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THE MECHANISMS AND FUNCTIONAL IMPLICATIONS OF ALTERED MICROVASCULAR VASOREACTIVITY IN HEALTHY AND ESSENTIAL HYPERTENSIVE MEN AND WOMEN

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ABSTRACT

Cutaneous vasodilation enables nutrient delivery to the skin and effectively transfers heat away from the body core to the environment for thermoregulatory purposes. Direct local skin heating elicits a hyperemic response that is reflective of global changes in microcirculation and in endothelial function. Local skin heating is commonly used as a microvascular vasoreactivity test because it is largely reliant on nitric oxide (NO)-dependent mechanisms to elicit vasodilation. NO-dependent vasodilation elicited from local skin heating is reduced in populations with cardiovascular risk factors such as aging and essential hypertension, but further studies are needed to characterize the up- and down-stream mechanisms mediating NO-dependent signaling during local skin heating. Further, the effects of systemic cardiovascular therapies on microvascular function can be examined using skin vasoreactivity tests in both healthy and clinical populations. Drug therapies that alter microvascular function could have functional thermoregulatory consequences during heat stress. The purpose of this series of studies was to investigate and characterize the mechanisms mediating NO-dependent vasodilation in middle-aged and essential hypertensive men and women. In addition, we planned to investigate the functional thermoregulatory consequences of systemic antithrombotic therapy during rest and exercise in the heat.

In the first study we investigated the specific nitric oxide synthase (NOS) isoforms responsible for mediating NO-dependent vasodilation during local skin heating in healthy young and middle-aged men and women. Human aging is associated with attenuated cutaneous vasodilation but little is known about the middle-aged cohort, which is commonly used for comparison with clinical populations. We tested the hypotheses that endothelial NOS (eNOS) is the primary isoform mediating NO production during local heating and that eNOS-dependent vasodilation would be reduced in middle-aged skin. Vasodilation was induced by local heating (42°C) and during acetylcholine dose-response (Ach-DR: 0.01, 0.1, 1.0, 5.0, 10.0, 50.0, 100.0mmol/L) protocols. Four intradermal microdialysis fibers were placed in the skin of 12 middle-aged (53±1yrs) and 12 young (23±1yrs) men and women. Sites served as control, non-selective NOS inhibited (NOS-I; L-NAME), inducible (i)NOS inhibited (1400W), and neuronal (n)NOS inhibited (NPLA). After full expression of the local heating response, L-NAME was perfused at all sites. Laser-Doppler flowmetry (LDF) was used to measure cutaneous vascular conductance (LDF flux/mean arterial pressure) and normalized to maximum (%CVC_{max}: sodium

nitroprusside). L-NAME reduced %CVC_{max} at baseline, all phases of the local heating response, and at all Ach concentrations compared to all other sites. iNOS inhibition reduced the initial peak (53±2vs. 60±2%CVCmax; p<0.001); however, there were no other differences between control, nNOS, and iNOS inhibited sites during the phases of local heating or Ach-DR. When age cohorts were compared, middle-aged men and women had reduced NO-dependent vasodilation during local heating (52±6 vs. 68±4%CVC_{max}; P = 0.013) and Ach perfusion (50mmol/L: 83±3 vs. 93±2; 100mmol/L: 83±4 vs. 92±3%CVC_{max}; both p=0.03). There were no differences in NOS isoform expression obtained from skin biopsy samples between groups (all p>0.05). These data suggest that eNOS mediates the production of NO during local heating and that it is attenuated in middle-aged skin.

In the second study we used spectral analysis (fast Fourier transformation) to characterize the skin flowmotion in LDF recordings during local heating-induced vasodilation before and after NOS-I (L-NAME) in essential hypertensive (HTN) and age-matched normotensive (NTN) men and women. We hypothesized that HTN reduces total power spectral densities (PSD), specifically in the frequency intervals (FI) associated with intrinsic endothelial and neurogenic control of the vasculature. Furthermore, we hypothesized that NOS-I would attenuate the endothelial FI in both groups. LDF recordings during local heating experiments from 18 HTN (MAP: 108±2mmHg) and 18 NTN (MAP: 88±2mmHg) men and women were analyzed. Local heating-induced vasodilation increased total PSD for all FI (all p<0.001). HTN lowered total PSD (HTN:30±6AU^2, NTN:14±3AU^2, p=0.03) and neurogenic FI (HTN:6±1AU^2, NTN:17±3AU^2, p<0.01) versus NTN. As a percentage of total PSD, HTN had reduced neurogenic (HTN:41±3%, NTN:51±1%, p<0.001) and higher myogenic contributions (HTN:29±3%, NTN:24±1%, p=0.04) to the total spectrum. NOS-I decreased total PSD (p<0.001) for both groups, but HTN exhibited lower endothelial (p<0.01), neurogenic (p<0.05), and total PSD (p<0.001) FI compared to NTN. These data suggest that HTN results in altered neurogenic and NO-dependent control of skin flowmotion and support the use of spectral analysis as a non-invasive technique to study vasoreactivity.

Antithrombotic therapy with low dose aspirin (ASA) or clopidogrel bisulfate (CLO; Plavix®) is associated with attenuated skin vasodilator response and a greater rate of rise in core temperature in healthy middle-aged individuals during passive heating in a water perfused suit. In the third study we hypothesized that ASA and CLO therapy would have negative

thermoregulatory and cardiovascular consequences during rest and exercise in a hot environment. This was a double-blind, placebo-controlled, crossover study that examined the functional consequences of 7 days of ASA (81mg), CLO (75mg), and placebo (sucrose) in 14 healthy, middle-age (50–65 yr) men and women during passive heating in air (40 min at 30ºC, 40% relative humidity) followed by exercise (60% $\dot{V}O_{2peak}$). Oral temperature (T_{or}) was measured in the antechamber (23.0ºC±0.1ºC) before entering a warm environmental chamber. After 40 min of rest, subjects cycled on a recumbent cycle ergometer for up to 120 min. Esophageal temperature (T_{es}) and laser- Doppler flux were measured continuously, and assessed as $\%CVC_{max}$. Before entry into the environmental chamber there were no differences in T_{or} among treatments. However, after 40 min of rest in the heat, T_{es} was significantly higher for ASA and CLO versus placebo (37.2ºC±0.1ºC, 37.3ºC±0.1ºC, vs. 37.0ºC±0.1ºC, both $P<0.001$), a difference that persisted throughout exercise ($P<0.001$ vs. placebo). The mean body temperature thresholds for the onset of cutaneous vasodilation were shifted to the right for both ASA and CLO during exercise ($P<0.05$). These data suggest that ASA and CLO elevate core temperatures during passive heat stress and shift the onset of peripheral thermoeffector mechanisms toward higher body temperatures during exercise in the heat.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Acetylcholine	Ach
Acetylcholine Dose-Response	Ach-DR
Aspirin	ASA
Adenosine Diphosphate	ADP
Adenosine Triphosphate	ATP
Calcitonin-Gene Related Peptide	CGRP
Calcium-Activated Potassium Channels	KCa
Cardiovascular Disease	CVD
Clopidogrel Bisulfate (Plavix®)	CLO
Cutaneous Vascular Conductance	CVC
Cutaneous Vascular Conductance % Maximum	$\%CVC_{max}$
Cyclooxygenase	COX
Endothelial Nitric Oxide Synthase	eNOS
Endothelium-Derived Hyperpolarizing Factor	EDHF
Epoxyeicosatrienoic Acids	EETs
Essential Hypertensive Men and Women	HTN
Fast Fourier Transformation	FFT
Frequency Intervals	FI
Hydrogen Peroxide	H_2O_2
Inducible Nitric Oxide Synthase	iNOS
Laser Doppler Flowmetry	LDF
Mean Arterial Pressure	MAP
Microdialysis	MD
Neuropeptide Y	NPY
Neuronal Nitric Oxide Synthase	nNOS
Nitric Oxide	NO
Nitric Oxide Synthase	NOS
Nitric Oxide Synthase Inhibition	NOS-I
Norepinephrine	NE
Normotensive Men and Women	NTN
Post Occlusion Reactive Hyperemia	PORH

Postural Orthostatic Tachycardic Syndrome	POTS
Power Spectral Density	PSD
Reactive Oxygen Species	ROS
Serotonin	5-HT
Thromboxane A_2	TXA_2
Transient Receptor Vanilloid Type-1	TRPV-1
Vasodilator-Simulated Phosphoprotein	pVASP

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Chapter 1

INTRODUCTION

Background and Significance

The microcirculation consists of the small resistance vessels (less than ~150 μm in diameter) that are involved in nutrient exchange and blood flow regulation. The mechanisms that regulate blood flow in the microcirculation consist of arteriolar myogenic regulation, endothelial signaling to the vascular smooth muscle, and neural stimulation of the vascular smooth muscle and endothelium. Altered regulation of the microcirculation has been observed during healthy human aging and in cardiovascular and chronic inflammatory disease pathologies. Impaired microvascular function is a systemic process that occurs in a similar fashion in multiple tissue beds throughout the body before signs and symptoms of cardiovascular disease, and has the potential for early identification of disease and monitoring of disease progression (Abularrage et al. 2005a, Abularrage et al. 2005b).

The human cutaneous circulation is a model circulation to examine microvascular dysfunction *in vivo* because it is an easily accessible vascular bed in which non- to minimally invasive stimuli provide a means to assess the mechanisms regulating microvascular blood flow (Minson 2010b, Holowatz, Thompson-Torgerson and Kenney 2008, Roustit and Cracowski 2013, Rossi et al. 2008b). Specifically, changes in the skin vasoreactivity have shown to correlate with renal (Coulon, Constans and Gosse 2012, Stewart et al. 2004) and coronary (Khan et al. 2008, Sax et al. 1987) clinical outcomes (Kruger et al. 2006, RG et al. 2003). Multiple skin vasoreactivity tests have been developed to examine microvascular function and have been mechanistically well characterized using laser Doppler flowmetry during concurrent delivery of pharmacological agents with the use of intradermal microdialysis to pharmacodissect the mechanisms regulating cutaneous blood flow. Skin vasoreactivity tests like local skin heating and reactive hyperemia have been extensively used in both healthy and diseased populations to

examine nitric oxide (NO)-dependent and endothelial derived hyperpolarizing factor (EDHF)-dependent vasodilation, respectively. Further, skin vasoreactivity tests have provided a means to assess microvascular function after oral drug therapies, which has potential for the monitoring of different drug therapies in clinical populations (Holowatz and Kenney 2011, Dahmus et al. 2013, Rossi et al. 2012, Blaise et al. 2010, Blaise et al. 2011).

Local Skin Heating

The cutaneous circulation is highly reactive to changes in skin temperature. Local skin heating elicits a highly reproducible, biphasic skin blood flow response that is marked by an initial peak in blood flow followed by a slower secondary rise that eventually plateaus (Brunt and Minson 2011, Minson 2010b, Minson, Berry and Joyner 2001b). The rapid initial increase in skin blood flow is an axon reflex primarily mediated by sensory-nerves. The slower secondary rise in skin blood flow is about 60-70% reliant on NO-dependent mechanisms (Minson et al. 2001b, Kellogg et al. 1999a, Kellogg, Zhao and Wu 2008a). The large NO-dependent vasodilation elicited from local skin heating has implications for assessing vascular function in those with cardiovascular disease. NO is essential for maintaining vascular health and is a vasoprotective gasotransmitter that inhibits platelet aggregation, smooth muscle proliferation, and monocyte adhesion.

The ability to assess NO-dependent vasodilation allows for local skin heating to be used to study how cutaneous vascular reactivity is affected during primary aging and in those with cardiovascular disease risk factors that affect endothelial function. Inhibition of nitric oxide synthase (NOS), the enzyme responsible for producing endogenous NO, can be used to assess NO-dependent vasodilation to evaluate vascular health. NO-dependent vasodilation is reduced in primary aging (>65 years old) (Minson et al. 2002) and populations with cardiovascular disease risk factors such as smokers (Fujii et al. 2013), essential hypertensive (Smith et al. 2011b), and hypercholesterolemic men and women (Holowatz and Kenney 2011).

Acetylcholine Dose Response

Acetylcholine stimulates endothelium-dependent vasodilation primarily through cyclooxygenase (COX)-dependent signaling with the vascular smooth muscle with a smaller role for NO (Holowatz et al. 2005, Noon et al. 1998). Acetylcholine-mediated vasodilation in the vasculature is attenuated with advancing age and essential hypertension, likely due to a reduction in COX-dependent vasodilation and an increase in thromboxane vasoconstrictor activity (Taddei et al. 1995, Buus et al. 2000, Heymes et al. 2000). However, the role of NO in the cutaneous vasculature is more apparent during dose-dependent perfusion of acetylcholine with NOS inhibition. This protocol has been used in many studies to examine reduced endothelium-derived NO-dependent vasodilation in populations with known microvascular dysfunction such as smokers (Fujii et al. 2013), essential hypertensive (Smith et al. 2011b), and postural tachycardia syndrome (POTS) (Stewart et al. 2011). The use of intradermal microdialysis to deliver increasing concentrations of acetylcholine is beneficial for examining endothelium-derived NO production.

Spectral Analysis

Skin blood flow is often assessed with laser Doppler flowmetry (LDF) during various skin vasoreactivity tests. Within LDF recordings there are low frequency periodic oscillations (vasomotion) that correspond with vasodilation and vasoconstriction of the cutaneous arterioles, which are the primary regulators of skin microcirculatory blood flow (also called skin flowmotion) (Rossi et al. 2008b). The LDF recordings of skin flowmotion can be decomposed so that the periodic oscillations can be separated into their characteristic frequencies using spectral analysis, providing insight into the physiological mechanisms mediating skin flowmotion (Kastrup, Bulow and Lassen 1989, Salerud et al. 1983, Stefanovska, Bracic and Kvernmo 1999, Kvernmo et al. 1999, Rossi et al. 2008b). A characteristic, or basic, frequency is the frequency contained in the LDF signal that has amplitudes that are significantly greater than other frequencies contained within the signal. Initially, LDF recordings are expressed in the time domain as laser Doppler flux (in arbitrary units). When the LDF signal undergoes spectral analysis, such as fast Fourier transformations or wavelet analysis, the data is transformed from the time domain into the characteristic frequencies that make up the periodic oscillations.

Oscillations in the frequency interval of 0.009-1.6Hz have been suggested to reflect five components of skin flowmotion (Rossi et al. 2008b), representing the influence of heart beat (0.6-2.0Hz) (Rossi et al. 2008b), respiration (0.15-0.6Hz), myogenic (~0.05-0.15Hz) (Stefanovska et al. 1999), neurogenic (~0.02-0.05Hz) (Kastrup et al. 1989, Soderstrom et al. 2003), and endothelial influence on vascular smooth muscle relaxation (~0.0095-0.02Hz) (Kvernmo et al. 1999, Rossi et al. 2008a, Gustafsson, Mulvany and Nilsson 1993, Kvandal et al. 2003). The two higher frequency intervals are due to hemodynamic modifications that occur during heart rate and respiration which transmit into the skin microcirculation (Rossi et al. 2008b). The lower frequencies are associated with local regulation of skin blood flow. Specifically, the myogenic frequency interval relates to the intrinsic myogenic signaling within the vascular smooth muscle that autoregulates the vascular tone to maintain a steady tissue blood flow with changing perfusion pressure. Neurogenic signaling comprises the sympathetic and sensory nerve contribution to vasomotion and the lowest frequency interval corresponds to endothelium-dependent production of NO and EDHFs that regulate vasomotor tone. Therefore, the use of spectral analysis may provide further mechanistic insights during the evaluation of cutaneous vasoreactivity in populations with altered microvessel tone such as essential hypertensives.

Skin Blood Flow and Platelet Inhibition

The cutaneous microcirculation has provided a means to monitor microvessel function after systemic drug therapies (Holowatz and Kenney 2011, Rossi et al. 2012, Roustit et al. 2012, Holowatz et al. 2010a). Mechanistic studies examining the effects of antithrombotic therapy on microvascular and thermoregulatory function have found that platelet inhibition with the two most common platelet inhibitors, namely daily low dose aspirin (ASA; 81mg) and clopidogrel bisulfate (75mg, Plavix®), alter cutaneous microvascular reactivity. Although ASA and clopidogrel inhibit platelets irreversibly for the life of the platelet (~10 days) through *independent* mechanisms (COX-1 and $P2Y_{12}$ adenosine diphosphate [ADP] receptors, respectively), both treatments attenuate reflex cutaneous vasodilation in middle-age men and women during passive heat stress. Specifically, aspirin and clopidogrel attenuated skin blood flow and reduced the time to increase core temperature 1°C during passive heat stress in a water perfused suit (Holowatz et al. 2010a), which was independent of local vascular COX-1 and 2 inhibition (Holowatz et al. 2009, Holowatz et al. 2010a). Further, clopidogrel was shown to augment the vasodilatory

response during reactive hyperemia, suggesting it may augment EDHF-dependent vasodilation in the skin, which further highlights the ability of the skin to monitor microvessel function after systemic antithrombotic therapy.

Summary

The three studies that comprise this dissertation utilize the cutaneous vascular bed to further characterize the mechanisms that mediate the skin blood flow response to local skin heating and to examine how systemic antithrombotic therapy affects skin blood flow and thermoregulatory effector mechanisms during exercise in a warm environment. While it is known that the skin blood flow response to local skin heating is predominantly mediated by NO-dependent vasodilation, the specific nitric oxide synthase (NOS) isoform that mediates this response during healthy, middle-aging (40-60) was unknown. The first study examined the specific NOS isoforms involved in producing endogenous NO-dependent vasodilation during local skin heating and used acetylcholine dose-responses to determine if this isoform was endothelium-dependent. The second study utilized spectral analysis and local skin heating during concurrent pharmacological inhibition of NOS to examine the role of NO in middle-aged and essential hypertensive men and women. The third study examined the functional consequences of antithrombotic therapy during passive warm air exposure and exercise in the heat.

Specific Aims and Hypothesis

Specific Aim 1: The purpose of the study, "Endothelial nitric oxide synthase mediates cutaneous vasodilation during local skin heating and is attenuated in middle-aged human skin," was to determine the specific NOS isoform(s) responsible for mediating vasodilation during local skin heating in healthy, young and middle-aged men and women. We perfused specific NOS inhibitors into the skin using intradermal microdialysis during local skin heating to delineate the NOS isoform(s) responsible for mediating this response. We then used intradermal microdialysis to perfuse acetylcholine in a dose-dependent manner with the specific NOS inhibitors to determine which NOS isoform(s) mediated endothelium-dependent vasodilation. Because NO-dependent (Minson et al. 2002) and acetylcholine-mediated vasodilation (Holowatz et al. 2005)

are attenuated with advanced aging (>65 years), we wanted to examine if attenuated skin blood flow was detectable in healthy, middle-aging. We complemented our *in vivo* functional analysis with *in vitro* analysis of NOS expression and the downstream molecular vasodilatory signaling molecule, vasodilator-simulated phosphoprotein (pVASP), from skin biopsy samples.

Hypothesis 1a: The vasodilation elicited from local skin heating and acetylcholine perfusion would be mediated by endothelial NOS (eNOS).

Hypothesis 1b: eNOS-dependent vasodilation would be attenuated in middle-aged men and women.

Specific Aim 2: The purpose of the study, "Altered skin flowmotion in hypertensive humans," was to evaluate skin vasoreactivity using spectral analysis during local heating in essential hypertensive and age-matched normotensive men and women before and after NOS inhibition. Essential hypertension results in attenuated skin blood flow during local skin heating due to a reduction in eNOS-dependent vasodilation (Smith et al. 2011b). Therefore, we wanted to evaluate the frequency intervals associated with intrinsic endothelial, neurogenic, and myogenic regulation of the microvasculature during basal blood flow, local skin heating, NOS inhibition, and perfusion of sodium nitroprusside (a NO donor) to further our understanding of the mechanisms regulating microvascular control in hypertension.

Hypothesis 2a: Essential hypertension would result in reduced total power spectral density (PSD; an index of skin flowmotion) as well as reduced frequency intervals corresponding to endothelial and neurogenic signaling during local skin heating.

Hypothesis 2b: Within site inhibition of NOS during local skin heating would result in reduced PSD in the frequency intervals associated with endothelial signaling, which would be restored after perfusion of an exogenous NO donor (sodium nitroprusside) in both essential hypertensive and normotensive men and women.

Specific Aim 3: The purpose of the study, "Aspirin and clopidogrel alter core temperature and skin blood flow during heat stress," was to examine the effect of ASA and clopidogrel on core temperature and thermoeffector mechanisms during whole body heat stress at rest and during exercise in a warm environment. We performed a randomized, double-blind, crossover study design after 7 days of ASA, clopidogrel, and placebo treatment.

Hypothesis 3a: ASA and clopidogrel would result in a greater rate of rise in core temperature during heat stress in ambient warm air versus placebo.

Hypothesis 3b: Reflex cutaneous vasodilation would be attenuated with ASA and CLO, resulting in a rightward shift in the skin blood flow-core temperature relation during exercise in the heat.

Figure 1-1

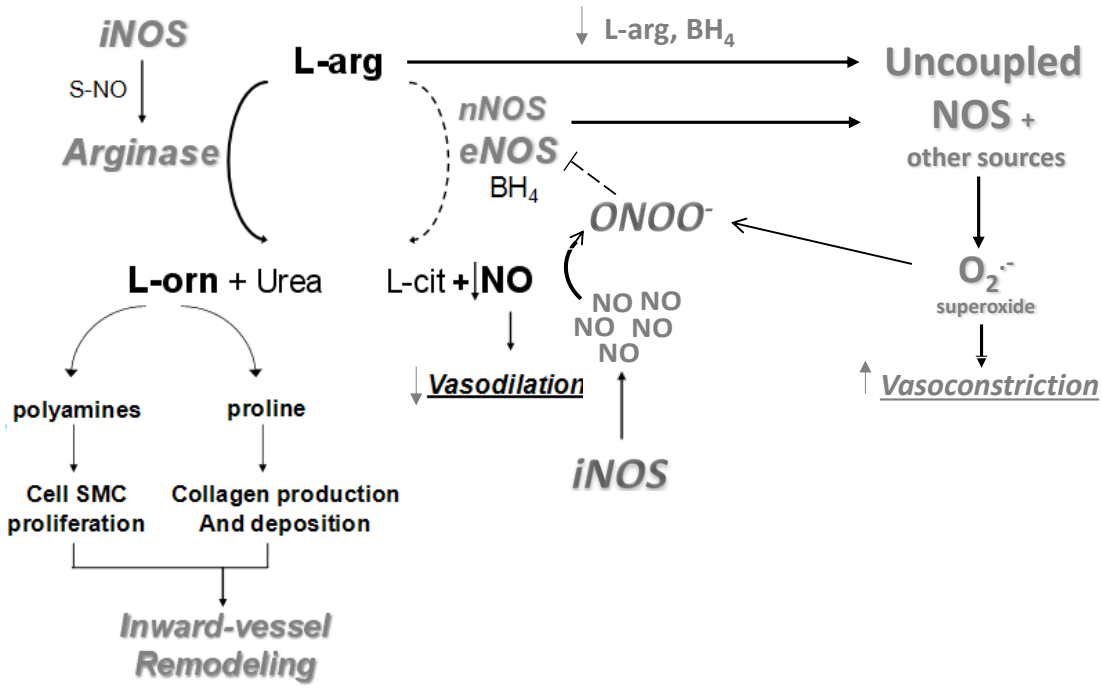


Figure **1-1**. Schematic depicting ways in which primary human aging and essential hypertension can reduce NO bioavailability and attenuate local cutaneous vasodilation during direct local skin heating. The signaling mechanisms which act to reduce NO bioavailability are in gray. L-orn: L-ornithine; L-cit: L-citrulline; BH_4: tetrahydrobiopterin; L-arg: L-arginine; ONOO: peroxyntirite; S-NO: S-nitrosating, NOS: nitric oxide synthase.

