23±1	25±1
$VO_{2\ max,}$ ml/kg/min	40±2	28±2*
Total Cholesterol, mg/dl	158±9	209±10*
HDL, mg/dl	55±3	57±5
LDL, mg/dl	87±9	120±7*
MAP, mmHg	89±2	93±2

Table **4-2**: Thresholds for Cutaneous Vasodilatation: (absolute T_{or} °C, ΔT_{or} °C). Values are means±SEM; T_{or} °C, oral temperature; ΔT_{or} °C, change in oral temperature from baseline; NOS, nitric oxide synthase; L-arg, L-arginine. * Significant difference vs. young subject group ($p<0.05$). ‡ Significant difference vs. control site within the young subject group ($p<0.05$). † Significant difference vs. control site within the older subject group ($p<0.05$).

Site	**Young**		**Older**	
	T_{or} °C	**ΔT_{or} °C**	**T_{or} °C**	**ΔT_{or} °C**
Control	36.74±0.10	0.41±0.07	36.76±0.09	0.57±0.06*
NOS Inhibited	36.89±0.08‡	0.56±0.04‡	36.94±0.06†	0.75±0.05*†
Arginase Inhibited	36.73±0.11	0.40±0.06	36.69±0.07	0.50±0.05
L-arg Supplemented	36.95±0.13	0.52±0.05	36.70±0.09	0.52±0.05
Arginase Inhibited + L-arg Supplemented	36.75±0.10	0.42±0.06	36.73±0.09	0.54±0.07*

Figure 4-1

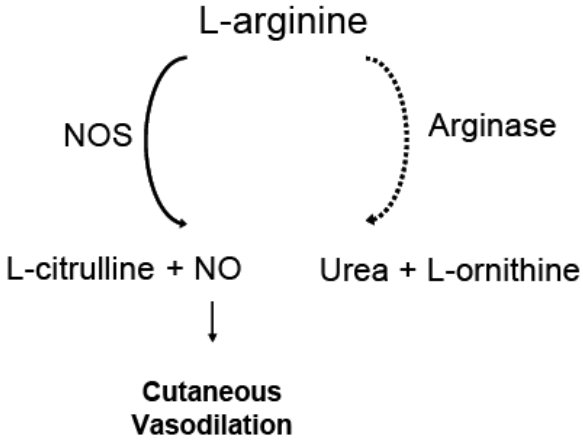

Figure **4-1**: Schematic representation of the L-arginine pathway in the cutaneous vasculature. L-arginine is the common substrate for both nitric oxide synthase (NOS) and arginase. Arginase catalyzes the conversion of L-arginine to urea and L-ornithine with is the precursor to proline and is important in cell growth and repair. NOS catalyzes the conversion of L-arginine to L-citrulline and nitric oxide (NO). NO is required for full expression of reflex cutaneous vasodilatation.

Figure 4-2

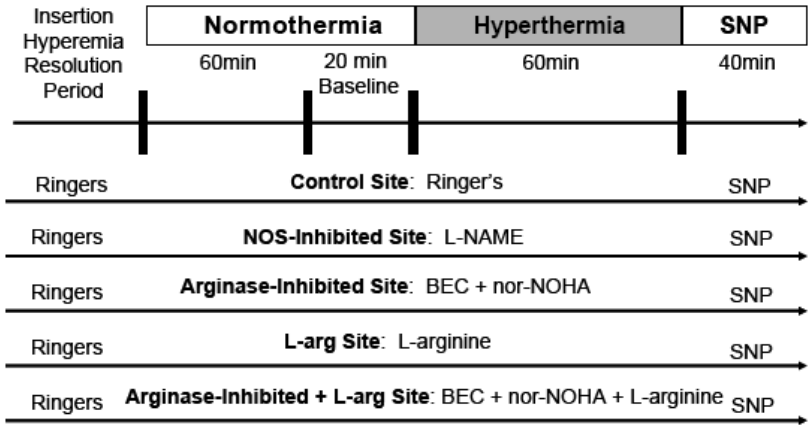

Figure **4-2**: A schematic illustration of the protocol. Each arrow represents a microdialysis treatment site. NOS, nitric oxide synthase; BEC, (S)-(2boronoethyl)-L-cysteine; nor-NOHA, N-hydroxy-nor-L-arginine; L-arg, L-arginine; SNP, sodium nitroprusside.

Figure 4-3

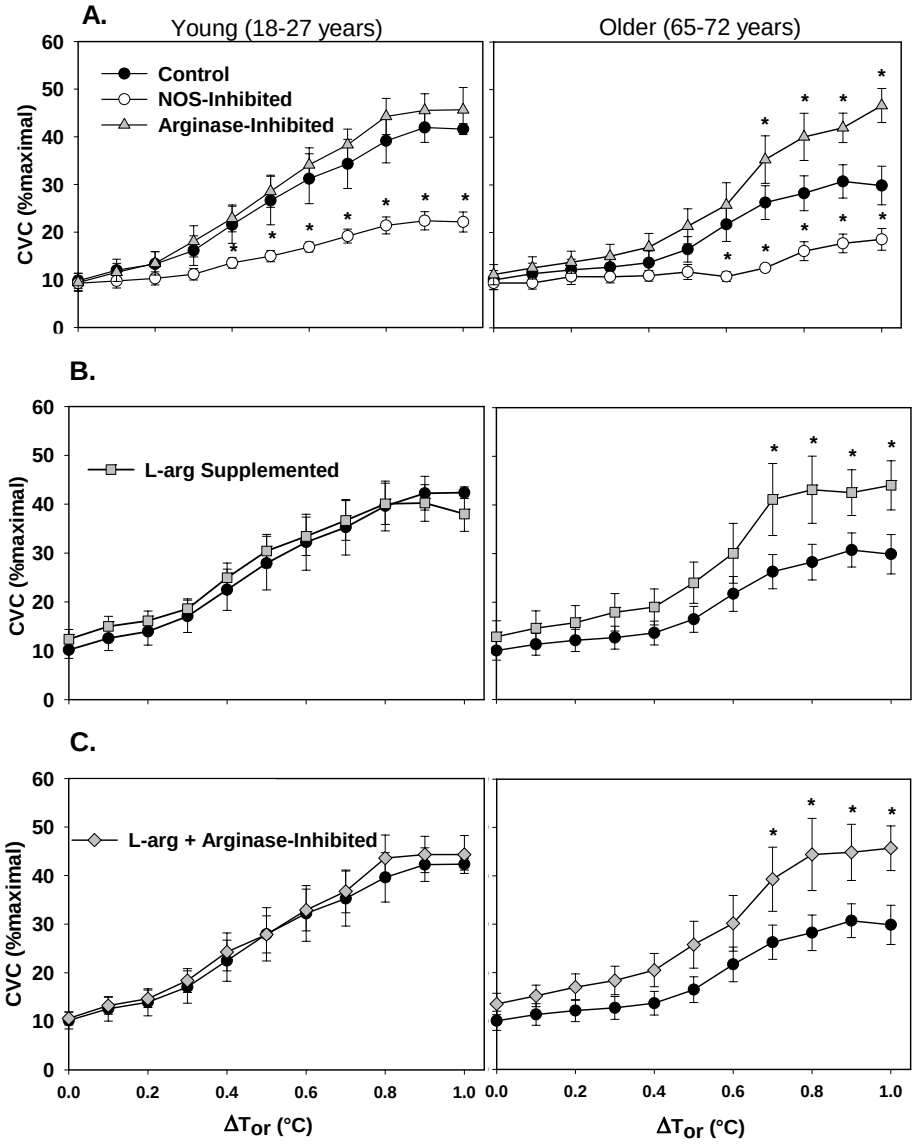

Figure **4-3**: Group mean ± SEM cutaneous vascular conductance (CVC) as a percent of maximal response during passive whole body heating. Young subject responses are displayed in the left panel and older subject responses are in the right panel. Panel A shows the arginase-inhibited site (▲) and nitric oxide synthase (NOS)-inhibited site (○). Panel B shows the L-arginine (L-arg) supplemented site (▪). Panel C shows the combined L-arg +Arginase-inhibited site (♦). CVC (%maximal) during the rise in oral temperature (ΔT_{or}, °C) in the control site (●) is illustrated in all of the panels for comparison. Arginase inhibition, L-arg supplementation, and combined treatments augmented CVC in old but not young subjects. *$p<0.05$ significant difference vs. the control site within subject groups.

Figure 4-4

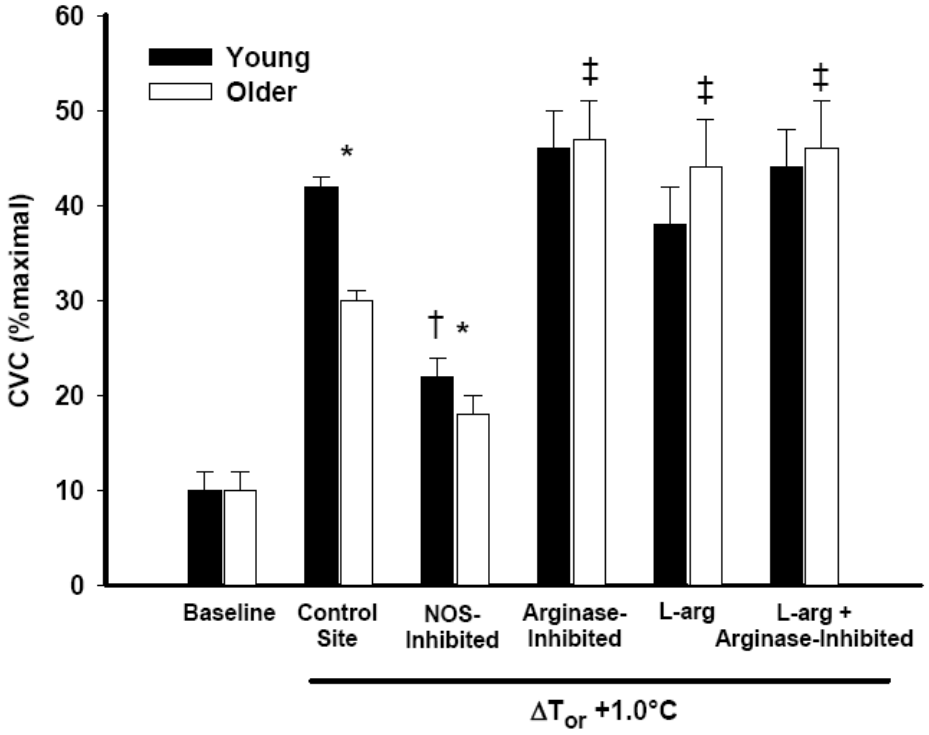

Figure **4-4**: Group mean ± SEM cutaneous vascular conductance (CVC) as a percent of maximal vasodilatation with a 1.0°C in oral temperature (ΔT_{or},°C) in all drug treatment sites. Baseline CVC (%maximal) illustrated for visual comparison. Solid bars represent the young subject group and the open bars represent the older subject group. Arginase inhibition, L-arginine supplementation, and combined treatments augmented CVC in old but not young subjects. * $p<0.05$ significant difference between age groups, †$p<.001$ significant vs. control site young subject group, ‡ $p<.001$ significant vs. control site older subject group.

Chapter 5

ACUTE ASCORBATE SUPPLEMENTATION ALONE OR COMBINED WITH ARGINASE INHIBITION AUGMENTS REFLEX CUTENEOUS VASODILATION IN AGED HUMAN SKIN

Introduction

Skin blood flow is controlled by dual sympathetic innervation comprising an adrenergic vasoconstrictor system and active vasodilator system (Grant & Holling, 1938). With increasing body core temperature, skin blood flow is reflexly increased by an initial withdrawal of tonic adrenergic vasoconstriction and upon reaching a specific temperature threshold is further increased by the active vasodilator system (Roddie *et al.*, 1957a). Cutaneous active vasodilation is purportedly mediated by the cotransmission of acetylcholine and an unknown neurotransmitter(s) from the sympathetic vasodilator system (Kellogg *et al.*, 1995). Furthermore, nitric oxide (NO) is required for full expression of cutaneous active vasodilation and contributes approximately 30% of the total vasodilatory response, where histamine and vasoactive intestinal peptide contribute to active vasodilation through NO-dependent mechanisms (Kellogg *et al.*, 1998a; Shastry *et al.*, 1998; Bennett *et al.*, 2003; Wilkins *et al.*, 2004a; Wong *et al.*, 2004; McCord *et al.*, 2006).

Human aging in the absence of overt pathology is associated with attenuated reflex cutaneous vasodilation (Kenney, 1988; Kenney *et al.*, 1997). Aged humans have a reduced functional cotransmitter(s) contribution to the increase in skin blood flow during hyperthermia and rely predominantly on NO-dependent mechanisms (Holowatz *et al.*, 2003), despite the fact that cutaneous NO-dependent vasodilation is compromised with advancing age (Minson *et al.*, 2002). Arginase is upregulated with advanced age and preferentially metabolizes the common substrate L-arginine in the final step of the urea cycle resulting in reciprocal regulation of endothelial NO-synthase (eNOS) (Berkowitz *et*

al., 2003). Furthermore, restoring L-arginine availability for NO synthesis through eNOS by acute arginase inhibition augments reflex vasodilation in aged human skin (Holowatz *et al.*, 2006b).

In addition to arginase upregulation, other age-related alterations in the mechanisms affecting NO bioavailability may also contribute to attenuated reflex cutaneous vasodilation. One alternative mechanism potentially contributing to impaired NO bioavailability in skin involves an age-related increase in oxidative stress (Lu *et al.*, 1999). Reactive oxygen species (ROS), including superoxide, increase in the skin with advancing age via both an increase in production and a decrease in degradation by a reduction in superoxide scavenging enzymes (Kohen, 1999; Lu *et al.*, 1999). In vascular endothelial cells superoxide can react with NO to form peroxynitrite up to four times faster than what can be metabolized, resulting in decreased NO bioavailability and therefore a reduced NO-dependent vasodilation (Beckman, 1996). Additionally, eNOS itself can uncouple and become a source of ROS in the presence of inadequate substrate and/or essential co-factor availability (Munzel *et al.*, 2005).

Acute ascorbate (Asc) supplementation in aged human forearm vasculature restores attenuated NO-dependent vasodilation through direct superoxide scavanging and by stabilizing the essential NOS cofactor tetrahydrobiopterin (BH_4) without effecting NOS activity (Heller *et al.*, 2001; Taddei *et al.*, 2001). Moreover in human skin, topical administration of 3-8% ascorbate solutions reduces oxidant stress associated with aging (Kohen, 1999; Sauermann *et al.*, 2004) and induced by exposure to ultaviolet irradiation (Dreher *et al.*, 1998; Leveque *et al.*, 2005). Therefore, the aim of this study was to determine the effect of acute ascorbate administration in attenuated reflex cutaneous vasodilation in aged skin. We hypothesized that Asc supplementation would augment reflex cutaneous vasodilation in aged skin. We further hypothesized that increasing L-arginine availability for NO synthesis through NOS by inhibiting arginase in combination with Asc supplementation would enhance reflex vasodilation over Asc supplementation alone.

Methods

Subjects

Experimental protocols were approved by the Institutional Review Board at The Pennsylvania State University and conformed to the guidelines set forth by the Declaration of Helsinki. Verbal and written consent was voluntarily obtained from all subjects prior to participation. Studies were performed on 11 young (18-27 years) and 10 older (65-72 years) men and women. Each subject underwent a complete medical screening, including blood chemistry, lipid profile evaluation (Quest Diagnostics Nichol Institute, Chantily, VA, USA), physical examination, and an assessment of maximal oxygen uptake (VO_{2max}) (SensorMedics Corperation, Yorba Linda, CA, USA). All subjects were screened for the presence of cardiovascular, dermatological, renal, and neurological disease. Subjects were healthy normally active not endurance trained (30-70th percentile values for maximal aerobic power normalized for age (2000)), normotensive, non-diabetic, healthy non-smokers who were not taking medications, including aspirin therapy, hormone replacement therapy or oral contraceptives. All young female subjects were studied on days 2-7 of the early follicular phase of their menstrual cycle. Experimental testing was conducted between September and February.

Instrumentation and Measurements

All protocols were performed in a thermoneutral laboratory with the subject in the supine position with the experimental arm at heart level. Upon arrival to the laboratory between the hours of 0700-0900, subjects were instrumented with four intradermal microdialysis fibers (10 mm, 20 kDa cutoff membrane, MD 2000, Bioanalytical Systems, IN, USA) in the skin on the right ventral forearm. Microdialysis sites were at least 4.0 cm apart to ensure no cross-reactivity of pharmacological agents between sites. Microdialysis fibers were placed at each site by first inserting a 25 gauge needle through

unanaesthetized skin using sterile technique. The entry and exit points were ~2.5 cm apart. The microdialysis fiber was then threaded through the internal lumen of the needle, and the needle was withdrawn, leaving the fiber in place. The microdialysis fibers were taped in place and perfused with lactated Ringer's solution during the insertion trauma resolution period at a rate of 2.0μL min^{-1} (Bee Hive controller and Baby Bee microinfusion pumps, Bioanalytical Systems, IN, USA) for 60-90 minutes until resolution of the insertion trauma.

To obtain an index of skin blood flow, cutaneous red blood cell (RBC) flux was measured with an integrated laser-Doppler flowmeter probe placed in a local heater (MoorLAB, Temperature Monitor SH02, Moor Instruments, Devon, UK) on the skin directly above each microdialysis membrane. All laser-Doppler probes were calibrated using Brownian standard solution. Cutaneous vascular conductance (CVC) was calculated as RBC flux divided by mean arterial pressure.

To control whole body temperature, subjects wore a water-perfused suit that covered the entire body except head, hands, and experimental arm and a water-impermeable rain suit to minimize evaporative heat loss. Electrocardiogram output was monitored throughout the protocol, and blood pressure was measured via brachial auscultation every 5 minutes. Oral temperature (T_{or}) was continuously monitored during baseline and throughout whole body heating with a thermister placed in the sublingual sulcas as an index of body core temperature. The subjects were instructed to keep the thermister in the same location in the sublingual sulcus and not to open their mouths or speak during the protocol. Mean skin temperature was calculated as the unweighted average of six copper-constantan thermocouples placed on the chest, middle back, abdomen, upper arm, thigh, and calf. During the insertion trauma resolution and baseline periods, thermoneutral water (34°C) was perfused through the suit to clamp whole body temperature. During whole body heating, 50°C water was perfused through the suit to raise T_{or} 0.8°C above baseline body temperature. Upon reaching a plateau in skin blood flow, the temperature of the water perfusing the suit was decreased to 46°C to clamp skin temperature for an additional 10 minutes which corresponded with a ΔT_{or} of 0.8°C.

Experimental Protocol

RBC flux over each microdialysis site was monitored during the insertion trauma resolution period (60-90 minutes). Following this period, microdialysis sites were randomly assigned to receive 1) lactated Ringers solution to serve as control, 2) 10.0mM N^G-nitro-L-arginine (L-NAME; Calbiochem San Diego, CA) to competitively inhibit NO production by NOS, 3) 10mM L-ascorbate (Sigma, St. Louis, MO) to supplement antioxidants, and 4) the combination of 10mM L-ascorbate and 5.0mM (s)-(2-boronoethyl)-L-cysteine-HCl (BEC) and 5.0mM N omega-hydroxy-nor-L-arginine (nor-NOHA) to supplement antioxidants and to inhibit arginase (Calbiochem, San Diego, CA, USA). All pharmacological agents were dissolved in lactated Ringers solution.

Our laboratory previously showed that a 10.0mM dose of L-NAME and a 5mM BEC + 5mM nor-NOHA was sufficient to maximally inhibit NOS and arginase in both subject groups, respectively (Minson *et al.*, 2002; Holowatz *et al.*, 2006b). Extensive pilot work was conducted to determine the final concentration of Asc used in the protocol. In three middle age (49±3) pilot subjects varying concentrations (1mM, 5mM, 7.5mM, 10.0mM, and 20.0mM) of Asc were delivered at a rate of 2 μL min^{-1} to different skin microdialysis sites for 90 minutes prior to and then throughout a standardized local heating protocol described elsewhere (Minson *et al.*, 2001). Although the mechanisms for the local heating induced rise in skin blood flow are different from reflex cutaneous vasodilation, the local heating protocol was chosen because the plateau phase of the local heating response is largely mediated by NO-dependent mechanisms. After the established plateau in skin blood flow during local heating 10mM L-NAME was infused until a skin blood flow decreased to a stable plateau. Concentrations greater than 7.5mM Asc did not further increase the NO-dependent plateau phase of the local heating response. Furthermore, the delta value from the established plateau in skin blood flow prior to L-NAME infusion and the post L-NAME plateau was significantly increased by Asc supplementation at doses greater than 5mM (Asc: 82±2 vs. Control: 60±2 $\%CVC_{max}$; $p<0.001$).

All microdialysis sites were perfused with assigned pharmacological agents continuously for at least 60 minutes prior to the start of the baseline and during the baseline and heating periods at a rate of 2.0μL min^{-1}. Baseline data were collected for 20 minutes prior to the start of whole body heating. After the baseline data collection period, whole body heating was conducted to raise T_{or} by 0.8°C. Following a 0.8°C rise in Tor, body temperature was clamped for 10 minutes. At the end of the heating protocol, each microdialysis site was perfused with 28.0mM sodium nitroprusside (SNP; Nitropress, Abbot Laboratories, Chicago, IL, USA) at a rate of 4.0μL min^{-1} to achieve maximal CVC. Local heating of the skin to 43°C was conducted simultaneously with SNP infusion to ensure maximal CVC had been obtained.

Data Acquisition and Analysis

Data were acquired using Labview software and National Instruments data acquisition system (Austin, TX, USA). The data were collected at 40 Hz, digitized, recorded and stored on a personal computer for further analysis. CVC data were averaged over 3-minute periods for baseline and every 0.1°C rise in T_{or} and are presented as a percent of maximal CVC (%CVC_{max}).

Student's t-tests were used to determine significant differences between the young and older groups for physical characteristics and for baseline absolute T_{or}. A three-way repeated measures mixed model analysis of variance (ANOVA) was conducted to detect differences between subject groups at the pharmacological treatment sites over the rise in T_{or} (SAS, version 9.1). Post hoc comparisons with Bonferroni corrections were performed when appropriate to determine where differences between groups and drug treatments occurred. The level of significance was set at α=0.05 for main effects and α=0.016 after Bonferroni correction. Values are presented as means ± SEM.

Results

Subject physical characteristics are presented in Table 5-1. There was no significant difference between subject groups for body mass index, systolic blood pressure, diastolic blood pressure, or for mean arterial pressure. The older subjects group had significantly lower maximal aerobic capacity and higher total cholesterol and low density lipoproteins. There was no significant difference between the groups for high density lipoproteins. Baseline T_{or} was not different between the subject groups ($p=0.43$)

Figure 5-1 shows the CVC response as a function of the rise in body core temperature for all of the treatment sites. The control site was significantly attenuated beginning at a ΔT_{or} of 0.3°C in the older subject group compared to the young subject group (Panel A). NOS inhibition significantly decreased CVC compared to the control site in both subject groups ($p<0.001$). Additionally, compared to the young subject group CVC was attenuated in the older subject group in the NOS-inhibited site at $\Delta T_{or} \geq 0.6$°C ($p<0.001$). There was no significant difference between the Asc site and the control site for the young subject group. However, the aged subjects had significantly higher CVC with Asc compared to the control site at $\Delta T_{or} > 0.7$°C ($p<0.05$) (Panel B). There were no significant differences between subject groups for the Asc site. Similarly, combined arginase inhibition and Asc augmented CVC above the level of the control site in the older subject group at $\Delta T_{or} > 0.6$°C ($p<0.001$), such that CVC was not significantly different in the from the young groups control site (Panel C). Combined Asc + arginase inhibition did not significantly change CVC compared to the control site in the young subjects.

Figure 5-2 summarizes the CVC responses in all of the treatment sites during the plateau in skin blood flow with a 0.8°C rise in body core temperature. CVC at baseline is illustrated for visual comparison. Compared to the young subject group, vasodilation was significantly attenuated in the control and NOS inhibited sites in the aged subject group($p<0.001$). Asc and combined Asc + arginase inhibition augmented CVC in the aged subject group above the level of their control site ($p<0.001$) but did not change CVC compared to control in the young subject group. With the addition of arginase inhibition

to Asc treatment, CVC significantly increased above the level of the site that received Asc alone in the aged subject group (p=0.001).

Discussion

The principal finding of the present study was that Asc alone selectively augmented reflex cutaneous vasodilation in aged subjects. Moreover, Asc combined with arginase inhibition (to increase L-arginine availability for NO synthesis through NOS) resulted in an additional increase in cutaneous vasodilation compared to sites treated with Asc alone. These treatments were effective at increasing reflex vasodilation only in the aged subject group with significant increases in body core temperature at ΔT_{or}>0.7°C and ΔT_{or}>0.6°C for Asc supplementation and Asc + arginase inhibition, respectively. Collectively, these data suggest that 1) age-associated increases in oxidative stress and arginase activity both limit cutaneous vasodilation in aged skin and that 2) reflex vasodilation can be augmented with antioxidant supplementation and acute arginase inhibition.

Our results show that acute Asc supplementation increases reflex cutaneous vasodilation in aged human skin. Given the non-specific antioxidant mechanism of action of ascorbate it is likely that the increase in skin blood flow in the age subject group was due to an increase in NO. NO is required for full expression of cutaneous active vasodilation in young skin and contributes approximately 40-50% to the total skin blood flow response (Kellogg *et al.*, 1998a; Shastry *et al.*, 1998). Due to a functional reduction in cotransmitter(s)-mediated vasodilation older subjects rely predominantly on NO-dependent vasodilation to increase skin blood flow during heat stress, with co-transmitter(s) contributing only modestly with significant increases in body core temperature (Minson *et al.*, 2002; Holowatz *et al.*, 2003). Attenuated reflex vasodilation in aged humans is likely due, in part, to an age-related increase in oxidative stress, supported by the finding that acute administration of supra-physiological doses of Asc was able to augment reflex cutaneous vasodilation.

Asc alone and combined with arginase inhibition only augmented skin blood flow in our aged subject group with significant increases in body core temperature. Whereas, NOS inhibition resulted in an attenuated skin blood flow response in both subject groups much earlier in whole body heating. Although we cannot discount the fact that Asc and the arginase inhibitors may have had other non-specific effects on the aged vasculature and may have augmented vasodilation through other non-NO dependent pathways, given the mechanism of action of Asc and the arginase-inhibitors it is likely that these treatments increased skin blood flow through NO-dependent mechanisms. Since we did not observe an increase in skin blood flow in the young subjects group, these findings suggest that potential age-related changes in either eNOS protein concentrations or activity may be present. However, evidence from human cell culture studies and animal models evaluating age-associated changes in eNOS expression, absolute concentration, and activity are divergent (Cernadas *et al.*, 1998; Matsushita *et al.*, 2001) and there has not been a direct assessment of age-related changes in eNOS protein concentration and activity in human skin. Another likely explanation for our findings is that there are different mechanisms mediating the early and late phase of reflex vasodilation and that our interventions were selective for the later phase. Although, NO is involved in both the early and the late phase of active vasodilation the upstream pathways mediating NO release may be different with acetylcholine, and VIP contributing to the early phase and H1 receptor activation contributing to the later phase through NO-dependent mechanisms (Shibasaki *et al.*, 2002; Wilkins *et al.*, 2004a; Wong *et al.*, 2004; McCord *et al.*, 2006).

NO bioavailability in the vasculature is dictated by the balance of NO production and degradation. In aged human skin, there is an increase in superoxide production and a decrease in degradation through attenuated superoxide dismutase and glutathione peroxidase activity leading to overall increased oxidative stress (Lu *et al.*, 1999). Superoxide directly decreases NO bioavailability by reacting with NO forming peroxynitrite at a rate up to four times faster than superoxide metabolism by superoxide dismutase (Beckman, 1996). Furthermore, peroxynitrite is capable of oxidizing critical cofactors for NOS, including tetrahydobiopterin (BH_4) leading to NOS uncoupling. During NOS uncoupling NOS itself becomes dysregulated and electron flow from the

reductase domain to the oxidase domain of the enzyme is directed toward molecular oxygen instead of the NO substrate L-arginine, resulting in superoxide production instead of NO (Heinzel *et al.*, 1992; Toth *et al.*, 2002; Kuzkaya *et al.*, 2003; Landmesser *et al.*, 2003). NOS uncoupling also occurs from compromised L-arginine availability (Vasquez-Vivar *et al.*, 1998). In the context of the present study, since acute Asc supplementation was able to augment reflex vasodilation in the aged these data suggest that overall oxidative stress is increased in aged human skin and impairs NO bioavailability through an increase in free radical production and aberrant metabolism which may include NOS uncoupling.

We were able to augment cutaneous vasodilation with acute antioxidant supplementation using the potent non-specific antioxidant L-ascorbate. These findings are consistent with the forearm muscle vascular bed, where NO-dependent vasodilation during infusions of endothelium-dependent agonists is restored in the presence of supra-physiological levels of Asc (Taddei *et al.*, 2001; Eskurza *et al.*, 2004). Interestingly, Asc is capable of increasing NO via several different mechanisms, including 1) directly scavenging ROS and 2) stabilizing the essential NOS cofactor BH_4 by recycling oxidized $BH_3^{\cdot}$ to BH_4, thereby inhibiting the production of superoxide through uncoupled NOS without directly altering NOS activity (Heller *et al.*, 2001; Kuzkaya *et al.*, 2003). In the aged human forearm muscle vasculature, BH_4 deficiency contributes to attenuated endothelium-dependent vasodilation, while acute BH_4 supplementation restores the vasodilatory responses to intraarterial infusions of acetycholine through NO-dependent mechanisms (Higashi *et al.*, 2006). In relation to the current data, increased reflex vasodilation in aged skin during Asc supplementation is likely due to a combination of direct ROS scavenging effects and stabilization of BH_4. However, direct supplementation with BH_4 in aged human skin is necessary to more fully elucidate its role in NOS uncoupling and oxidative stress in aged human skin.

In addition to BH_4 deficiency, NOS can also uncouple from inadequate L-arginine availability (Vasquez-Vivar *et al.*, 1998; Munzel *et al.*, 2005). We have recently shown that L-arginine availability for NO synthesis is compromised in aged skin and that restoring the available pool of L-arginine for NO synthesis through arginase inhibition

and/or L-arginine supplementation augments reflex cutaneous vasodilation (Holowatz *et al.*, 2006b). Arginase competes for the common NOS substrate L-arginine and is upregulated with advanced age (Berkowitz *et al.*, 2003). In the present study, combined Asc + arginase inhibition significantly increased CVC greater than the Asc supplemented site alone in aged skin. These findings suggest that both oxidative stress and limited L-arginine bioavailability independently contribute to attenuated reflex cutaneous vasodilation. Alternatively, these mechanisms may be linked and upregulated arginase activity may directly contribute to age-related increases in oxidative stress through NOS uncoupling due to limited L-arginine availability.

If oxidative stress and upregulated arginase activity independently contribute to attenuated cutaneous vasodilation, the resulting cutaneous vasodilation during combined antioxidant supplementation and arginase inhibition should be greater than the vasodilation during the individual treatments. However, if the current data are compared to an arginase-inhibited site from our previous study which used many of the same subjects and utilized the same protocol, there is no difference between the arginase-inhibited site and combined Asc + arginase-inhibited site (arginase-inhibited: 40±5 vs Asc + arginase inhibited: 41±3%CVC_{max}; p=0.71). It is possible that the arginase inhibition alone maximized the capacity of the cutaneous vessels vasodilate at this level of hyperthermia (+0.8°C), approaching a "ceiling effect" of the vessel, and a greater vasodilatory stimulus may unmask differences between the drug treatment sites. Thus, it is difficult to tease out the precise contributions and interactions of oxidative stress and upregulated arginase activity to decreased NO-dependent vasodilation with our model.

The use of other cutaneous vasodilator stimuli, such as prolonged local heating which induces NO-dependent vasodilation, may provide further insight into these questions.

Limitations

We did not directly show that the dose of Asc delivered to the cutaneous vasculature through microdialysis reduced oxidative stress in aged skin. However, the dose of Asc utilized in this study was more than double compared to studies where topical administration of an 8.0% ascorbate solution decreased markers of oxidative stress in human skin (Leveque *et al.*, 2004; Leveque *et al.*, 2005). Moreover, topical administration of this dose reached peak concentration and demonstrated effective antioxidant capabilities within one hour of administration. We delivered an 18% (10mM) ascorbate solution directly to the cutaneous vasculature through intradermal microdialysis and let this solution perfuse the microdialysis fiber for at least one hour prior to the start of baseline measurements.

We cannot discount the possibility that augmented reflex cutaneous vasodilation in the aged subjects may have been the result of non-specific effects of ascorbate on non-NO-dependent pathways. ROS act as signaling molecules to mediate cold-induced constriction of the vascular smooth muscle (Bailey *et al.*, 2005) and may also mediate vasoconstriction through a variety of pathways. Non-NO-dependent effects of antioxidant treatment on the cutaneous vasculature may have been revealed if we had simultaneously inhibited NOS and supplemented with antioxidants.

Summary

In summary, acute Asc supplementation increased reflex cutaneous vasodilation in aged skin. Asc supplementation combined with arginase inhibition, to increase L-arginine availability for NO synthesis through NOS, resulted in a greater increase in skin blood flow during hyperthermia. These treatments did not alter reflex cutaneous vasodilation in young subjects. Collectively, these data indicate that age-related increases in oxidative stress and upregulated arginase activity may contribute to attenuated reflex cutaneous vasodilation.

Table **5-1**: Subject Characteristics. Values are means±SEM; HDL, high density lipoprotein; LDL, low density lipoprotein; MAP mean arterial pressure; T_{or}, oral temperature. *Significant difference vs. young subject group ($p<0.05$)

Variable	Young	Older
Sex (M, F)	7, 4	5, 5
Age, years	22±1	68±1*
BMI, kg/m^2	23±1	25±1
$VO_{2\ max,}$ ml/kg/min	46±3	29±3*
Total Cholesterol, mg/dl	150±7	205±10*
HDL, mg/dl	51±3	57±5
LDL, mg/dl	83±8	127±7*
SBP, mmHg	114±3	120±4
DBP, mmHg	73±2	77±2
MAP, mmHg	87±2	91±2
Baseline T_{or}, °C	36.40±0.08	36.32±0.08

Figure 5-1

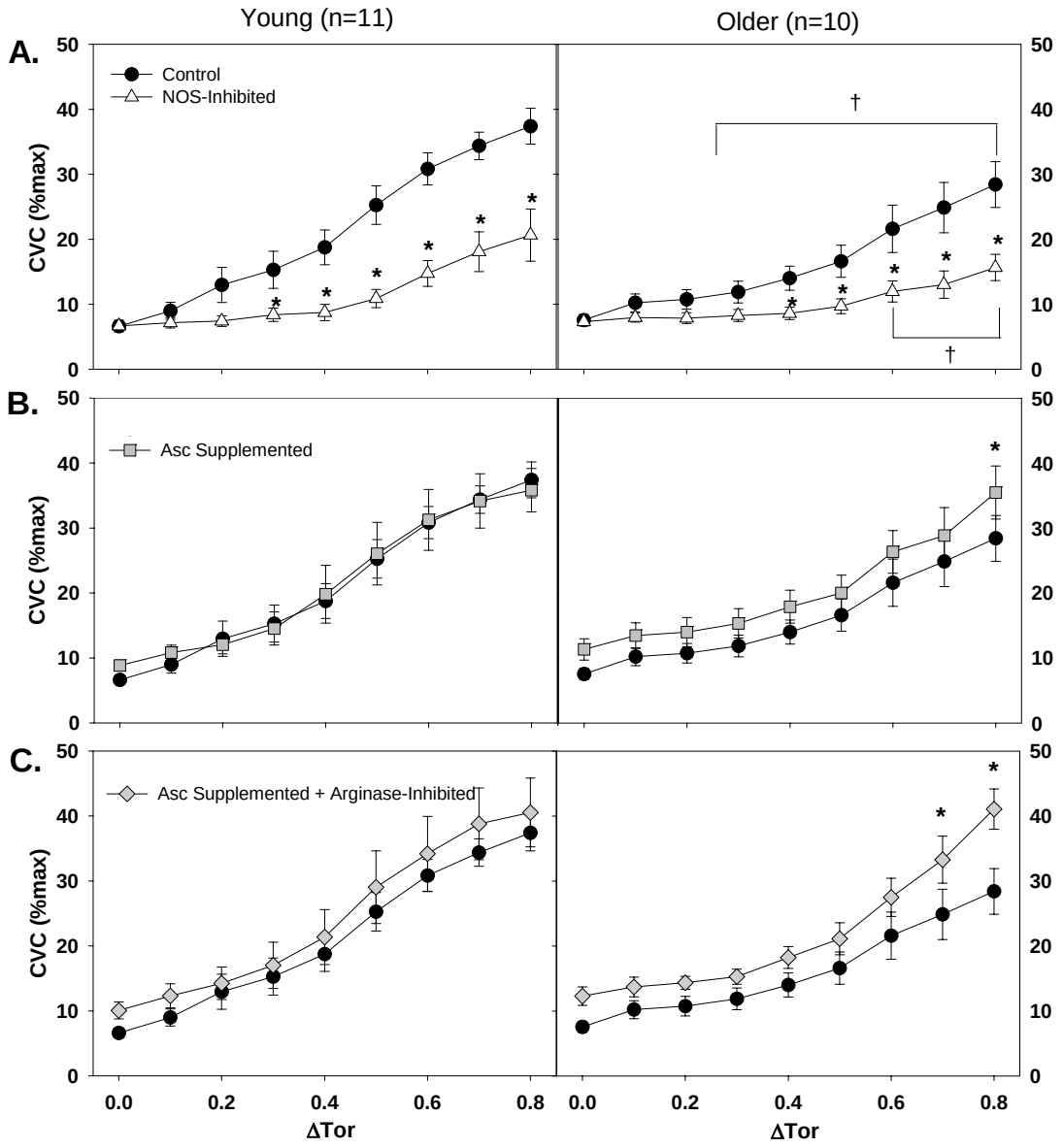

Figure **5-1**: Group mean ± SEM cutaneous vascular conductance (CVC) as a percent of maximal response during passive whole body heating. Young subject responses are displayed in the left panel and older subject responses are in the right panel. Panel A shows the nitric oxide synthase (NOS)-inhibited site (○). Panel B shows the Ascorbate supplemented (Asc) supplemented site (▪). Panel C shows the combined Asc +Arginase-inhibited site (♦). CVC (%maximal) during the rise in oral temperature (ΔT_{or}, °C) in the control site (●) is illustrated in all of the panels for comparison. *$p<0.05$ significant difference vs. the control site within subject groups, †$p<0.05$ significant difference between age groups

Figure 5-2

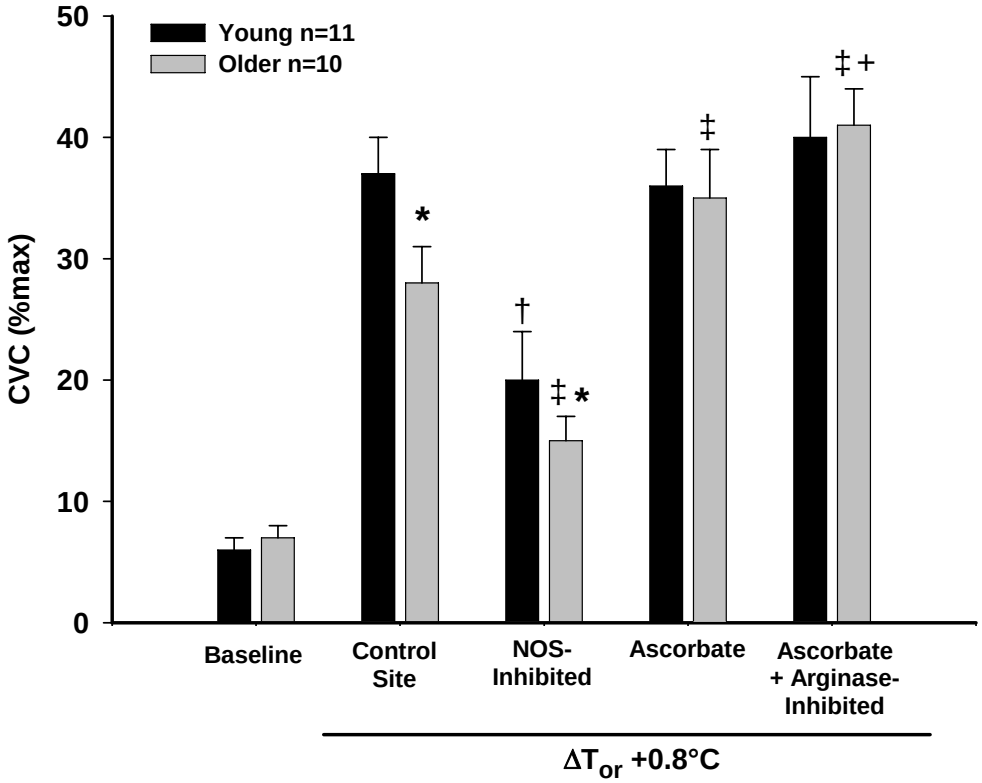

Figure **5-2**: Group mean ± SEM cutaneous vascular conductance (CVC) as a percent of maximal vasodilation with a 0.8°C in oral temperature (ΔT_{or},°C) in all drug treatment sites. Baseline CVC (%maximal) illustrated for visual comparison. Black bars represent the young subject group and the grey bars represent the older subject group. Ascorbate supplementation (Asc) and combined Asc + Arginase-inhibition augmented CVC in old but not young subjects. * $p<0.05$ significant difference between age groups, †$p<0.001$ significant vs. control site young subject group, ‡ $p<0.001$ significant vs. control site older subject group, + $p<0.05$ significant difference from Asc site in the older subject group.

Chapter 6

UPREGULATION OF ARGINASE ACTIVITY CONTRIBUTES TO ATTENUATED REFLEX CUTANEOUS VASODILATATION IN HYPERTENSIVE HUMANS

Introduction

Skin blood flow is controlled by two branches of the sympathetic nervous system, an adrenergic vasoconstrictor system and an active vasodilator system(Grant & Holling, 1938). With rising body core temperature skin blood flow initially increases by a release of vasoconstrictor tone and upon reaching a specific threshold skin blood flow further increases by the active vasodilator system (Roddie *et al.*, 1957a). Active vasodilatation is mediated by sympathetic cholinergic co-transmission where acetylcholine and an unknown vasodilator are co-released from sympathetic nerves (Kellogg *et al.*, 1995). Acetylcholine primarily mediates sweating but can also modulate cutaneous vasodilatation (Kellogg *et al.*, 1995, Shibasaki *et al.*, 2002). Vasoactive intestinal peptide (Bennett *et al.*, 2003), histamine 1 (H1) receptor activation (Wong *et al.*, 2004), and substance P (Wong & Minson, 2006) have all been implicated as potential co-transmitter vasodilator pathways contributing to active vasodilatation. Moreover, these pathways cause cutaneous vasodilatation in part through nitric oxide (NO)-dependent mechanisms, which is required for full expression of active vasodilatation (Kellogg *et al.*, 1998a; Shastry *et al.*, 1998).