Chapter 2

REVIEW OF THE LITERATURE

Vascular dysfunction is one pathological change that occurs with aging and cardiovascular disease. Several non-invasive methods are currently available to assess endothelial and vascular smooth muscle function of larger conduit arteries and have shown prognostic value for early detection and evaluation of arterial vascular disease (Martin and Anderson 2009, Heitzer et al. 2005, Lerman and Zeiher 2005). However, resistance vessel function may be more important for early detection and identification of the pathological changes that occur with aging and arterial vascular disease because changes in resistance vessel function occur systemically and precede structural changes that occur in the conduit circulation (Lind et al. 2011, Padilla et al. 2011). Endothelial dysfunction and the resulting reduction in nitric oxide (NO) bioavailability in resistance vessels contributes to greater retrograde and oscillatory shear in the upstream conduit arteries (Padilla et al. 2011), creating unfavorable shear patterns for development of atherosclerotic lesions and structural changes in vascular smooth muscle. Pathological changes that occur during aging and cardiovascular disease result in vascular smooth muscle dysfunction due to thickening and loss of vascular smooth muscle elasticity. Modifiable cardiovascular risk factors such as hypertension accelerate both endothelial and vascular smooth muscle dysfunction compared to healthy, age-matched individuals (Taddei et al. 1997, Heitzer et al. 2005). Further, vasoreactivity tests that could monitor resistance vessel function *in vivo* could enable clinicians to test the efficacy of therapeutic interventions.

The cutaneous circulation is emerging as a model circulation to examine global resistance vessel dysfunction because non- to minimally-invasive stimuli can be used to assess endothelial and vascular smooth muscle vasoreactivity (Holowatz et al. 2008, Minson 2010b, Roustit and Cracowski 2013). Laser Doppler flowmetry coupled with intradermal microdialysis can be used to assess skin blood flow while delivering localized pharmacological agents to elucidate the mechanisms that are altered with various cardiovascular and inflammatory diseases. In addition, mechanistic changes in cutaneous resistance vessel vasoreactivity can be examined after systemic

cardiovascular therapies (Holowatz and Kenney 2011, Rossi et al. 2012, Holowatz et al. 2010a). The cutaneous circulation is highly reactive to changes in skin temperature, which has allowed for local skin heating and cooling to be used to examine the mechanisms regulating microvascular cutaneous blood flow. The first part of this review will focus on the mechanisms mediating skin blood flow during local thermal stress with primary aging and essential hypertension.

In addition to delivering oxygen and nutrients to the skin, changes in cutaneous blood flow is one of the primary effector responses in thermoregulation for the maintenance of body core temperature. Skin blood flow is increased in a mechanistically distinct manner during whole body heating versus direct local skin heating. Reflex cutaneous vasodilation elicited from increases in body core temperature is purportedly mediated by the co-transmission of acetylcholine and an unknown neurotransmitter(s) from sympathetic cholinergic active vasodilator nerves (Kellogg et al. 1995). Primary human aging and essential hypertension results in attenuated reflex cutaneous vasodilation during heat stress. In addition, medications that alter cutaneous blood flow may have deleterious or beneficial effects on temperature regulation and cardiovascular outcomes. The second half of this review will focus on examining how skin blood flow is altered with antithrombotic therapies and the functional thermoregulatory and cardiovascular consequences associated with their use.

Mechanisms Mediating Vasodilation during Local Skin Heating

The hyperemic response to local skin heating is dependent on ambient/skin temperature (Abraham et al. 2013) and rate of skin heating (Houghton et al. 2006, Hodges et al. 2009). The local heating protocol that has most commonly been used to evaluate NO-dependent vasodilation involves increasing skin temperature from 33°C up to 42°C at a rate of 0.5°C•5 sec^{-1}, which elicits a highly reproducible, biphasic skin blood flow response (Brunt and Minson 2011, Minson 2010b, Minson et al. 2001b). The rapid initial increase in skin blood flow is primarily mediated by sensory-nerves, followed by a brief nadir, and then a slower secondary rise in skin blood flow that is about 60-70% reliant on NO-dependent mechanisms (Minson et al. 2001b, Kellogg et al. 1999a, Kellogg et al. 2008a).

The initial peak in skin blood flow is an axon reflex primarily mediated by local sensory nerves that likely release calcitonin-gene related peptide (CGRP) (Minson et al. 2001b, Munce

and Kenney 2003a, Stephens et al. 2001) and substance P (Wong and Minson 2011, Wong, Tublitz and Minson 2005). These neuropeptides act on the vascular smooth muscle causing relaxation in response to thermal stimuli. The axon reflex is also partially mediated by NO (Houghton et al. 2006) and endothelium-derived hyperpolarizing factor (EDHF)-dependent (Brunt and Minson 2012) signaling pathways elicited from activation of transient receptor vanilloid type-1 (TRPV-1) channels on sensory nerves (Wong and Fieger 2010) and/or interaction with sympathetic nerves (Hodges et al. 2009).

The slower secondary plateau in skin blood flow is predominantly reliant (~60-70%) on NO for full expression (Kellogg et al. 2008a, Minson et al. 2001b) with the remainder coming from EDHF-dependent mechanisms (Brunt and Minson 2012). Inhibition of NO-dependent vasodilation with nitric oxide synthase (NOS) inhibitors can be used to assess NO-dependent vasodilation in those with cardiovascular risk factors. In the cutaneous circulation the two constitutively expressed NOS isoforms are endothelial NOS (eNOS) and neuronal NOS (nNOS). During local skin heating, both eNOS and nNOS have been reported to mediate the vasodilatory response to local skin heating. eNOS has been reported to mediate the vasodilatory response in the forearm skin of healthy, young men and women (Kellogg et al. 2008a), whereas, nNOS has been implicated in mediating this response in the calf of postural orthostatic tachycardia syndrome (POTS) patients (Stewart et al. 2011). These conflicting findings may be due to regional and/or patient population differences. A third NOS isoform, inducible NOS (iNOS) is upregulated during inflammation and may decrease constitutive NOS production of NO. Inducible NOS is Ca^{2+}-independent due to tight binding of calmodulin, resulting in continuous production of NO. Large quantities of NO can create oxidative stress when it interacts with other NO molecules, forming peroxynitrite. Peroxynitrite can then uncouple the constitutively expressed NOS isoforms, resulting in the production of pro-constrictor reactive oxygen species (ROS) instead of the vasodilatory gasotransmitter NO. In addition, iNOS has been reported to reduce NO-dependent vasodilation through posttranslationally S-nitrosating arginase which increases its activity. Upregulated arginase activity can deplete the NOS substrate L-arginine and reduce NO production through eNOS (Dunn et al. 2011). There is also evidence to suggest that sensory and/or sympathetic nerves are involved in the slower secondary rise in skin blood flow during local heating, increasing NO-dependent vasodilation through adenosine receptor (Fieger and Wong 2010) and TRPV-1 channel activation (Wong and Fieger 2010).

The other vasodilator molecule(s) involved with increasing skin blood flow during local skin heating include EDHF. EDHF-dependent vasodilation in the skin is mediated by hydrogen peroxide (H_2O_2) (Medow et al. 2011) and epoxyeicosatrienoic acids (EETs) working through calcium-activated potassium channels (KCa) (Brunt and Minson 2012). There is a great deal of redundancy in the vasodilatory signaling pathways during local skin heating and it has been suggested that NO and EDHF may be compensatory. For example, in situations where NO-mediated relaxation is reduced and reactive oxygen species (ROS) increased, EDHF-dependent vasodilation is augmented to elicit endothelium-dependent vasodilation to compensate for the loss of NO (Shimokawa and Matoba 2004). The ability to assess NO-dependent and EDHF-dependent vasodilation allows for local skin heating to be used to study how cutaneous vascular reactivity is affected during primary aging and in those with cardiovascular disease risk factors such as essential hypertension.

Primary Aging and Cutaneous Vasodilation during Local Skin Heating

Healthy human aging, even in the absence of cardiovascular risk factors, causes a progressive impairment of the cardiovascular system, which results in altered vascular function. Age-related changes in microvascular function result in attenuated vasodilatory responses to direct local skin heating. During standardized local skin heating there is an attenuated vasodilatory response, including a reduction in the axon reflex and local skin heating plateau (Minson et al. 2002).

The reduction in the axon reflex observed during primary aging is not due to the modest NO-contribution to this response (Minson et al. 2001b). The reduction in the axon reflex during aging could be the result of 1) a reduced sensory nerve response to changing skin temperature, 2) desensitization of sensory/sympathetic nerves due to heightened oxidative stress and sympathetic nerve activity with aging, 3) a reduction in the release of neurotransmitters from sensory and/or sympathetic nerves eliciting vasodilation, and/or 4) a reduced vascular responsiveness to the neurotransmitters released. Pretreatment of the skin with capsaicin before local skin heating has been used to examine sensory nerve activity with aging. Capsaicin pretreatment desensitizes heat sensitive TRPV-1 channels on local sensory nerves and in aged subjects does not affect skin blood flow during local skin heating (Munce and Kenney 2003a). The axon reflex was also

found to be reduced with aging in the superficial malleolar gaiter skin, which may have clinical implications for examining neuropathies in skin regions where ulcerations are more common (Millet et al. 2012).

In aged skin the secondary plateau in blood flow during local heating is also reduced, partially through attenuated NO-dependent vasodilation (Minson et al. 2002). However, the primary NOS isoform responsible for mediating the skin blood flow through the aging spectrum is unknown. In mouse models of aging, iNOS S-nitrosates and activates arginase 1, leading to age-associated vascular dysfunction (Dunn et al. 2011) and providing a putative mechanism for the reduction in NO-dependent vasodilation with aging. To date, the mechanisms mediating the age-associated reduction in NO-dependent vasodilation with local skin heating have not been elucidated. Putative mechanisms reducing NO-dependent vasodilation with aging that have been demonstrated in the skin with reflex cutaneous vasodilation include upregulated arginase activity (Holowatz, Thompson and Kenney 2006b), increased oxidative stress (Holowatz, Thompson and Kenney 2006a), depleted NOS substrate (Holowatz et al. 2006b), and reduced availability of the cofactors needed for NOS production of NO (Stanhewicz et al. 2012). Alternatively, the moderate contribution of EDHF-to local skin heating could be reduced with aging; however, this has yet to be investigated.

Age-related structural changes and vascular smooth muscle hypertrophy in the microvasculature reduce maximal-heat induced cutaneous vasodilation (Martin, Loomis and Kenney 1995). Some of the structural changes reducing skin blood flow with aging include, vascular smooth muscle hypertrophy, stiffening of the blood vessels through increased polyamine and proline synthesis, and capillary rarefaction associated with flattening of the underside of the epidermis reducing microcirculatory capillary loops (Holowatz, Thompson-Torgerson and Kenney 2010b). Increased vascular smooth muscle activity with aging results from a shift of the endothelium from a prodilatory to a proconstrictor milieu, with increased production of cyclooxygenase (COX)-derived, endothelium-dependent contracting factors (EDCF) (Taddei et al. 2006). The age-related decline in maximal heat-induced skin blood flow is linear from young adulthood through old age (Martin et al. 1995), which warrants assessment of the skin blood flow response throughout the age-spectrum.

Essential Hypertension and Cutaneous Vasodilation during Local Skin Heating

Essential hypertension results in vascular dysfunction involving a complex interaction of inflammation, a loss of endothelium-dependent vasodilation, reduced bioactivity of endothelium-derived NO, hyperresponsiveness to vasoconstrictor stimuli, and structural changes within the microvasculature comparable to an accelerated form of the vascular changes observed in aging (Taddei et al. 1997, Taddei et al. 2006, Feihl et al. 2006). *In vivo* assessment of skin blood flow in essential hypertensive humans corroborates this statement. Specifically, essential hypertension results in an attenuated vasodilatory response to local skin heating similar to aged participants (Smith et al. 2011b, Carberry, Shepherd and Johnson 1992).

Essential hypertension results in attenuated cutaneous eNOS-dependent vasodilation during local skin heating and perfusion of the endothelium-dependent agonists (Smith et al. 2011b). In addition, iNOS inhibition restores eNOS-dependent vasodilation in essential hypertensive humans, suggesting that upregulated iNOS activity is involved in reducing functional NO-dependent vasodilation. Furthermore, maximal cutaneous vasodilation elicited by forearm heating (Carberry et al. 1992) and whole body heat stress (Kenney, Kamon and Buskirk 1984, Holowatz and Kenney 2007b, Holowatz and Kenney 2007a) is significantly reduced in humans with essential hypertension. Many of the mechanisms mediating reduced NO bioavailability during local skin heating in essential hypertensive humans have not been elucidated but have been examined during whole body heating. Similar to aging, reduced NO bioavailability may arise from upregulated arginase activity (Holowatz and Kenney 2007b), increased oxidative stress (Holowatz and Kenney 2007a), and potentially reduced NOS substrate and bioavailability of the cofactors needed for NOS production of NO. In contrast, the stimulus activating arginase and intracellular regulation of NO substrate pools, including impairments in cationic amino acid transporters, are different with aging and essential hypertension (Holowatz et al. 2010b).

Essential Hypertension and Skin Flowmotion

Microcirculatory vasomotion is important for optimal distribution of blood flow in the microvascular bed, reducing microvascular resistance and promoting the functional recruitment

of previously inactive capillary beds (Rossi et al. 2008b). Increased resistance to microcirculatory blood flow in hypertensive humans is an important cause of high blood pressure. Therefore, studying skin flowmotion in essential hypertensive humans may provide valuable insight into the mechanisms increasing vascular resistance in essential hypertensive humans.

Spectral analysis of the periodic oscillations contained in skin blood flow recordings using laser Doppler flowmetry (LDF) allows for examination of the mechanisms contributing to the skin flowmotion. The periodic oscillations contained within the LDF signal can be characterized into five frequency intervals relating to different components of vasomotor control: heart beat (0.6-2.0Hz) (Rossi et al. 2008b), respiration (0.15-0.6Hz), myogenic (~0.05-0.15Hz) (Stefanovska et al. 1999), neurogenic (~0.02-0.05Hz) (Kastrup et al. 1989, Soderstrom et al. 2003), and endothelial (~0.0095-0.02Hz) control (Kvernmo et al. 1999, Rossi et al. 2008a, Gustafsson et al. 1993, Kvandal et al. 2003). The two higher frequency intervals are due to central hemodynamic modifications that occur during beating of the heart and respiration which transmit into the skin microcirculation (Rossi et al. 2008b) while the lowest frequencies relate to local control of vasomotor tone. Essential hypertension attenuates vasomotor control, likely due to 1) endothelial dysfunction due to a shift from NO and EDHF-dependent vasodilatory signaling to an EDCF proconstrictor state, 2) increased myogenic tone and vascular stiffening, and 3) altered neural signaling to the endothelium and vascular smooth muscle.

Reduced cutaneous endothelial function, specifically through reductions in NO-dependent vasodilation is well documented in essential hypertension (Smith et al. 2011b, Taddei et al. 1997). Reduced NO bioavailability and increased sensitization to EDCFs likely increase the resistance of the microcirculation in essential hypertensive humans. Increased resistance in the microcirculation is also related to the prevalence of myogenic tone in the vessels. Myogenic tone is an intrinsic property of the vascular smooth muscle that is independent of neural or humoral control, eliciting contraction in response to stretching as a way to maintain constant blood flow in the face of changing blood pressure. Essential hypertension results in increased blood pressure and thus myogenic tone to protect distal capillaries against the deleterious effects of hypertension. One putative mechanism for the increase in inflammatory responses is through heightened perivascular nerve activity, especially from sympathetic nerves, contributing to release of neurokinins from primary afferent nerves increasing the inflammatory response in essential hypertension. However, these mechanisms have not been studied yet in the cutaneous circulation.

The few studies that have used spectral analysis to examine skin flowmotion in essential hypertension used post occlusion reactive hyperemia (PORH) and local skin heating as a means to assess microvascular vasoreactivity. Skin flowmotion responses in thermoneutral situations where skin temperature is not controlled are not altered in humans with essential hypertension (Gryglewska et al. 2010b, Gryglewska et al. 2010a, Rossi et al. 2011, Rossi et al. 2006). However, masked hypertension, or populations that have normal clinical blood pressure readings with frequently high ambulatory blood pressure readings, have higher neurogenic and myogenic resting flowmotion (Gryglewska et al. 2010b), suggesting altered mechanisms of vascular dysfunction in this population.

PORH skin flowmotion is attenuated in newly hypertensive and longstanding hypertensive men and women (Rossi et al. 2006). PORH is a skin-specific vasoreactivity test, which is primarily dependent on 1) sensory nerves (Lorenzo and Minson 2007), 2) stimulation of large-conductance Ca^{2+} Dependent K^{+} (BKCa) channels (Lorenzo and Minson 2007), and 3) possible modulation of locally derived COX products (Medow, Taneja and Stewart 2007). In normotensives, PORH resulted in increased total PSD and the frequency intervals corresponding to endothelial, neural, and myogenic signaling. PORH did not increase total PSD, endothelial, or neurogenic frequency intervals in newly, unmedicated essential hypertensive or longstanding essential hypertensive men and women who had been taken off their antihypertensive medication for one day. In addition, longstanding essential hypertension increases the frequency interval associated with myogenic signaling (Rossi et al. 2006). This suggests that newly essential hypertensive individuals may have reduced endothelial and neurogenic control of vasomotion, with more long-standing hypertension resulting in irreversible heightened intrinsic myogenic responsiveness due to prolonged exposure to high blood pressure and/or inward vessel remodeling. With what is known about the mechanisms mediating PORH, it is likely that both essential hypertensive groups had attenuated endothelial-dependent vasodilation (EDHF-mediated) and sensory nerve responses during ischemic stress. Conversely, treatment with antihypertensive medication restored vasomotor responses after PORH in newly essential hypertensive men and women who were previously naïve to pharmacotherapy (Rossi et al. 2011), suggesting that if essential hypertension is treated early enough microvascular function can be restored.

Essential hypertension did not negatively affect skin flowmotion during local skin heating (Gryglewska et al. 2010a). However, in the latter study local skin heating was only performed for eight minutes, which is not enough time for the full expression of the NO-dependent vasodilatory response to local skin heating. Therefore, more studies using well-characterized and experimentally controlled skin-vasoreactivity tests should be used to examine the effects of essential hypertension on skin flowmotion.

Platelet Inhibition and Skin Blood Flow

The cutaneous circulation allows for evaluation of microvessel function after systemic drug therapies. The skin has been used to examine how the two most commonly used antithrombotic therapies, namely low dose aspirin (81mg, ASA) and clopidogrel bisulfate (75mg, Plavix®) alter thermoregulatory and microvascular function in healthy, middle-aged men and women.

Daily over-the-counter low dose ASA is the gold standard anticoagulatory therapy for primary and secondary prevention of atherothrombotic disease. ASA has been increasingly used due to recommendations by the American Academy of Chest Physicians that all men and women over fifty years old, even in the absence of cardiovascular risk factors, use daily ASA for prophylactic prevention of primary atherothrombotic disease (Vandvik et al. 2012). In low doses, ASA irreversibly inhibits platelet COX-1 in the pre-systemic portal circulation, virtually suppressing all platelet production of the potent vasoconstrictor thromboxane A_2 (TXA_2) (Patrono and Rocca 2012, Tuleja et al. 2003, Kyrle et al. 1987). ASA-induced COX-1 inhibition lasts for the life of the platelet (~8-10 days) because platelets are anucleate and cannot regenerate COX-1 enzymes (Patrono and Rocca 2012). In contrast to its effects on platelets, ASA does not affect endothelial COX-dependent PGI_2 synthesis because it does not reach the vascular endothelium in sufficient concentrations or for an adequate period of time to fully inhibit endothelial COX. If ASA does reach the vascular endothelium, COX-1 can be rapidly resynthesized within six hours after acute inhibition (Patrono et al. 2008, Patrono et al. 1985). However, larger doses of ASA (>600 mg) are capable of inhibiting both COX-1 and -2 in the vascular endothelium (Patrono and Rocca 2012, Patrono et al. 1985).

Clopidogrel, which inhibits platelets in an independent manner from low dose ASA, irreversibly inhibits platelet adenosine diphosphate (ADP)-$P2Y_{12}$ receptors for the life of the platelet. Clopidogrel is the most widely used prescription antiplatelet therapy and has been used in over 115 million people worldwide (Partnership 2012). It is recommended by the American College of Chest Physicians that long-term antithrombotic therapy (either ASA or clopidogrel) be used in patients with established coronary artery disease(Vandvik et al. 2012).

Interest in the effects of antithrombotic therapies on cutaneous vasoreactivity began when it was found that chronic low-dose aspirin therapy consistently and significantly attenuated reflex cutaneous vasodilation (Holowatz and Kenney 2009). At the time, it was known that healthy human aging results in attenuated reflex cutaneous vasodilation due to a reduction in both NO- and co-transmitter-dependent vasodilation (Holowatz et al. 2003, Kenney et al. 1997b). Therefore, it was hypothesized that platelet inhibition with ASA attenuated skin blood flow by 1) reducing COX-derived vasodilators, 2) reducing the release of known vasodilators from platelets (i.e. NO, adenosine triphosphate (ATP), ADP, and serotonin (5-HT)) that have been implicated in reflex vasodilation (Holowatz et al. 2010a), and/or 3) altering whole blood viscoelastic properties, thus decreasing shear stimulus on the cutaneous endothelium (Holowatz et al. 2010a). However, localized, non-specific inhibition of cutaneous microvascular COX-1 and 2 did not affect reflex cutaneous vasodilation in healthy, middle-aged men and women, suggesting that cutaneous blood flow was likely altered through platelet-specific mechanisms.

Skin-specific tests were used to examine some of the putative mechanisms altering cutaneous vasodilation with low-dose ASA and clopidogrel. Specifically, reactive hyperemia and slow local skin heating were performed with seven days of ASA and clopidogrel therapy in a randomized, double-blind, placebo controlled fashion to examine how these drugs affect cutaneous vasoreactivity. Surprisingly, clopidogrel augmented local cutaneous reactive hyperemic response during PORH, suggesting that clopidogrel may alter sensory or EDHF-dependent vasodilation. Conversely, ASA had no effect on cutaneous reactivity during these mechanistic studies. In addition, sympathetic activity was assessed through local bretylium blockade, which was not affected with either treatment. One limitation to this study was the slow local heating rate used (which was chosen to mimic the slower dilation to simulate shear stress during whole body heating), which is less reliant on NO-dependent vasodilation (Houghton et al. 2006) and may not tax the NO-reserve to see differences in NO-dependent vasodilation observed

during standardized local skin heating. Further mechanistic studies are needed to elucidate the effects of ASA and clopidogrel on NO-dependent vasodilation, sensory nerves, and the ability of these therapies to alter whole blood viscoelastic properties. In addition, the functional thermoregulatory and cardiovascular implications of low dose ASA and clopidogrel during a more natural environmental heat stress such as exposure to hot ambient air or exercise in the heat are unknown.

Figure 2-1

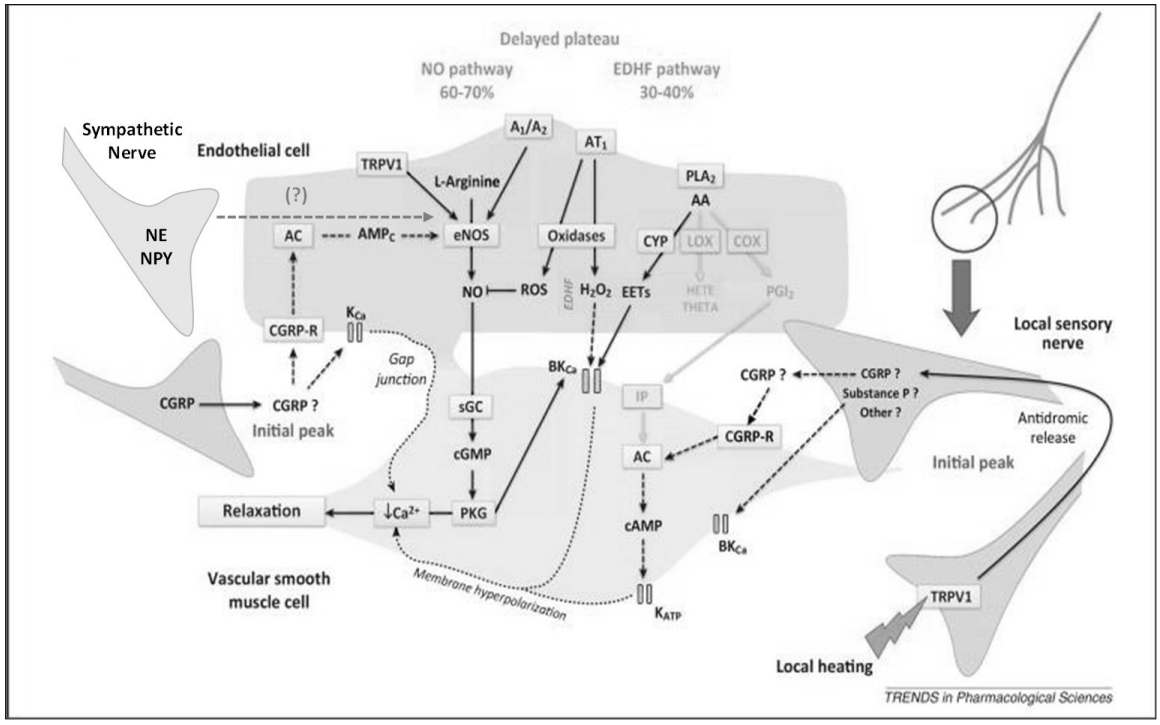


Figure **2-1**. Schematic illustrating the mechanisms mediating the thermal hyperemic response to direct local skin heating. Both primary aging and essential hypertension result in attenuated NO-dependent dilation during local skin heating. Further studies are needed to characterize pathological changes in neural, myogenic, and endothelial control of cutaneous vasodilation during local skin heating in aged and essential hypertensive populations. Adapted from Roustit and Cracowski 2013.