Essential hypertension is associated with attenuated cutaneous vasodilatation during local (Carberry *et al.*, 1992) and systemic thermal stress (Kenney *et al.*, 1984). Chronically elevated systemic vascular resistance causes impairments in vasodilatory responses resulting from reduced NO-dependent vasodilatation and structural maladaptations including vascular smooth muscle hypertrophy (Taddei *et al.*, 1998). The incidence of essential hypertension increases with advancing age (AHA, 2006),

suggesting that hypertensive pathology-associated decreases in NO bioavailability coupled with healthy age-related deficits in non-NO-dependent mechanisms (Holowatz *et al.*, 2003) may combine to attenuate reflex cutaneous vasodilatation. However, the precise contribution of NO to reflex cutaneous vasodilatation and the involvement of mechanisms limiting NO bioavailability in hypertensive human skin remain unclear.

One potential mechanism limiting NO-dependent vasodilatation with hypertension is the upregulation of vascular arginase activity. Arginase is constitutively expressed in two isoforms and catalyzes the conversion of L-arginine to L-ornithine and urea in the final step of the urea cycle. Furthermore, upregulated arginase is mechanistically linked to the pathogenesis of vascular dysfunction with hypertension through increases in the polyamine and proline precursor L-ornithine, which contributes to vascular smooth muscle cell proliferation and intimal thickening (Wu & Morris, 1998; Durante *et al.*, 2001; Durante *et al.*, 2006). Arginase is also capable of reciprocally regulating NO synthesis through preferentially utilizing the common NO-synthase substrate L-arginine. In several different animal models of hypertension, inhibition of arginase augments vasodilatory responses to endothelium-dependent agonists through NO-dependent mechanisms (Johnson *et al.*, 2004; Zhang *et al.*, 2004a; Demougeot *et al.*, 2005; Demougeot *et al.*, 2006). Additionally, in aged human skin acute inhibition of arginase augments cutaneous vasodilatation (Holowatz *et al.*, 2006b, a). Taken together, these data suggest that upregulated arginase activity may limit L-arginine availability for NO synthesis in hypertensive vasculature.

Therefore, the purpose of this study was to determine the role of arginase in reflex cutaneous vasodilatation in an unmedicated essential hypertensive subject sample. Since arginase is upregulated in the skin with primary human aging (65-85 years) (Holowatz *et al.*, 2006b), we sought to control for this by investigating the role of arginase in reflex vasodilatation in an age- and sex-matched healthy control group. We hypothesized that (1) reflex vasodilatation in hypertensive subjects would be attenuated due to a reduced NO contribution and (2) acute arginase inhibition alone or with concurrent L-arginine supplementation would significantly augment reflex vasodilatation in hypertensive skin while only modestly increasing reflex vasodilatation in the age-matched control group.

Methods

Subjects

Experimental protocols were approved by the Institutional Review Board at The Pennsylvania State University and conformed to the guidelines set forth by the Declaration of Helsinki. Verbal and written consent was voluntarily obtained from all subjects prior to participation. Studies were performed on 9 age- and sex-matched control subjects (57±3 years, 6 men, 3 women) and 8 unmedicated subjects with essential hypertensive (57±4 years, 6 men, 2 women). Each subject reported to the laboratory on three separate occasions at least 1 week apart for blood pressure measurements via brachial auscultation with a mercury sphygmomanometer before experimental protocols were performed. During each blood pressure measurement visit the subject sat quietly with their feet on the floor and underwent three serial blood pressure measurements with five minutes between readings. Subjects were considered hypertensive if two of the three measurements during each visit was greater than 140/90 mmHg. After determining the subject's blood pressure status each subject underwent a complete medical screening, including blood chemistry, lipid profile evaluation (Quest Diagnostics Nichol Institute, Chantily, VA, USA), resting electrocardiogram, and physical examination. All subjects were screened for the presence of cardiovascular disease other than hypertension, dermatological, and neurological disease. Subjects were normally active, non-diabetic, healthy non-smokers who were currently not taking medications, including aspirin therapy, hormone replacement therapy or oral contraceptives. All premenopausal female subjects were studied on days 2-7 of the follicular phase of their menstrual cycle.

Instrumentation and Measurements

All protocols were performed in a thermoneutral laboratory with the subject in the semi-supine position with the experimental arm at heart level. Upon arrival at the laboratory, subjects were instrumented with five intradermal microdialysis fibres (MD 2000, Bioanalytical Systems, IN, USA) (10 mm, 20 kDa cutoff membrane) in the skin on the right ventral forearm. Microdialysis sites were at least 4.0 cm apart to insure no cross-reactivity of pharmacological agents being delivered to the skin. Microdialysis fibres were placed at each site by first inserting a 25 gauge needle through unanaesthetized skin using sterile technique. The entry and exit points were ~2.5 cm apart. The microdialysis fibre was then threaded through the needle, and the needle was withdrawn, leaving the fibre in place. The microdialysis fibres were taped in place and perfused with lactated Ringer's solution during the insertion trauma resolution period at a rate of 2.0μL min^{-1} (Bee Hive controller and Baby Bee microinfusion pumps, Bioanalytical Systems, IN, USA) for 60-90 minutes.

To obtain an index of skin blood flow, cutaneous red blood cell (RBC) flux was measured with an integrated laser-Doppler flowmeter probe placed in a local heater (MoorLAB, Temperature Monitor SH02, Moor Instruments, Devon, UK) on the skin directly above each microdialysis membrane. All laser-Doppler probes were calibrated using Brownian standard solution. Cutaneous vascular conductance (CVC) was calculated as RBC flux divided by mean arterial pressure.

To control whole body temperature, subjects wore a water-perfused suit that covered the entire body except head, hands, and experimental arm and a water-impermeable rain suit to minimize evaporative heat loss. The subject's electrocardiogram was monitored throughout the protocol, and blood pressure was measured via brachial auscultation every 5 minutes. Oral temperature (T_{or}) was continuously monitored during baseline and throughout whole body heating with a thermistor placed in the sublingual sulcus as an index of body core temperature. The subjects were instructed to keep the thermistor in the same location in the sublingual sulcus and not to open their mouths or speak during the protocol. Mean skin temperature

was calculated as the unweighted average from six copper-constantan thermocouples placed on the chest, middle back, abdomen, upper arm, thigh, and calf. During the insertion trauma resolution and baseline periods, thermoneutral water (34°C) was perfused through the suit to clamp body temperature. During whole body heating, 50°C water was perfused through the suit to raise subject's T_{or} 1.0°C above baseline body temperature. Local skin temperature over each microdialysis site was maintained at 33°C during baseline and whole body heating (Moor Instruments SHO2, Devon UK)

Experimental Protocol

RBC flux over each microdialysis site was monitored during the insertion trauma resolution period (60-90 minutes). Following this period, microdialysis sites were randomly assigned to receive 1) 10.0mM N^G-nitro-L-arginine (L-NAME) to inhibit NO production by NO-synthase (Kellogg *et al.*, 1999; Minson *et al.*, 2002; Holowatz *et al.*, 2005), 2) the combination of 5.0mM (s)-(2-boronoethyl)-L-cysteine-HCl (BEC) and 5.0mM N omega-hydroxy-nor-L-arginine (nor-NOHA) to inhibit arginase (Holowatz *et al.*, 2006b) (Calbiochem, San Diego, CA, USA), 3) 10.0mM L-arginine (Sigma) to supplement the substrate for NO-synthase and arginase (Holowatz *et al.*, 2006b), or 4) 5.0mM BEC + 5.0mM nor-NOHA + 10.0mM L-arginine to inhibit arginase and supplement the substrate for NO-synthase and arginase. A fifth microdialysis site was perfused with only lactated Ringers to serve as control. All pharmacological solutions were mixed just prior to usage, dissolved in lactated Ringer's solution, and sterilized using syringe microfilters (Acrodisc, Pall, Ann Arbor, MI, USA).

A cocktail or arginase inhibitors was used to insure that both isoforms of arginase that are present in human skin were adequately inhibited. Both nor-NOHA and BEC do not inhibit NOS making them beneficial for the study of arginase NOS interactions (Tenu *et al.*, 1999; Colleluori & Ash, 2001). Additionally, the binding characteristics of this cocktail of inhibitors is ideal for quick-onset, long lasting acute arginase inhibition (Colleluori & Ash, 2001). BEC is a slow binding, long lasting, competitive inhibitor of arginase I and II (Cox *et al.*, 1999), while nor-NOHA is a quick acting, potent, non-

specific arginase inhibitor (Tenu *et al.*, 1999). Additionally, extensive pilot work was conducted to ensure that the concentrations of arginase inhibitors maximally inhibited the arginase pathway. Briefly, varying concentrations (0.1mM, 1.0mM, 2.5mM, 5.0mM 10.0mM) of each BEC + nor-NOHA were delivered to different skin microdialysis sites during a standardized local heating protocol described elsewhere (Minson *et al.*, 2001). Increasing concentrations above 2.5mM BEC + 2.5mM nor-NOHA did not further increase the NO-dependent plateau phase of the local heating response.

All microdialysis sites were perfused with assigned pharmacological agents continuously for at least 60 minutes prior to the start of the baseline and during the baseline and heating periods at a rate of 2.0μL min^{-1}. Baseline data were collected for 20 minutes prior to the start of whole body heating. After the baseline data collection period, whole body heating was conducted to raise T_{or} by 1.0°C. At the end of the heating protocol, each microdialysis site was perfused with 28.0mM sodium nitroprusside (SNP; Nitropress, Abbot Laboratories, Chicago, IL, USA) at a rate of 4.0μL min^{-1} to achieve maximal CVC. Local heating of the skin to 43°C was conducted over each microdialysis site simultaneously with SNP infusion to ensure maximal CVC had been obtained.

Data Acquisition and Analysis

Data were acquired using Labview software and National Instruments data acquisition system (Austin, TX, USA). The data were collected at 40 Hz, digitized, recorded and stored on a personal computer for further analysis. CVC data were averaged over 3-minute periods for baseline and every 0.1°C rise in T_{or} and are presented as a percent of maximal CVC (%CVC_{max}). Absolute maximal CVC in each microdialysis site was calculated as the average of the stable plateau in laser-Doppler flux during 28mM SNP infusion and local heating to 43C divided by mean arterial pressure. The ΔCVC (%maximal) between the control site and the NO-synthase inhibited site was calculated at every 0.1°C rise in body core temperature.

Student's unpaired t-tests were used to determine significant differences between the groups for physical characteristics. Two-way repeated measures analysis of variance (ANOVA) was conducted to detect 1) differences due to blood pressure and pharmacological treatment on maximal CVC and 2) differences due to blood pressure on the $\Delta\%CVC_{max}$ between the control and the NO-synthase inhibited sites for every 0.1°C rise in T_{or}. A mixed models three-way repeated measures ANOVA was conducted to detect differences in $\%CVC_{max}$ between subject groups at the pharmacological treatment sites over the rise in T_{or} (SAS, version 8.01). Tukey post hoc tests, were performed when appropriate to determine where differences between groups and drug treatments occurred. The level of significance was set at $\alpha=0.05$. Values are presented as means ± SEM.

Results

The physical characteristics of the subjects are presented in Table 6-1. The subjects were well matched for age, body mass index, total cholesterol, and high density and low density lipoproteins. Resting systolic, diastolic, and mean arterial pressure were significantly elevated in the hypertensive group (all $p<0.001$).

Figure 6-1 illustrates the $\%CVC_{max}$ response for all sites to the rise in body core temperature in both subject groups. There were no significant differences in $\%CVC_{max}$ between the groups at the control site (Panel A). NO-synthase inhibition significantly decreased CVC compared to the control site in both subjects groups ($P<0.05$). However, the hypertensive group had significantly higher $\%CVC_{max}$ in the NO-synthase inhibited site beginning at $\Delta T_{or}>0.7$°C ($p<0.05$) compared to the normotensive group. In the normotensive group, arginase inhibition did not change $\%CVC_{max}$ compared to the control site. In contrast, arginase inhibition significantly increased $\%CVC_{max}$ compared to the control site in the hypertensive group at $\Delta T_{or}>0.6$°C (Panel A). There were no significant differences between the control site and the L-arginine supplemented site in either subject group with the rise in ΔT_{or} (Panel B: $p>0.05$). Combined arginase-inhibition + L-arg significantly increased $\%CVC_{max}$ compared to control sites in both

subject groups starting at ΔT_{or}>0.2°C in the normotensive and ΔT_{or}>0.4°C in the hypertensive group, respectively (Panel C: both p<0.05).

Figure 6-2 summarizes the $\%CVC_{max}$ responses in each microdialysis treatment site with a 1.0°C rise in body core temperature. There were no differences between the groups at the control site (p>0.05). NO-synthase inhibition decreased the $\%CVC_{max}$ in both subject groups compared to the control site (both p<0.001); however $\%CVC_{max}$ was significantly higher in the hypertensive group (p<0.001). Arginase-inhibition increased $\%CVC_{max}$ in the hypertensive (p<0.001) but not the normotensive group. There were no differences between the L-arginine supplemented site and the control site in either group (p>0.05), but combined arginase-inhibition + L-arginine supplementation augmented $\%CVC_{max}$ in both groups (both p<0.001).

Figure 6-3 illustrates the $\Delta\%CVC_{max}$ between the control site and the NO-synthase inhibited site across the rise in body core temperature. The $\Delta\%CVC_{max}$ was attenuated in the hypertensive group with ΔT_{or}>0.5°C compared to the normotensive group (P<0.001).

Figure 6-4 shows the absolute maximal CVC at all microdialysis sites for both subject groups. With in each subject group there was no effect of pharmacological treatment on maximal CVC. However, maximal CVC was attenuated by approximately 25% in the hypertensive group compared to the normotensive group (p=0.001).

Discussion

The principal findings of this study were that humans with essential hypertension have attenuated reflex cutaneous vasodilatation due, in part, to reduced NO-dependent vasodilatation, and that acute arginase inhibition-- but not L-arginine supplementation-- significantly augmented reflex vasodilatation in hypertensive skin. In age-matched normotensive subjects, increasing L-arginine availability through either arginase inhibition or L-arginine supplementation did not significantly alter skin blood flow but together these treatments augmented reflex vasodilatation with ΔT_{or}>0.2°C. These findings suggest that arginase is upregulated in the skin of hypertensive subjects and

contributes to reduced NO-dependent vasodilatation by limiting intracellular L-arginine availability of NO synthesis. Since direct L-arginine supplementation in hypertensive skin failed to result in an increase in cutaneous vasodilatation, these data suggest that either (1) freely exchangeable extracellular L-arginine does not limit NO synthesis (2) there is diminished transport of L-arginine through the cationic amino acid transporter (CAT), or (3) caveolar NO-synthase activity associated with the CAT is decreased in hypertensive skin.

Our results confirm earlier reports that hypertensive humans have attenuated reflex vasodilatation during systemic hyperthermia. While there were no significant differences between the groups in the control site when data are expressed as a percentage of maximal CVC, the hypertensive group exhibited reduced absolute maximal CVC. Therefore, reflex cutaneous vasodilation is attenuated in the hypertensive group but this reduction is masked when data are normalization to a percent of maximal vasodilatation. Due to capillary density and site to site variability in raw laser-Doppler flux measurements (Braverman *et al.*, 1990), we chose to represent the data as a percentage of maximal CVC. Using a different experimental paradigm to induce hyperthermia, we have previously demonstrated that unmedicated humans with essential hypertensive have attenuated skin blood flow (Kenney *et al.*, 1984). However, similar to the present study, others (Kellogg *et al.*, 1998b) have failed to demonstrate attenuated reflex vasodilatation during passive heat stress when data are represented as a percentage of maximal CVC, but have shown reduced maximal CVC during whole arm local heating with laser-Doppler and forearm blood flow measurements (Carberry *et al.*, 1992). Similar reductions in absolute maximal CVC (~20-25%) are observed in other disease states associated with microvascular dysfunction, such as Type II diabetes (Wick *et al.*, 2006; Sokolnicki *et al.*, 2007). Collectively, our data show that reflex vasodilatation is attenuated in humans with essential hypertensive due to impairments in vascular signalling. Moreover, the reduction in maximal CVC likely reflects additional structural alterations in the cutaneous vasculature that limit absolute vasodilator capacity

Our data show that there is an attenuated reduction in $\%CVC_{max}$ with NO-synthase inhibition (Figures 6-1 and 6-2) and that the $\Delta\%CVC_{max}$ between the control site

and the NO-synthase inhibited site is reduced (Figure 6-3) in hypertensive subjects. Hence, cutaneous NO-dependent vasodilatation is reduced in humans with hypertension. There are several putative mechanisms that may contribute to decreased NO production in hypertensive skin including (1) upregulated arginase activity, and (2) decreased L-arginine availability in relation to intracellular NO-synthase localization.

Our findings implicate a role for upregulated vascular arginase in limiting cutaneous vasodilatation in humans with established essential hypertension. Arginase is also upregulated with aging-related endothelial dysfunction (Berkowitz *et al.*, 2003). In aged human skin, arginase inhibition also augments reflex cutaneous vasodilatation (Holowatz *et al.*, 2006b); however the precise stimulus mediating the upregulation of vascular arginase with either aging or hypertension is unknown. It is likely that there are several inducers of arginase expression and/or agents that modify arginase activity that are unique to the vascular disease state whether it is aging or hypertension. Recently, upregulated expression and activity of vascular arginase has been observed prior to the onset and throughout the development of hypertension in the spontaneously hypertensive rat (SHR) model, implicating arginase in the pathogenesis of essential hypertension. Further, pharmacologically preventing the rise in blood pressure blunted arginase upregulation, suggesting that increased hemodynamic forces act as an inducer of arginase expression (Demougeot *et al.*, 2006). However, increased hemodynamic forces alone did not fully explain the hypertension-associated increase in vascular arginase expression.

In addition to hemodynamic forces mediating the upregulation of arginase, there is another recently discovered mechanism that stimulates arginase activation. In cultured endothelial cells, arginase is activated by dissociation from the microtubule cytoskeleton (Ryoo *et al.*, 2006). There are several mediators capable of causing microtubule disruption with hypertension included activation of the small GTPase, Rho kinase. Rho kinase also modulates Ca2+ sensitivity in vascular smooth muscle and is capable of downregulating NO-synthase (Ming *et al.*, 2002; Wettschureck & Offermanns, 2002) and arginase (Ming *et al.*, 2004) expression and activity. Further Rho kinase is known to be upregulated with human essential hypertension (Masumoto *et al.*, 2001). In relation to human cutaneous vasculature, we have recently found that Rho kinase mediates

cutaneous vasoconstriction (Thompson-Torgerson *et al.*, 2006). Collectively, these data point to a potential mechanism for activation of arginase through Rho kinase-mediated disruption of the microtubules cytoskeleton.

In the present study we found that although NO-dependent vasodilatation is attenuated in hypertensive human skin, exogenous L-arginine supplementation directly to the cutaneous vasculature through intradermal microdialysis did not significantly alter the $\%CVC_{max}$ during whole body heating. One potential explanation for this finding is that the dose of L-arginine delivered to the cutaneous vasculature through intradermal microdialysis may have been insufficient to observe an increase in skin blood flow during hyperthermia. However, the dose of L-arginine per gram of tissue delivered through intradermal microdialysis was comparable, or greater than, what is commonly administered during whole limb arterial infusion studies (Taddei *et al.*, 1997). Additionally, we have previously used this dose of L-arginine using the same experimental techniques and we observed augmented cutaneous vasodilatation in a healthy aged population (Holowatz *et al.*, 2006b). In the present study we also observed a significant effect of L-arginine treatment when added to arginase inhibition in the normotensive control group suggesting that the dose of L-arginine delivered through the microdialysis fibre was efficacious. Thus, although it is possible, it is unlikely that higher doses of L-arginine would have produced different results.

A more likely explanation for the inability of exogenous L-arginine supplementation to augment cutaneous vasodilatation involves the CAT transporters and the cellular localization of microdomains of L-arginine accessible to NO-synthase. There are at least three potential microdomains of L-arginine including: (1) a domain that is associated with the CAT that is freely exchangeable with the extracellular space and associated with caveolar NO-synthase, (2) a domain in the cytosolic fraction where arginase is localized when activated that is non-freely exchangeable (Topal *et al.*, 2006), and (3) an intracellular domain consisting of L-arginine synthesized from conversion of L-citrulline through argininosuccinate synthase (McDonald *et al.*, 1997; Flam *et al.*, 2001; Huynh & Chin-Dusting, 2006; Ryoo *et al.*, 2006). In our experimental design localized cutaneous L-arg supplementation most likely affected the freely exchangeable

microdomain of L-arginine whereas arginase inhibition likely increased the availability of L-arginine in the cytosolic microdomain not associated with the CAT. Our results indicate that either the concentration of L-arginine in the freely exchangeable pool is sufficient to support NO production through caveolar associated NO-synthase, although caveolar-associated NO-synthase may be decreased with hypertension (Forstermann & Munzel, 2006), and/or that L-arginine transport through the CAT is impaired with hypertension. Radiotracer studies in the human forearm muscle circulation have demonstrated that subjects with essential hypertension, and those genetically predisposed to hypertension, have impaired L-arginine transport through the CAT (Schlaich *et al.*, 2004). Future investigations in the cutaneous circulation measuring NO-synthase expression and activity, and L-arginine transport through the CAT are necessary to resolve these questions.

In the age-matched normotensive subject group, we found that although arginase inhibition or L-arginine supplementation did not affect cutaneous vasodilatation independently, the combination of these treatments significantly augmented vasodilatation with relatively small increases in body core temperature (ΔT_{or}>0.2°C). In middle aged subjects L-arginine availability appears sufficient to support full expression of reflex vasodilatation. Therefore, it was unexpected that combined L-arginine supplementation and arginase inhibition augmented cutaneous vasodilatation. These data suggest that it takes a robust increase in L-arginine in potentially multiple microdomains to augment cutaneous vasodilatation in middle-aged humans. In contrast, we have previously reported that these combined treatments did not result in a further increase skin blood flow greater than the individual treatments in healthy aged skin (65-85 years) (Holowatz *et al.*, 2006b). However, the subjects from the previous aging study were significantly older and had a greater attenuation in skin blood flow under control conditions compared to the age-matched normtensive subjects from the present study. In addition to age-related decreases in L-arginine availability, another possible explanation for this difference is that there is greater vasodilatory reserve in subjects of advanced age.

Our present data show that middle-aged normotensive subjects rely heavily on NO-dependent mechanisms to increase skin blood flow during hyperthermia. In young

healthy human subjects, NO contributes approximately 30-40% to the total reflex cutaneous vasodilatory response (Kellogg *et al.*, 1998a; Kellogg *et al.*, 1998b; Shastry *et al.*, 1998). Subjects of more advanced age display an impaired cotransmitter contribution to reflex vasodilatation and also rely heavily on NO-dependent mechanisms to increase skin blood flow. Similar to subjects of advanced age, our data shows that deficits in cotransmitter contribution are evident in healthy middle-aged skin.

Limitations

We performed extensive pilot work to determine the dosage of the arginase inhibitors we utilized in the present study. The concentration that we delivered through the microdialysis fibre was significantly greater than the highest dose commonly used in *in vitro* preparations (5×10^{-3}M vs. 10×10^{-5}M) that maximally inhibit the arginase pathway (Berkowitz *et al.*, 2003). Because of the inherent uncertainly of the concentration of the inhibitor delivered through the microdialysis fiber that reaches the intradermal space, we chose in increase the concentration of arginase inhibitors to ensure that an efficacious dose would be administered. The results of our pilot testing suggest that the arginase pathway was fully inhibited at concentrations greater than 2.5×10^{-3}M, but there is the possibility that the arginase pathway was not completely inhibited with the concentrations utilized in this investigation.

We did not directly show that the interventions employed in this study augmented cutaneous vasodilatation via NO-dependent mechanisms. However, our chosen pharmacological treatments specifically targeted the arginase-L-arginine-NO pathway (Cox *et al.*, 1999; Tenu *et al.*, 1999). Although we did not quantify the NO contribution within each microdialysis treatment site, we did evaluate the time course of the interventions. Additional data from NO-synthase inhibition during the plateau phase of the rise in skin blood flow would have helped to clarify the pharmacological action of our chosen arginase inhibitors.

Summary

In summary, humans with essential hypertension exhibit attenuated reflex cutaneous vasodilatation due to a reduced NO-dependent vasodilatation. Acute arginase inhibition but not L-arginine supplementation augmented reflex vasodilatation in hypertensive skin. These data suggest that arginase is upregulated in hypertensive vasculature and that L-arginine supplementation alone is insufficient to augment cutaneous vasodilatation suggesting either (1) L-arginine concentrations in the freely exchangeable microdomain are sufficient to support NO production through the available NO-synthase, or (2) the CAT is dysfunctional and limits L-arginine transport into the cell with hypertensive pathology. Finally, in aged-matched normotensive control subjects L-arginine supplementation or arginase inhibition individually did not alter the skin blood flow response during hyperthermia but a combination of these two treatments significantly augmented reflex vasodilatation.

Table **6-1**: Subject Characteristics. Values are means±SEM; HDL, high density lipoprotein; LDL, low density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP mean arterial pressure. *Significant difference vs. normotensive subject group ($p<0.05$)

Variable	Normotensive	Hypertensive
Sex (M, F)	6, 3	6, 2
Age, years	57±3	57±4
BMI, kg/m^2	24.0±1.0	26.5±1.0
Total Cholesterol, mg/dl	183±9	195±15
HDL, mg/dl	58±6	54±5
LDL, mg/dl	110±9	119±11
SBP, mmHg	116±3	150±3*
DBP, mmHg	76±1	94±1*
MAP, mmHg	81±10	112±2*

Figure 6-1

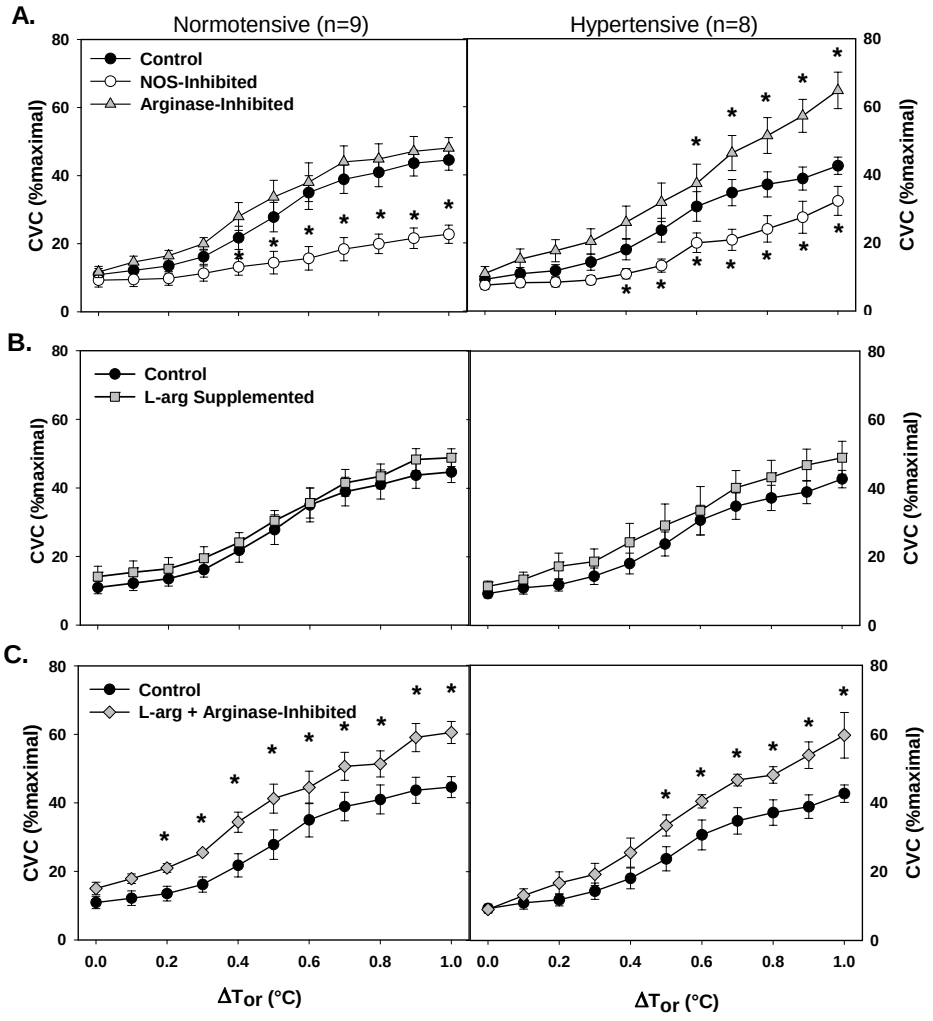

Figure **6-1**: Group mean ± SEM cutaneous vascular conductance (CVC) as a percent of maximal response during passive whole body heating. Age-matched normotensive subject responses are displayed in the left panel and essential hypertensive subject responses are in the right panel. Panel A shows the arginase-inhibited site (▲) and nitric oxide synthase (NOS)-inhibited site (○). Panel B shows the L-arginine (L-arg) supplemented site (▪). Panel C shows the combined L-arg +Arginase-inhibited site (♦). CVC (%maximal) during the rise in oral temperature (ΔT_{or}, °C) in the control site (●) is illustrated in all of the panels for comparison. Arginase inhibition and combined arginase inhibition + L-arg supplementation augmented $\%CVC_{max}$ in essential hypertensive subjects. Arginase inhibition + L-arg supplementation combined but not the individual treatments augmented CVC in the age-matched normotensive subjects. *$p<0.05$ significant difference vs. the control site within subject groups.

Figure 6-2

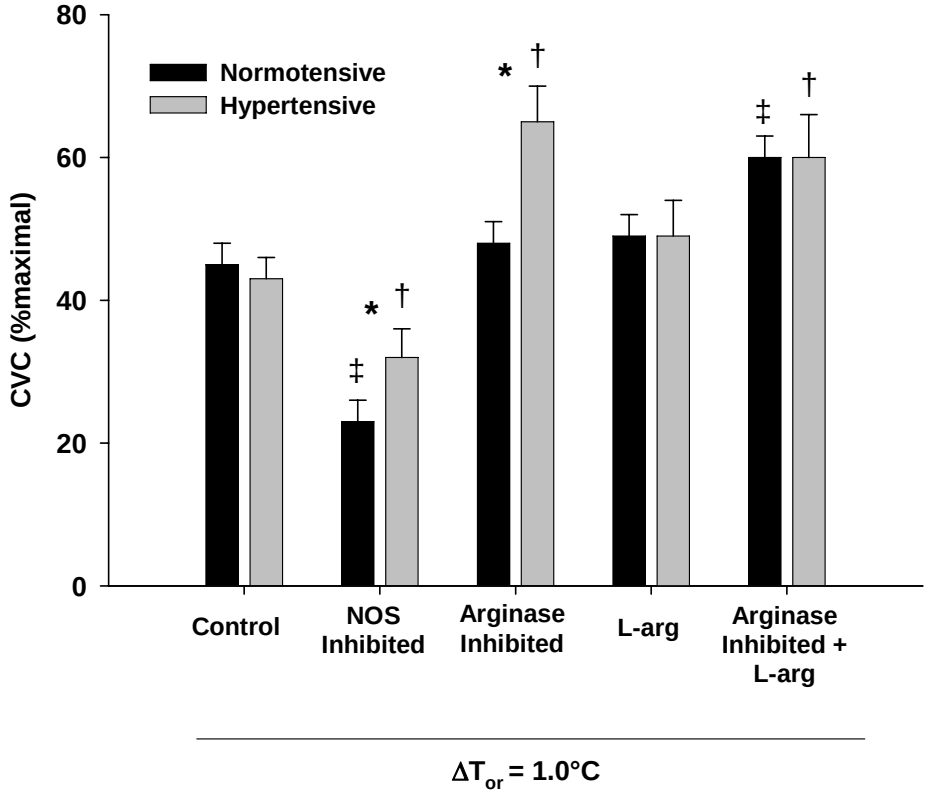

Figure **6-2**: Group mean ± SEM cutaneous vascular conductance (CVC) as a percent of maximal vasodilatation with a 1.0°C in oral temperature (ΔT_{or},°C) in all drug treatment sites. Solid bars represent the age-matched normotensive subject group and the open bars represent the hypertensive subject group. The hypertensive subject group had a greater $\%CVC_{max}$ in the NO-synthase inhibited site compared to the age-matched normotensive group. Arginase inhibition, and combined treatments augmented $\%CVC_{max}$ in essential hypertensive subjects but only combined treatments augmented $\%CVC_{max}$ in the age-matched normotensive subjects. * $p<0.05$ significant difference between blood pressure groups, †$p<.001$ significant vs. control site hypertensive group, ‡ $p<.001$ significant vs. control site age-matched normotensive group.

Figure 6-3

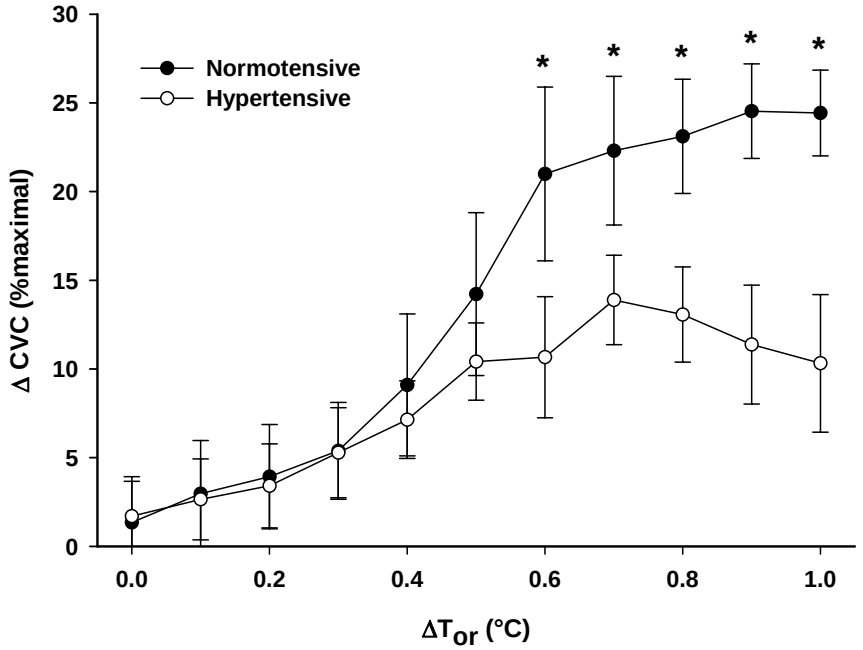

Figure **6-3**: Group mean ± SEM change in cutaneous vascular conductance (ΔCVC) as a percent of maximal vasodilatation between the control site and the NO-synthase inhibited site across the rise in body core temperature (ΔT_{or},°C) in the age-matched normotensive subject group (●) and essential hypertensive subject group (○). The essential hypertensive group exhibited attenuated $\Delta\%CVC_{max}$ with ΔT_{or}>0.5°C. * $p<0.001$ vs. age-matched normotensive group.

Figure 6-4

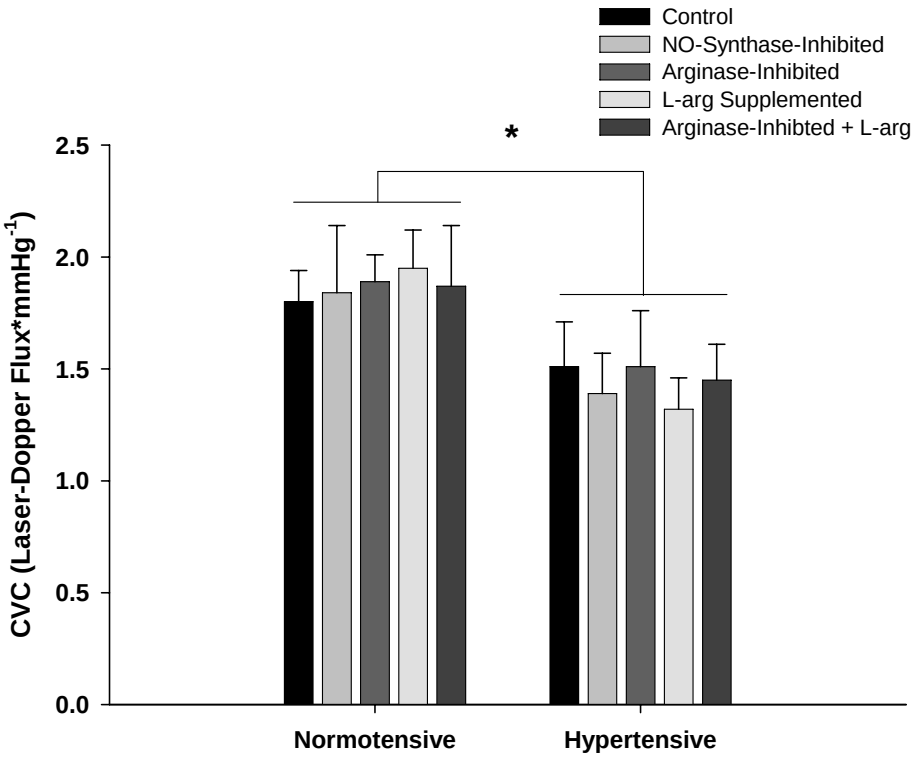

Figure **6-4**: Group mean ± SEM for absolute maximal cutaneous vascular conductance (CVC, laser-Doppler flux*mmHg^{-1}). There were no significant effects of pharmacological treatments on maximal CVC, but there was a significant group blood pressure effect on maximal CVC. * $p<0.001$ between blood pressure groups.

Chapter 7

ASCORBATE SENSITIVE OXIDANTS CONTRIBUTE TO ATTENUATED NO- AND NON-NO-DEPENDENT REFLEX CUTAENOUS VASODILATION IN HYPERTENSIVE HUMANS

Introduction

Skin blood flow is reflexly controlled by dual sympathetic innervation consisting of an adrenergic vasoconstrictor system and an active vasodilator system (Grant & Holling, 1938). With rising body core temperature, skin blood flow is initially increased by a withdrawal of adrenergic vasoconstrictor tone and is further increased by the active vasodilator system (Roddie *et al.*, 1957b). Active vasodilation is mediated by sympathetic co-transmission of acetylcholine and an unknown neurotransmitter which mediate sweating and cutaneous vasodilation, respectively (Kellogg *et al.*, 1995). Vasoactive intestinal peptide (Bennett *et al.*, 2003), histamine 1 receptor activation (Wong *et al.*, 2004), and substance P (Wong & Minson, 2006) all contribute to active vasodilation in part through NO-dependent pathways which are required for full expression of active vasodilation, contributing approximately 30-40% to the total vasodilatory response (Kellogg *et al.*, 1998a; Shastry *et al.*, 1998).

Essential hypertension is associated with attenuated cutaneous vasodilation during local (Carberry *et al.*, 1992) and systemic thermal stress (Kenney *et al.*, 1984; Holowatz & Kenney, 2007). We have recently shown that NO-dependent reflex cutaneous vasodilation is attenuated in hypertensive skin and that increasing L-arginine availability through acute arginase inhibition augments reflex vasodilation (Holowatz & Kenney, 2007). Arginase catalyzes the conversion of L-arginine to L-ornithine and urea in the final step of the urea cycle and is capable of limiting NO production by competing for the common NO-synthase substrate L-arginine (Hecker *et al.*, 1995). In addition to arginase

decreasing NO production, there are likely additional mechanisms impairing NO bioavailability in hypertensive cutaneous vasculature.

One potential mechanism contributing to the reduced NO bioavailability implicated in the pathogenesis of hypertensive vascular pathology is increased oxidative stress (Warnholtz *et al.*, 2004). In essential hypertensive vasculature, there is increased production of reactive oxygen species (ROS) coupled with decreased degradation of ROS through dysfunctional enzymatic antioxidant defense mechanisms (Lassegue & Griendling, 2004). Furthermore, augmented ROS (superoxide) production reduces NO bioavailability by readily reacting with newly synthesized NO forming peroxynitrite which potentiates superoxide production by uncoupling endothelial NO-synthase (eNOS) (Forstermann & Munzel, 2006). eNOS uncoupling results from peroxynitrite-mediated oxidization of the essential NOS cofactor tetrahydrobiopterin (BH_4) and/or inadequate L-arginine availability (Munzel *et al.*, 2005). Taken together, these data suggest that hypertension-associated upregulated arginase activity may not only contribute to decreased NO production though limiting L-arginine but may also increase oxidant stress through uncoupled eNOS.

Acute ascorbate supplementation in the forearm muscle vasculature of hypertensive humans increases endothelium-dependent vasodilation through NO-dependent mechanisms (Taddei *et al.*, 1998), acting directly as an antioxidant and acting indirectly to stabilize the essential NOS cofactor BH_4 (Heller *et al.*, 2001; Taddei *et al.*, 2001). The purpose of the present study was to determine the relative role of oxidant stress and augmented arginase activity on attenuated NO-dependent vasodilation in human hypertensive cutaneous vasculature. We sought to control for age-related increases in oxidant stress and arginase activity (Holowatz *et al.*, 2006b, a) by matching unmedicated essential hypertensive subjects to age- and sex-matched healthy normotensive control subjects. We hypothesized that (1) acute antioxidant (ascorbate) supplementation in the skin would augment NO-dependent reflex cutaneous vasodilation and (2) arginase inhibition combined with ascorbate supplementation would further enhance NO-dependent reflex vasodilation over ascorbate supplementation alone.

Methods

Subjects

Experimental protocols were approved by the Institutional Review Board at The Pennsylvania State University. Verbal and written consent was voluntarily obtained from all subjects prior to participation. Studies were performed on 9 unmedicated essential hypertensive subjects (57±4 years, 6 men, 3 women) and 9 age- and sex-matched control subjects (57±3 years, 6 men, 3 women). Each subject reported to the laboratory on three separate occasions at least 1 week apart for blood pressure measurements via brachial auscultation with a mercury sphygmomanometer to determine their blood pressure status before experimental protocols were performed. During each blood pressure measurement visit the subject sat quietly with their feet on the floor and underwent three serial blood pressure measurements with five minutes between each reading. Subjects were considered hypertensive if two of the three measurements during each visit was greater than 140/90 mmHg. After determining the subject's blood pressure status each subject underwent a complete medical screening, including blood chemistry, lipid profile evaluation (Quest Diagnostics Nichol Institute, Chantily, VA, USA), resting electrocardiogram, and physical examination. All subjects were screened for the presence of cardiovascular disease other than hypertension, dermatological, and neurological disease. Subjects were normally active, non-diabetic, healthy non-smokers who were currently not taking medications, including aspirin therapy, hormone replacement therapy or oral contraceptives. All premenopausal women were studied on days 2-7 of the follicular phase of their menstrual cycle. Seventeen of the eighteen subjects had also participated in a previous study in our laboratory (Holowatz & Kenney, 2007).