Figure 2-2

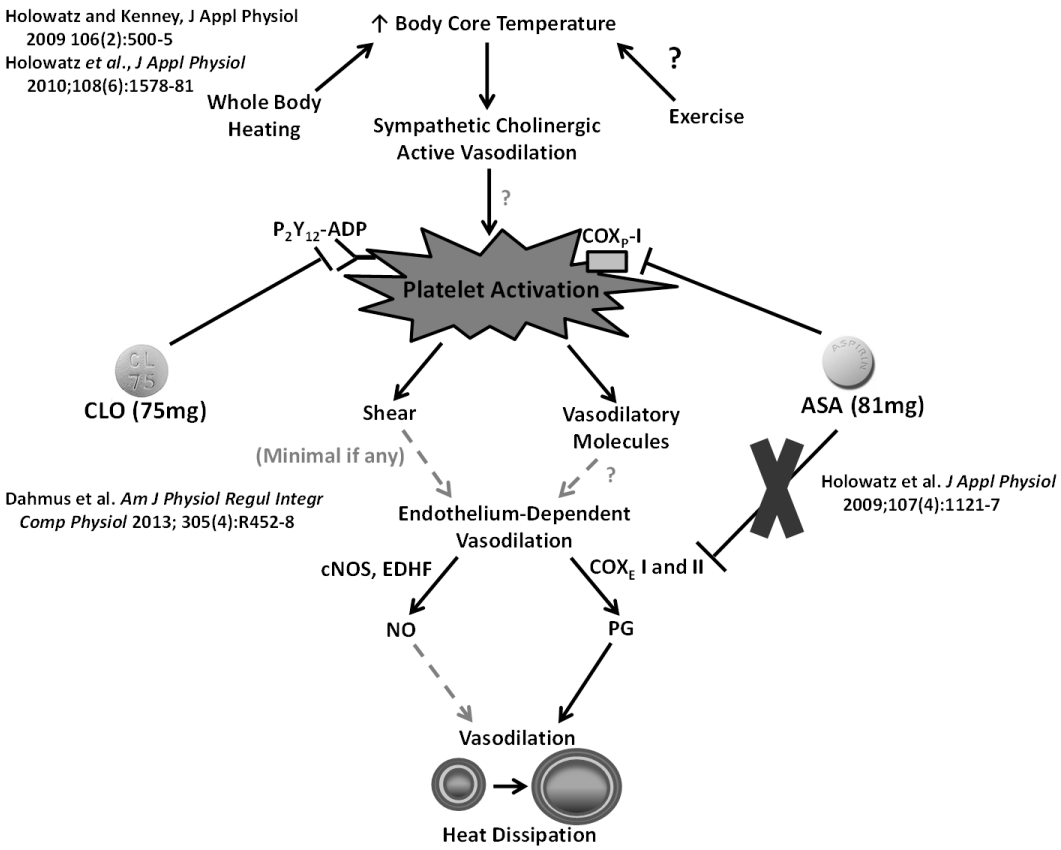


Figure **2-2**. Schematic illustrating the putative mechanisms and thermoregulatory consequences of altered microvascular function during systemic platelet inhibition with low dose aspirin (ASA; 81mg) and clopidogrel bisulfate (CLO, 75mg).

Chapter 3

ENDOTHELIAL NITRIC OXIDE SYNTHASE MEDIATES CUTANEOUS VASODILATION DURING LOCAL SKIN HEATING AND IS ATTENUATED IN MIDDLE-AGED HUMAN SKIN

Introduction

The cutaneous microvasculature provides an easily accessible, minimally-invasive circulation to examine vascular function (Cracowski et al. 2006, Abularrage et al. 2005c, Holowatz et al. 2008). Several methods are available to locally induce vasodilation in the cutaneous microvasculature. Direct local warming of the skin just below the pain threshold to 42°C elicits near maximal vasodilation in young, healthy individuals because at this temperature the vascular smooth muscle is completely relaxed and gives a highly reproducible skin blood flow response that (Kellogg et al. 1999b, Minson, Berry and Joyner 2001a, Minson 2010a) has been increasingly utilized to examine microvascular dysfunction in clinical populations (Minson et al. 2001b, Kellogg et al. 1999b, Holowatz et al. 2011, Smith et al. 2011a). The skin blood flow response to local heating is biphasic, consisting of an initial sensory nerved-mediated rapid increase in skin blood flow, followed by a slower secondary rise to a plateau that is approximately 70% reliant on nitric oxide (NO)-dependent mechanisms (Johnson and Kellogg 2010a, Kellogg et al. 1999b, Minson et al. 2001a). In addition to local heating, perfusion of the endothelium-dependent agonist acetylcholine (Ach) in a dose-dependent manner can be utilized to examine receptor-mediated endothelial function (Medow, Glover and Stewart 2008b, Smith et al. 2011a).

All three nitric oxide synthase (NOS) isoforms (endothelial, neuronal, and inducible) have been purported to contribute to the skin local heating response in different patient populations (Stewart et al. 2007b), (Kellogg, Zhao and Wu 2008b). Significant controversy exists as to which is the primary isoform mediating the production of NO during the plateau phase of the skin local heating response. We have recently shown that an upregulation of the pro-inflammatory signaling mediator inducible nitric oxide synthase (iNOS) contributes to attenuated

NO-dependent cutaneous vasodilation in essential hypertensive humans (Smith et al. 2011a) potentially through S-nitrosylation of arginase-1, which competes for the NOS substrate L-arginine (Santhanam et al. 2007b). Whether iNOS may contribute to the local heating response in non-pathological states is unclear.

Changes in both resistance vessel structure and decrements in NO-mediated dilation occur with aging and the development of cardiovascular disease (Holowatz 2008a, Rizzoni et al. 1996). Primary human aging, even in the absence of cardiovascular disease, is associated with attenuated skin blood flow responses including reduced plateau and relative NO-dependent cutaneous vasodilation during local heating and perfusion of endothelium-dependent receptor agonists (Kenney et al. 1997a, Minson et al. 2002). However, little is known about these mechanisms along the aging continuum, specifically, in the middle-aged cohort often used for comparison with clinical disease populations.

Considering 1) the important role of NO in vascular health, 2) the increased utilization of local skin heating to assess microvascular function, 3) the conflicting evidence for both eNOS (Kellogg et al. 2008b) and nNOS (Stewart et al. 2007b) involvement in NO synthesis during local heating, and 4) possible changes to these mechanisms with healthy middle aging, the primary aim of this study was to identify the predominant NOS isoform mediating cutaneous vasodilation during local heating. A secondary aim was to determine if there are any changes in these mechanisms with primary middle-aging. We hypothesized 1) that eNOS would be the predominant NOS isoform mediating cutaneous vasodilation during local heating, 2) that eNOS-mediated vasodilation would be attenuated in healthy, middle-aged humans. In addition to functional *in vivo* studies, *in vitro* analysis of skin biopsy samples were also performed to examine NOS isoform expression and the downstream molecular vasodilatory target vasodilator-simulated phosoprotein (pVASP) as an index of NOS activity.

Methods

Subjects. All experimental procedures were pre-approved by The Pennsylvania State University Institutional Review Board and met the guidelines set by the Declaration of Helsinki. Twelve

middle-aged and twelve younger men and women voluntarily participated in the study after giving written and verbal informed consent. All subjects were screened prior to participating in the experiment to ensure they were nonsmokers, and free of dermatologic, metabolic and cardiovascular diseases. In addition, all subjects' blood pressures were monitored for 24 hours using an ambulatory blood pressure monitor (Ambulo 2400, Portland, OR, USA) to ensure they were normotensive. Participants were normally active and were not taking any cardiovascular medications or supplements, oral contraceptives, or hormonal replacement therapy. All women were either postmenopausal or were tested in the low hormone phase of their menstrual cycles.

Instrumentation and Measurements. Subjects arrived in the room temperature (23°C) laboratory between 7:00 and 9:00 am dressed in comfortable clothing typically exercise pants and a T-shirt for both protocols.Upon arrival subjects were instrumented with four intradermal microdialysis (MD) fibers (10 mm, 20 kDa cutoff membrane, MD 2000 Bioanalytical Systems, West Lafayette, IN, USA) as previously described (Smith et al. 2011a). Once inserted, the MD agents were perfused at a rate of 2.0 uL/min (Bee Hive control and Baby Bee microinfusion pumps; Bioanalytical Systems) for 60-90 minutes to ensure resolution of local hyperemia from needle insertion trauma.

In Vivo Analysis of Vascular Function

To obtain an index of skin blood flow, laser Doppler flux (millivolts) was measured (LDF; MoorLAB, Temperature Monitor SHO2, Moor Instruments, Devon, UK) placed in local heating units directly over the membrane portion of each MD fiber. The local heaters were set at 33°C to clamp local skin temperature during baseline measurements in the local heating protocol and throughout the Ach dose-dependent perfusion protocol. This localized temperature was used to ensure that the changes in $\%CVC_{max}$ were due to the perfusion of Ach. Arterial blood pressure was measured every 5 minutes in the right arm throughout the experiment using a Cardiocap blood pressure monitor and was verified with brachial auscultation. Mean arterial pressure (MAP) was calculated as the diastolic blood pressure plus one-third the pulse pressure. Cutaneous vascular conductance (CVC) was calculated as red blood cell flux divided by MAP.

Experimental Protocols. Pharmalogical agents were perfused during the 60-90 minute resolution of the local hyperemic response from needle insertion trauma. After the local hyperemic

response subsided, baseline recordings were measured for 20 minutes or until the hyperemic response had subsided. Each subject participated in 2 protocols (local heating and Ach perfusion in a dose-dependent manner) that took place on two different days and were separated by a minimum of one week. During the protocols MD fiber sites were randomly assigned 1) lactated Ringer's solution to serve as control, 2) 20 mM N^G –nitro-L-arginine (L-NAME; Tocris, Ellisville, MO) to non-selectively inhibit all NOS isoforms (NOS-I), 3) 0.1 mM N-[3-(aminomethyl)benzyl] acetamidine (1400W; Calbiochem, San Diego, CA) to selectively inhibit iNOS (iNOS-I), and 4) 5 mM N^w-propyl-L-arginine (NPLA; Tocris, Ellisville, MO) to selectively inhibit nNOS (nNOS-I). All inhibitors were dissolved in lactated Ringer's solution and were sterilized using syringe microfilters (Acrodisc, Pall, Ann Arbor, MI, USA). The efficacy of the isoform specific antagonism and concentrations of the pharmacological agents used in this study have been demonstrated in other microdialysis studies (1400W Ki = 7 nM, NPLA Ki = 57 nM) (Santhanam et al. 2007a)(Stewart et al. 2007a, Holowatz et al. 2005, Garvey et al. 1997, Medow, Glover and Stewart 2008a, Zhang et al. 1997, Cooper, Mialkowski and Wolff 2000). Furthermore, the efficacy of NPLA was also piloted with a whole body heating protocol in four young subjects to determine if the concentration used attenuated reflex vasodilation similar to those reported by Kellogg et al. (Kellogg, Zhao and Wu 2009a).

Local Heating. After 20 minutes of baseline measurements, the local heaters were increased at a rate of 0.5°C every 5 seconds to a temperature of 42°C. The sites were held at 42°C until a stable CVC plateau was reached (~30-40 minutes). After the stable plateau, while the local heater temperature was maintained at 42°C 20 mM L-NAME was perfused at a rate of 4 uL/min for 40-50 minutes at all sites to determine the reduction in CVC from inhibition of all NOS isoforms. This allowed for the within site calculation of the percent reduction in CVC after NOS inhibition. The total local heating time was between 70-90 minutes.

Acetylcholine Dose-Response. After the baseline measurements Ach perfusion in a dose-dependent manner was performed to assess endothelial derived eNOS-mediated dilation (Khan et al. 1997, Morris and Shore 1996, Holowatz et al. 2005). Just prior to the Ach perfusion, Ach doses (0.01, 0.1, 1, 5, 10, 50, and 100 mM) were diluted with the site-specific pharmalogical agents. The doses were administered for 5 minutes in increasing concentrations (~40 minutes). This amount of time elicited a stable plateau of at least 3 minutes. At the end of each protocol, all sites were perfused with 28 mM sodium nitroprusside (SNP; Nitropress, Abbott Laboratories,

Chicago, IL) at a rate of 4 uL/min and were locally heated to 43°C to ensure maximal CVC had been achieved.

In Vitro Analysis of Vascular Function

Ventral forearm skin samples were obtained from the opposite arm on a separate day from the *in vivo* functional assessment of vasoreactivity. The skin was anesthetized using 2% lidocaine without epinephrine. Using sterile technique two 3 mm diameter skin samples were obtained. Samples were rinsed in lactated Ringers and immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Western blot analysis. After centrifugation of skin homogenates twice at 15000g at 4°C for 20 minutes, protein concentration was determined using a Bio-Rad DC protein assay. For Western blot analysis, 25 μg (for eNOS and nNOS) and 50 μg (for iNOS and pVASP) proteins were fractionated by SDS/PAGE and electrotransferred to a nitrocellulose membrane (Hybond-ECL, Amersham Life Sciences). The membranes were blocked for 1 hour at room temperature (5% nonfat dry milk, in Tris Buffered Saline containing 0.1% Tween-20; TBST) and incubated with a primary antibody to eNOS (SantaCruz Biotech; 1:1000); nNOS (BD Bioscience, 1:1000); iNOS (BD Bioscience, 1:1000), pVASP (1:1000; Cell Signaling). Bound antibody was detected with HRP-conjugated IgG secondary antibody (1:1000) (Santa Cruz Biotechnology) and visualized using enhanced chemiluminescence. Next, the pVASP blot was stripped using Restore Plus Western Blot Stripping Buffer (Thermo Scientific) and re-probed with VASP antibody (Cell Signaling). GAPDH was used as loading control. Densitometry analyses were performed using Image J software (NIH).

Data Acquisition and Statistical Analysis

Skin blood flow data were normalized as a percentage of maximal CVC (%CVC_{max}). Data were collected at 40 Hz, digitized, recorded, and stored in a personal computer until data analysis (Windaq, Dataq Instruments, Akron, OH, USA). CVC data were averaged over a stable 5 minutes of baseline, local heating plateau, post L-NAME plateau, and maximal vasodilation.

The initial sensory-nerve peak was calculated as the highest average values (30 second intervals). The vasodilation due to NO at the plateau was calculated from the difference between the plateau and the post-L-NAME plateau. CVC for each Ach-dose was averaged over the last 3 minute of perfusion of each concentration and normalized to maximal vasodilation which was obtained from the most stable 5 minute period where skin blood flow had peaked with the infusion of 28 mM SNP and with simultaneous locally heating to 43°C.

Student's unpaired *t*-tests were used to determine significant differences between age groups for physical characteristics and the reduction in NO-mediated dilation after perfusion of L-NAME during the local heating protocol. Data were initially analyzed using a three-way mixed model repeated measures analysis of variance (ANOVA: group x microdialysis treatment site x phase of the local heating response or Ach dose) (SAS, version 9.2, Cary, NC, USA). When the groups were collapsed data were analyzed using a two-way mixed models repeated measures ANOVA (microdialysis treatment site x phase of local heating response or Ach dose) Apriori specific planned comparisons were performed when appropriate to determine where differences between groups and localized drug treatments existed. The level of significance was set at $\alpha = 0.05$ for main effects. All values are expressed as mean ± SEM.

Results

Subject characteristics are presented in Table 3-1. A total of 24 subjects were tested. Age groups were well matched for height, BMI, MAP, and cholesterol ratio (total cholesterol/HDL cholesterol). Absolute maximal CVC are presented in Table 3-2. There was no effect of age or localized microdialysis drug treatment on absolute maximal CVC (all $p>0.05$).

During our initial pilot experiments to determine the efficacy of the nNOS inhibitor NPLA, relative NO-dependent vasodilation (assessed with L-NAME) was attenuated with pre-treatment of 5mM NPLA (Control: 28±8 vs. NPLA: 8±5 $\%CVC_{max}$).

Figure 3-1 shows the time course of the local heating response in a control site from a representative subject. All of the distinct phases of the response are labeled.

There was no interaction between age and NOS isoform specific inhibitor during local heating or Ach dose-response; therefore, data for both age groups were combined to illustrate the differences due to specific NOS isoform inhibition. Figure 3-2 shows the mean data for the distinct phases of the local heating response in all drug treatment sites. Non-specific NOS inhibition with L-NAME decreased baseline (panel A), the initial peak (panel B) and the plateau (panel C) compared to all other sites (all $P<0.001$). iNOS inhibition reduced the initial sensory nerve-mediated peak middle-aged versus young (53±2 vs. 60±2%CVC_{max}; panel B, $P<0.001$), but there were no other differences between specific NOS-inhibited treatment sites. Figure 3 illustrates the age-group combined %CVC_{max} response for all drug treatment sites with increasing concentrations of the endothelium-receptor agonist Ach. L-NAME decreased %CVC_{max} across all concentrations of Ach (all $P<0.001$). Compared to the control site there were no other differences with isoform specific NOS inhibition.

Because there was no difference between sites with specific NOS isoform inhibition (NPLA and 1400W) data for the control site for the two age groups are presented in Figures 3-4 and 3-5. Figure 3-4 shows the NO-dependent plateau and the post-L-NAME plateau of the local heating response. A quantification of total within site NO-dependent vasodilation is illustrated with arrows and values provided. There were no differences in the plateau, but the middle-age group had an augmented post L-NAME plateau. Therefore, the within site NO-dependent vasodilation was reduce in the middle-aged group (52±6 vs. 68±4%CVC_{max}; $P=0.013$). Figure 3-5 shows the %CVC_{max} response with increasing concentrations of Ach separated by age. The middle-aged group had attenuated Ach-induced vasodilation at the highest concentration (100mmol/L: 83±4 vs. 92 ± 3%CVC_{max}; both $P=0.03$).

Figure 3-6 shows densitometric analysis and representative western blots of nNOS, iNOS, eNOS, and pVASP from the skin biopsy samples for both groups. There were no differences in eNOS, nNOS or iNOS expression between the groups. Further, there was no difference in the downstream vasodilatory target pVASP between groups.

Discussion

The principal finding of the present study was that eNOS is the primary isoform mediating cutaneous NO-dependent vasodilation during local heating and perfusion of the endothelium-dependent agonist Ach. There was no effect of selective nNOS or iNOS inhibition compared to the control sites on the plateau response to local heating or during the acetycholine dose response but there was a significant attenuation when all NOS isoforms were inhibited with L-NAME. Further, during local heating iNOS contributed to the sensory nerve-mediated initial peak suggesting an upregulation in iNOS activity associated with sensory nerve activation. A secondary finding of the present study was that deficits in functional NO-dependent vasodilation can be detected in middle-aged skin. These deficits were only apparent during 1) local heating when NO-dependent vasodilation was quantified using a within-site perfusion of L-NAME and not from a comparison of the plateau $\%CVC_{max}$ values or 2) at the highest concentration of Ach (100mmol/L). There was no difference in NOS isoform expression or downstream vasodilatory molecular targets (pVASP) suggesting the modest changes observed in function are likely due to changes in NO metabolism.

In young human skin there is controversy pertaining to the precise NOS isoform mediating the production of NO during the skin local heating response (Kellogg et al. 2008b, Stewart et al. 2007b). The use of different pharmacological inhibitors, patient populations, and regional skin circulations examined contributes to the inconsistent findings with these studies. The results from the present study suggest that eNOS is the primary NOS isoform mediating the production of NO during the plateau phase of the local heating response in young and middleaged non-glaborous forearm skin. This is evidenced from sites treated with the specific nNOS and iNOS inhibitors having no effect on attenuating the plateau response and that non-specific NOS inhibition with L-NAME at the plateau had a similar effect in all sites. It is possible that that the concentration of the inhibitors used were not efficacious at blocking the specific NOS isozymes. However, we used the same concentration of NPLA used by Kellogg et al. (Kellogg, Zhao and Wu 2009b), finding similar results during local heating. We also verified the efficacy of this inhibitor in pilot work using a whole body heating protocol and observed an attenuation in the NO-mediated portion of the reflex vasodilatory response. Furthermore, we used the same concentration of 1400W (iNOS-inhibitor) detecting significant effects specific to iNOS mechanisms in an essential hypertensive population. Together, these data confirm that the NO-

mediated portion of the plateau response during local heating is likely produced via eNOS-dependent mechanisms.

One unexpected finding from the present study was that iNOS inhibition with 1400W reduced the initial sensory nerve-mediated peak in skin blood flow during local heating. Based on the putative role of iNOS as an inflammatory mediator that reduces eNOS-dependent mechanisms (Santhanam et al. 2007b, Smith et al. 2011a) we did not anticipate that iNOS inhibition would attenuate the initial peak in healthy subjects. The initial peak is partially mediated by the production of NO, but there is a significant portion that is due to the release of neurogenic vasodilator peptides acting on neurokinin 1 receptors including the putative neurotransmitters substance P and CGRP (Wong and Minson 2011). Moreover, it appears that adrenergic neurotransmitters also modulate this response (Hodges et al. 2008). Our data suggest that iNOS-mediated NO production also contributes to the initial peak. Interestingly, in other tissues substance P has been shown to augment NO production through iNOS (Jeon et al. 1999).

In the present study we utilized two skin-specific protocols to examine possible age-related changes in cutaneous microvascular function. These techniques are increasingly being utilized to examine mechanisms of microvascular dysfunction in clinical populations. Many of the disease states under investigation have an onset in middle-age and therefore clinical populations are often compared to a healthy middle-aged cohort. Furthermore, much of the work in the aging literature has compared a group of subjects of advanced age (65-85 years) contrasted to a group of young healthy control subjects (18-30 years) (Minson et al. 2002). In this study we have specifically examined a middle- aged group (46-57 years). Our data indicate that deficits in cutaneous NO-mediated vasodilation can be detected in this middle-aged population. These deficits only become apparent when NO-dependent vasodilation is quantified within site by perfusing L-NAME during the plateau phase of the local heating response. Without this direct quantification there are no differences in the absolute plateau phase of the response which is in contrast to what we observe with advanced age or in hypercholesterolemic (Holowatz et al. 2011) and essential hypertensive patients (Smith et al. 2011a). . These data suggest that a secondary NO-independent pathway is upregulated to compensate for the decrease in NO with healthy middle aging. In the context of the aging continuum this is an intriguing finding because it appears that the middle aged are able to compensate and that redundancy is lost in advanced age.

In middle-aged skin we also observed that endothelium-dependent vasodilation to the agonist Ach was reduced but only at the highest concentration tested (100 mmol/L). There was also no difference in the Ach-dose response in sites treated with the non-specific NOS inhibitor L-NAME between age groups. In contrast in an essential hypertensive population of the same age group where there is more clinically significant endothelial dysfunction we observe an augmented Ach-dose responses in sites continuously treated with L-NAME. Taken together, these data show that modest age-related changes in cutaneous microvascular function specific to NO can be detected in a healthy middle-aged skin.