Instrumentation and Measurements

All protocols were performed in a thermoneutral laboratory with the subject in the semi-supine position with the experimental arm at heart level. Upon arrival to the laboratory, subjects were instrumented with four intradermal microdialysis fibers (MD 2000, Bioanalytical Systems, IN, USA) (10 mm, 20 kDa cutoff membrane) in the skin on the right ventral forearm. Microdialysis sites were at least 4.0 cm apart to insure no cross-reactivity of pharmacological agents being delivered to the skin. Microdialysis fibers were placed at each site by first inserting a 25 gauge needle through unanaesthetized skin using sterile technique. The entry and exit points were ~2.5 cm apart. The microdialysis fiber was then threaded through the needle, and the needle was withdrawn, leaving the fiber in place. The microdialysis fibers were taped in place and perfused with lactated Ringer's solution during the insertion trauma resolution period at a rate of 2.0μL min^{-1} (Bee Hive controller and Baby Bee microinfusion pumps, Bioanalytical Systems, IN, USA) for 60-90 minutes.

To obtain an index of skin blood flow, cutaneous red blood cell (RBC) flux was measured with an integrated laser-Doppler flowmeter probe placed in a local heater (MoorLAB, Temperature Monitor SH02, Moor Instruments, Devon, UK) on the skin directly above each microdialysis membrane. All laser-Doppler probes were calibrated using Brownian standard solution. Cutaneous vascular conductance (CVC) was calculated as RBC flux divided by mean arterial pressure (MAP). MAP was calculated as diastolic pressure plus one third pulse pressure.

To control whole body temperature, subjects wore a water-perfused suit that covered the entire body except head, hands, and experimental arm and a water-impermeable rain suit to minimize evaporative heat loss. The subject's electrocardiogram was monitored throughout the protocol, and blood pressure was measured via brachial auscultation every 5 minutes. Oral temperature (T_{or}) was continuously monitored during baseline and throughout whole body heating with a thermister placed in the sublingual sulcas as an index of body core temperature. The subjects were instructed to keep the thermister in the same location in the sublingual

sulcus and not to open their mouths or speak during the protocol. Mean skin temperature was calculated as the unweighted average of six copper-constantan thermocouples placed on the chest, middle back, abdomen, upper arm, thigh, and calf. During the insertion trauma resolution and baseline periods, thermoneutral water (34°C) was perfused through the suit to clamp body temperature. During whole body heating, 50°C water was perfused through the suit to raise T_{or} 0.8°C above baseline body temperature. Upon reaching a 0.8°C rise in oral temperature, skin and oral temperatures were clamped by perfusing the suit with 46°C for the remainder of the heating protocol.

Local skin temperature over each microdialysis site was maintained at 33°C during baseline and whole body heating (Moor Instruments SHO2, Devon UK).

Experimental Protocol

RBC flux over each microdialysis site was monitored during the insertion trauma resolution period (60-90 minutes). Following this period, microdialysis sites were randomly assigned to receive 1) lactated Ringers solution to serve as control, 2) 10.0mM N^G-nitro-L-arginine (L-NAME; Calbiochem San Diego, CA) to competitively inhibit NO production by NOS, 3) 10mM L-ascorbate (Asc: Sigma, St. Louis, MO) to supplement antioxidants (Holowatz *et al.*, 2006a), or 4) the combination of 10mM L-ascorbate and 5.0mM (s)-(2-boronoethyl)-L-cysteine-HCl (BEC) and 5.0mM N omega-hydroxy-nor-L-arginine (nor-NOHA) to supplement antioxidants and to inhibit arginase (Asc+A-I: Calbiochem, San Diego, CA, USA) (Holowatz *et al.*, 2006b; Holowatz & Kenney, 2007). All pharmacological solutions were mixed just prior to usage, dissolved in lactated Ringer's solution, sterilized using syringe microfilters (Acrodisc, Pall, Ann Arbor, MI, USA), and wrapped in foil.

All microdialysis sites were perfused with assigned pharmacological agents continuously for at least 60 minutes prior to the start of the baseline and during the baseline and heating periods at a rate of 2.0μL min^{-1}. Baseline data were collected for 20 minutes prior to the start of whole body heating. After the baseline data collection period, whole body heating was conducted to raise T_{or} by 0.8°C. Following a 0.8°C rise

in T_{or}, body temperature was clamped for 30-40 minutes. After 10 minutes of stable laser-Doppler flux values, 10mM L-NAME was perfused through all microdialysis fibers at a rate of 2.0μL min^{-1} to inhibit NOS within each treatment site. L-NAME infusions were discontinued after laser-Doppler flux decreased to a stable plateau (Figure 1). At the end of the heating protocol, each microdialysis site was perfused with 28.0mM sodium nitroprusside (SNP; Nitropress, Abbot Laboratories, Chicago, IL, USA) at a rate of 4.0μL min^{-1} to achieve maximal CVC. Local heating of the skin to 43°C was conducted simultaneously with SNP infusion to ensure maximal CVC had been obtained.

Data Acquisition and Analysis

Data were acquired using Labview software and National Instruments data acquisition system (Austin, TX, USA). The data were collected at 40 Hz, digitized, recorded and stored on a personal computer for further analysis. CVC data were averaged over 3-minute periods for baseline and every 0.1°C rise in T_{or} and are presented as a percent of maximal CVC (%CVC_{max}). The ΔCVC (%maximal) in the control, Asc, and Asc + A-I sites was calculated as the difference between the plateau values in %CVC_{max} before and after NOS inhibition.

Student's unpaired t-tests were used to determine significant differences between the groups for physical characteristics. Two-way repeated measures analysis of variance (ANOVA) was conducted to detect differences due to blood pressure and pharmacological treatment on maximal CVC and on the within site Δ%CVC_{max} due to NOS inhibition. A mixed models three-way repeated measures ANOVA was conducted to detect differences between subject groups at the pharmacological treatment sites over the rise in T_{or} (SAS, version 8.01). Tukey post hoc tests were performed when appropriate to determine where differences between groups and drug treatments occurred. The level of significance was set at $\alpha=0.05$. Values are presented as means ± SEM.

Results

Subject characteristics are presented in Table 7-1. The subjects were well matched for age, sex, body mass index, total cholesterol, and high density and low density lipoproteins. The hypertensive subjects had significantly higher resting systolic, diastolic, and mean arterial pressure (all $p<0.001$).

Figure 7-1 illustrates the timeline of L-NAME administration with representative tracings in control microdialysis sites from a normotensive and a hypertensive subject.

Figure 7-2 shows the $\%CVC_{max}$ response for all sites to the rise in body core temperature in both subject groups. $\%CVC_{max}$ in the control site was attenuated in the hypertensive subjects starting with $\Delta T_{or} \geq 0.6°C$ compared to the normotensive group ($p<0.05$). NOS inhibition throughout whole body heating attenuated $\%CVC_{max}$ in both subject groups starting at $\Delta T_{or} \geq 0.3°C$ in the hypertensive group and $\Delta T_{or} \geq 0.2°C$ in the normotensive group (both $p<0.05$). Asc and Asc+A-I significantly increased $\%CVC_{max}$ in the hypertensive group at $\Delta T_{or} \geq 0.4°C$ ($p<0.05$ vs. Co), but did not alter $\%CVC_{max}$ in the normotensive group ($p>0.05$ vs. Co). There was not a significant difference between the Asc and Asc+A-I treated sites in either subject group ($p>0.05$).

Figure 7-2 also illustrates the mean $\%CVC_{max}$ after NOS inhibition within the control, Asc, and Asc+A-I sites at $\Delta Tor = 0.8°C$. NOS inhibition within the control site decreased $\%CVC_{max}$ in both subject groups ($p<0.05$). Further, there was not a significant difference between the groups in $\%CVC_{max}$ in the control site after NOS inhibition (hypertensive 29±4 vs. normotensive: 34±4 $\%CVC_{max}$, $p>0.05$). Asc and Asc + A-I increased $\%CVC_{max}$ after NOS inhibition in the hypertensive group (Asc: 41±4, Asc+A-I: 40±4$\%CVC_{max}$, $p<0.05$ vs. Co) but not in the normotensive group (Asc: 29±3, Asc+A-I: 30±4$\%CVC_{max}$, $p>0.05$ vs. Co).

Figure 7-3 summarizes the $\%CVC_{max}$ responses in all of the treatment sites during the plateau in skin blood flow with a 0.8°C rise in body core temperature before and after within-site NOS inhibition. Compared to the normotensive group, vasodilation at the control site was attenuated in the hypertensive group ($p<0.001$) and there was a blunted decrease with NOS inhibition (hypertensive: -8±1 vs. normotensive: -14±2 $\Delta\%CVC_{max}$,

$p<0.001$). Compared to their respective control sites, Asc and combined Asc + A-I further decreased the $\Delta\%CVC_{max}$ in the hypertensive group (Asc: -19±4, Asc+A-I: -17±4%CVC_{max}, $p<0.05$ vs. Co), but not in the normotensive subject group (Asc: -11±3, Asc+A-I: -15±3%CVC_{max}, $p>0.05$ vs. Co).

Maximal absolute CVC was attenuated in the hypertensive group compared to the normotensive group (normotensive: 1.32 ±0.11 vs. hypertensive: 1.78±0.2 flux/mmHg; $p=0.001$). With in each subject group there was no effect of pharmacological treatment on maximal absolute CVC ($p>0.05$).

Discussion

The primary findings of this study were that humans with essential hypertension have (1) attenuated reflex cutaneous vasodilation that can be augmented with acute ascorbate supplementation alone or combined with arginase inhibition and (2) the increase in cutaneous vasodilation with these treatments is mediated by both NO- and non-NO-dependent mechanisms. In age-matched normotensive subjects acute ascorbate supplementation did not alter the overall skin blood flow response or the relative contribution of downstream NO and non-NO-dependent pathways to reflex vasodilation. These data suggest that increased ROS associated with hypertension directly decrease NO-dependent vasodilation and may augment vasoconstrictor tone in cutaneous vessels. Contrary to our hypothesis, concurrent ascorbate supplementation and arginase inhibition did not result in a greater increase in vasodilation than ascorbate alone suggesting that (1) there is redundancy in the underlying cellular mechanisms that these treatments affect and/or 2) that ascorbate supplementation alone maximized the capacity of the cutaneous vessels to vasodilate at this level of hyperthermia.

Our data confirm earlier reports (Kenney *et al.*, 1984) that reflex cutaneous vasodilation is attenuated in essential hypertensive subjects. In the present study, cutaneous vasodilation at the control site was only modestly reduced when expressed as a percentage of maximal vasodilation, however absolute maximal CVC was significantly attenuated. We chose to represent the data as a percentage of maximal CVC to account

for variability in capillary density under each laser-Doppler flowmeter, allowing for comparisons between microdialysis treatment sites (Braverman *et al.*, 1990). Others (Carberry *et al.*, 1992) have consistently observed a reduction in maximal CVC using both venous occlusion plethysmography to measure absolute and laser-Doppler flowmetry to measure relative changes in skin blood flow during whole arm local heating. Taken together, these data suggest that reflex cutaneous vasodilation is significantly attenuated with hypertension with a reduction in maximal CVC, suggesting structural maladaptations of the cutaneous vessels that limit absolute vasodilator capacity.

Our data show that in hypertensive skin there is a blunted reduction in $\%CVC_{max}$ after NOS inhibition in the control site (Figure 7-2 and 7-3). Thus, reduced NO-dependent vasodilation contributes to the overall attenuated skin blood flow response during hyperthermia in hypertensive humans. Ascorbate supplementation alone or combined with arginase inhibition increased both the absolute increase in $\%CVC_{max}$ with rising body temperature but also the $\Delta\%CVC_{max}$ after NOS inhibition within these sites. These data demonstrate that these interventions specifically targeted the NO pathway and increased cutaneous vasodilation through NO-dependent mechanisms. Additionally, because the antioxidant ascorbate increased NO-dependent vasodilation, these data suggest that increased oxidant stress contributes to attenuated NO-dependent vasodilation in essential hypertensive skin.

A pro-oxidant vascular environment and resulting decreased NO bioavailability is implicated in the pathogenesis of hypertensive vascular disease (Warnholtz *et al.*, 2004). NO bioavailability is dictated by the balance of ROS production and clearance through antioxidant defense mechanisms. With essential hypertension ROS production, particularly superoxide, in the vasculature increases predominantly through NAD(P)H-oxidase, xanthine oxidase, and uncoupled eNOS (Warnholtz *et al.*, 2004). In this deleterious cycle, superoxide decreases functional NO by reacting to form peroxinitrite at a rate that is four times faster than it can be cleared by superoxide dismutase (Beckman, 1996). Peroxynitrite is then capable of oxidizing critical cofactors for NOS, including BH_4 leading to NOS uncoupling. This contributes to further superoxide production while also limiting functional NO synthesis (Heinzel *et al.*, 1992;Toth *et al.*, 2002; Kuzkaya *et*

al., 2003; Landmesser *et al.*, 2003). In the present study we chose to directly supplement antioxidants to the cutaneous vasculature to examine a functional link between hypertension-associated increased oxidant stress and reduced NO-dependent vasodilation. Because of the generalized antioxidant mechanisms of action of ascorbate, this antioxidant potentially increased NO bioavailability through a variety of mechanisms including (1) direct scavenging of superoxide, and/or (2) stabilization of the essential NOS cofactor BH_4 enabling recoupling of NOS and functional NO production (Heller *et al.*, 2001; Taddei *et al.*, 2001). However, with the present data we are unable to delineate which of these mechanisms led to the increase in NO-dependent vasodilation.

Other mechanisms, including inadequate L-arginine availability, can also lead to eNOS uncoupling (Vasquez-Vivar *et al.*, 1998). We have recently shown that acute inhibition of arginase augments vasodilation in hypertensive skin, suggesting that arginase activity is upregulated in hypertensive cutaneous vasculature. Taken together, these data suggest that hypertension-associated upregulated arginase activity may be linked not only to decreased NO production but to superoxide synthesis through eNOS. We originally hypothesized that concurrent inhibition of arginase and ascorbate supplementation would result in a greater increase in NO-dependent vasodilation than ascorbate alone, however our data indicate that the resulting cutaneous vasodilation from the combined treatments is not additive. One possible explanation for this finding is that there is redundancy in the underlying cellular mechanisms of action of these treatments, where both treatments likely facilitated recoupling of eNOS resulting in increased functional NO synthesis and decreased superoxide production. Additional research investigating the role of BH_4 availability is necessary to more fully understand the mechanisms leading to uncoupled eNOS and decreased NO-dependent vasodilation in hypertensive skin.

An alternative explanation for the inability of combined ascorbate supplementation and arginase inhibition to further augment cutaneous vasodilation is that ascorbate supplementation alone may have maximized the capacity of the hypertensive cutaneous vessels to vasodilate reaching a “ceiling” effect with this given hyperthermic stimulus (T_{or}=Δ0.8°C). It is possible, but not likely, that a further increase in cutaneous

vasodilation with the combined treatments could be unmasked with a more significant hyperthermic stimulus.

An unexpected finding from the present study was that compared to control sites, ascorbate supplementation alone and combined with arginase inhibition increased the $\%CVC_{max}$ after NOS was inhibited in hypertensive skin (Figure 3). These data suggest that ascorbate supplementation augmented cutaneous vasodilation in hypertensive vasculature by non-NO-dependent as well as NO-dependent mechanisms. One potential explanation for this finding is that a pro-oxidant environment in hypertensive vasculature mediates enhanced vasoconstrictor tone. In human cutaneous vascular smooth muscle cells ROS act as signaling molecules to mediate cold-induced vasoconstriction through rho-kinase-dependent mechanisms (Bailey *et al.*, 2005). Because upregulated rho-kinase signaling is implicated in hypertensive vascular pathology (Masumoto *et al.*, 2001), it is possible that ROS mediated enhanced vasoconstriction in the absence of a cold stimulus through rho-kinase dependent mechanisms. Thus, providing antioxidants would inhibit the ROS-mediated enhanced vasoconstrictor tone resulting in augmented non-NO-dependent vasodilation.

An alternative explanation for the increase in non-NO-dependent vasodilation with ascorbate treatment in hypertensive skin, involves a potential synergistic interaction in downsteam signaling between NO and the putative sympathetic neurotransmitter(s) mediating reflex cutaneous vasodilation. NO is capable of mediating cutaneous vasodilatation synergistically with sympathetic cotransmitters, resulting in a combined vasodilatation that is greater than the sum of the individual contributions (Wilkins *et al.*, 2003). The synergistic effect may be mediated by downstream cross-talk between NO mediated cGMP-dependent and sympathetic neurotransmitter(s) mediated cAMP-dependent mechanisms (Farrell & Bishop, 1995, 1997), or alternatively, NO may prejunctionally enhance the release of sympathetic neurotransmitter(s) (Grider *et al.*, 1992). While the precise mechanism and potential interactions between NO and sympathetic neurotransmitter(s) involved in active cutaneous vasodilation remains elusive, our data suggests that augmenting NO bioavailability in a clinical population

with compromised endothelial function can also increase non-NO-dependent vasodilation.

Summary

In conclusion, NO-dependent reflex cutaneous vasodilation is attenuated in essential hypertensive subjects and antioxidant supplementation alone or combined with arginase inhibition augments cutaneous vasodilation during hyperthermia through NO- and non-NO dependent mechanisms. These data suggest that oxidant stress is increased in hypertensive cutaneous vasculature which decreases functional NO bioavailability and may enhance relative vasoconstrictor tone. Finally, in hypertensive subjects the resulting vasodilation with concurrent ascorbate supplementation and arginase inhibition was not greater than the vasodilation with ascorbate alone, suggesting redundancy in their mechanism of action through uncoupled eNOS and/or that ascorbate maximized the cutaneous vasodilatory capacity as this level of hyperthermia.

Table **7-1**: Subject characteristics. Values are means±SEM; HDL, high density lipoprotein; LDL, low density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP mean arterial pressure. *Significant difference vs. normotensive subject group ($p<0.05$).

Variable	**Normotensive**	**Hypertensive**
Sex (M, F)	6, 3	6, 3
Age, years	57±3	57±4
BMI, kg/m^2	24.0±1.0	26.3±1.0
Total Cholesterol, mg/dl	183±9	189±16
HDL, mg/dl	58±6	53±5
LDL, mg/dl	110±9	114±11
SBP, mmHg	116±3	159±3*
DBP, mmHg	76±1	94±1*
MAP, mmHg	81±10	112±1*

Figure 7-1

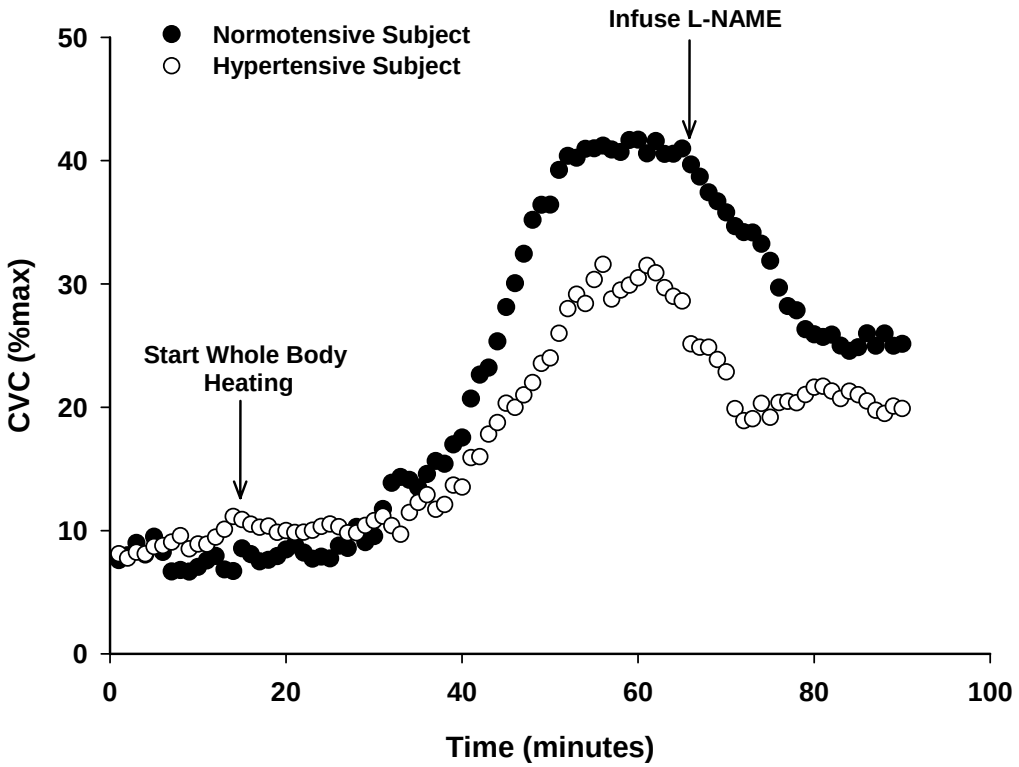

Figure **7-1**: Representative tracings of cutaneous vascular conductance (CVC) as a percentage of maximal vs. time from the control site in a normotensive (●) and a hypertensive subject (○). Whole body heating started at minute 10 and L-NAME was perfused through the microdialysis sites after oral temperature increased 0.8°C (minute 65 in this example).

Figure 7-2

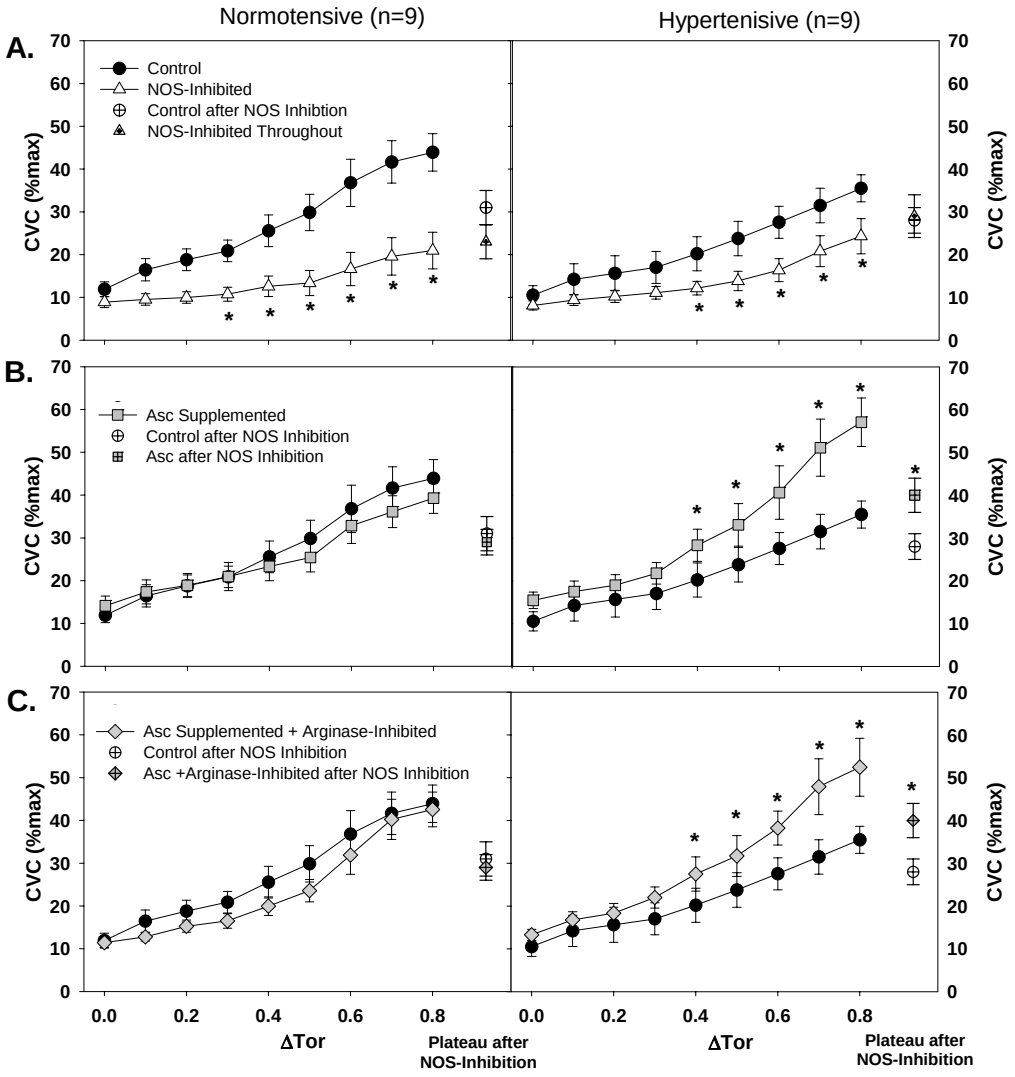

Figure **7-2**: Group mean ± SEM cutaneous vascular conductance (CVC) as a percent of maximal response during passive whole body heating. Age-matched normotensive subject responses are displayed in the left panel and hypertensive subject responses are in the right panel. Panel A shows the nitric oxide synthase (NOS)-inhibited site (Δ) throughout heating and during the plateau with ΔT_{or}=0.8°C. Panel B shows the Ascorbate supplemented (Asc) supplemented site (□) throughout heating and after NOS inhibition. Panel C shows the combined Asc +Arginase-inhibited site (◊) throughout heating and after NOS inhibition. The control site (●) throughout heating and after NOS inhibition is illustrated in all of the panels for comparison. Asc and Asc +Arginase inhibition augmented cutaneous vasodilation during heating and after within site NOS inhibition in the hypertensive subject group. *$p<0.05$ significant difference vs. the control site within subject groups.

Figure 7-3

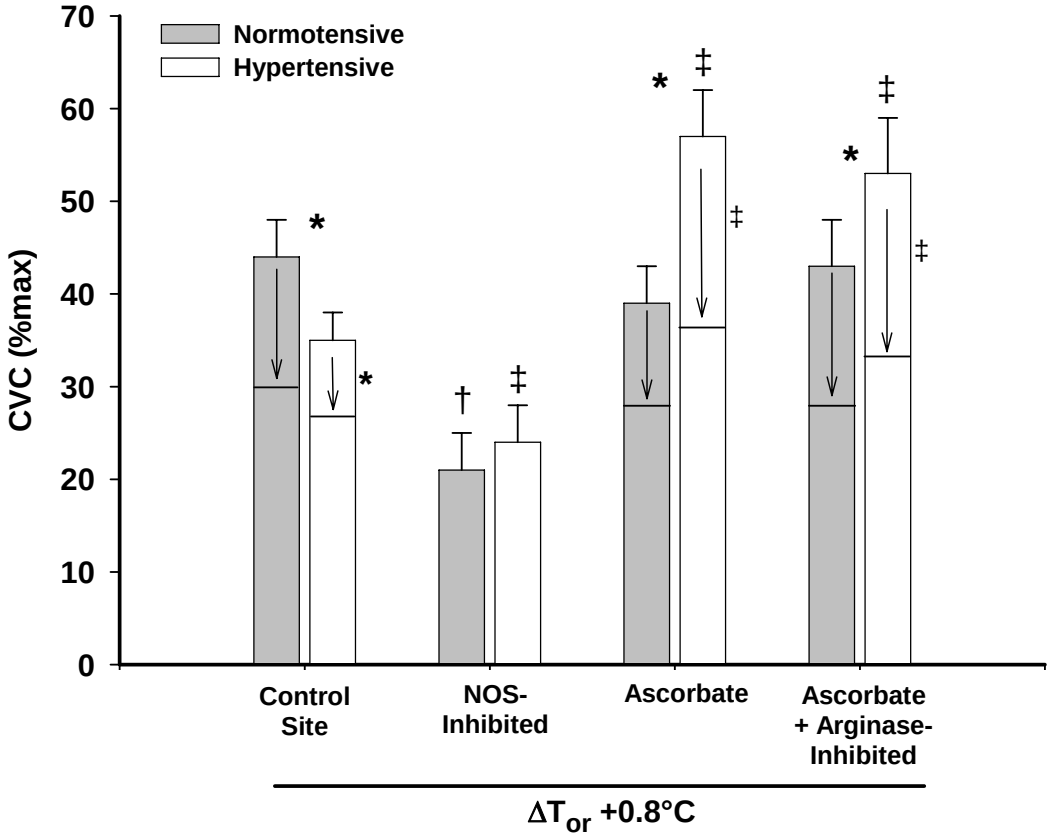

Figure **7-3**: Group mean ± SEM cutaneous vascular conductance (CVC) as a percent of maximal vasodilation with a 0.8°C in oral temperature (ΔT_{or},°C) in all drug treatment sites. Grey bars represent the age-matched normotensive subject group and the open bars represent the hypertensive subject group. The decrease in CVC after NOS inhibition is illustrated with arrows. Ascorbate supplementation (Asc) and combined Asc + Arginase-inhibition augmented CVC and the change in CVC after NOS inhibition in hypertensive but not normotensive subjects. * $p<0.05$ significant difference between blood pressure groups, †$p<0.001$ significant vs. control site age-matched normotensive subject group, ‡ $p<0.001$ significant vs. control site hypertensive subject group.

Chapter 8

CONCLUSIONS AND FUTURE DIRECTIONS

The five studies comprising this dissertation were designed to explore the mechanisms underlying primary human aging- and hypertensive vascular pathology-related changes to reflex cutaneous vasodilation. Specifically, these studies investigated (1) age-related changes to acetylcholine-mediated cutaneous vasodilation and (2) the putative pathways in which nitric oxide (NO) bioavailability may be impaired with primary aging and hypertension. This chapter is intended to summarize the results of these studies and to place them into a broader integrated physiological context. Finally, future avenues of research that may further clarify the effects of aging and hypertension on impaired cutaneous vasoreactivity will be discussed.

Acetylcholine-Mediated Cutaneous Vasodilation

The primary finding of this study was that cutaneous vasodilation to exogenous acetylcholine is mediated by prostanoid and non-NO, non-prostanoid dependent pathways. NO did not directly contribute to cutaneous vasodilation in either age group. In the older subjects, there was a significant increase in baseline cutaneous vascular conductance ($\%CVC_{max}$) in sites where cyclooxygenase (COX) was inhibited, suggesting an age-related increase in COX derived vasoconstrictors contributes to basal cutaneous vasomotor tone. Finally, prostanoid dependent cutaneous vasodilation to exogenous acetylcholine infusion was attenuated in aged skin, but there was a compensatory increase in the non-NO-non-prostanoid dependent contribution to acetylcholine-mediated vasodilation.

Implications

These findings have several implications for both the basic mechanism of cutaneous active vasodilation in young skin, and the age-related attenuation in reflex vasodilation during heat stress. Shibasaki *et al.* hypothesized that actylcholine modulated the initial rise in skin blood flow in young skin through NO-dependent mechanisms. Acetylcholinesterase inhibition augmented the initial rise and concurrent NO-synthase (NOS) inhibition decreased the initial rise (Shibasaki *et al.*, 2002). However, an alternative hypothesis consistent with our data is that acetylcholine modulates the initial rise in skin blood flow during heat stress through primarily prostanoid-dependent mechanisms. Subsequent investigations suggest that COX-derived vasodilators contribute significantly and independently of NO mechanisms to reflex cutaneous vasodilation (McCord *et al.*, 2006). Considering the potential for multiple sources of NO independently contributing to the different phases of active vasodilation, and possible downstream interactions, it is likely that there is a great deal of redundancy functioning to preserve cutaneous vasodilatory functions. Additionally, pharmaco-dissection of the contributing vasodilatory pathways will likely be difficult considering the possibility of compesensatory upregulation when one or more vasodilators is inhibited.

Future Directions

1.) Our data suggests that there is a shift in COX derived vasodilators to vasoconstrictors in aged human skin. This finding has interesting thermoregulatory implications concerning the factors that modulate basal cutaneous vasomotor tone and heat loss in thermoneutral conditions. Together with the data from McCord et al., these findings suggest that aged individuals taking COX-inhibitors may have a higher skin blood flow under thermoneutral conditions and significantly attenuated skin blood flow during hyperthermia. The effects of chronic COX-inhibition may have deleterious thermoregulatory consequences including an increased risk for developing hypothermia and hyperthermia in response to hot and cold environments, respectively.

2.) We found that non-NO-non-prostanoid vasodilators contribute substantially to acetycholine-induced vasodilation in human skin. The microinfusion protocol that was developed for the present study may be useful for the identification of potential endothelium derived hyperpolarization factors in the cutaneous vasculature.
3.) This study was designed to examine age-related changes to the contributions of NO-synthase- (NOS) and COX-derived vasodilators to acetycholine-mediated cutaneous vasodilation. We were unable to detect a significant direct contribution of NO to acetylcholine-mediated vasodilation in either age group. We designed the microinfusion of acetylcholine to produce cutaneous vasodilation similar to what is observed during the initial rise in skin blood flow during whole body heating. We also performed two microinfusions of the same dose of acetylcholine to examine reproducibility of the skin blood flow response. However, contrary to our findings, Kellogg *et al.* reported a significant NO contribution to acetylcholine-mediated cutaneous vasodilation (Kellogg *et al.*, 2005). This group continuously infused a concentration of acetylcholine that was significantly larger than what was infused in the present study and did not measure skin blood flow as the vasodilatory response to acetylcholine resolved. These contrasting results could be clarified if a similar experiment to ours was performed with the same antagonists but if instead complete acetylcholine dose response curves were obtained in young and aged skin.

Arginase in aged human skin

The principal finding of this study was that acute arginase inhibition, L-arginine supplementation or both augment reflex cutaneous vasodilation in aged skin. These treatments did not alter the skin blood flow response to whole body heating in young subjects. These results suggest that arginase is upregulated in aged human skin and limits intracellular L-arginine availability of NO synthesis through NOS. Cumulatively, these data demonstrate that the age-related deficit in reflex cutaneous vasodilation can be restored by either inhibiting arginase to replenish the available pool for L-arginine for

NO synthesis, or by directly supplementing supraphysiological concentrations of L-arginine to effectively saturate both the NOS and arginase pathways.

Ascorbate in aged human skin

The primary findings of this study were that in aged human skin 1) ascorbate supplementation alone selectively increased reflex cutaneous vasodilation, 2) ascorbate supplementation combined with arginase inhibition elicits an additional increase in cutaneous vasodilation compared to sites treated with ascorbate alone. These treatments did not affect the skin blood flow response in young subjects and only augmented skin blood flow with significant elevations in body core temperature ($\Delta T_{or} \geq 0.6°C$) in aged subjects. These findings suggest that both increased oxidant stress and upregulated arginase activity decrease cutaneous vasodilation in aged human skin.

Arginase in hypertensive human skin

The principal findings of this study were that humans with essential hypertension have (1) attenuated reflex cutaneous vasodilation due, in part, to reduced NO-dependent vasodilation and (2) acute arginase inhibition-- but not exogenous L-arginine supplementation-- significantly augmented reflex vasodilation in hypertensive skin. In age-matched normotensive subjects, increasing L-arginine availability through either arginase inhibition or L-arginine supplementation did not significantly alter skin blood flow but together these treatments augmented reflex vasodilation with $\Delta T_{or} > 0.2°C$. These findings suggest that arginase is upregulated in the skin of hypertensive subjects and contributes to reduced NO-dependent vasodilation by limiting intracellular L-arginine availability for NO synthesis. Since direct L-arginine supplementation in hypertensive skin failed to result in an increase in cutaneous vasodilation, these data suggest that either (1) freely exchangeable extracellular L-arginine does not limit NO synthesis (2) there is diminished transport of L-arginine through the cationic amino acid

transporter (CAT), or (3) caveolar NOS activity associated with the CAT is decreased in hypertensive skin.

Oxidant stress in hypertensive human skin

The primary findings of this study were that humans with essential hypertension have (1) attenuated reflex cutaneous vasodilation that can be augmented with acute ascorbate supplementation alone or combined with arginase inhibition and (2) the increase in cutaneous vasodilation with these treatments is mediated by both NO- and non-NO-dependent mechanisms. In age-matched normotensive subjects, acute ascorbate supplementation did not alter the overall skin blood flow response or the relative contribution of downstream NO and non-NO-dependent pathways to reflex vasodilation. These data suggest that increased reactive oxygen species (ROS) associated with hypertension directly decrease NO-dependent vasodilation and may augment vasoconstrictor tone in cutaneous vessels. Contrary to our hypothesis, concurrent ascorbate supplementation and arginase inhibition did not result in a greater increase in vasodilation than ascorbate alone suggesting that (1) there is redundancy in the underlying cellular mechanisms that these treatments affect (replenishing L-arginine availability and stabilizing tetrahydrobiopterin effectively re-coupling NOS) and/or 2) that ascorbate supplementation alone maximizes the capacity of the cutaneous vessels to vasodilate at this degree of hyperthermia.

Implications

Healthy aged subjects rely predominantly on compromised NO function to increase SkBF during hyperthermia, due to a reduced functional cotransmitter contribution to the increase skin blood flow during heat stress. Increasing NO bioavailability through a variety of mechanisms, including acute antioxidant supplementation and/or increasing L-arginine availability for NO synthesis, augments

reflex vasodilation and effectively abolishes the age-related deficit in skin blood flow. Furthermore, these findings suggest that the arginase/L-arginine/NO pathway may be a potential therapeutic target to improve NO bioavailability in aged vasculature.

Essential hypertensive vascular pathology is associated with globally compromised endothelial function, namely significant reductions in NO bioavailability. Hypertension-induced vascular dysfunction coupled with the normal aging process severely impairs cutaneous vasodilatory function in this clinical population, rendering them more vulnerable to complications arising from reduced skin blood flow, such as hyperthermia and impaired wound healing. Our *in vivo* data suggests that upregulated arginase activity and increased oxidant stress are two primary mechanisms limiting NO-dependent cutaneous vasodilation. Interestingly, the results from the ascorbate study suggest that non-NO-dependent vasodilation can be increased with ascorbate supplementation. One potential explanation is that oxidants may augment basal cutaneous vasoconstrictor tone that competes with vasodilatory signals during heat stress and/or that oxidants produced via NOS uncoupling significantly contribute to attenuated cutaneous vasodilation. Currently, it is not possible to measure NOS uncoupling *in vivo*, and there is doubt that *in vitro* techniques to visualize the coupled state of NOS provide a true index of superoxide production. NOS can be spatially coupled but can still produce superoxide (Forstermann & Munzel, 2006).

One unexpected difference between the primary human aging studies and the hypertension studies was that L-arginine supplementation was effective at increasing skin blood flow in the aged population but was ineffective in the hypertensive population. This divergent finding indicates that the underlying mechanisms, as they pertain to freely exchangeable L-arginine, mediating reduced NO bioavailability are different between these groups. Our results suggest that there is a reduction in the concentration of L-arginine in the freely exchangeable microdomain with aging, however with hypertension either (1) there is a sufficient concentration of L-arginine in this microdomain to support NOS synthesis, implicating reduced NOS activity with hypertension, or (2) there is a reduction in L-arginine transport from the CAT and/or (3) there is a dissociation of caveolar NOS from the CAT. These findings have important clinical applications

pertaining to the efficacy of therapeutic L-arginine supplementation with aging and in vascular disease.

In order to examine the effects of oxidant stress on NO-dependent vasodilation, ascorbate was used to acutely supplement antioxidants. I*n vivo* human vascular studies commonly utilize ascorbate for this purpose. However, one difficulty in interpreting results from these studies is that ascorbate acts directly as an antioxidant and it prevents oxidation of the essential NOS cofactor tetrahydriobiopterin (BH_4) (Toth *et al.*, 2002). In order to more fully understand the role of BH_4 in NOS uncoupling in human skin with aging and hypertension, direct supplementation studies need to be performed. Along similar lines, our studies only examined the end product of oxidant stress in aged and hypertensive cutaneous vasculature. A systematic investigation into the putative enzymatic sources of reactive oxygen species is necessary.

In both the aging studies and the hypertension studies we found that acute arginase inhibition augmented cutaneous vasodilation, suggesting that arginase activity is upregulated. However, the precise signal that mediates arginase upregulation and whether it is similar in both of these populations is unclear. Several potential inducers have been identified including (1) pressure-induced arginase activation (Demougeot *et al.*, 2006) and (2) rapid arginase activation by dissociation from the microtubule cytoskeleton (Ryoo *et al.*, 2006). There may be a common mechanism mediating arginase activation through microtubule disruption mediated by activation of the small GTPase, Rho kinase. Rho kinase also modulates Ca2+ sensitivity in vascular smooth muscle and is capable of downregulating NOS (Ming *et al.*, 2002; Wettschureck & Offermanns, 2002) and arginase (Ming *et al.*, 2004) expression and activity. Rho kinase is known to be upregulated in primary aged and essential hypertensive human vasculature (Masumoto *et al.*, 2001). In the cutaneous circulation we have recently demonstrated that Rho kinase-dependent pathways mediate cutaneous vasoconstriction (Thompson-Torgerson *et al.*, 2006) and furthermore are upregulated with primary aging (Thompson-Torgerson *et al.*, 2007). Thus, Rho kinase mediated microtubule disruption may be a common mechanism by which arginase is upregulated with vascular dysfunction.

The series of studies that comprise this dissertation contribute to emerging evidence pertaining to the complex interactions of oxidant stress, arginase activation, and reduced NO bioavailability in vascular dysfunction. The state of the art experimental techniques utilized in these studies have allowed us to translationally examine the putative mechanisms mediating vascular dysfunction in aged and hypertensive populations. Future studies with these techniques in the cutaneous vasculature coupled with an *in vitro* examination of eNOS, arginase, and Rho-kinase expression, activity and activation is necessary to more fully understand the complex interactions between these signaling pathways and their contribution to vascular dysfunction.

Future Directions

1.) The studies presented in this dissertation have provided insight into some of the basic mechanisms that mediate reduced NO bioavailability with primary aging and hypertension. Our model delivered specific arginase inhibitors, and supraphysiological concentrations of L-arginine and ascorbate directly to the cutaneous vasculature. It is necessary to determine whether oral L-arginine and ascorbate supplementation could produce similar effects in the cutaneous vasculature during heat stress. Previous oral supplementation studies have failed to show a positive vascular benefit of L-arginine or ascorbate therapy. It may be necessary to provide a cocktail of supplements to functionally recouple NOS, with ascorbate and BH_4, while also increasing NOS substrate concentrations by providing L-arginine, the endogenous arginase inhibitor valine, and L-citrulline which can be converted into L-arginine via arginosuccincate intracellularly.
2.) We have demonstrated that we can functionally restore the age-related deficit in reflex vasodilation with either local arginase inhibition or L-arginine supplementation. However, there are central cardiovascular impairments that occur with primary human aging that likely limit the rise in skin blood flow during hyperthermia (Minson *et al.*, 1998). It is unknown whether the aged left ventricle could support full expression of reflex cutaneous vasodilation throughout the cutaneous vasculature. In order to address this question it would be necessary to systemically infuse L-arginine during whole body

heat stress while measuring central cardiovascular response (cardiac output, renal and splanchnic blood flow).