In vitro analysis of skin biopsy samples shows that there was no difference in the expression of the NOS isoforms between age groups. Furthermore, there was no difference in the downstream vasodilatory target pVASP/VASP for Ser239, which is the major site at which the cGMP-dependent kinase PKG phosphorylates VASP. Functionally we detected modest declines in endothelium-dependent vasodilation in the middle-aged group. The biochemical expression data do not show a significant difference between NOS isoform protein content or differences in pVASP between age groups, but these data are not reflective of mechanisms affecting NO such as increased oxidant stress. It is not surprising that we were unable to differences in pVASP as these proteins were measures in un-stimulated whole skin homogenates and functionally only modest differences in eNOS-dependent vasodilation were measureable.

Limitations

Our aim with the present study was to examine the specific isoforms mediating cutaneous vasodilation during local heating. We further wanted to determine if there were any age-related changes to these mechanisms in middle aged skin. We have previously examined similar mechanisms with primary advanced aging. In the current study it would have been ideal to have three age groups to determine the age-related changes along the aging continuum. While we are relying on historical data collected by our research group for the advanced age group, this does not detract from our ability to make conclusions about the mild cutaneous microvascular dysfunction that we measured in the middle-aged group. Moreover, this study enables us to put these middle-age related changes in context with those changes that we observe in other clinical populations (Holowatz 2011b, Holowatz et al. 2011, Minson et al. 2002, Smith et al. 2011a).

We have utilized a standardized (Minson et al. 2001a, Minson et al. 2002, Holowatz 2011a, Holowatz 2011b, Holowatz et al. 2011, Smith et al. 2011a) local heating protocol to induce NO-dependent vasodilation using the non-specific NOS-inhibitor L-NAME to quantify vasodilation due to the production of NO during heating. In order to do this we continually heated the skin for 70-90 minutes. Hodges et al. noted that with adrenergic blockade that heating for this length of time resulted in a "die-away phenomenon" (Hodges et al. 2009). Because we did not include a time control it is possible that part of what we are attributing as vasodilation due to NO production may be accounted for with the die-away phenomenon. However, if this were the case the error would have been systematic across all sites and therefore does not change the main interpretation of the local heating data.

In addition to the functional data collected with isoform specific inhibitors, we have analyzed skin samples from the same subjects to examine NOS isoform protein expression. Unfortunately, we were not able to measure NOS activity in these samples because of 1) limited protein recovery from the skin homogenates and 2) there are many non-NOS dependent endogenous sources of nitrate and nitrite in human skin resulting from ultraviolent light exposure (Mowbray et al. 2009). It would have also been ideal to obtain western blot data of phosphorylated eNOS. This would have yielded greater insight into our results showing attenuated cutaneous vasodilation in middle-aged skin. However, because of the labile nature of phosphorylated proteins and the time laps between sample analyses we were unable to make these measurements.

Finally, our conclusions are based off of establishing the efficacy of our isoform specific inhibitors. There is evidence in knockout animal models and with prolonged isoform specific inhibition that other isoforms may be upregulated. We cannot exclude this as a possibility, although we think it is unlikely because of the relatively short time course of our isoform specific inhibition would not be sufficient to observe an increase in protein expression. In regard to the specific isoform inhibitor efficacy we rigorously tested these inhibitors including performing additional whole body heating experiments to verify the results of Kellogg et al. and relied on historical data from other laboratories to determine final inhibitor concentrations. We have also seen efficacious inhibition of iNOS with this concentration of 1400W in an essential hypertensive population which was supported with molecular data (Smith et al. 2011a). However, it is possible that isoform-specific NOS blockade was not fully achieved. In young subjects our data showed

that at the intermediate concentrations of acetycholine that NPLA modestly inhibited vasodilation. Although, we did see and attenuation in NO-dependent vasodilation during reflex heating in our pilot work with young subjects and no effect on the local heating response in the present study, these data suggest to us that NPLA at this concentration may have also partially inhibited other NOS isoforms, likely eNOS.

Summary

In summary, our main finding was that eNOS is the primary isoform mediating cutaneous NO-dependent vasodilation during local heating and perfusion of the endothelium-dependent agonist Ach. Using these skin-specific techniques we found that modest deficits in functional NO-dependent vasodilation were present in middle-aged human skin. Additional, NO derived from iNOS contributed to the sensory nerve-mediated initial peak. Finally, from the skin biopsy analysis we observed no differences in NOS isoform expression or downstream vasodilatory molecular targets suggesting the modest changes observed in function are likely due to changes in enzyme activity.

Table **3-1**. Subject Characteristics. Values are means±SE. *Significant difference from younger subjects ($P<0.05$).

Variable	**Young**	**Middle-Aged**
Sex (Men, Women)	7, 5	3, 9
Age (Years)	23±1	53±1*
Height (cm)	168±3	174±3
Weight (kg)	81±5	68±3*
Body Mass Index ($kg \cdot m^{-2}$)	27±1	25±1
Resting Mean Arterial Pressure (mmHg)	87±2	85±2
Fasting glucose ($mg \cdot dL^{-1}$)	89±1	85±2
Total Cholesterol ($mg \cdot dL^{-1}$)	148±5	190±5*
Low density lipoproteins ($mg \cdot dL^{-1}$)	86±7	108±4*
High density lipoproteins ($mg \cdot dL^{-1}$)	44±3	61±5*
Cholesterol ratio (total/HDL)	3.4±0.3	3.2±0.2

Table **3-2**. Absolute Maximal Cutaneous Vascular Conductance. Group mean±SE of absolute maximal cutaneous vascular conductance (flux/mmHg) for the four treatment sites in young and middle-aged groups.

Site	**Young**	**Middle-Aged**
Control	1.70±0.22	1.87±0.23
NPLA (nNOS-inhibited)	1.94±0.28	1.75±0.18
1400W (iNOS-inhibited)	1.92±0.22	1.85±0.23
L-NAME (all NOS-inhibited)	1.86±0.21	1.75±0.20

Figure 3-1

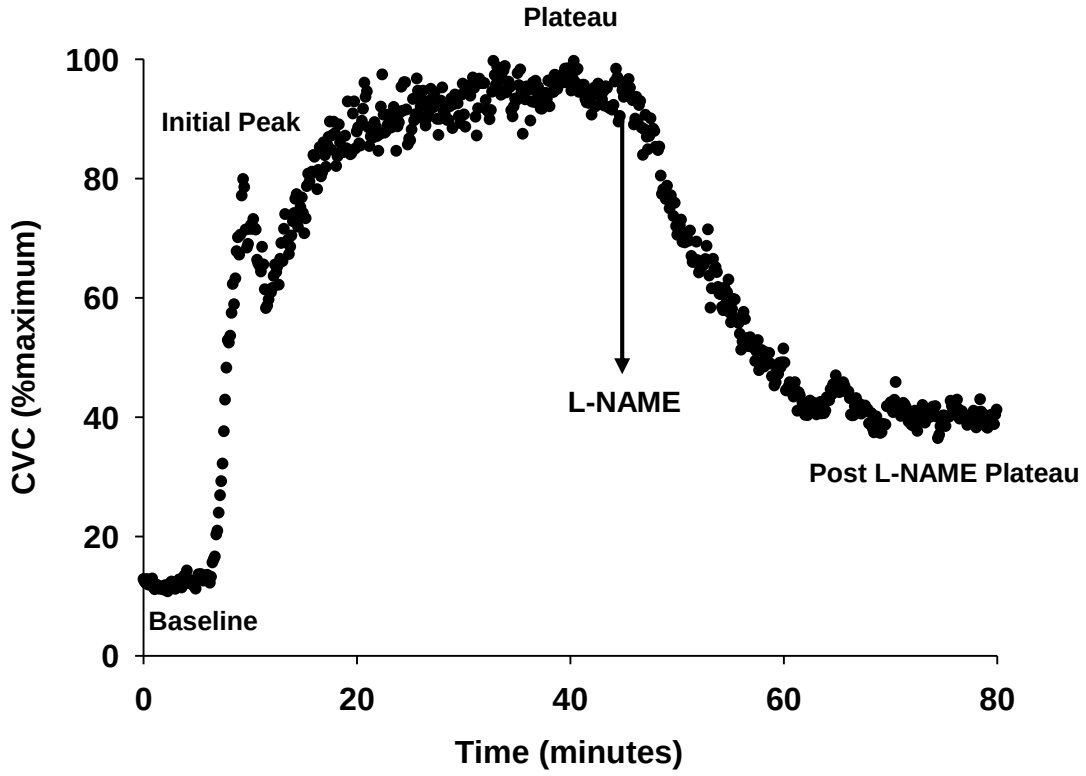


Figure **3-1**. A time course representative tracing of the control site during the local heating protocol. The baseline, initial peak, plateau, and post-L-NAME plateau are identified.

Figure 3-2

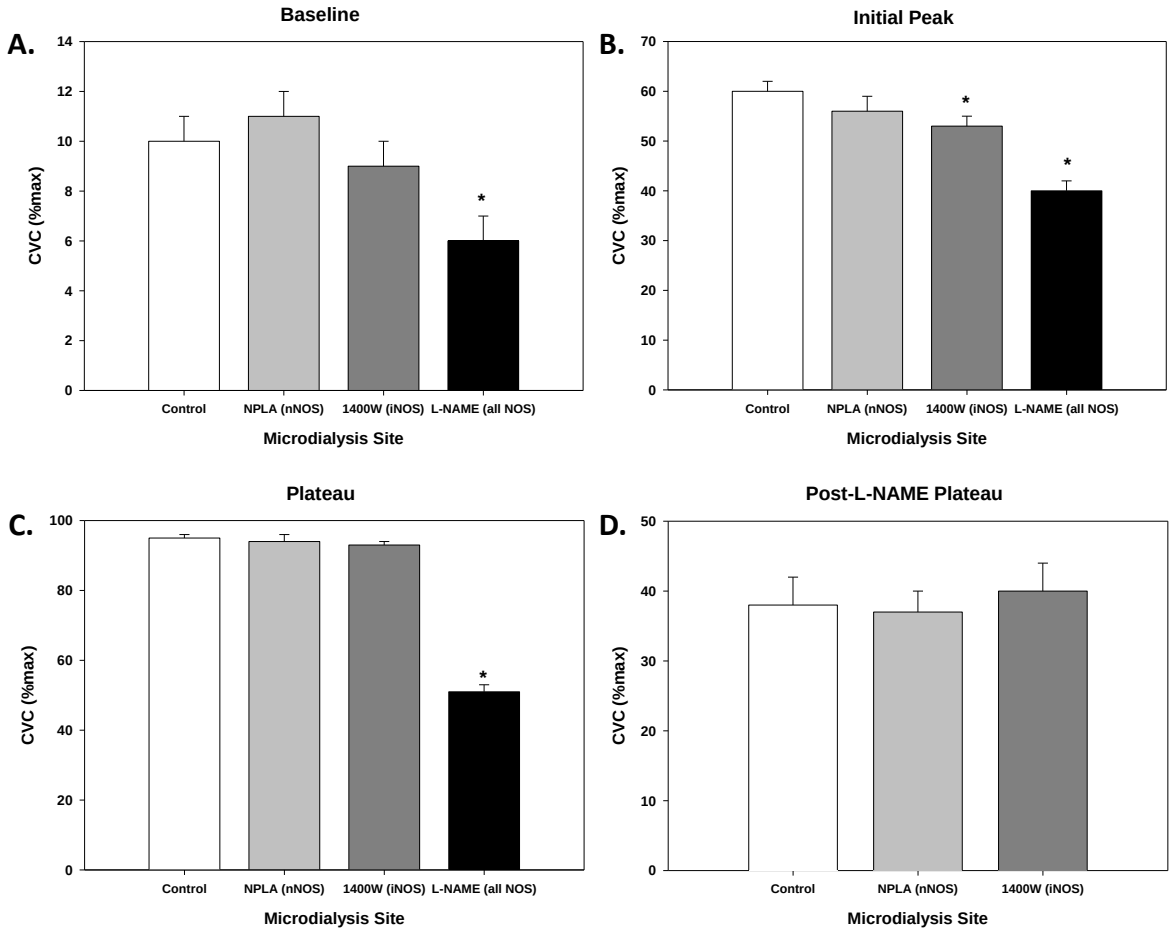


Figure **3-2**. Mean±SE as a percentage of maximal cutaneous vascular conductance (%CVC_{max}) during A) baseline, B) initial peak, C) plateau, and D) non-specific NOS inhibition with L-NAME for the microdialysis drug treatment sites. iNOS inhibition with 1400W decreased the initial peak and non-specific NOS inhibition with L-NAME decreased baseline, initial peak, and the plateau during local heating. *P<0.001 Significant difference from the control site.

Figure 3-3

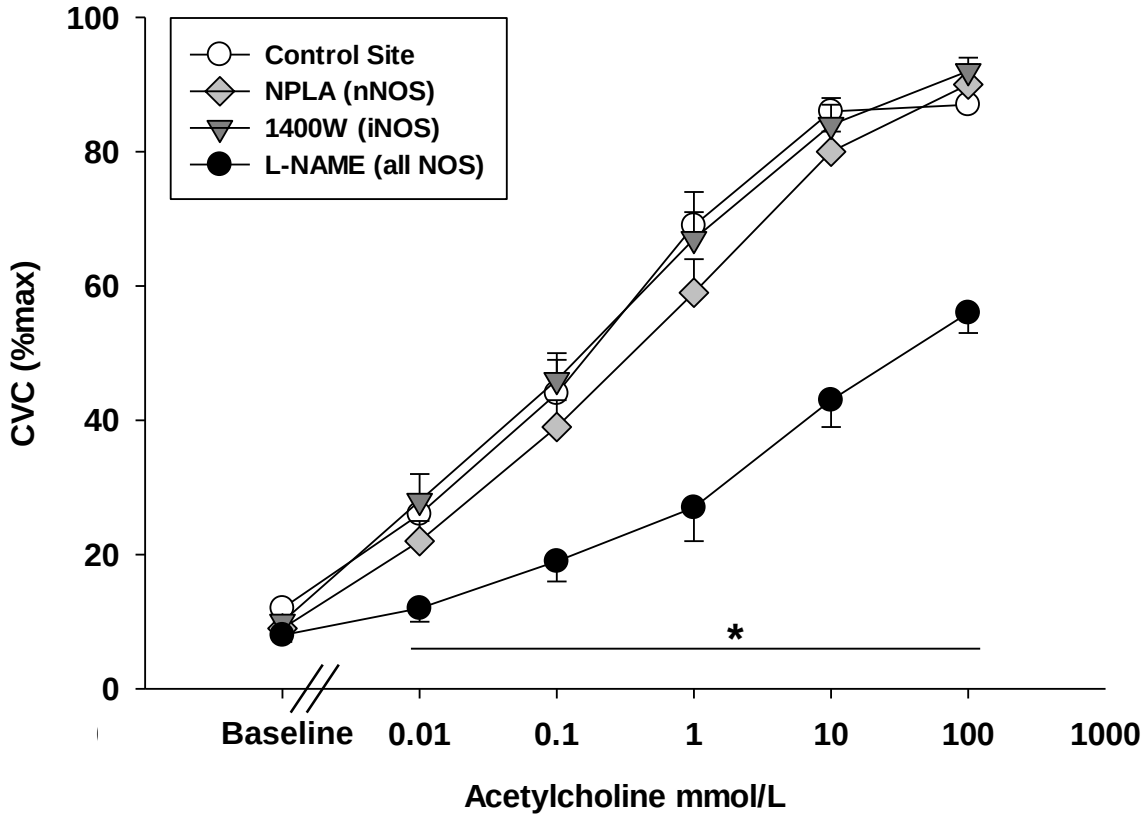


Figure **3-3**. Mean±SE as a percentage of maximal cutaneous vascular conductance (%CVC_{max}) during an acetylcholine (Ach) dose response. There was no effect of selective nNOS or iNOS inhibition. Non-selective NOS inhibition with L-NAME attenuated %CVC_{max} at all concentrations of Ach. *P<0.001 Significant difference from the control site.

Figure 3-4

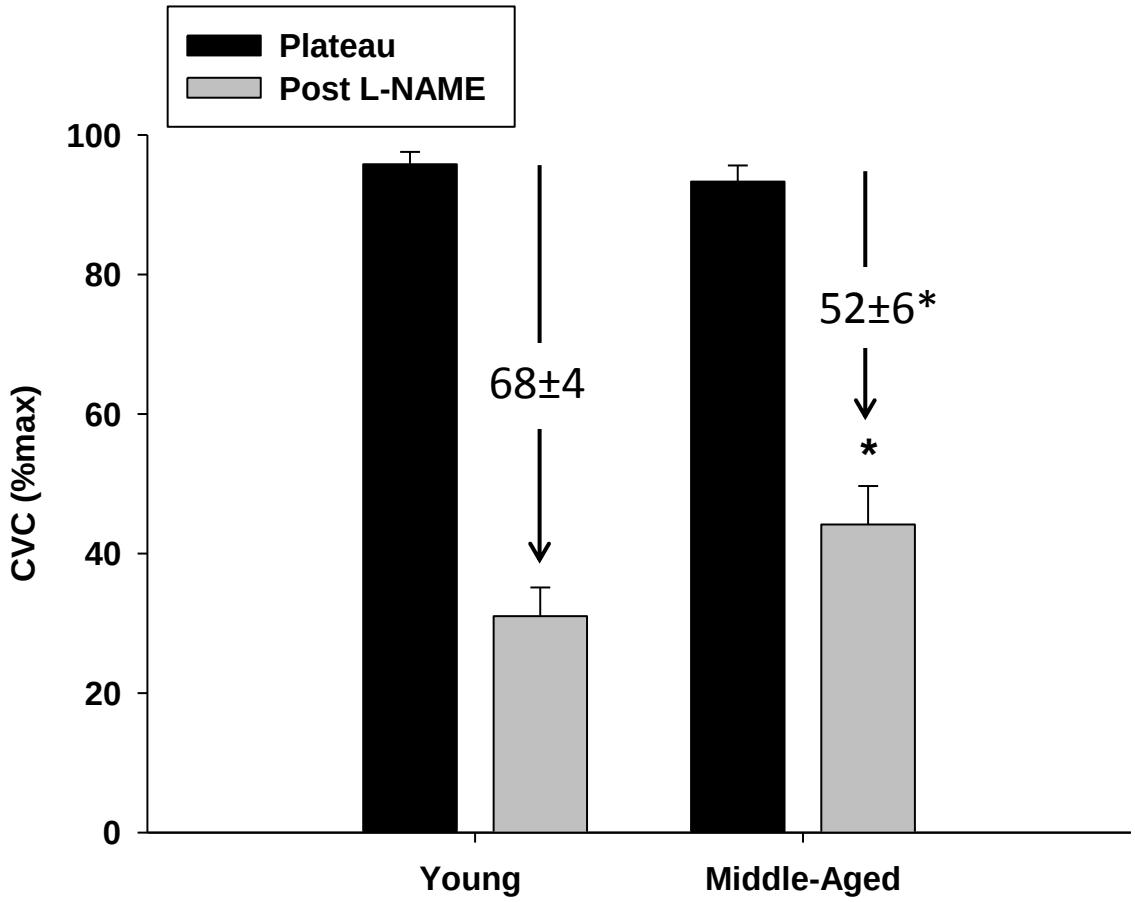


Figure **3-4**. Group mean±SE as a percentage of maximal cutaneous vascular conductance (%CVC_{max}) during the plateau and post-L-NAME plateau of the local heating response. The post-L-NAME plateau was increased and total NO-dependent vasodilation, illustrated as the difference between the plateau and the post L-NAME plateau was attenuated in the middle aged group. *P=0.013 Significant difference versus young.

Figure 3-5

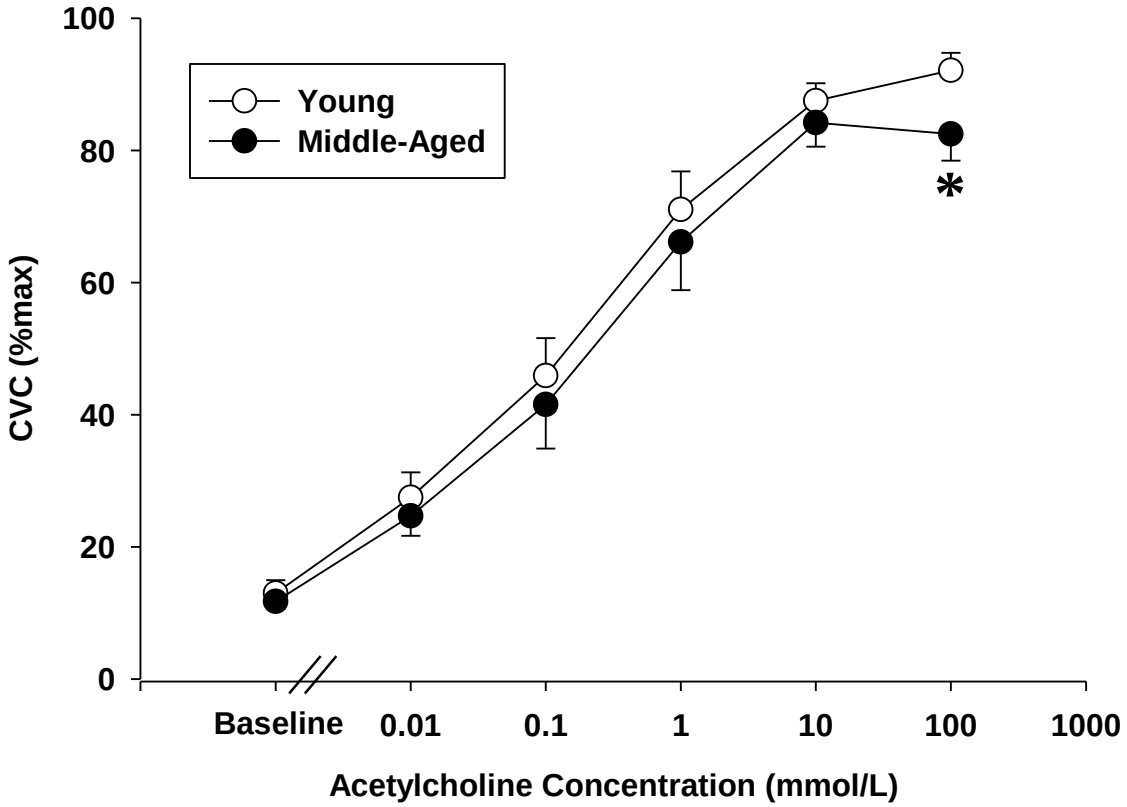


Figure **3-5**. Group mean±SE as a percentage of maximal cutaneous vascular conductance (%CVC_{max}) during an acetylcholine (Ach) dose response in the control sites. Ach-induced vasodilation was attenuated in the middle-aged at the two highest concentrations of Ach (50 and 100mM). *P=0.03 Significant difference versus young.

Figure 3-6

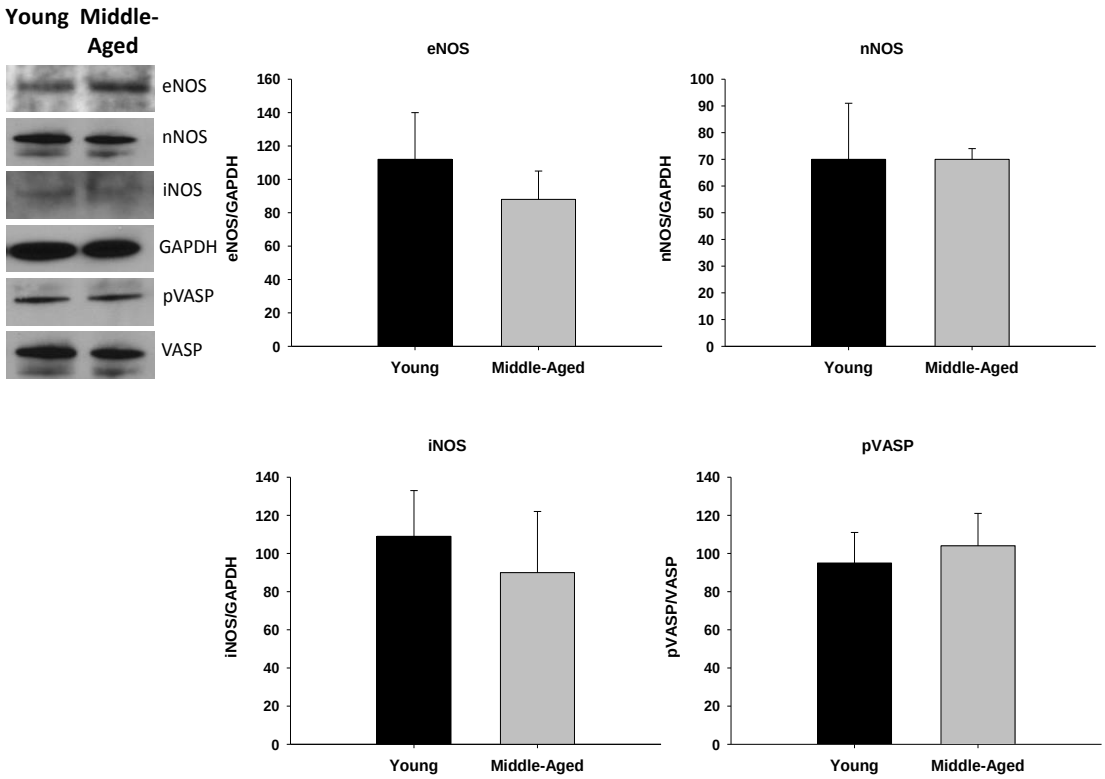


Figure **3-6**. iNOS, nNOS, eNOS, VASP, and pVASP. Expression of the three NOS enzymes, and VASP, and pVASP activity were determined by western blotting. GAPDH was used as loading control. Sample blot is shown in first panel. Densitometry analysis was performed using ImageJ software (NIH).