3.) Emerging evidence suggests that there is differential regulation of the L-arginine microdomains accessible to NOS. Examination of the mechanisms that modulate L-arginine accessibility to NOS within each of these microdomains is necessary to more fully understand the role of arginase and freely exchangeable L-arginine in reducing NO-dependent vasodilation with aging and essential hypertension (Flam *et al.*, 2001). One approach to examining the regulation of the freely exchangeable pool of L-arginine would be to inhibit the CAT using L-lysine (Topal *et al.*, 2006). In aged skin these experiments would clarify the potential interactions between freely exchangeable L-arginine and intracellular arginase. In hypertensive cutaneous vasculature, similar experiments would clarify whether there is a hypertension-associated dysregulation of the CAT and/or potential dissociation of caveolar NOS from the CAT.

4.) The techniques employed in this series of studies have allowed us to functionally examine the effect of arginase inhibition and antioxidant supplementation on reflex cutaneous vasodilation. These data lend support to our hypotheses pertaining to the mechanisms mediating age- and hypertension-associated reduction in skin blood flow, but we have not directly measured oxidant stress, NOS uncoupling, or arginase activity. *In vitro* studies examining eNOS, arginase I and II, and Rho-kinase expression, activity, and mechanisms for activation in human cutaneous vasculature would contribute to a more complete understanding of the mechanisms underlying decreased NO bioavailability with primary human aging and hypertensive vascular pathology.

5.) Oxidized low density lipoprotein (Ox-LDL) rapidly activates arginase II by causing disruption of the microtubule cytoskeleton (Ryoo *et al.*, 2006). There is a mechanistic link between augmented vascular arginase activity and the pathogenesis of atherosclerosis through an increase in the polyamine and proline precursor L-ornithine which contributes to vascular smooth muscle cell proliferation and intimal thickening (Wu & Morris, 1998; Durante *et al.*, 2001). Thus Ox-LDL may initiate this deleterious cascade. Because reductions in cutaneous vasoreactivity are clearly evident in hypercholesterolemic clinical populations (Binggeli *et al.*, 2003), a thorough assessment

of the underlying mechanisms mediating reduced skin blood flow is necessary. To this end, studies examining hypercholesterolemia-mediated reductions in skin blood flow with interventional HMG-CoA-reductase inhibitor (statin) therapy may provide insight into *in vivo* activation and possible recovery of NO signaling. One of the pleiotropic effects of statin therapy is to increase blood flow by inhibiting Rho-mediated cytoskeletal changes which subsequently leads to stabilization of eNOS (Laufs *et al.*, 2000; Rikitake *et al.*, 2005). Considering that Rho induces arginase activity in atherosclerotic animal models (which can be inhibited by statins or Rho kinase inhibitors (Ming *et al.*, 2004), and the reciprocal relationship between NOS and arginase (Berkowitz *et al.*, 2003); one putative mechanism for the increase in endothelial NO bioavailability with statin drugs is through a decrease in arginase activity. Future investigations with a hypercholesterolemic clinical population would clarify the role of arginase in endothelial dysfunction and may provide *in vivo* evidence that arginase is a therapeutic target for the prevention and treatment of atherosclerosis.

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Appendix A

INFORMED CONSENT FORMS

Acetylcholine Study

INFORMED CONSENT FORM FOR CLINICAL RESEARCH STUDY
The Pennsylvania State University

ORP USE ONLY:
The Pennsylvania State University
Office for Research Protections
Approval Date: 12/11/04 T. Kahler
Expiration Date: 12/10/05 T. Kahler
Biomedical Institutional Review Board
IRB#17411

Title of Project: **Cutaneous Mechanisms of Acetylcholine-Mediated Vasodilation**

Principal Investigator: **W. Larry Kenney, Ph.D.**
Address: 102 Noll Laboratory
Phone: (814) 863-1672
Other Investigators: **Lacy Holowatz, M.S., Doctoral Candidate**
Jane Pierzga, M.S., Research Assistant

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 under the supervision of Dr. W. Larry Kenney.

1. Purpose of the study: 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, you may become more prone to illness in extreme heat. This study explores whether the natural chemicals and/or your blood vessels' ability to respond to them change as you age. To do this, the study uses "microdialysis." 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 cm2 (0.4 inch2), nickel-sized area of skin at each tubing site. One substance (acetylcholine) is a natural chemical made by your skin's nerves. Others, L-NAME (NG-nitro-L-arginine methyl ester) and Toradol, stop the vessels from responding to acetylcholine (ACH). Also, SNP (sodium nitroprusside) causes your blood vessels to get as large as they can. We measure the flow in vessels near the tubing with a machine that shines a low energy laser light onto your skin. We explore if the blood vessels in your skin are unable to respond to acetylcholine and why.

2. Procedures: You may request personnel of the same gender to perform procedures.

Screening Day 1: You will not eat or drink after midnight during the night before your exam. You report to the General Clinical Research Center (GCRC) for your appointment. When you arrive, the nurses will draw 15 ml (1 Tbsp) of blood from a vein in your arm. You will have an examination by the GCRC medical staff that includes blood pressure, check-up, height, weight, and ECG. If you are a woman of childbearing-age, you will submit a urine sample for a pregnancy test.

Screening Day 2: You will report to the GCRC for a medical history and graded exercise test (GXT).
For the GXT, you exercise on a bicycle to measure your fitness level. GCRC clinical staff is present. The electrical activity of your heart and your blood pressure are measured. During the test, you wear a nose clip and breathe into a tube to measure the oxygen and carbon dioxide you breathe out. You will help the researcher adjust 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). At first, you will pedal easily. If the seat is uncomfortable, you will ask the researcher to adjust it. Pedaling becomes a little harder every 2 minutes. The test will be most accurate if you do your best to pedal for as long as you can. However, you can stop whenever you want to stop. The test is 10-20 minutes long.

Acetylcholine (ACH) Characterization Day 3: When you arrive at the laboratory, you will wash your forearm and pat it dry. The researcher will place a tight band around your upper arm so your veins are easily seen. The researcher will mark places on your arm 2.5 cm (1 inch) apart and away from veins where the tubing will enter and exit your skin. You will have 4 sets of marks on your arm. The tight band is removed. The researcher will clean your arm with an orange-colored Betadine fluid, and alcohol. If you wish, the researcher will place an ice bag on your arm for 5 minutes to numb your skin. Then the researcher will insert a thin needle into your skin at each entry mark. The needle's tip will travel between the layers of skin for 2.5 cm (1 inch) and leave your skin at the matching exit mark. The tubing is threaded through the needle. Next, the researcher will withdraw the needle leaving the tubing in your skin. Any redness of your skin will subside in 30-45 minutes. You will have up to 4 sets of tubing in your skin.

A pencil-sized probe will be taped on your arm at each site where there is thin tubing in your skin. The probes will measure your skin's blood flow using a very low energy laser light. Also, a machine that shines laser light onto your skin without touching your skin may be used. The researcher will start a plain fluid (Lactated Ringer's solution) flowing through the tubing in your skin. Then the study and data collection will begin. During the study, the researcher will measure your heart rate and blood pressure.

You will rest for 20-minutes. Then the plain fluid will still flow through one set of tubing. Ringer's + L-NAME, Ringer's + Toradol, and Ringer's + L-NAME + Toradol will flow through the other tubing. After 45 minutes, ACH is added to all fluids for 1 minute. Then the ACH is stopped, and the fluids still flow through the tubing while skin blood flow is measured for about 1.5 hours. During ACH and for 1.5 hours afterward, the fluid flowing out of the tubing is collected. Later, this fluid is tested for a chemical that your body makes. Lastly, Ringer's + SNP will flow through all tubing for 30 – 45 minutes. Then the researcher heats the laser probes' holders to 42°C (108°F) for about 20 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. A bag of ice is placed on your arm for 10 minutes to reduce any bruising that may occur. You will have your blood pressure and heart rate measured, and your arm examined by the medical staff of the GCRC before leaving.

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, heart attack (< 0.05%), and death (< 0.02%). Severe reactions are rare.
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, you may 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. But you may feel a little pain when the tubing is removed from your skin. You may become lightheaded or may faint. Although rare, it is possible for the tubing to break during removal from your skin. Then the researcher removes the tubing by pulling on the other end of it. This produces no additional risk for you. Even more rare, the tubing could break so that a piece of the tubing is left under your skin. In this case, any tubing remaining in your skin would be treated like a splinter. The thin layer of skin over the tubing may have to be cut to allow the tubing's removal. If slight bleeding occurs, applying mild pressure with sterile gauze will stop it. Infection is possible. Sterile techniques and supplies like those used in hospital will be used to keep the risk minimal. A sterile bandage will be placed on the site after the experiment. You will be told how to take care of the site.
Fluid flowing through the tubing: The substances flowing through the tubing only go to a 2.5 cm2 (0.4 inch2) area of skin at each tubing site. 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. Other researchers used these substances with microdialysis in skin. The researchers have not reported that these substances caused bad reactions. If a bad reaction should occur, medical help will be summoned.
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, Toradol: These substances stop the action of my body's natural chemicals upon the blood vessels in the skin. A small amount of these substances will enter the skin around the tubing. This only affects the blood flow in the vessels in that small area of skin. The effect of these substances is gone within an hour after the experiment. Other researchers have used these substances with microdialysis in skin. The researchers have not reported that these substances caused bad reactions.
ACH (acetylcholine): ACH is like one of the natural chemicals made by the nerves in the skin. A small amount of these substances will enter the skin around the tubing. This only affects the blood flow in the vessels in that small area of skin. The effect of ACH is gone within an hour after the experiment. Other researchers used ACH with microdialysis in skin. The researchers have not reported that ACH caused bad reactions.

Sodium Nitroprusside (SNP): Only a small amount of SNP will enter your skin around the tubing. SNP increases the blood flow in the vessels and reddens that small area of skin. This effect is gone within an hour after the experiment. Other researchers have used SNP with microdialysis in skin. They have reported no bad reactions with SNP.
Laser Doppler Flowmetry: Weak lasers can hurt your eye if you stare into the light for a long time. The laser is not turned on until the probes are taped to a surface. The design of the other laser machine hides the laser source from view. The red light you see on the surface of your skin is harmless. The tape may irritate your skin.
Blood Pressure: Your blood pressure is measured using the method common in a doctor's office. A cuff will be inflated on your upper arm. As the cuff slowly deflates, a person listens with a stethoscope at the bend in your elbow. During the short time the cuff is inflated, your arm may feel numb.
ECG: This machine measures the electrical activity of your heart. You will have 12 wires from the machine taped to spots on your body. There have been no adverse effects from this measure. The tape may irritate your skin.
Betadine: Hospitals and the researcher 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 or shellfish. You will inform the researcher if you have these allergies so that alcohol is used instead. This bad 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.
Latex: Some gloves and medical materials are made of latex rubber. You will inform the researcher 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 medical staff uses the same techniques used in hospitals.
Local Heating: The researcher will 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

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 will help us to know the changes that happen with aging that impair the body's resistance to heat stress. Knowing these changes may lead to ways to prevent these impairments to avoid heat illness and death in older people. This could lead to improvements in health and quality of life for the individual. A greater percent of our population is becoming aged. As this happens, the health and welfare of older people has an even greater impact on society, as a whole.

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 3 days. The first day is for the first part of the screening that should last no longer than 1/2 hour. The second day is for the 2nd part of the screening that should last no more than 1 hour. The third day is for the experiment, which should last no longer than 4.5 hours.

7. Statement of confidentiality: The data is available only to the investigators. 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 for Research Protections and the Biomedical Institutional Review Board (IRB) may review records related to this project.

8. Right to ask questions: If you have any questions or concerns about the research or your participation in the present investigation, you may contact Lacy Holowatz (W: 814-863-2948, 861-6255) or Jane Pierzga (W: 814-865-1236, H: 814-692-4720). If there are findings during the research that could relate to your wanting to help with the study, you will be told of the findings. You may contact the Office for Research Protections, 212 Kern Graduate Building, University Park, PA 16802, (814) 865-1775 for additional information concerning your right as a research participant.
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.

9. Compensation: You will receive $15.00 for each of the microdialysis tubing inserted in your arm on Day 3 (maximum $60.00). You will receive $30.00 more for completing the study on Day 3. You will receive a lab T-shirt.

If you are an employee of Penn State University, the compensation you receive for participation will be treated as taxable income and therefore taxes will be taken from the total amount. If you are not employed by Penn State University, 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: Medical care is available in the event of injury resulting from research but neither financial compensation nor free medical treatment is provided. You are not waiving any rights that you may have against the University for injury resulting from negligence of the University or investigators.

11. Voluntary participation: Your participation in this study is voluntary, and you may withdraw from this study at any time by notifying the investigator. Your withdrawal from this study or your refusal to participate will in no way affect your care or access to medical services. You may decline to answer specific questions. However, your acceptance into the study may be contingent upon answering these questions. Your helping with the study may be ended 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.

12. In the event that abnormal test results are obtained, you will be apprised of the results immediately and recommended to contact your private medical provider for follow-up.

This is to certify that I am 18 years of age or older and I consent to and give permission for my participation as a volunteer in this program of investigation. I understand that I will receive a signed copy of this consent form. I have read this form, and understand the content of this consent form.

_____

Volunteer Date

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

_____

Investigator Date

INFORMED CONSENT FORM FOR CLINICAL RESEARCH STUDY
The Pennsylvania State University

Title of Project: **Cutaneous Mechanisms of Acetylcholine-Mediated Vasodilation- addendum**

Principal Investigator: **W. Larry Kenney, Ph.D.**
Address: 102 Noll Laboratory
Phone: (814) 863-1672
Other Investigators: **Lacy Holowatz, M.S., Doctoral Candidate**
Jane Pierzga, M.S., Research Assistant

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 under the supervision of Dr. W. Larry Kenney.

Purpose: In your first study, we put Toradol into your skin at some microdialysis (MD) sites to stop the blood vessels from responding to acetylcholine. At the end, we put sodium nitroprusside (SNP) through all of the MD probes to cause maximal skin blood flow. We saw that the increase in skin blood flow caused by SNP was lower than we expected in skin exposed to Toradol. No one has seen this effect before now. Toradol seems to change your ability to obtain a true maximal blood flow with SNP. We have used local heating to obtain maximal blood flow in other studies that do not use MD. Adding local heating to this study will allow us to get a true maximal blood flow at the sites exposed to Toradol. Also, local heating will allow us to explore the effect of Toradol on the skin blood flow produced by SNP. We want you to repeat the earlier study with local heating added. We will compare your first and second studies.
Most of the information in your first informed consent still applies. You may not have to repeat the screening unless your health status has changed since you first screened for the study. The researcher will talk with you to decide if your health status has changed. If you are a woman of child-bearing age, you will submit a urine sample for another pregnancy test.

1 Acetylcholine (ACH) Characterization: When you arrive at the laboratory, you will wash your forearm and pat it dry. The researcher will place a tight band around your upper arm so your veins are easily seen. The researcher will mark places on your arm 2.5 cm (1 inch) apart and away from veins where the tubing will enter and exit your skin. You will have 4 sets of marks on your arm. The tight band is removed. The researcher will clean your arm with an orange-colored Betadine fluid, and alcohol. If you wish, the researcher will place an ice bag on your arm for 5 minutes to numb your skin. Then the researcher will insert a thin needle into your skin at each entry mark. The needle's tip will travel between the layers of skin for 2.5 cm (1 inch) and leave your skin at the matching exit mark. The tubing is threaded through the needle. Next, the researcher will withdraw the needle leaving the tubing in your skin. Any redness of your skin will subside in 30-45 minutes. You will have up to 4 sets of tubing in your skin.

A pencil-sized probe will be taped on your arm at each site where there is thin tubing in your skin. The probes will measure your skin's blood flow using a very low energy laser light. Also, a machine that shines laser light onto your skin without touching your skin may be used. The researcher will start a plain fluid (Lactated Ringer's solution) flowing through the tubing in your

skin. Then the study and data collection will begin. During the study, the researcher will measure your heart rate and blood pressure.

You will rest for 20-minutes. Then the plain fluid will still flow through one set of tubing. Ringer's + L-NAME, Ringer's + Toradol, and Ringer's + L-NAME + Toradol will flow through the other tubing. After 45 minutes, ACH is added to all fluids for 1 minute. Then the ACH is stopped, and the fluids still flow through the tubing while skin blood flow is measured for about 1.5 hours. During ACH and for 1.5 hours afterward, the fluid flowing out of the tubing is collected. Later, this fluid is tested for a chemical that your body makes. Lastly, Ringer's + SNP will flow through all tubing for 30 – 45 minutes. Then the researcher heats the laser probes' holders to 42°C (108°F) for about 20 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. A bag of ice is placed on your arm for 10 minutes to reduce any bruising that may occur. You will have your blood pressure and heart rate measured, and your arm examined by the medical staff of the GCRC before leaving.

2. Discomforts and risks:

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, you may 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. But you may feel a little pain when the tubing is removed from your skin. You may become lightheaded or may faint. Although rare, it is possible for the tubing to break during removal from your skin. Then the researcher removes the tubing by pulling on the other end of it. This produces no additional risk for you. Even more rare, the tubing could break so that a piece of the tubing is left under your skin. In this case, any tubing remaining in your skin would be treated like a splinter. The thin layer of skin over the tubing may have to be cut to allow the tubing's removal. If slight bleeding occurs, applying mild pressure with sterile gauze will stop it. Infection is possible. Sterile techniques and supplies like those used in hospital will be used to keep the risk minimal. A sterile bandage will be placed on the site after the experiment. You will be told how to take care of the site.
Fluid flowing through the tubing: The substances flowing through the tubing only go to a 2.5 cm2 (0.4 inch2) area of skin at each tubing site. 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. Other researchers used these substances with microdialysis in skin. The researchers have not reported that these substances caused bad reactions. If a bad reaction should occur, medical help will be summoned.
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, Toradol: These substances stop the action of my body's natural chemicals upon the blood vessels in the skin. A small amount of these substances will enter the skin around the tubing. This only affects the blood flow in the vessels in that small area of skin. The effect of these substances is gone within an hour after the experiment. Other researchers have used these

substances with microdialysis in skin. The researchers have not reported that these substances caused bad reactions.
ACH (acetylcholine): ACH is like one of the natural chemicals made by the nerves in the skin. A small amount of these substances will enter the skin around the tubing. This only affects the blood flow in the vessels in that small area of skin. The effect of ACH is gone within an hour after the experiment. Other researchers used ACH with microdialysis in skin. The researchers have not reported that ACH caused bad reactions.
Sodium Nitroprusside (SNP): Only a small amount of SNP will enter your skin around the tubing. SNP increases the blood flow in the vessels and reddens that small area of skin. This effect is gone within an hour after the experiment. Other researchers have used SNP with microdialysis in skin. They have reported no bad reactions with SNP.
Laser Doppler Flowmetry: Weak lasers can hurt your eye if you stare into the light for a long time. The laser is not turned on until the probes are taped to a surface. The design of the other laser machine hides the laser source from view. The red light you see on the surface of your skin is harmless. The tape may irritate your skin.
Blood Pressure: Your blood pressure is measured using the method common in a doctor's office. A cuff will be inflated on your upper arm. As the cuff slowly deflates, a person listens with a stethoscope at the bend in your elbow. During the short time the cuff is inflated, your arm may feel numb.
Betadine: Hospitals and the researcher 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 or shellfish. You will inform the researcher if you have these allergies so that alcohol is used instead. This bad 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.
Latex: Some gloves and medical materials are made of latex rubber. You will inform the researcher if you are allergic to latex and decline to participate in the study.
Local Heating: The researcher will 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.

4. a. Benefits to me: You could gain some knowledge about how your body works.

b. Potential benefits to society: This study will help us to know the changes that happen with aging that impair the body's resistance to heat stress. Knowing these changes may lead to ways to prevent these impairments to avoid heat illness and death in older people. This could lead to improvements in health and quality of life for the individual. A greater percent of our population is becoming aged. As this happens, the health and welfare of older people has an even greater impact on society, as a whole.

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: This experiment should last no longer than 4.5 hours

7. Statement of confidentiality: The data is available only to the investigators. 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 for Research Protections and the Biomedical Institutional Review Board (IRB) may review records related to this project.

8. Right to ask questions: If you have any questions or concerns about the research or your participation in the present investigation, you may contact Lacy Holowatz (W: 814-863-2948, 861-6255) or Jane Pierzga (W: 814-865-1236, H: 814-692-4720). If there are findings during the research that could relate to your wanting to help with the study, you will be told of the findings. You may contact the Office for Research Protections, 212 Kern Graduate Building, University Park, PA 16802, (814) 865-1775 for additional information concerning your right as a research participant.
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.

9. Compensation: You will receive $15.00 for each of the microdialysis tubing inserted in your arm (maximum $60.00). You will receive $30.00 more for completing the study.

If you are an employee of Penn State University, the compensation you receive for participation will be treated as taxable income and therefore taxes will be taken from the total amount. If you are not employed by Penn State University, 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: Medical care is available in the event of injury resulting from research but neither financial compensation nor free medical treatment is provided. You are not waiving any rights that you may have against the University for injury resulting from negligence of the University or investigators.