Chapter 4

ALTERED SKIN FLOWMOTION IN HYPERTENSIVE HUMANS

Introduction

The cutaneous circulation is an accessible vascular bed which allows for non-to minimally invasive studies of endothelial, neurovascular, and vascular smooth muscle vasoreactivity *in vivo* (Minson 2010b, Holowatz et al. 2008, Roustit and Cracowski 2013). A number of skin vasoreactivity tests have been used to study microvascular function in populations that are at risk for or currently have cardiovascular disease (CVD). Local skin heating coupled with laser Doppler flowmetry is a common test of microvascular function which elicits highly reproducible skin blood flow responses that have been mechanistically well characterized. Local skin heating causes a hyperemic response that is predominantly reliant on endothelium-dependent nitric oxide (NO)-mediated vasodilation (~70%). This response has been studied in a range of healthy (Minson et al. 2001b, Kellogg et al. 2008a, Bruning et al. 2012) and disease populations (Smith et al. 2011b, Holowatz and Kenney 2011).

Our laboratory has examined unmedicated stage 1 essential hypertensive men and women without other co-morbidities, yet exhibit endothelial dysfunction during local skin heating, marked by a reduction in endothelial nitric oxide synthase (eNOS)-dependent vasodilation (Smith et al. 2011b). There is growing evidence that spectral analysis of the low frequency periodic oscillations in blood flux measurements using laser Doppler flowmetry can provide non-invasive mechanistic information on microvascular control mechanisms (Salerud et al. 1983, Kastrup et al. 1989, Stefanovska et al. 1999). These periodic oscillations, or skin flowmotion, represent the influence of heart beat (0.6-2.0Hz), respiration (0.15-0.6Hz), myogenic (~0.05-0.15Hz) (Stefanovska et al. 1999), neurogenic (~0.02-0.05Hz) (Kastrup et al. 1989, Soderstrom et al. 2003), and endothelial influence on vascular smooth muscle relaxation (~0.0095-0.02Hz) (Kvernmo et al. 1999, Rossi et al. 2008a, Gustafsson et al. 1993, Kvandal et al. 2003). Spectral analysis has been performed on populations with known microvascular dysfunction such as

diabetes (Schmiedel, Schroeter and Harvey 2007), chronic smokers (Avery et al. 2009, Rossi et al. 2007), hypercholesterolemics (Rossi et al. 2009) and essential hypertensive men and women (Rossi et al. 2006, Gryglewska et al. 2010b, Gryglewska et al. 2010a). While these studies saw altered control of skin flowmotion, many of them utilized skin vasoreactivity tests and subject populations in which the mechanisms mediating the skin blood flow response are not thoroughly understood.

The aim of the present study was to utilize spectral analysis to evaluate skin vasoreactivity in unmedicated essential hypertensive and age-matched normotensive men and women before and after pharmacological inhibition of NO-dependent signaling (Smith et al. 2011b). We hypothesized that essential hypertension would result in reduced total power spectral density (PSD) around the frequency intervals of interest during local heating, due to reduced intrinsic endothelial and neurogenic signaling. Furthermore, we hypothesized that *within* site inhibition of NO production would result in reduced endothelial signaling, which would be restored after perfusion of an exogenous NO donor (sodium nitroprusside) in both essential hypertensive and normotensive subjects.

Materials and Methods

Subjects

All 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 were voluntarily obtained from all subjects prior to participation. We performed Fourier transform-based power spectral analysis on previously collected local skin heating studies in unmedicated essential hypertensive subjects from our laboratory (pilot data and (Smith et al. 2011b)). Fast Fourier transformations (FFT) were applied to laser Doppler recordings during local skin heating in 18 essential hypertensive and 18 age-matched normotensive men and women. Subject characteristics are presented in Table 1. Subject's blood pressures were classified in accordance with the guidelines set forth by the American Heart Association (Chobanian et al. 2003) during three separate visits and further explored using an ambulatory 24-hour blood pressure monitor. Subjects underwent a complete medical screening including a resting ECG, physical examination, lipid profile and blood chemistry (Quest Diagnostics, Pittsburgh, PA) and were otherwise healthy with the exclusion of stage 1 essential hypertension. All subjects were normally active, non-diabetic, non-smokers who

were not taking any prescription medications with primary or secondary vascular effects, including antihypertensive pharmacotherapy, vitamins and supplements. Seventeen of the 18 essential hypertensive subjects were naïve to antihypertensive therapy, and 1 subject had stopped antihypertensive therapy >1 year prior to participating in the protocol. All of the premenopausal women (n=4) were studied on days 2 to 7 of their menstrual cycle, and postmenopausal women (n=14) reported that it had been ≥1 year since the cessation of their last menses.

Local Skin Heating and Assessment of Skin Blood Flow

The local heating protocols were performed in a thermoneutral laboratory with the subject in a semi-supine position and the experimental arm at heart level. Intradermal microdialysis fibers (MD 2000, Bioanalytical Systems, West Lafayette, IN) were inserted into the ventral side of the forearm skin as previously described (Smith et al. 2011b). Local heaters (SHO2, Moor Devon UK) were used to control skin temperature and mount the laser Doppler flowmeter probes (MoorLAB, Moor Devon UK), which measured skin blood flux over the microdialysis sites. We performed Fourier transform-based power spectral analysis on the control microdialysis sites. The control site had lactated Ringer's solution perfused throughout insertion hyperemia (60-90 minutes), a baseline where local skin temperature was clamped at 33°C (20 min), during standardized local skin heating to 42°C (0.5°C every 5 s), and post local skin heating to record at least 30 minutes of a stable, heat-induced hyperemic skin blood flow response (Smith et al. 2011b, Minson et al. 2001b, Kellogg et al. 1999a, Johnson and Kellogg 2010b). The latter skin blood flow plateau is predominantly mediated by NO from eNOS (Kellogg et al. 2008a, Bruning et al. 2012, Johnson and Kellogg 2010b, Minson et al. 2001b). The NO-dependent vasodilation was assessed *within* each control site by perfusing 20mM of a non-specific NOS inhibitor (N^G-nitro-*L*-arginine methyl ester (L-NAME); Tocris) through the microdialysis fiber until the skin blood flow was reduced for a stable 20 minute period (L-NAME plateau). After the L-NAME plateau, local skin temperature was increased to 43°C and 28mM of sodium nitroprusside (SNP) was perfused to elicit maximal vasodilation for data normalization and to test endothelium-independent vasodilator responsiveness (Holowatz et al. 2005).

Power Spectral Density (PSD) Analysis

The skin blood flow data was collected using a MoorLAB laser Doppler flowmetry system. This system uses 785nm solid state laser diodes as the laser light source. In the MoorLAB device, the laser Doppler signal was band-pass filtered in the range of 20Hz to 14.9kHz. The skin blood flow data from the local heating protocols were digitized and saved at a sampling frequency of 40Hz using WinDAQ software (Dataq Instruments, Akron OH). These files were later converted to Microsoft Excel files and each skin blood flow response was divided into four periods: baseline, local heating plateau, L-NAME plateau, and SNP plateau for further analysis. The regions of interest converted into Microsoft Excel files were determined by visually assessing 600 seconds (24001 data points) of stable, flat portions of the laser Doppler recordings that were devoid of motion artifacts.

Estimating PSD. The frequencies of oscillations contained in the laser Doppler recordings from the four periods were analyzed using Fourier transform-based power spectral analysis as described by Avery et al, 2009. All laser Doppler recordings were measured in arbitrary flow units (AU) prior to analysis. Periodograms were derived from the FFT of the laser Doppler recordings in MATLAB® (version 2013a) and the average of the periodograms were used to estimate the PSD (Avery et al. 2009). The power (in AU^2) was calculated around the 0.01Hz (0.008-0.02Hz), 0.04Hz (0.02-0.05Hz) and 0.1Hz (0.05-0.15Hz) frequency intervals, considered to correspond to endothelial, neurogenic, and myogenic activity, respectively (Avery et al. 2009, Meyer et al. 2003, Rossi et al. 2006). During a given period (i.e. baseline, local heating plateau, etc.) the frequency intervals of interest were normalized relative to that period's total power to assess the contribution of each frequency during standardized physiological perturbations.

Statistical Analysis

The Anderson-Darling test was used to assess normality of the data. All normally distributed data are presented as mean±SE and non-normally distributed data are presented as median and interquartile ranges [IQR]. A one-way analysis of variance (ANOVA) was used to test the differences in mean PSD between baseline, local heating plateau, L-NAME plateau, and SNP plateau within a group (i.e. normotensive or hypertensive) when data were normally

distributed, whereas the non-parametric Kruskal-Wallis test was used to assess differences in the median PSD when data were non-normal. The Wilcoxon rank sum test was used to determine differences between each group's frequency intervals where appropriate. The level of significance was set at $\alpha=0.05$, with post hoc Bonferroni corrections for the specific planned comparisons. All statistical analysis was performed using Minitab statistical software (version 16.2.4, State College, PA).

Results

Subject characteristics are presented in Table 1. Subject groups were well matched for age, body mass index, fasting glucose, hemoglobin A1c, triglycerides, total cholesterol, and high- and low-density lipoproteins. Hypertensive subjects had higher systolic, diastolic and mean arterial pressure compared to the normotensive controls ($P<0.001$).

The mean PSD from FFT of the laser Doppler recordings are presented in figure 4-1 for a visual representation of how the mean PSD changes during a standardized local heating protocol in normotensive and essential hypertensive subjects. The mean PSD were much lower across all frequency intervals during baseline versus the local heating plateau, L-NAME plateau, and SNP-induced max in the normotensive and essential hypertensive subjects. Mean PSD was lower during L-NAME perfusion versus local skin heating and SNP perfusion for both groups ($P<0.001$). Visual inspection of the mean PSD plots illustrates that normotensive subjects had a higher PSD in the lower frequency intervals (~0.01-0.06Hz) during the local heating plateau (figure 4-1b) and L-NAME plateaus (figure 4-1c) versus essential hypertensive subjects. However, after SNP-induced maximum vasodilation (figure 4-1d), the PSD are nearly identical between normotensive and essential hypertensive subjects.

Figure 4-2 presents the PSD calculated from the frequency intervals corresponding to endothelial, neurogenic, myogenic, and total power from the mean PSD plots (figure 4-1). Local heating, L-NAME perfusion (post heating), and SNP perfusion increased PSD in all of the frequency intervals and total power versus baseline (figure 4-2a) in normotensive and essential hypertensive subjects ($p<0.001$). There were no differences in any of the frequency intervals of interest or total power between the normotensive and essential hypertensive groups during baseline ($p>0.05$). However, after locally heating the skin (figure 4-2b), the essential

hypertensive subjects had a lower total power versus normotensive subjects (P=0.03). Specifically, the essential hypertensive subjects had lower PSD in the neurogenic frequency intervals (P<0.01) with a trend towards lower endothelial PSD (P=0.07) compared to the normotensive group. During the L-NAME plateau PSD was lower across all frequency intervals and total power versus the heating plateau and SNP-induced max (P<0.001). After NOS-inhibition, the essential hypertensive subjects had a lower PSD in the endothelial (P<0.01) and neurogenic frequency intervals (P=0.04) as well as the in the calculated total power (P<0.001) compared to the normotensive group. During the NO-independent vasodilation elicited from perfusion of SNP, there were no differences in any of the frequencies of interest or total power in the normotensive or essential hypertensive groups (P>0.05). However, the endothelial frequency interval was higher during the SNP-induced max in the essential hypertensive group versus baseline, heating plateau, and L-NAME plateau (P<0.05).

Figure 4-3 illustrates the endothelial, neurogenic, and myogenic PSD as a percentage of total power for each given period of the local heating response. The pie charts for the heating plateau, L-NAME plateau, and SNP max are drawn to scale according to their absolute total power. The baseline charts were increased in size for visual purposes. The normotensive group exhibited an elevated neurogenic contribution to the total power during local skin heating versus any other phase of the local heating response (P<0.001). However, there were no differences in the contribution of endothelial and myogenic frequency intervals to total power for any of the phases of local heating (P>0.05). There were no differences in the endothelial, neurogenic, or myogenic contributions to the total power for any of the local heating phases in the hypertensive group (P>0.05). However, the hypertensive subjects had a reduced neurogenic contribution to total power during the local heating response compared to the normotensive group (P<0.001). The hypertensive group had a higher myogenic contribution to the total power during the local heating plateau versus the normotensive group (P=0.04). After NOS inhibition, hypertensives had less of an endothelial contribution to the total power versus the normotensive group (P=0.016). There were no differences in vasomotor-control during baseline or SNP perfusion in between groups.

Discussion

The principle finding from the present study is that hypertensive subjects exhibited an attenuated vasomotor response to local skin heating, which was marked by a lower total power and reduced neurogenic control of vasomotor tone. After NOS inhibition, hypertensive subjects exhibited lower total power, endothelial and neurogenic frequency intervals than normotensives, suggesting that hypertension results in altered neurogenic and NO-dependent control of skin flowmotion. Perfusion of SNP, a NO-donor, restored vasomotor control similar to normotensive subjects, suggesting endothelium-independent control of vasomotor tone was still intact in these newly hypertensive subjects. This supports a growing body of evidence suggesting that endothelial dysfunction is observed prior to structural and functional changes of the vascular smooth muscle (Smith et al. 2011b). Finally, spectral analysis of laser Doppler flux from standardized local skin heating is able to detect mechanistic changes in the control of skin blood flow similar to those observed during pharmacological assessment of vasoreactivity.

Similar to our mechanistic studies in this patient population (Smith et al. 2011b), in the current study we did not observe any difference in baseline skin blood flow using spectral analysis. Other studies that have performed spectral analysis on laser-Doppler flux similarly found no difference in the PSD during baseline conditions in either early stage (Rossi et al. 2006, Gryglewska et al. 2010b) or long standing essential hypertension (Rossi et al. 2006). In contrast, Gryglewska et al. 2010 found that individuals who have masked hypertension, or normal in-clinic blood pressure recordings but have consistently high ambulatory blood pressure readings, have altered basal blood flow suggesting they may have mechanistically-distinct vascular adaptations with their disease progression (Gryglewska et al. 2010b).

In the present study, essential hypertensive subjects exhibited a lower total power with a reduction in the frequency interval associated with neurogenic signaling during local skin heating. This corroborates our previous finding analyzing skin blood flow normalized to cutaneous vascular conductance (CVC; CVC=laser Doppler flux/mean arterial pressure). We found that essential hypertension had a lower skin blood flow during the local heating plateau when expressed as absolute CVC (Smith et al. 2011b) and when normalized as a percentage of CVC maximum (Smith et al. 2011b). Gryglewska et al. did not see any differences in PSD between normotensives and early stage essential hypertensives during local skin heating, but noticed a

reduced total power and reductions in the frequency interval associated with myogenic activity in those with family history of hypertension (Gryglewska et al. 2010a). However, in the latter study only 8 minutes of laser Doppler recordings were measured post-heating, which may not be enough time to reach full expression of local eNOS-mediated heating-induced vasodilation.

Our original hypothesis was that we would see a significant reduction in PSD in the frequency interval associated with endothelial signaling in essential hypertensive men and women. However, we did not observe a statistically significant difference in the endothelial frequency interval even though essential hypertensives showed a trend toward lower endothelial signaling (P=0.07). We may not have observed a difference in the lowest frequency interval because the essential hypertensive participants were relatively healthy with the exception of high blood pressure classification. In addition, this was a retrospective analysis of previously collected data so we were underpowered with only eighteen subjects based on our power calculations for detecting statistically significant differences in spectral density for a given frequency interval. Our preliminary power analysis suggested an N of twenty seven to detect an effect size of 25% for a given frequency interval.

Interestingly, essential hypertensive subjects exhibited reductions in the frequency interval corresponding to neurogenic signaling. Neurogenic regulation of skin blood flow involves activation of heat-sensitive afferent sensory nerves and/or sympathetic activity that is reliant on local skin heating rate (Houghton et al. 2006, Hodges et al. 2009). The standard rate of heating we used results in the well characterized biphasic response marked by an axon reflex-mediated peak followed by slower prolonged vasodilation that is primarily reliant on NO. The axon reflex is largely mediated by C-fiber afferent sensory nerves that are hypothesized to release substance P (Wong and Minson 2011, Wong et al. 2005) and CGRP (Stephens et al. 2001, Munce and Kenney 2003a, Minson et al. 2001b) which can then act on the endothelium and/or vascular smooth muscle to induce vasorelaxation. NO and endothelial derived hyperpolarization factors (EDHF) also appear to play a role in the axon reflex (Brunt and Minson 2012). The secondary plateau of the skin blood flow response is largely dependent on eNOS production of NO for full expression (~60-70%) (Minson et al. 2001b, Bruning et al. 2012, Kellogg et al. 2008a) with the remainder coming from EDHF-dependent mechanisms (Brunt and Minson 2012). There is growing evidence to suggest that NO-dependent vasodilation elicited during local heating interacts with both sympathetic and/or sensory nerves for full expression, providing a putative

mechanism for the observed lower neurogenic frequency interval in our hypertensive subjects. Specifically, Hodges et al found that norepinephrine (NE) and neuropeptide Y (NPY) from sympathetic noradrenergic nerve activation caused vasodilation through eNOS-dependent pathways, which is further supported by other studies examining sympathetic nerves and vasodilation (Tew et al. 2011, Carter and Hodges 2011, Hodges and Sparks 2013, Houghton et al. 2006). During local heating when sensory nerves are inhibited with topical anesthetic cream the secondary plateau is significantly reduced (Minson et al. 2001b). Further, populations that have attenuated NO-dependent vasodilation exhibit lower vasodilatory responses during stimulation of capsaicin-sensitive sensory nerves (Stephens et al. 2001) (Munce and Kenney 2003b, Munce and Kenney 2003a) (Krishnan and Rayman 2004, Stansberry et al. 1999). Therefore, it is plausible that impaired sympathetic and sensory nerve activation may be involved in the observed reduction in neurogenic signaling we observed in the essential hypertensive subjects. Further pharmacological studies are needed to elucidate if hypertension reduces sensory and/or sympathetic nerve-induced vasodilation because this could have clinical implications for the prevention of peripheral neuropathies in this population.

When we assessed the frequency intervals regulating vasomotor control as a percentage of total power, local skin heating increased the PSD in the frequency interval associated with neurogenic control of blood flow. Essential hypertensive subjects had a lower total power, with significantly less neurogenic contribution to this response. In addition, essential hypertensive men and women had a larger myogenic contribution to the vasomotor control, which may be indicative of less neurogenic-induced vascular smooth muscle relaxation or a compensatory means of regulating vasomotor tone.

After NOS inhibition with L-NAME there was a reduction in the total power in all of the frequency intervals for both groups. When assessed relative to the total power, the local heating-induced elevation of the neurogenic frequency interval observed in the normotensive subjects was restored to baseline values after NOS-inhibition. This supports sympathetic and/or sensory nerve interaction with NO in this group. We originally hypothesized that L-NAME would reduce PSD in the lowest frequency interval associated with endothelial signaling when expressed in absolute and relative terms. While we did see a reduction in the endothelial frequency when expressed as absolute PSD, we did not observe this when normalized as a percentage of total power.

Other studies have used endothelium-dependent agonists to characterize the endothelial frequency interval in different populations. Iontophoretic application of Ach and SNP increases PSD in all of the frequency intervals governing vasomotor control, but when assessed relative to total power, Ach selectively increases the endothelial frequency interval (Stefanovska et al. 1999, Kvernmo et al. 1999, Kvandal et al. 2006). When NOS inhibition with N^G-monomethyl-L-arginine (L-NMMA) was intra-brachially infused after Ach and SNP application, it abolished the difference between the Ach and SNP treatments in the endothelial frequency interval 0.0095-0.021 Hz (Kvandal et al. 2006). We may not have observed a selective reduction in the lowest frequency interval when normalized relative to total power after NOS inhibition for several reasons. One is that we used local heating instead of a pure endothelial-dependent agonist. We observed that the local heating response is highly neurogenic and likely increases NO-dependent vasodilation through a mechanistically different pathway than pure pharmacological stimulation of the endothelium. Second, we also used a different method for delivering the NOS inhibitors than the aforementioned studies. In the present study, we used intradermal microdialysis to locally deliver the non-selective NOS inhibitor to the site where laser Doppler flux was assessed, whereas in the latter studies brachial artery infusions of L-NMMA (Kvandal et al. 2006, Kvandal et al. 2003). Finally, the latter studies used a Morlet wavelet analysis (Kvandal et al. 2006, Kvandal et al. 2003) instead of traditional fast Fourier transformations, which have been commonly used to examine skin vasoreactivity.

Interestingly, NOS inhibition resulted in a lower contribution from the endothelial frequency interval to total power in the essential hypertensive group. This reduced endothelial contribution was not different than the other periods of the local heating response. In addition, we did not observe an increase in the neurogenic frequency interval during local skin heating, suggesting reduced neurogenic activation of NO-signaling pathways. In our previous pharmacological assessment study (Smith et al. 2011b), the impaired NO-dependent vasodilator system could be restored with inhibition of inducible NOS (iNOS) with *N*-(3-(Aminomethyl)benzyl)acetamidine (1400w) (Smith et al. 2011b). Together our results suggest that essential hypertensive humans may have chronic inflammation which may 1) desensitize the sympathetic and/or sensory nerves to stimuli such as heat, 2) cause chronic neurogenic inflammation and thus reduce post synaptic control of neurogenic vascular signaling, 3) increase oxidative stress causing eNOS-uncoupling and reducing NO production, and/or 4) increased

oxidative stress creating a favorable environment for the generation of pro-constrictor endothelium derived contracting factors.

Finally, when SNP was perfused to assess endothelial-independent vasodilation, the total power was restored in essential hypertensive subjects similar to the normotensive group. Further, when normalized as a percentage of total power there was no difference between the groups suggesting that in early hypertension NO-independent mechanisms are not altered.

Limitations. Sex and ethnic differences exist in the pathophysiology of endothelial dysfunction associated with hypertension, and in the present study we included men and women as well as a mixture of ethnicities. In the present study, the groups were not evenly matched for sex and were predominantly Caucasian. However, we did not observe any sex differences, but were underpowered to make this full comparison.

In conclusion, spectral analysis of the laser Doppler recordings during local skin heating corroborates *in vivo* pharmacological assessment of the mechanisms mediating skin blood flow and may provide additional mechanistic information. Specifically, essential hypertension reduces the neurogenic and total power contributing to skin flowmotion during local skin heating, suggesting that they have attenuated neural responses to local thermal stress. Further, after NOS inhibition, essential hypertension reduced endothelial, neurogenic, and total power suggesting that they have reduced NO-independent redundant vasodilator mechanisms contributing to skin flowmotion during local skin heating. Essential hypertension did not affect the skin vasomotor response to SNP, supporting that skin flowmotion can detect altered neural and endothelial vasomotor control before there is measurable vascular smooth muscle dysfunction.

Table **4-1**. Subject Characteristics. Values are mean±SE. BMI, body mass index; BP, blood pressure; MAP, mean arterial pressure; HDL, high-density lipoprotein, LDL, low-density lipoprotein. * P<0.01, ** P<0.001 Significantly different than normotensive men and women.