11. Voluntary participation: Your participation in this study is voluntary, and you may withdraw from this study at any time by notifying the investigator. Your withdrawal from this study or your refusal to participate will in no way affect your care or access to medical services. You may decline to answer specific questions. However, your acceptance into the study may be contingent upon answering these questions. Your helping with the study may be ended 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.

12. In the event that abnormal test results are obtained, you will be apprised of the results immediately and recommended to contact your private medical provider for follow-up.

This is to certify that I am 18 years of age or older and I consent to and give permission for my participation as a volunteer in this program of investigation. I understand that I will receive a signed copy of this consent form. I have read this form, and understand the content of this consent form.

_____

Volunteer Date

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

_____

Investigator Date

Whole body heating aging studies

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**

Principal Investigator: **Lacy Holowatz, M.S., Doctoral Candidate**
Address: 129 Noll Laboratory
Phone: 814-863-2948
Faculty Advisor: **W. Larry Kenney, Ph.D.**
Address: 102 Noll Laboratory
Phone: 814-863-1672
Research Assistant: **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 under the supervision of Dr. W. Larry Kenney.

1. Purpose of the study: 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 cm2 (0.4 inch2), nickel-sized area of skin at each tube. These substances are like some natural chemicals found in your body. The substances are:

1. L-arginine HCl - one of the building blocks of the proteins found in your body
2. Vitamin C – shuts down waste products in cells that have reactive oxygen.
3. Tempol (4-hydroxy-2,2,6,6-tetrametyl piperidinoxyl) - shuts down waste products in cells that have reactive oxygen.
4. L-NAME (NG-nitro-L-arginine methyl ester) – like a natural protein found in your cells. It stops chemical reactions that involve that protein.

5. BEC ((s)-(2-boronoethyl)-L-cysteine-HCl) – like a natural protein found in your cells. It stops chemical reactions that involve that protein.
6. nor-NOHA (N-hydroxy-nor-L-arginine) – like a natural protein found in your cells. It stops chemical reactions that involve that protein.
7. 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 General Clinical Research Center (GCRC) for your appointment. When you arrive, the staff draws 15 ml (1 Tbsp) of blood from a vein in your arm. You have an examination by the GCRC 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.
_____ initial Screening Day 2: You report to the GCRC for a medical history and graded exercise test (GXT). Bring clothes in which you can exercise. You may use clothing we provide. GCRC clinical staff is present or in the building. 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 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 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 Trial 1, "Role of Arginase" (Day 3 or 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, 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: 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. Another method, Finapres, uses a small cuff that fits on your finger. The last method, Colin, uses a cuff that fits on your wrist. 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 + L-arginine HCl
Probe 5. Lactated Ringer's + BEC + nor-NOHA + L-arginine HCl

After 60 minutes, we perform another set of measurements. 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. 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 42°C (108°F) for 30 – 45 minutes. This creates the greatest amount of blood flow possible. Then the trial 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. We place a sterile bandage 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. The GCRC's medical staff measures your blood pressure and heart rate before you leave.

_____ **initial Trial 2**, "Effect of ROS" (Day 3 or 4): The measurements and preparation are the same as that described for the other experiment. There are 4 MD probes placed in your skin for this trial.

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 + Tempol + Vitamin C
Probe 4. Lactated Ringer's + Tempol + Vitamin C + BEC + nor-NOHA

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. We increase the temperature of the water flowing through the suit to 48°C (118.4°F). 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 3 and 4. Heating ends when the skin blood flow at probes 3 and 4 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 42°C (108°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. The GCRC's medical staff measures 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, heart attack (< 0.05%), and death (< 0.02%). Severe reactions are rare. We monitor you closely during the test.

Treadmill: It is possible for you to stumble or fall on the treadmill 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 and watch you closely during the test. We make all changes in speed slowly, and assist you on and off the treadmill.

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 cm2 (0.4 inch2) 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, L-arginine HCl, Vitamin C, Tempol, 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 most of these substances (L-NAME, L-arginine HCl, Vitamin C, SNP) in human skin. There have been no reports of bad reactions. Tempol, nor-NOHA, and BEC have been used in animals and in human cell culture. 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 coated the wire placed under your tongue to make it smooth. However, 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: We measure your blood pressure using the method common in a doctor's office. A cuff inflates on your upper arm. As the cuff slowly deflates, we listen with a stethoscope at the bend in your elbow. During the short time we inflate the cuff, your arm may feel numb or tingly.
Blood Pressure (Finapres): The small cuff on your finger pulses with your blood pressure. In time, your finger may feel numb or tingly. We can move the cuff to another finger or stop the reading to rest you finger.
Blood Pressure (Colin): When we remove the sensor on your wrist, a mark may remain. The mark goes away after a few minutes. Your arm may feel tingly or numb while the cuff on the arm inflates.
ECG: This machine measures the electrical activity of your heart. You will have 3-12 wires from the machine taped to spots on your body. 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 or shellfish. 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: You should not worry if your answer is "right enough." The only "right" answer is one that best describes how you feel.
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, 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.

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.

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 or 4 - Trial 1, "Role of Arginase,": 4.5 hours.
_____ initial Day 3 or 4 - Trial 2, "Effect of ROS," : 5.0 hours.

7. Statement of confidentiality: The data is available only to the investigators. 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 following may review and copy records related to this research: The Office of Human Research Protections in the U.S. Department of Health and Human Services; The U.S. Food and Drug Administration (FDA) if applicable; The Penn State University Biomedical Institutional Review Board (IRB); The Penn State University Office for Research Protections (ORP).

8. Right to ask questions: If you have any questions or concerns about the research or your participation in the present investigation, you may contact Lacy Holowatz (W: 814-863-2948,

814-861-6255) or Jane Pierzga (W: 814-865-1236, H: 814-692-4720). If there are findings during the research that could relate to your wanting to help with the study, you will be told of the findings. You may contact the Office for Research Protections, 201 Kern Graduate Building, University Park, PA 16802, (814) 865-1775 for additional information concerning your right as a research participant.

9. Compensation: You will receive a lab T-shirt.
"Role of Arginase": You will receive $15.00 for each of the 5 MD probes inserted in your arm (maximum $75.00). You will receive $30.00 more for completing the study.
"Effect of ROS": You will receive $15.00 for each of the 4 MD probes inserted in your arm on (maximum $60.00). You will receive $30.00 more for completing the study.
For each trial, you are paid an amount of money equal to the part of the trial that you complete. For instance, if you complete only half of a trial you will be paid for each probe that was inserted plus $15.00 for that trial. This is because $15.00 is one half of $30.00. You may be asked to repeat a trial. If you agree to repeat a trial, you will be paid for the repeated trial as stated above.

If you are an employee of Penn State University, the compensation you receive for participation will be treated as taxable income and therefore taxes will be taken from the total amount. If you are not employed by Penn State University, 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: Medical care is available in the event of injury resulting from research but neither financial compensation nor free medical treatment is provided. You are not waiving any rights that you may have against the University for injury resulting from negligence of the University or investigators.

11. Voluntary participation: Your participation in this study is voluntary, and you may withdraw from this study at any time by notifying the investigator. Your withdrawal from this study or your refusal to participate will in no way affect your care or access to medical services. You may decline to answer specific questions. However, your acceptance into the study may be contingent upon answering these questions. Your helping with the study may be ended 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.

12. In the event that abnormal test results are obtained, you will be apprised of the results immediately and recommended to contact your private medical provider for follow-up.

This is to certify that I am 18 years of age or older and I consent to and give permission for my participation as a volunteer in this program of investigation. I understand that I will receive a signed copy of this consent form. I have read this form, and understand the content of this consent form.

I have been given an opportunity to ask any questions I may have, and all such questions or inquiries have been answered to my satisfaction.

_____

Volunteer Date

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

_____

Investigator Date

Whole body heating hypertension studies

INFORMED CONSENT FORM FOR CLINICAL RESEARCH STUDY
The Pennsylvania State University

Title of Project: **Thermoregulatory mechanisms of nitric oxide-mediated cutaneous vasodilation in mild essential hypertensive humans - Part 1 Whole-Body Heating**

Principal Investigator: **Lacy Holowatz, M.S., Doctoral Candidate**
Address: 129 Noll Laboratory
Phone: 814-863-2948
Faculty Advisor: **W. Larry Kenney, Ph.D.**
Address: 102 Noll Laboratory
Phone: 814-863-1672
Research Assistant: **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 under the supervision of Dr. W. Larry Kenney.

Note: This study has 2 parts. Each part has 2 experiments. You may choose to help with either or both parts. If you were to complete both parts, you would have 4 experiments total. You must read and sign a separate informed consent form to help with Part 1.

1. Purpose of the study: 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. People with high blood pressure cannot increase the blood flow in their skin as well as those with normal blood pressure. So, high blood pressure can make you more prone to illness in extreme heat. This study looks at whether the change in skin blood flow with high blood pressure 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 cm2 (0.4 inch2), nickel-sized area of skin at each tube. These substances are like some natural chemicals found in your body. The substances are:

1. L-arginine HCl - one of the building blocks of the proteins found in your body
2. Vitamin C – shuts down waste products in cells that have reactive oxygen.

3. L-NAME (NG-nitro-L-arginine methyl ester) – like a natural protein found in your cells. It stops chemical reactions that involve that protein.
4. BEC ((s)-(2-boronoethyl)-L-cysteine-HCl) – like a natural protein found in your cells. It stops chemical reactions that involve that protein.
5. nor-NOHA (N-hydroxy-nor-L-arginine) – like a natural protein found in your cells. It stops chemical reactions that involve that protein.
6. 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.

If we think that you may have high blood pressure, we will measure your blood pressure on three occasions within a 2-week period. This will make sure that you belong in the high blood pressure group. We can make these readings at a High Blood Pressure Clinic, on separate visits to the Noll Lab, and/or during your screening visits.

It is possible that your pressure could fall below our limits for the High Blood Pressure Group during the study. In this unlikely event, we would measure your blood pressure on three occasions within a 2-week period again. If your blood pressure remains below our limits, you will not complete the study.

_____ **initial Screening Day 1:** You do not eat or drink after midnight during the night before your exam. You report to the General Clinical Research Center (GCRC) for your appointment. When you arrive, the staff draws 15 ml (1 Tbsp) of blood from a vein in your arm. We send the blood sample to a lab that tests it for wellness markers. The lab destroys the sample after testing it. You have an examination by the GCRC medical staff that includes blood pressure, check-up, height, weight, and 12-lead ECG. Your percent body fat is measured using a tool that looks like tongs. The tongs gently measures the thickness of skin folds at several places on your body. If you are a woman of childbearing-age, you submit a urine sample for a pregnancy test.

_____ **initial Screening Day 2:** You report to the GCRC for a medical history and exercise test. Bring clothes and shoes in which you can exercise. You may use clothing we provide, but you must provide your own shoes. GCRC clinical staff is present or in the building. For the exercise test, you will walk on a treadmill. 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).

If you are in the high blood pressure group, you may choose to exercise on a bike instead. You walk on the treadmill or ride a bike for 30 minutes at an easy pace during the test. For instance, a 70 kg (154 lb) person would walk at 4 km/hr (2.5 mph) and 2.5% grade on the treadmill.

If you have normal blood pressure, the treadmill's grade 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.

You can stop the test whenever you want to stop. The test is 10-20 minutes long.

_____ **initial Trial 1**, "Role of Arginase" (Day 3 or 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, 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 thin probe and its holder 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: We place 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. Another method, Finapres, uses a small cuff that fits on your finger. The last method, Colin, uses a cuff that fits on your wrist. 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 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 + L-arginine HCl
Probe 5. Lactated Ringer's + BEC + nor-NOHA + L-arginine HCl

After 60 minutes, we perform another set of measurements. 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. 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 skin under the laser probes' holders to 42°C (108°F) for 30 – 45 minutes. This creates the greatest amount of blood flow possible. Then the trial 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. We place a sterile bandage 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. The GCRC's medical staff measures your blood pressure and heart rate before you leave.

_____ **initial Trial 2,** "Effect of ROS" (Day 3 or 4): The measurements and preparation are the same as that described for the other experiment. There are 4 MD probes placed in your skin for this trial.

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 + Vitamin C
Probe 4. Lactated Ringer's + Vitamin C + BEC + nor-NOHA

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. We increase the temperature of the water flowing through the suit to 48°C (118.4°F). 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 3 and 4. Heating ends when the skin blood flow at probes 1, 3 and 4 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 skin under the laser probes' holders to 42°C (108°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. The GCRC's medical staff measures your blood pressure and heart rate before you leave.

3. Discomforts and risks:

Exercise Test :
If you have high blood pressure: The exercise is very mild. It is like walking 4 – 4.8 km/hr (2.5 – 3.0 mph) in the mall while holding less than 10 kg (25 lbs) of gifts. You will have increased heart rate. You may also have tiredness, sweating, and breathlessness. You may have foot or muscle fatigue. Although unlikely, you may also have lightheadedness, fainting, nausea, or muscle cramps. Severe reactions, such as irregular heartbeat, heart attack, or death can occur with mild exercise. Severe reactions with this level of exercise are very rare. We monitor you closely during the test.
If you have normal blood pressure: 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, heart attack (< 0.05%), and death (< 0.02%). Severe reactions are rare. We monitor you closely during the test.
Treadmill: It is possible for you to stumble or fall on the treadmill 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 and watch you closely during the test. We make all changes in speed slowly, and assist you on and off the treadmill.
Bike: It is possible for you to fall from the bike leading to cuts, scrapes, dislocations, broken bones, head injury, abnormal heart rhythms, or even death. We will watch you closely during the test and assist you on and off the 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 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 cm2 (0.4 inch2) 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, L-arginine HCl, Vitamin C, 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 most of these substances (L-NAME, L-arginine HCl, Vitamin C, SNP) in human skin. There have been no reports of bad reactions. Nor-NOHA, and BEC have been used in animals and in human cell culture. 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 coated the wire placed under your tongue to make it smooth. However, 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: We measure your blood pressure is using the method common in a doctor's office. A cuff inflates on your upper arm. As the cuff slowly deflates, we listen with a stethoscope at the bend in your elbow. During the short time we inflate the cuff, your arm may feel numb or tingly.
Blood Pressure (Finapres): The small cuff on your finger pulses with your blood pressure. In time, your finger may feel numb or tingly. We can move the cuff to another finger or stop the reading to rest you finger.
Blood Pressure (Colin): When we remove the sensor on your wrist, a mark may remain. The mark goes away after a few minutes. Your arm may feel tingly or numb while the cuff on the arm inflates.
ECG: This machine measures the electrical activity of your heart. You will have 3-12 wires from the machine taped to spots on your body. 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 or shellfish. 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: You should not worry if your answer is "right enough." The only "right" answer is one that best describes how you feel.

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, 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: You may feel embarrassed having this measure. The researcher makes this measure in a private and professional way.

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 high blood pressure. These results could suggest ways to prevent or treat the changes that make people with high blood pressure prone to heat illness. This could help to prevent heat illness and death in such people. It is estimated that one quarter to one third of the people in the United States is being treated for or do not know that they have high blood pressure. Therefore, this health issue could impact many of people in our country.

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 Visit(s) for blood pressure readings.
_____ 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 or 4 - Trial 1, "Role of Arginase,": 4.5 hours.
_____ initial Day 3 or 4 - Trial 2, "Effect of ROS," : 5.0 hours.

7. Statement of confidentiality: The data is available only to the investigators. 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 Office for Research Protections at Penn State and the Biomedical Institutional Review Board may review records related to this project.

8. Right to ask questions: If you have any questions, complaints, or concerns about the research or your participation in the present investigation, you may contact Lacy Holowatz (W: 814-863-2948, 814-861-6255) or Jane Pierzga (W: 814-865-1236, H: 814-692-4720). You may also call these numbers if you feel this study has harmed you. If there are findings during the research that could relate to your wanting to help with the study, you will be told of the findings. You may contact the Office for Research Protections, 201 Kern Graduate Building, University Park, PA 16802, (814) 865-1775 for additional information concerning your right as a research participant.
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.

9. Compensation: You will receive a lab T-shirt.
"Role of Arginase": You will receive $15.00 for each of the 5 MD probes inserted in your arm (maximum $75.00). You will receive $30.00 more for completing the study.
"Effect of ROS": You will receive $15.00 for each of the 4 MD probes inserted in your arm on (maximum $60.00). You will receive $30.00 more for completing the study.

For each trial, you are paid an amount of money equal to the part of the trial that you complete. For instance, if you complete only half of a trial you will be paid for each probe that was inserted plus $15.00 for that trial. This is because $15.00 is one half of $30.00. You may be asked to repeat a trial. If you agree to repeat a trial, you will be paid for the repeated trial 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: Medical care is available in the event of injury resulting from research but neither financial compensation nor free medical treatment is provided. You are not waiving any rights that you may have against the University for injury resulting from negligence of the University or investigators.

11. Voluntary participation: Your participation in this study is voluntary, and you may withdraw from this study at any time by notifying the investigator. Your withdrawal from this study or your refusal to participate will in no way affect your care or access to medical services. You may decline to answer specific questions. However, your acceptance into the study may be contingent upon answering these questions. Your helping with the study may be ended 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.

12. In the event that abnormal test results are obtained, you will be apprised of the results immediately and recommended to contact your private medical provider for follow-up. If your blood pressure is above normal, we will advise you to inform your doctor. High blood pressure is a condition that develops over many years, and you have probably had this condition for a long time. You will likely need medical treatment for this condition. You may wish to talk with your doctor before being in our study. We will give the results of our measurements to you or, upon your request, send them to your doctor.

This is to certify that I am 18 years of age or older and I consent to and give permission for my participation as a volunteer in this program of investigation. I understand that I will receive a signed copy of this consent form. I have read this form, and understand the content of this consent form.

_____

Volunteer **Date**

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

_____

Investigator **Date**

VITA

Lacy Marie Alexander Holowatz

Education

The Pennsylvania State University, Ph.D., Dept. of Kinesiology	2007
University of Oregon, Master's of Science, Dept. of Human Physiology	2002
University of Oregon, Bachelor's of Science, Dept. of Human Physiology	2000

Fellowships

American Heart Association predoctoral Fellowship, Pennsylvania Delaware Affiliate	2005-2007

Awards

Dept. of Kinesiology, Graduate Research Award, The Pennsylvania State University	2005
American College of Sports Medicine, Carl V. Gisolfi Memorial Student Research Grant	2005
American Physiological Society, Caroline tum Suden Professional Opportunity Award	2006
Dept. of Kinesiology, Dissertation Award, The Pennsylvania State University	2006
American Physiological Society, Caroline tum Suden Opportunity Award	2007
American Physiological Society, Exercise and Environmental Physiology Predoctoral Recognition Award	2007
Alumni Association Dissertation Award, The Pennsylvania State University	2007

Selected Peer Reviewed Publications out of 16 Total Publications

1. Minson CT, **Holowatz LA**, Wong BJ, Kenney WL & Wilkins BW. (2002). Decreased nitric oxide- and axon reflex-mediated cutaneous vasodilation with age during local heating. *J Appl Physiol* **93,** 1644-1649.

2. Holowatz LA, Houghton BL, Wong BJ, Wilkins BW, Harding AW, Kenney WL, et al. (2003). Nitric oxide and attenuated reflex cutaneous vasodilation in aged skin. *Am J Physiol Heart Circ Physiol* **284,** H1662-1667.

3. 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.

4. Wong BJ, Wilkins BW, **Holowatz LA** & Minson CT. (2003). Nitric oxide synthase inhibition does not alter the reactive hyperemic response in the cutaneous circulation. *J Appl Physiol* **95,** 504-510.

5. Holowatz LA, Thompson CS, Minson CT & Kenney WL. (2005). Mechanisms of acetylcholine-mediated vasodilatation in young and aged human skin. *J Physiol* **563,** 965-973.

6. Houghton BL, **Holowatz LA** & Minson CT. (2005). Influence of progestin bioactivity on cutaneous vascular responses to passive heating. *Med Sci Sports Exerc* **37,** 45-51; discussion 52.

7. Thompson CS, **Holowatz LA** & Kenney WL. (2005a). Attenuated noradrenergic sensitivity during local cooling in aged human skin. *J Physiol* **564,** 313-319.

8. Thompson CS, **Holowatz LA** & Kenney WL. (2005b). Cutaneous vasoconstrictor responses to norepinephrine are attenuated in older humans. *Am J Physiol Regul Integr Comp Physiol* **288,** R1108-1113.

9. Holowatz LA, Thompson CS & Kenney WL. (2006a). Acute ascorbate supplementation alone or combined with arginase inhibition augments reflex cutaneous vasodilation in aged human skin. *Am J Physiol Heart Circ Physiol* **291,** H2965-2970.

10. Holowatz LA, Thompson CS & Kenney WL. (2006b). L-Arginine supplementation or arginase inhibition augments reflex cutaneous vasodilatation in aged human skin. *J Physiol* **574,** 573-581.

11. Thompson-Torgerson CS, **Holowatz LA**, Flavahan NA & Kenney WL. (2006). Cold-induced cutaneous vasoconstriction is mediated by Rho kinase in vivo in human skin. *Am J Physiol Heart Circ Physiol* **epu**b, Dec 15.

12. Holowatz LA & Kenney WL. (2007). Upregulation of arginase activity contributes to attenuated reflex cutaneous vasodilatation in hypertensive humans. *J Physiol* **e-pub**, March 8.

13. Holowatz LA, Thompson-Torgerson CS & Kenney WL. (2007). Altered Mechanisms of Vasodilation in Aged Human Skin. *Exercise and Sport Sciences Reviews* **35**.