	Normotensives	**Essential Hypertensives**
Subjects (male, female)	(5,13)	(13,5)
Age (years)	53±1	52±1
BMI	25.3±0.7	26.5±0.5
Systolic BP (mmHg)	113±3	139±2**
Diastolic BP (mmHg)	76±2	92±2*
MAP (mmHg)	88±2	108±2**
Fasting Glucose ($mg \cdot dL^{-1}$)	91±2	93±2
HbA1c	5.5±0.1	5.5±0.1
HDL ($mg \cdot dL^{-1}$)	56±4	57±3
LDL ($mg \cdot dL^{-1}$)	107±5	110±5
Triglycerides ($mg \cdot dL^{-1}$)	94±12	91±16

Figure 4-1

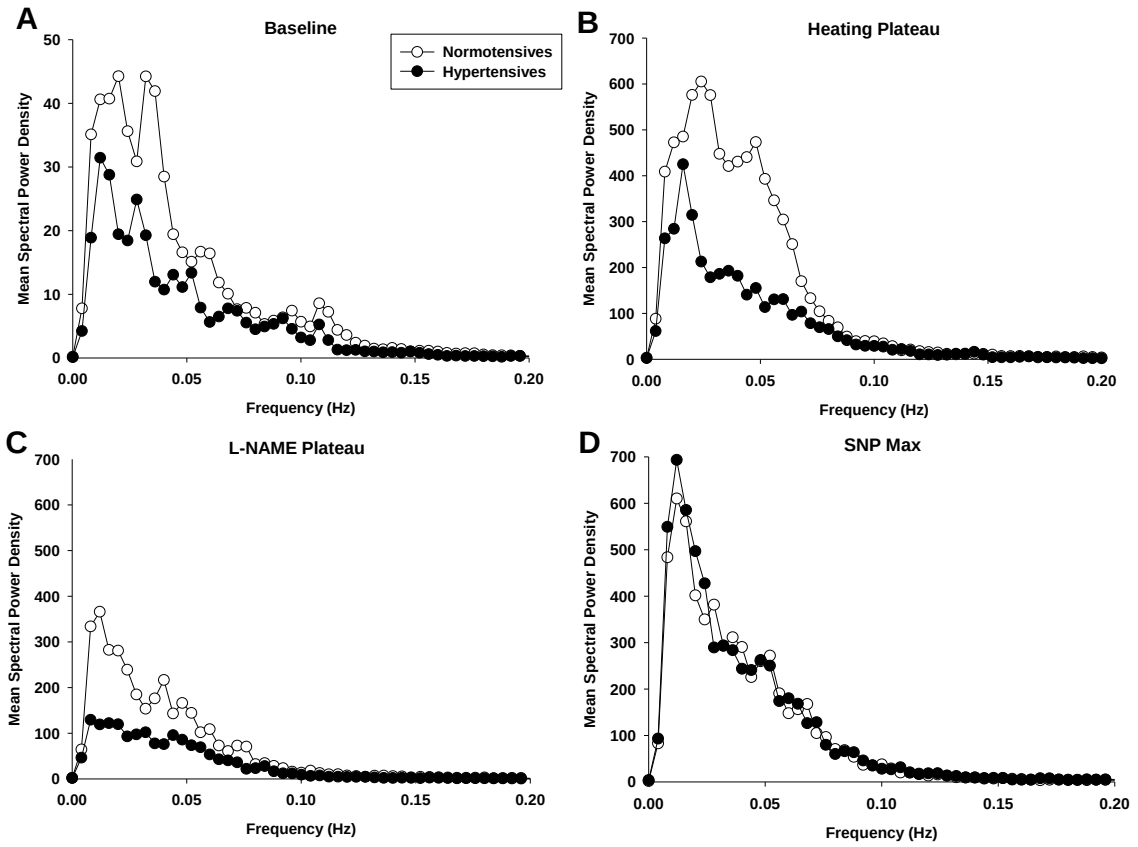


Figure **4-1**. Mean power spectral density plots for (A) baseline, (B) local skin heating, (C) non-specific nitric oxide synthase inhibition with L-NAME, and (D) SNP-induced maximum laser Doppler recordings during a local skin heating protocol in normotensive (unfilled circles) and essential hypertensive (black circles) men and women. Spectra were estimated from the fast Fourier transformation of the laser Doppler recordings. The frequency intervals 0.01Hz (0.008-0.02Hz), 0.04Hz (0.02-0.05Hz), and 0.1Hz (0.05-0.15Hz) are considered to correspond to endothelial, neurogenic, and myogenic activity, respectively.

Figure 4-2

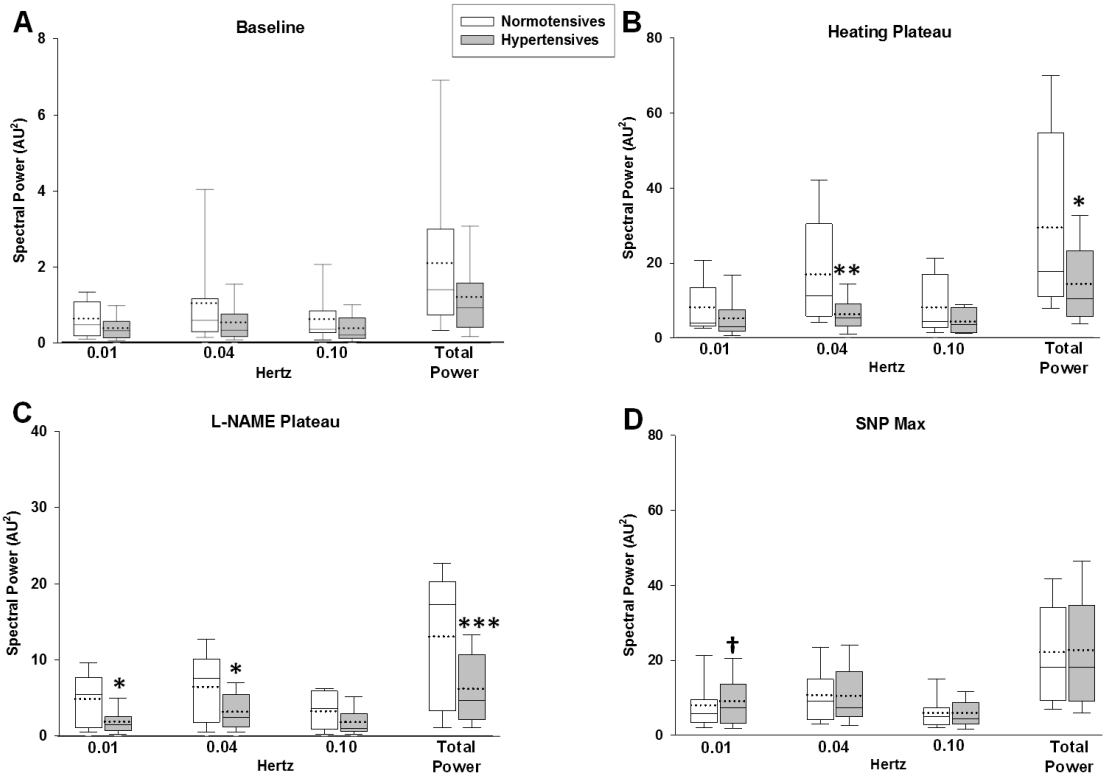


Figure **4-2**. Power spectral density (PSD) during (A) baseline, (B) local skin heating, (C) non-specific NOS-I with L-NAME, and (D) SNP-induced maximum laser Doppler responses during local skin heating protocol in normotensive (white bars) and essential hypertensive (gray bars) men and women. The power was calculated from the fast Fourier transformation of the laser Doppler signal around 0.01Hz (0.008-0.02Hz), 0.04Hz (0.02-0.05Hz), and 0.1Hz (0.05-0.15Hz) frequency intervals (FI), which are considered to correspond to endothelial, neurogenic, and myogenic activity, respectively. The total power was calculated from the of the non-overlapping FI. Baseline spectral power in all FI were lower than all other periods of the local heating response in both groups (P<0.001). L-NAME plateau spectral power was lower for all FI compared to the heating plateau and SNP-induced max (P<0.05). *P<0.05, **P<0.01, ***P<0.001 Significantly different than normotensive men and women. †P<0.05 Significantly different than baseline, local skin heating, and reduction with L-NAME for the endothelial FI in essential hypertensive subjects. Data are represented as box and whisker plots with the whiskers representing the 95th percentile. The solid lines within the box represent the median PSD and the dashed lines represent the average PSD.

Figure 4-3

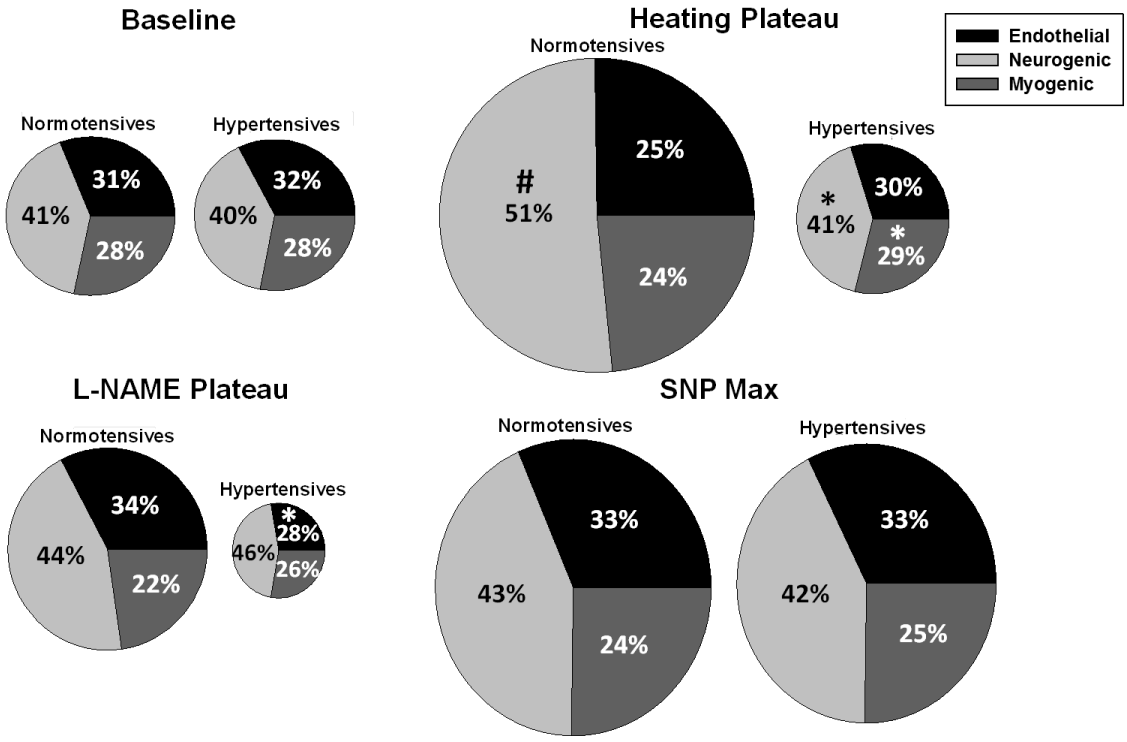


Figure **4-3**. Pie chart representing the mean relative power spectral density (PSD) as a percent contribution of the calculated total power during (A) baseline, (B) local skin heating, (C) non-specific NOS inhibition with L-NAME, and (D) SNP-induced maximum laser Doppler recordings. The relative energy contribution of each frequency interval (FI) was assessed around the 0.01Hz (0.008-0.02Hz), 0.04Hz (0.02-0.05Hz), and 0.1Hz (0.05-0.15Hz) FI, considered to correspond to endothelial, neurogenic, and myogenic activity, respectively. The total power was calculated from the of the non-overlapping FI. The size of the pie chart is proportional to the absolute total power with the exception of the baseline period which was increased in size so the data would be visible (proportional between the normotensive and essential hypertensive group). #P<0.05 Significantly different than the neurogenic FI in the baseline, L-NAME plateau, and SNP max periods. *P<0.05 Significantly different than normotensives.

Chapter 5

ASPIRIN AND CLOPIDOGREL ALTER CORE TEMPERATURE AND SKIN BLOOD FLOW DURING HEAT STRESS

Introduction

Primary human aging results in a diminished ability to vasodilate cutaneous arterioles in response to increasing ambient and/or body core temperatures (Martin et al. 1995, Kenney et al. 1997b). Reflex cutaneous vasodilation is mediated in part by an unknown cholinergic cotransmitter (Kellogg et al. 1995) and is dependent on nitric oxide (NO) synthase (Kellogg et al. 1998, Shastry et al. 1998) and cyclooxygenase (COX)–dependent signaling (McCord, Cracowski and Minson 2006) for full expression. Age-related impairments in reflex cutaneous vasodilation result from reduced nitric oxide (NO) and cotransmitter-mediated vasodilation and a shift from local vascular cyclooxygenase (COX)-derived vasodilators to vasoconstrictors (Holowatz et al. 2003, Holowatz et al. 2009).

Aging is also associated with an increase in cardiovascular disease and in the use of cardiovascular medications, including systemic platelet inhibitors for both primary and secondary prevention of thromboembolic disease (Cannon et al. 2010, Roger et al. 2011). Aspirin (ASA) is the most common over the counter anti-platelet medication and is recommended for all men over 45 and women over 50 years of age with one or more cardiovascular disease risk factors (Patrono et al. 2008). Clopidogrel bisulfate (CLO, Plavix®; 75 mg) is the most widely prescribed anti-platelet medication for the secondary prevention of thromboembolic events, prescribed to over 115 million individuals (Plavix website). While ASA and CLO inhibit platelets via different mechanisms (COX-1 and P2Y12 ADP receptors, respectively), both treatments independently attenuate reflex cutaneous vasodilation in middle-aged men and women during passive-whole body heat stress (Holowatz et al. 2010a).

In the previous study (Holowatz et al. 2010a), we noted that treatment with either of these orally-administered platelet inhibitors decreased the time required to raise oral temperature (Tor) by 1°C during passive heating in a water-perfused suit. However, the significant thermal strain imposed by a water-perfused suit, where skin temperature is clamped and the core-to-skin gradient is reversed, does not accurately replicate a natural environmental heat stress, e.g. resting or exercising in a warm environment. The functional thermoregulatory and cardiovascular implications of ASA versus CLO therapies during environmental heat stress and/or exercise in a warm environment have not been systematically explored.

The purpose of this study was to examine the effect of two commonly used antiplatelet medications, ASA and CLO, on core temperature and thermoeffector mechanisms during whole body heat stress at rest and during exercise in a warm environment. We performed a randomized double-blinded, crossover study design after 7 days of platelet COX-1 inhibition with systemic low-dose ASA (81 mg), 7 days of specific platelet P_2Y_{12} ADP-receptor inhibition with CLO (75 mg), and placebo. We hypothesized that use of the ASA and CLO would result in a greater rise in body core temperature during heat stress in ambient warm air versus placebo. We further hypothesized that reflex cutaneous vasodilation would be attenuated with ASA and CLO, resulting in a rightward shift in the skin blood flow:core temperature relation during exercise in the heat.

Methods

This study was approved by the Institutional Review Board at The Pennsylvania State University and conformed by the guidelines set forth by the Declaration of Helsinki. Verbal and written consent were voluntarily obtained from each subject before participation. Participants aged 50-65 years were studied because systemic platelet inhibitor therapy is most commonly prescribed for this age cohort.

All subjects underwent a complete medical screening which included a resting electrocardiogram (ECG), physical examination, blood chemistry and lipid profile (Quest Diagnostics Nichol Institute, Chantilly, VA), and coagulation study (prothrombin time and international normalization ratio (INR); iSTAT°1 Analyzer, Abbott, Abbott Park, IL). A $\dot{V}O_{2peak}$ test (ParvoMedics, Salt Lake City, UT) with a 12-lead ECG was performed to ensure that subjects were free of any potential underlying cardiovascular disease. For the subsequent exercise studies,

a $\dot{V}O_{2peak}$ test was performed on an electromagnetically braked recumbent cycle ergometer (Lode Corival, Groningen, Netherlands) and an exercise workload (watts) that elicited 60% $\dot{V}O_{2peak}$ was determined. No subject was taking ASA or CLO therapy or any other medication prior to the study, including any anti-inflammatory medications, hormone replacement therapy, oral contraceptives, vitamins, or nutritional supplements. All subjects were normally active, non-diabetic, and non-smokers. Five of the women studied were postmenopausal and the remaining two were studied in the early follicular phase of the menstrual cycle. Participants were asked to refrain from drinking alcohol for at least 24 hours and to refrain from consuming caffeine-containing products for at least 12 hours before the experiment.

Blinded Drug Treatments

Non-identifiable capsules were compounded by a registered pharmacist (Boalsburg Apothecary) and were given to subjects to take once daily over 7-10 days. The study design was a randomized, double-blind, crossover study with 81mg of ASA (Bayer®), 75mg of clopidogrel bisulfate (Plavix®, Bristol-Myers Squibb) and placebo (sucrose) treatments. The duration and dose of ASA and CLO used in the present study was chosen because ASA has shown to be efficacious for full platelet inhibition within 4-6 days (Hirsh et al. 1995), whereas CLO reaches a dose- and time-dependent inhibition of platelet aggregation (40-60%) after 3-5 days (Quinn and Fitzgerald 1999). In addition, the doses of ASA and CLO used in the present study are commonly used for primary and secondary prevention of thromboembolic events, respectively. Participants were instructed to take the experimental medications each morning with breakfast and their final pill was taken the morning of the experiment. The average circulation time of a platelet is 10 days (Mason et al. 2007); therefore, a minimum of a 3 week washout period separated each experimental trial to allow for full platelet recovery and removal of experimental medications (Hirsh et al. 1995).

Subjects

Fourteen middle-aged men and women (55±1yr; 7 men and 7 women) underwent seated passive heat stress in a warm environmental chamber followed by exercise in the same

environmental conditions. Because 1 man did not finish all 3 trials, data were analyzed for 13 subjects.

Experimental Protocol

Urine samples were obtained and urine specific gravity (Refractometer, Atago A300CL) and osmolality were measured upon arrival to the laboratory to ensure euhydration. Subjects then entered a thermoneutral antechamber (23.0±0.1°C) where they had their oral temperature (T_{or}) measured (WelchAllyn, Sure Temp Plus, Navan, Ireland) and a 20 gauge intravenous (IV) catheter placed in the antecubital vein for periodic blood sampling during the experiment. Participants were weighed prior to baseline measurements and post exercise to determine whole body sweat losses and calculate sweating rates.

Subjects then entered the environmental chamber (T_{db}=30°C; T_{wb}=22°C, 40% relative humidity) where they were instrumented (see instrumentation) and remained quietly seated on the recumbent cycle ergometer for 40 minutes. After 40 minutes of passive heat stress, subjects exercised at 60% of their $\dot{V}O_{2peak}$ for 2 hours or until they (1) requested to stop, (2) reached an esophageal temperature (T_{es}) of 39°C, or (3) reached 90% of their heart rate max. After cessation of exercise, subjects remained seated on the recumbent cycle ergometer for 30 additional minutes while a local heating protocol was performed to elicit maximal cutaneous vasodilation for normalization of laser-Doppler flowmetry data. During this time the chamber dry bulb temperature was decreased to T_{db}=23°C.

Instrumentation and Measurements

Upon entering the environmental chamber, the participants were instrumented and then rested for 40 minutes on the recumbent cycle ergometer in the warm environment. A copper-constantan thermocouple sealed in an infant feeding tube was inserted through the naris a distance ¼ of the subject's standing height to measure T_{es} at the level of the left atrium. Skin temperatures were measured using copper-constantan thermocouples at six sites: calf, thigh, abdomen, chest, back, and upper arm, and an unweighted mean of these sites were calculated ($\overline{T}_{sk}$) (Thompson and Kenney 2004). Mean body temperature ($\overline{T}_b$) was calculated as $\overline{T}_b=0.9T_{es}+0.1\overline{T}_{sk}$ (Stolwijk and

Hardy 1966) and the rate of rise in T_{es} was calculated during exercise as the slope of the T_{es} versus time for each experiment ($\Delta T_{es}/\Delta$time).

An index of skin blood flow was continuously measured using laser-Doppler flowmetry at two sites on the ventral surface of the right forearm. Laser-Doppler probes were held in place by local heaters, which were maintained at 34°C to locally clamp skin temperature to ensure changes in skin blood flow were of reflex origin (MoorLAB, Temperature Monitor SHO2, Moor Instruments, Devon, UK). Arterial blood pressure was measured by manual auscultation every 5 minutes. Cutaneous vascular conductance (CVC) was calculated as laser-Doppler flux divided by mean arterial pressure (MAP). In addition, beat-by-beat blood pressure and heart rate (HR) were measured continuously (Finapres® BP Monitor 2300, Ohmeda, Louisville, CO). Forearm blood flow (FBF) were measured during seated rest on the cycle ergometer and every 5 minutes during exercise by venous occlusion plethysmography using mercury-in-silastic strain gauge (EC6 Plethysmograph, Hokanson, Bellevue, WA) while blood flow to the hand was occluded (Whitney 1953). Forearm vascular conductance was calculated as FBF/MAP.

Thermal sensation was obtained during seated rest on the cycle ergometer in the environmental chamber and every 5 minutes of exercise (1-8 scale) (Young et al. 1987). Rating of perceived exertion (RPE) was obtained every 5 minutes during exercise (Borg RPE Scale, 6-20) (Borg 1970).

Blood samples were drawn every 40 min of seated rest on the cycle ergometer, during the final minute of exercise, and following the recovery period. Samples were immediately analyzed for hematocrit (microhematocrit centrifugation) and hemoglobin concentration (HemacueHb 201+) and the percent change in plasma volume (ΔPV) was calculated from hematocrit and hemoglobin using the Dill and Costill method (Dill and Costill 1974). All blood and urine samples were analyzed in triplicate.

Experimental Timeline

The study ran over the course of 18 months with subjects entering at random times to minimize seasonal effects on thermoregulatory effector mechanisms. Time of day was

standardized for each subject to prevent diurnal variations in body core temperature (Aoki, Stephens and Johnson 2001).

Data Acquisition and Statistical Analysis

Data were acquired using Windaq software and Dataq data-acquisition systems (Akron, OH). The data were collected at 40Hz, digitized, recorded and stored on a personal computer for future analysis. T_{es} data were averaged over 1 min intervals every 5 min of exercise. CVC data were averaged over 3 min intervals every 5 min during seated rest and exercise. Absolute maximal CVC was calculated as the average of a 5 minute stable plateau in laser-Doppler flux after locally heating the skin to 43°C (~30-40 minutes of heating) and CVC was calculated and represented as a percentage of maximum (%CVC_{max}). Slopes of the FBF data were calculated using the first derivative of the volume changes during each venous occlusion period using Windaq advance codas analysis software. Forearm vascular conductance is reported in units of milliliters per 100 milliliters of forearm per minute per 100mmHg (mL•100 mL^{-1}•min^{-1}•100 $mmHg^{-1}$).

Separate two-way mixed model analysis of variance (ANOVA) with repeated measures were conducted to determine differences between trials for (1) the %CVC_{max}, FVC, MAP, HR, $\overline{T}_{sk}$, T_{es}, and change in T_{es} responses versus time and (2) for the hematologic variables over the discrete sampling periods. Specific planned comparisons with Bonferroni corrections were performed when appropriate. The level of significance was set at α=0.05 and data are presented as mean ± SE.

Results

Subject characteristics are presented in Table 5-1. All subjects were healthy and moderately physically active.

The effects of ASA vs. CLO relative to placebo on T_{es} after 40 minutes of passive heat stress in the environmental chamber are shown for each individual in Figure 5-1. Both ASA (5-1A) and CLO (5-1B) treatment resulted in a significantly higher T_{es} after seated passive heat

exposure compared to the placebo trial (both $P<0.05$). Prior to this heat exposure, before entering the environmental chamber, T_{or} obtained in the thermoneutral antechamber was not different between the groups ($P=0.156$ main effect).

Figure 5-2 shows the mean core temperatures (T_c) responses during the time course of the experiment. Twenty five minutes before entering the environmental chamber T_{or} was measured in a thermoneutral antechamber. Upon entering the environmental chamber (time = 0), T_{es} responses were recorded for 35 minutes during seated rest (time = 5-40 minutes) and subsequent exercise in the heat (time=40-95 minutes). For clarity, data are presented until 25% of subjects dropped out of exercise ($n\geq4$), while all subjects are included in the recovery data (time=100, 110, and 120 minutes). The elevation in T_{es} that occurred during passive heating with both drugs compared to placebo persisted throughout exercise heat stress ($P<0.001$). There was no difference in the rate of rise in T_{es} during exercise between trials (Placebo: 0.05±0.01°C, ASA: 0.05±0.02°C, CLO: 0.05±0.01°C; $P=0.883$). During recovery from exercise, T_{es} remained significantly elevated with CLO treatment ($P<0.001$).

Figure 5-3 shows the mean $\%CVC_{max}$ (Figure 5-3A) and FVC responses (Figure 5-3B) after 35-40 minutes of seated rest followed by exercise in the heat (time 40-95 minutes). There were no differences between CLO treatment and placebo in either $\%CVC_{max}$ or FVC during passive heat exposure as a function of time ($P>0.05$). In contrast, ASA treatment significantly attenuated these responses compared to both the CLO and placebo trials for both $\%CVC_{max}$ and FVC responses. Both ASA and CLO treatments resulted in a rightward shift of $\%CVC_{max}$: mean body temperature relation (Figure 5-4), such that skin blood flow was lower for a given mean body temperature. Mean body temperature thresholds for reflex vasodilation were shifted for both ASA and CLO treatments (both 37.3±0.1°C) compared to placebo trials (37.1±0.1°C; $P<0.05$) during exercise. Finally, there were no differences in absolute CVC_{max} (flux/MAP) among treatments (ASA: 1.8±0.2, CLO: 2.1±0.3, and placebo: 1.8±0.3 flux/mmHg; $P>0.05$).

Table 5-2 shows the cardiovascular and performance variables during passive and exercise heat stress. There were no differences in heart rate, T_{sk}, or subjective thermal sensation during passive warm air exposure prior to the start of exercise. MAP prior to exercise was slightly higher with ASA treatment vs. placebo ($P<0.05$). There were no differences in the reason for terminating exercise, exercise duration, the final exercise T_{es}, heart rate, MAP, or T_{sk}. Likewise,

the change in plasma volume, whole body sweating rates, and thermal sensation ratings were similar among the trials.

Discussion

The principal findings from the present study were that ASA and CLO treatment did not affect thermoneutral core temperature as measured orally, but resulted in an elevated core temperature when the subjects sat in warm ambient conditions for 40 minutes. After the passive heat exposure, the increase in core temperature with ASA and CLO persisted throughout exercise. Treatment with ASA, but not CLO, attenuated the skin blood flow response during exercise heat stress compared to placebo as a function of time. Finally, ASA and CLO treatment shifted the thresholds for reflex vasodilation during exercise after a passive heat exposure toward higher mean body (and core) temperatures, such that skin blood flow was lower for a given mean body temperature.

Aspirin, Clopidogrel and Passive Thermal Stress

We previously demonstrated that reflex cutaneous vasodilation during whole body heating with a water-perfused suit was attenuated in subjects taking ASA and CLO versus no drug (Holowatz and Kenney 2009, Holowatz et al. 2010a). In the previous study examining the neurovascular signaling mechanisms mediating the reduction in skin blood flow with these drugs, we observed that the time it took subjects to increase their body core temperature by 1.0°C was significantly reduced when they were taking ASA or CLO. The aim of the current study was to determine the potential thermoregulatory and cardiovascular consequences of ASA and CLO therapy in natural warm air environment during both passive heat stress and exercise. Our present findings demonstrate that even mild heat exposure results in an increased resting core temperature when subjects are taking either ASA or CLO, as both of these treatments resulted in a higher body core temperature after 40 minutes of passive heat stress in a compensable warm air environment compared to placebo trials.

We previously reported that ASA and CLO consistently resulted in attenuated reflex cutaneous vasodilation during hyperthermia using the water-perfused suit versus no drug (Holowatz et al. 2010a). In the present study, after 40 minutes of passive heating in warm air there were no differences in the $\%CVC_{max}$ or the FVC responses among treatments. Considering the core temperature increase after the 40 minutes of warm ambient air exposure in the present study, the threshold for reflex cutaneous vasodilation had not been reached. Therefore it is not surprising that we were unable to detect a difference in skin blood flow responses during rest with the mild thermal stress utilized in the present study. It would be necessary to increase the heating stimulus, like that achieved in a water perfused suit, to reach the threshold and observe any potential differences.

Aspirin, Clopidogrel and Exercise in the Heat

The ASA- and CLO-related elevation in core temperature that appeared by 40 min of passive heating persisted throughout exercise heat stress and into recovery. Further, there was a shift in the threshold for the onset of reflex cutaneous vasodilation toward higher body temperatures with both ASA and CLO. While there was no increase in the rate of rise in body core temperature during exercise, there was a rightward shift in the skin blood flow ($\%CVC_{max}$):mean body temperature relation. These data indicate that given the same environmental conditions, ASA and CLO alter thermoregulatory effector mechanisms such that skin blood flow is lower for a given mean body temperature. Plotted against time (Fig. 5-3), ASA treatment resulted in lower skin blood flow as demonstrated by both $\%CVC_{max}$ and FVC responses versus placebo, an effect not observed with CLO treatment. The $\%CVC_{max}$ and FVC data were similar in both direction and magnitude for each of the given treatments. Based on previous skin blood flow data using the water-perfused suit model, we originally hypothesized that reflex vasodilation during exercise would be attenuated with CLO treatment. However in the present study, because core temperatures were somewhat higher after passive heat stress with CLO, differences in skin blood flow as a function of time are not different from placebo.

While we observed differences in skin blood flow with ASA and CLO in the present and previous studies (Holowatz et al. 2010a, Holowatz and Kenney 2009), there appeared to be no differences in fluid balance and evaporative heat loss among trials, as evidenced by similar changes in plasma volume and absolute whole body sweating rates. Although it is unlikely that ASA or CLO treatments affected initial absolute plasma volume, we did not measure total plasma

volume and are therefore relying on the relative change in plasma volume. Further studies using thermal modeling along with highly sensitive indirect calorimetric measurements are needed to more precisely measure changes in dry heat loss mechanisms while undergoing ASA and CLO therapy and the potential thermoregulatory consequences that may occur in more severe environmental temperatures like summer heat waves or saunas where the evaporation of sweat is limited.

Based on the present data, the mechanisms underlying the effect of these drugs on thermoregulation remain speculative. However, the consistency of findings across the present and previous studies (Holowatz et al. 2010a) using different modes of passive heating demonstrate altered mechanisms of temperature regulation and/or thermal balance with ASA and CLO during passive heat stress. A similar pattern of altered thermoregulatory responses is observed during the luteal phase of the menstrual cycle or with synthetic progesterone administration (Brooks-Asplund, Cannon and Kenney 2000, Charkoudian and Johnson 1997, Charkoudian and Johnson 1999). Because progesterone shifts the threshold for reflex vasodilation to higher mean body temperatures but does not alter the sensitivity of the response, this has been interpreted as an alteration in central thermoregulatory control, i.e. shifting the "set point" for activation of peripheral vasodilator skin sympathetic nerve activity (Nadel et al. 1970). Our data suggest that ASA and CLO may alter central hypothalamic thermoregulatory control, despite evidence for minimal transfer of CLO across the blood brain barrier in tissue distribution studies of CLO in rat models (Herbert et al. 1993). In addition to the direct drug actions of these antithrombotic drugs across the blood brain barrier, the central resetting for the onset of active vasodilation while undergoing ASA or CLO therapy could be mediated by altered neural afferent nerve signaling to the hypothalamus (Bradford et al. 2007). However, little is known about the afferent control of skin blood flow and much research in this area remains to be done.

In addition to central alterations in the regulation of body temperature, ASA and CLO could be exerting their effects peripherally through neural or humoral signaling to the cutaneous microvasculature. One putative mechanism for the reduction in skin blood flow with ASA and CLO is through their ability to reduce platelet activation. ASA and CLO independently attenuate platelet activation for the life of the platelet (~10 days). Specifically, ASA acetylates COX-1 in the portal circulation thereby inhibiting COX-mediated PGH_2 and thromboxane synthesis (Patrono et al. 1985) while CLO is metabolized in the liver and inhibits P_2Y_{12} ADP platelet

surface receptors (Hirsh et al. 1995). During hyperthermia platelets may be activated neurogenically through sensory nerves or platelet vessel wall interactions causing the release of platelet derived vasodilators such as 5-hydroxytryptamine (5-HT) and adenosine diphosphate (ADP) from platelet dense core granules (Holowatz et al. 2010a). The release of platelet-derived vasodilators may elicit endothelium-dependent vasodilations in a similar fashion as acetylcholine. Supporting peripheral neurogenic platelet vessel wall interactions, the axon reflex mediated neurogenic inflammation created by applying an anodal current to the skin is reduced with platelet COX-1 inhibition (Rousseau et al. 2008).

Other putative means of altering skin blood flow with ASA or CLO may be via altering direct cytokine and prostaglandin (ASA) signaling across the blood-brain barrier or through attenuation of local production of the latter pyrogenic molecules. In the present study, 81mg of ASA was chosen because it does not reach the vascular endothelium in significant concentrations or for an adequate period of time to fully inhibit vascular COX-1, like that of higher dose ASA regimens (600 mg), limiting COX-1 inhibition to the platelet (Patrono et al. 1985). In a previous study, localized vascular nonspecific COX inhibition with ketorlac did not alter cutaneous vasodilation during whole body heating in a water perfused suit, suggesting that local vascular COX was not involved in the attenuated reflex vasodilation observed with ASA therapy (Holowatz et al. 2009).

ASA may alter central or peripheral thermoregulatory mechanisms by increasing systemic exposure to low concentrations of its active metabolite salicylate. Jacobson and Bass demonstrated that oral treatment with much higher doses of sodium salicylate increased sweating rate in a compensable environment and tended to increase rectal temperature (T_{re}) in an uncompensible environment ($P<0.07$) (Jacobson and Bass 1964). Furthermore, salicylate attenuated the decrease in T_{re} with heat acclimation (Bass and Jacobson 1965). However, due to the low concentration of salicylate in the dose of ASA used in the present study, the direct action of salicylate on thermoregulatory effector mechanisms is likely minimal.

There have been a few other studies that have examined the effects of higher doses of ASA on body temperature during exercise that have found no effect on body temperature in thermoneutral (Downey and Darling 1962) and hot environments (Goldsmith, Fox and Hampton 1967). In the present study body temperature was elevated after 40 minutes of passive heat exposure resulting in the maintenance of higher body temperatures throughout exercise. The

aforementioned studies used up to 60 fold higher concentrations of ASA over a shorter duration which alters the mechanism of action of ASA through inhibition of both platelet and vascular endothelial COX. In addition, the environmental conditions, exercise modality, and means of assessing core temperature were different. In the present study, we measured esophageal temperature, which is considered to be the gold standard for assessing core temperature and has a high degree of sensitivity to measure small changes in the temperature of the blood that is perfusing the hypothalamic thermoregulatory control centers. These other studies used rectal (Downey and Darling 1962) and tympanic temperatures(Goldsmith et al. 1967), which are known to be influenced by lower body exercise, and have a significantly great lag time and variability (tympanic temperature).

Other anti-inflammatory drugs that have minimal antiplatelet effects, but alter prostaglandin production, have been used to study temperature regulation during exercise and whole body heat stress. Rofecoxib, a specific COX-2 inhibitor, *reduced* rectal and body temperature during treadmill exercise in a warm environment (Bradford et al. 2007), whereas acute ibuprofen, a reversible non-selective COX 1 and 2 inhibitor (Haag et al. 2008) did not alter the onset of reflex vasodilation during whole body heat stress in a water-perfused suit (Charkoudian and Johnson 1999). Similarly, these studies used different anti-inflammatory drugs that affect prostaglandin synthesis with a different mechanism of action, likely resulting in the disparate results. Further studies are needed to determine the effects of various dosages of anti-inflammatory medications on the control of body temperature.

Within our group of 13 participants, we detected greater variation in the CLO temperature data compared to ASA (Figure 5-1). Both of these medications have large inter- and intra-individual variability on their effectiveness as platelet inhibitors (Ellis et al. 2009, Momary and Dorsch 2010), and potential alterations in drug metabolism may have accounted for intra-individual variability. For example, CLO metabolism between individuals is variable due to genetic variations in the cytochrome P450 enzymes which metabolize the pro-drug clopidogrel to its active metabolite, and can also be affected by other nutritional factors that can either inhibit or induce drug metabolism by cytochrome P450 enzymes (Ma et al. 2010, Uchiyama 2011). In the present study subjects were instructed to maintain their normal diet and avoid the most common nutritional inhibitors of the cytochrome P450 enzymes including grapefruit juice. Within our subject group there were some individuals who exhibited more dramatic attenuation in $\%CVC_{max}$

and also had an increased rate of rise in T_{es} during exposure to mild thermal stress with CLO therapy, while others were relatively nonresponsive. Based on our power calculations a sample size of 10 subjects was needed to observe a meaningful physiological difference in skin blood flow (~12% CVC_{max} difference). Due to the large number of patients using CLO, a larger scale study may result in a more marked effect of this drug on thermoregulatory outcomes during exercise in the heat.

Limitations

Before participants entered the heated environmental chamber T_{or} was carefully measured in a thermoneutral antechamber and was not significantly different among trials. Once the participant entered the environmental chamber they were instrumented with an esophageal probe and T_{es} was measured. During esophageal probe placement T_{es} was already raised above placebo (Figure 5-2). We have corroborating data from our lab using the water-perfused suit model to induce passive heat stress that demonstrates that there was no significant difference in baseline T_{or} between platelet inhibitor treatments when measured continuously throughout heating (unpublished data).

We intentionally tested healthy subjects who have no underlying cardiovascular disease and who did not take ASA or CLO as a logical extension from our original studies. Because these drugs are intended for the primary and secondary prevention of thromboembolic disease our results may be significantly different had we tested the thermoregulatory effects of these drugs in populations with cardiovascular disease. However, following up on our previous observations our aim in the current study was to examine the functional thermoregulatory and cardiovascular consequences of the two most commonly used antithrombotic medications regimens during a hyperthermic stress in a more natural heat stress environment. Even though healthy subjects were tested these data are relevant because many healthy individuals engage in prophylactic ASA therapy.

In conclusion, oral administration of ASA and CLO treatment resulted in a higher core temperature during passive exposure to warm ambient temperatures. This elevation in core temperature persisted throughout exercise heat stress and into recovery. Furthermore, both antithrombotic drugs resulted in a threshold shift for the onset of reflex cutaneous vasodilation.

While the mechanisms underlying the increase in core temperature remain speculative, the consistency of findings across two studies with differing modes of heating provides substantial evidence for the effects of ASA and CLO on human temperature regulation. From a functional perspective, these data highlight the need for future work examining the effects of these commonly used antithrombotic regimens on thermoregulatory effector mechanisms during passive exposure to high heat and humidity such as saunas and/or hot tubs.

Table **5-1**. Subject Characteristics. Values are presented as mean±SE.

Sex (Men, Women)	7,7
Age (Years)	55±1
Body Mass Index ($kg \cdot m^{-2}$)	25.2±0.7
Mean Arterial Pressure (mmHg)	90±2
Total Cholesterol ($mg \cdot dL^{-1}$)	178±9
High-Density Lipoproteins ($mg \cdot dL^{-1}$)	60±3
Low-Density Lipoproteins ($mg \cdot dL^{-1}$)	101±7
Fasting Blood Glucose ($mg \cdot dL^{-1}$)	94±2
Glycosylated Hemoglobin (HbA1c, %)	5.5±0.1
$\dot{V}O_{2peak}$ ($L \cdot min^{-1}$)	2.32±0.21
$\dot{V}O_{2peak}$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	29.5±1.7

Table **5-2**. Subject Characteristics. Values are presented as mean±SE. *Significant difference versus placebo (P<0.05). VF, volitional fatigue; HRL, heart rate limit (90% HR max); T_{es} limit, esophageal temperature limit (39°C).

After 40 Minutes of Passive Heating	**Placebo**	**ASA**	**CLO**
Mean Arterial Pressure (mmHg)	85±2	89±1*	86±1
Mean Skin Temperature (°C)	34.7±0.1	34.5±0.2	34.7±0.2
Thermal Sensation (0-10 Scale)	4.2±0.1	4.2±0.1	4.5±0.1
At the End of Exercise	**Placebo**	**ASA**	**CLO**
Reason for Terminating Exercise	7VF, 4HRL, $2T_{es}$ limit	7VF, 4HRL, $2T_{es}$ limit	7VF, 4HRL $1T_{es}$ limit
Exercise Duration (minutes)	68±6	71±6	65±7
Esophageal Temperature (°C)	38.1±0.1	38.3±0.1	38.3±0.1
Heart Rate (beats•min^{-1})	143±3	143±4	144±4
Mean Arterial Pressure (mmHg)	95±3	97±3	95±2
Mean Skin Temperature (°C)	35.0±0.2	35.0±0.2	35.1±0.2
Change in Plasma Volume (%)	-10.5±0.6	-11.4±0.7	-10.4±0.9
Sweating Rate (g•h^{-1})	766±157	767±128	940±202
Thermal Sensation (0-10 Scale)	6.9±0.2	7.1±0.2	7.1±0.1
Borg Rating of Perceived Exertion	17±1	17±1	18±1

Figure 5-1

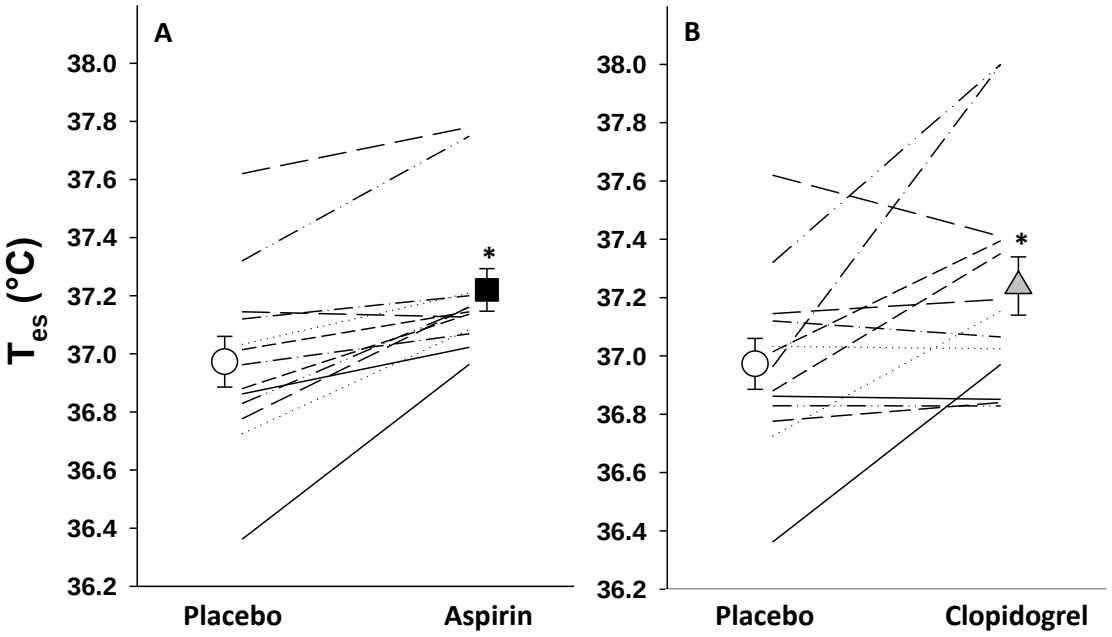


Figure **5-1**. Individual differences in T_{es} between placebo and systemic drug treatments after 40 minutes of mild passive heat stress (30°C, 40% relative humidity) in an environmental chamber. Mean responses±SE for low-dose ASA (graph A) and CLO (graph B) compared with placebo trials. Both low-dose ASA and CLO significantly increased T_{es} versus placebo ($P<0.05$).

Figure 5-2

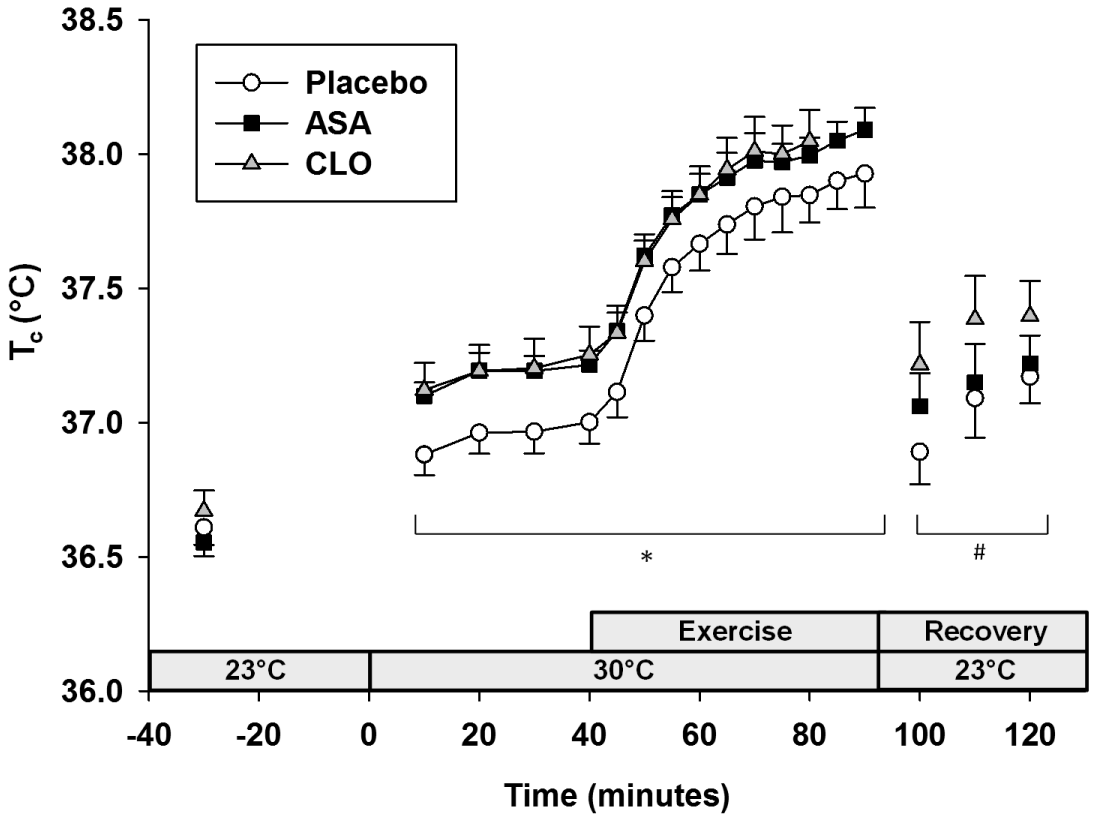


Figure **5-2**. Temperature profile±SEM during thermoneutral rest (23°C), passive warm ambient air exposure (30°C, 40% relative humidity), exercise (60% $\dot{V}O_{2peak}$ on a recumbent cycle ergometer) in a warm environment, and the recovery period after exercise (23°C) during systemic low-dose ASA, CLO, and placebo trials. In thermoneutral conditions before exercise, there was no significant difference in T_{or} with ASA or CLO therapy versus placebo. ASA and CLO treatment resulted in higher T_{es} during passive heat stress (0-40 min), which persisted throughout exercise (time= 40-90 min) in a warm environment. CLO therapy resulted in higher T_{es} during the recovery period after exercise versus ASA and placebo. Data are shown during exercise until 25% of subjects terminated exercise (n≥4). *Significant difference of drug treatment versus placebo (P<0.001). #Significant difference versus CLO (P<0.05).

Figure 5-3

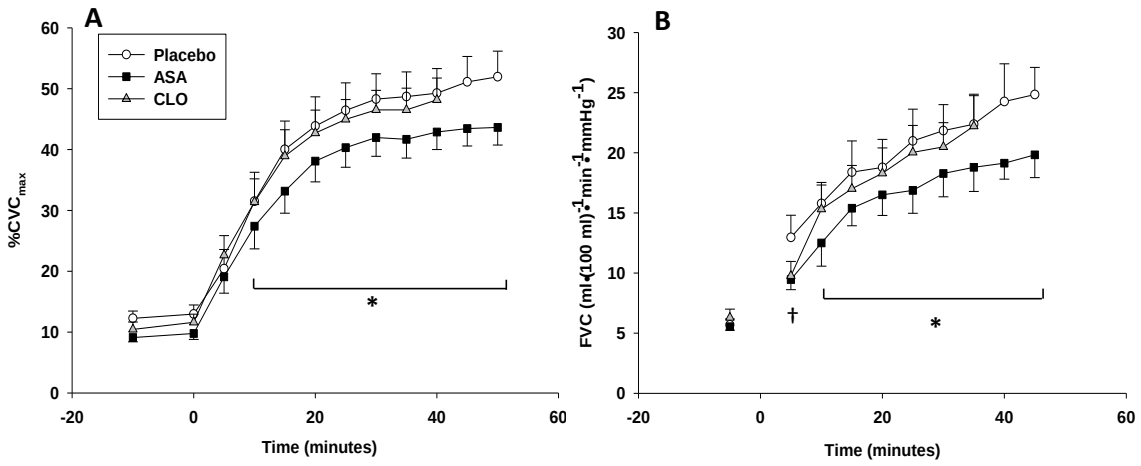


Figure **5-3**. Indices of skin blood flow measured by (A) laser-Doppler flowmetry and (B) venous occlusion plethysmography after 40 min of resting warm air exposure (30°C, 40% relative humidity) and exercise (60% $\dot{V}O_{2peak}$ on a recumbent cycle ergometer) in a warm environment. During resting warm air exposure, there was no significant difference in CVC (%CVC_{max}) or forearm vascular conductance (FVC) with low-dose ASA and CLO treatment versus placebo. After the initiation of exercise (40-90 min), ASA therapy resulted in lower %CVC_{max} and FVC responses versus CLO and placebo. Data are shown during exercise until 25% of subjects terminated exercise (n≥4). *Significantly different versus ASA (P<0.05) (main effect for FVC data). †Significantly different versus placebo (P<0.05).

Figure 5-4

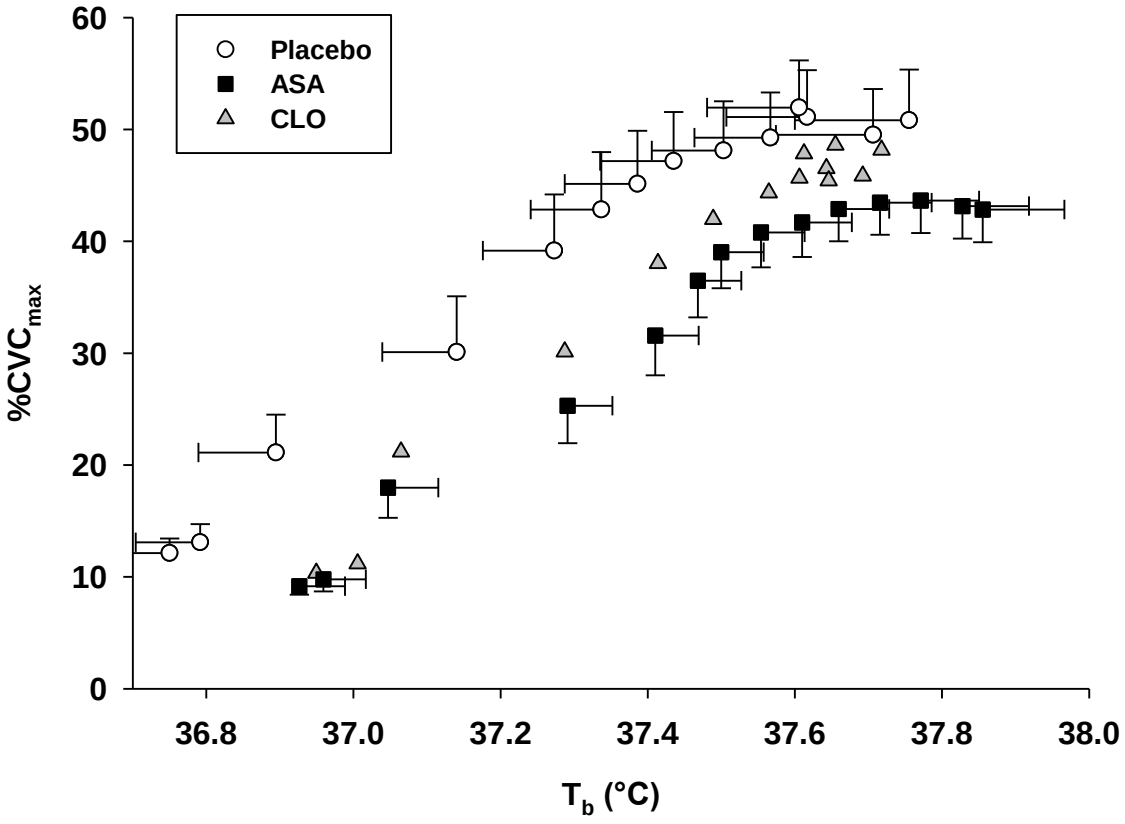


Figure **5-4**. CVC±SEM as a percentage of maximum (%CVC_{max}) versus mean body temperature ($\overline{T}_b$). Low-dose ASA and CLO resulted in a rightward shift for the onset of reflex vasodilation ($P<0.05$). Error bars were omitted for CLO trials for clarity.

Chapter 6

CONCLUSION AND FUTURE DIRECTIONS

The three studies comprising this dissertation utilized the cutaneous circulation to elucidate the mechanisms mediating nitric oxide (NO)-dependent vasodilation in middle-aged and essential hypertensive men and women, to characterize how skin blood flow is altered with advancing age, and to examine the functional thermoregulatory and cardiovascular implications of using common antithrombotic agents during both rest and exercise in the heat. Specifically, these studies (1) elucidated the nitric oxide synthase (NOS) isoforms responsible for mediating cutaneous NO-dependent vasodilation elicited from local skin heating and acetylcholine perfusion in middle-aging, (2) examined how essential hypertension alters the components mediating skin flowmotion during physiological (local skin heating) and pharmacological examination of NO-dependent mechanisms, (3) and examined the functional thermoregulatory and cardiovascular implications of using systemic antithrombotic therapies during rest and exercise in a hot environment. This chapter is intended to summarize the results of each of these three studies and discuss future research directions to further our understanding of how aging, essential hypertension, and antithrombotic therapies affect cutaneous microvascular function.

Nitric Oxide Synthase Isoforms and Local Cutaneous Vasodilation

The primary finding of this study was that endothelial NOS (eNOS) mediates cutaneous NO-dependent vasodilation during local heating and perfusion of endothelial-dependent agonist acetylcholine in healthy young and middle-aged men and women. During local skin heating NO derived from iNOS contributed to the sensory nerve-mediated axon reflex, suggesting that local skin heating may result in minor neurogenic inflammation. Further, modest deficits in functional NO-dependent cutaneous vasodilation were detectable in healthy middle-aged humans. There were no differences in expression of the NOS isoforms or downstream vasodilatory molecular targets, suggesting that the mild deficits in NO-dependent vasodilation observed in the middle-aged men and women were likely due to changes in NO metabolism or NOS activity.

Implications

These findings further elucidate the mechanisms mediating cutaneous vasoreactivity during local skin heating in healthy young and middle-aged men and women. Kellogg *et al*. found that local skin heating was mediated by eNOS-dependent mechanisms, whereas reflex cutaneous vasodilation was mediated by neuronal NOS (nNOS)-dependent mechanisms in the forearm skin of healthy, young men and women (Kellogg et al. 2008a). In essential hypertensive humans, Smith *et al*. found that eNOS-dependent mechanisms mediated cutaneous vasodilatory responses to local skin heating and acetylcholine perfusion; however, these eNOS-dependent mechanisms were attenuated due to upregulated iNOS activity. Conversely, Stewart *et al.* found that nNOS-dependent mechanisms mediated the local skin heating response in the calf skin of postural orthostatic tachycardia syndrome (POTS) women. This study supports the work by Kellogg *et al.* and suggests that in healthy young and middle-aged men and women eNOS-dependent mechanisms mediate cutaneous vasodilation during local skin heating. We also have pilot data in four young healthy subjects demonstrating that nNOS-dependent mechanisms play a large role in mediating reflex cutaneous vasodilation (see Figure 6-1). In summary, our findings support the use of the cutaneous circulation as a test of microvascular function because we were able to detect functional deficits in endothelium-dependent vasodilation prior to the development of cardiovascular risk factors and detectable changes in NOS protein expression in healthy middle-aged men and women.

Future Directions

1) Our data suggest that eNOS-dependent mechanisms mediate cutaneous vasodilation during local skin heating and perfusion of endothelium-dependent agonists in forearm skin. There is evidence to suggest that nNOS and eNOS have distinct physiological roles, facilitating alterations in basal vasomotor tone and blood flow distribution, respectively (Melikian et al. 2009). The findings that nNOS-dependent mechanisms mediate cutaneous vasodilation during local calf skin heating in POTS patients suggests that there may be pathological changes in NOS-mediated vasodilation in certain patient populations and/or regional skin differences in the NOS isoforms regulating cutaneous vasodilation.

2) This study found that middle-aged subjects had heightened skin blood flow after non-specific NOS inhibition, suggesting that redundant non-NO vasodilator pathways may be upregulated in

this age-group. The balance between NO and EDHF-dependent vasodilation throughout the aging spectrum should be further investigated during local skin heating, which is a vasoreactivity test known to be largely mediated by NO with the remaining vasodilation due to endothelium-derived hyperpolarizing factor (EDHF)-dependent mechanisms. In addition, future studies could examine the role of NOS isoforms, specifically inducible NOS (iNOS), in older men and women (>65), who exhibit a greater attenuation in NO-dependent vasodilation.

3) In this study iNOS inhibition attenuated the initial peak in healthy young and middle-aged men and women. However, in essential hypertensives iNOS inhibition augments the initial peak versus age-matched normotensives (Smith et al. 2011b), suggesting that neurogenic inflammation of sensory and/or sympathetic nerves may play a role in microvascular dysfunction with aging and cardiovascular diseases.

Altered Skin Flowmotion in Essential Hypertension

The principle finding of this study was that essential hypertension reduced the total power spectral density (PSD) contributing to skin flowmotion during local skin heating, specifically in the in the frequency intervals corresponding to neurogenic control of skin flowmotion. During local skin heating when the frequency intervals were normalized to total PSD, essential hypertensives did not exhibit an increase in neurogenic signaling and had a significantly higher myogenic contribution to skin flowmotion versus age-matched normotensive men and women. These findings suggest that essential hypertension may result in impaired sympathetic and/or sensory nerve activation during local skin heating and have increased intrinsic myogenic control of skin flowmotion. After NOS inhibition, essential hypertension reduced endothelial, neurogenic, and total PSD suggesting that they have reduced NO-independent redundant vasodilator mechanisms contributing to skin flowmotion during local skin heating.

Implications

This study furthered our knowledge of NO-dependent control of skin flowmotion after local skin heating. We specifically examined the components responsible for regulating skin

flowmotion after baseline, local skin heating, perfusion of a pharmacological NOS inhibitior, and after a NO donor. Essential hypertension attenuated the increase in total PSD and neurogenic control of skin flowmotion during local skin heating compared to age-matched normotensives. These findings corroborate and add to what has been found in previous studies that have examined cutaneous vascular responses during local skin heating in essential hypertension. Specifically, essential hypertensive men and women exhibited lower skin blood flow during the plateau phase of local skin heating, which is partially due to reduced eNOS-dependent vasodilation caused by upregulated iNOS activity (Smith et al. 2011b). In the previous study, the source of eNOS-dependent NO-production was hypothesized to be from the cutaneous endothelium because eNOS-mediated the vasodilatory response to perfusion of the endothelium-dependent agonist, acetylcholine. However, the role of sensory and/or sympathetic nerves was not examined so the effects of neurogenic activation of eNOS were unknown. Therefore, our findings that essential hypertension significantly attenuates neurogenic control of skin flowmotion during local skin heating are novel. It should be noted that we did observe a trend in reduced endothelial contribution to local skin heating ($p=0.07$), which may not have reached significance due to our limited number of subjects studied ($n=18$) when power calculations suggested an $n=23$ to identify a 25% difference in the frequency intervals contributing to skin flowmotion. Conversely, the findings that essential hypertension reduced total PSD and neurogenic signaling during local skin heating are in contrast to the findings of Gryglewska *et al*., who did not detect any differences in skin flowmotion during local skin heating in unmedicated essential hypertensive men and women (Gryglewska et al. 2010a). During the latter study, local skin heating was only performed for eight minutes, which is not enough time for full expression of cutaneous vasodilation during the given heating protocol and may account for these discrepant results.

The novelty of this study includes an examination of NO-dependent control of skin flowmotion with spectral analysis in essential hypertensive men and women. After NOS inhibition during local skin heating, essential hypertension resulted in a lower endothelial, neurogenic, and total PSD suggesting that they have reduced NO-independent redundant vasodilator mechanisms contributing to skin flowmotion during local thermal stress. However, non-NO and EDHF-mediated cutaneous vasodilator mechanisms have not yet been examined in essential hypertensive men and women *in vivo*. Finally, in newly hypertensive men and women we were able to restore total PSD and improve the frequency interval corresponding to

endothelial control of skin flowmotion in essential hypertensive men and women with an NO donor sodium nitroprusside, demonstrating that neurogenic and endothelial control of cutaneous vasodilation occurs in the microcirculation before significant vascular smooth muscle dysfunction and remodeling.

Future Directions

1) These data suggest that essential hypertension significantly reduces the total PSD and neurogenic response to local skin heating. We had previously found that eNOS-dependent production of NO was reduced with upregulated iNOS function. Together these results suggest that essential hypertensive humans may have chronic inflammation which may 1) desensitize the sympathetic and/or sensory nerves to stimuli such as heat, 2) cause chronic neurogenic inflammation and thus reduce post synaptic control of neurogenic vascular signaling, 3) increase oxidative stress causing eNOS-uncoupling and reduce NO production during neurogenic activation of NO-dependent pathways, and/or 4) increase oxidative stress creating a favorable environment for the generation of pro-constrictor endothelium derived contracting factors. Future studies should examine the role of sensory nerves and inflammation on endothelial dysfunction in essential hypertensive men and women.

2) We have found that essential hypertensive men and women have a lower endothelial, neurogenic, and total PSD during NOS inhibition. These data suggest that in addition to attenuated NO-dependent vasodilation, essential hypertension reduces the redundant pathways mediating cutaneous vasodilation during local skin heating. More mechanistic studies are needed to examine how EDHF-dependent vasodilation may be altered with newly, untreated essential hypertension to elucidate some of the pathological changes that occur during the development of this hypertension.

3) In the present study we focused on NO-dependent control of skin flowmotion. Since iNOS function is upregulated in essential hypertension and is likely involved in reducing eNOS-dependent production of NO, future studies should use spectral analysis to examine how iNOS inhibition affects the mechanisms regulating skin flowmotion during local skin heating. In addition, future studies could examine how antihypertensive therapies affect the regulation of skin flowmotion during local skin heating.

4) In the present study we used a standardized local skin heating vasoreactivity test to evaluate NO-dependent mechanisms regulating skin flowmotion; however, alternative skin-specific vasoreactivity tests can be used to examine different mechanisms mediating microvascular function. Local skin cooling elicits vasoconstriction, which becomes less adrenergic and more RhoA/Rho-Kinase (ROCK) dependent in essential hypertensive humans, which is reflective of a compensatory pathway to preserve vasoconstriction. ROCK is inhibitory towards eNOS pathways, and can disrupt the balance between the two systems creating a pro-constrictor vascular state (Smith, Santhanam and Alexander 2013, Holowatz and Kenney 2010). Therefore, spectral analysis of the skin blood flow response to local skin cooling may provide a means to assess altered endothelial and myogenic regulation of skin flowmotion in essential hypertensive humans and other populations with known microvascular dysfunction.

Thermoregulatory and Cardiovascular Consequences of Platelet Inhibition during Passive Rest and Exercise in the Heat

The primary finding of this study was that systemic low dose aspirin (ASA, 81mg) and clopidogrel bisulfate (Plavix®, 75mg) resulted in higher core temperatures during passive exposure to warm ambient temperatures, which persisted throughout exercise heat stress into recovery in healthy, middle-aged men and women. ASA and clopidogrel altered thermoregulatory effector mechanisms by increasing the threshold for the onset of reflex cutaneous vasodilation. However, the slope of the skin blood flow: body temperature relation was not different with ASA and CLO treatment during exercise. These data suggest that antithrombotic therapy does not change the sensitivity of the skin blood flow: body temperature relation but rather alters central thermoregulatory control shifting the “set point” for active cutaneous vasodilation to higher body temperatures. Core temperatures were elevated after passive heat stress with clopidogrel, therefore, differences in skin blood flow as a function of time may not have been observed because of the greater stimulus for increased skin blood flow with clopidogrel treatment.

In contrast to the thermoregulatory outcomes of this study, ASA and clopidogrel did not affect any of the cardiovascular outcomes (heart rate and blood pressure) or performance variables (rating of perceived exertion, thermal sensation, or exercise duration) associated with exercise and thermal stress. However, the environmental heat stress used in the present study was mild and may not have been great enough to examine the magnitude of thermoregulatory and

cardiovascular outcomes that may be observed in hot climates or in certain occupational settings. From a functional perspective, these data highlight the need for future work examining the effects of these commonly used antithrombotic therapies on thermoregulatory effector mechanisms during rest and work in hot humid environments.

Implications

The findings that ASA and clopidogrel resulted in higher core temperatures during passive heat stress corroborates previous studies examining these antithrombotic agents during whole body heating in a water perfused suit (Holowatz and Kenney 2009, Holowatz et al. 2010a). In the present study mild passive heat stress resulted in elevated core temperatures with ASA and clopidogrel therapy. Previous studies used a water-perfused suit, which is an unnatural environment resulting in a reversal of the core-to-skin temperature gradient causing increased blood flow to actually be detrimental to temperature regulation. In contrast to previous studies (Holowatz et al. 2010a, Holowatz and Kenney 2009), we did not detect any differences in skin or forearm blood flow with ASA and clopidogrel therapy. However, core temperature was not elevated to the same extent as the studies using a water perfused suit. In the present study we may not have reached the core temperature threshold for reflex cutaneous vasodilation. The results from the present and previous studies demonstrate that healthy, middle-aged men and women who use low dose aspirin for primary prevention of atherothrombotic disease may be at a greater risk for heat related injuries in situations where they are exposed to passive heat stress such as sitting in a hot tub or a sauna.

ASA and clopidogrel did not alter the rate of heat gain or sensitivity of the skin blood flow response to changes in body temperature or any of the cardiovascular and performance values during exercise. However, core temperatures were elevated during exercise with ASA and clopidogrel therapy due to the previous passive heat exposure and the onset of reflex vasodilation was shifted to higher body temperatures suggesting a difference in central thermoregulatory control. In addition, recovery core temperatures remained elevated in the clopidogrel trial, suggesting that exercise after heat exposure or during repeated bouts of work/exercise in the heat could result in greater heat storage with clopidogrel, resulting in negative cardiovascular or thermoregulatory outcomes.

Future Directions

1) We have found that ASA and clopidogrel therapy elevated core temperatures during forty minutes of passive exposure to warm air, and that core tempertures remained elevated throughout the subsequent exercise heat stress into recovery in healthy, middle-aged men and women. Future studies are needed to realize the full thermoregulatory and cardiovascular consequences of these antithrombotic therapies during passive exposure to high heat and humidity such as saunas or hot tubs. In addition, the functional thermoregulatory effects of these antithrombotic agents should be studied while performing work in uncompensable, hot, humid environments which are similar to some occupational working environments.

2) Clopidogrel therapy resulted in elevated core temperature during the exercise recovery period in a thermoneutral environment. The effects of ASA and clopidogrel therapy during repeated bouts of exercise should be examined to determine the functional thermoregulatory and cardiovascular outcomes of these commonly used antithrombotic therapies.

3) The present study examined the functional thermoregulatory and cardiovascular consequences of ASA and clopidogrel therapy in *healthy,* middle-aged men and women. These studies should be performed in populations with known atherothrombotic disease, such as those who are using these medications for the secondary prevention of stroke, to determine the functional thermoregulatory and cardiovascular implications for those who are already at a higher risk for heat-related morbidities.

Figure 6-1

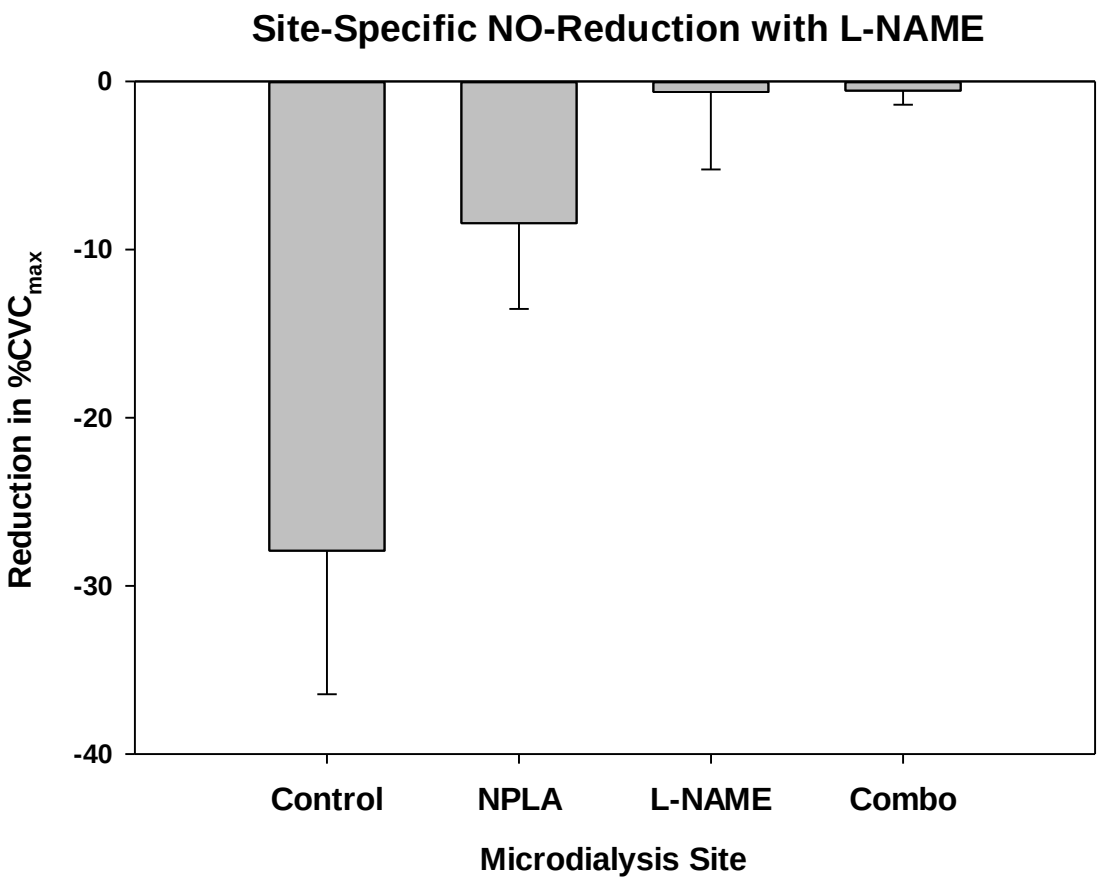


Figure **6-1**. Pilot data examining the reduction in CVC as a percentage of maximum (%CVC_{max}) with specific nitric oxide synthase (NOS) inhibitors during a 1°C rise in oral temperature in four young, healthy women. Values are mean±SE. In these four subjects, non-specific NOS inhibition showed the largest reduction in %CVC_{max}. Neuronal NOS inhibition with NPLA also reduced %CVC_{max}, whereas inducible NOS and the control site did not exhibit a reduction in %CVC_{max}.

Appendix A: NOVEL METHODOLOGY FOR ASSESSING AGE-RELATED CHANGES IN CUTANEOUS VASODILATION DURING LOCAL SKIN HEATING

Healthy human aging in the absence of overt cardiovascular disease reduces the capacity for cutaneous vasodilation during local skin heating (Minson et al. 2002, Martin et al. 1995) and is reflective of generalized age-associated microvascular dysfunction (Holowatz 2008b, Minson 2010b, Holowatz and Kenney 2010). Assessment of the skin blood flow response during rapid local skin heating is usually averaged over time and not over the physiological stimulus driving the response (i.e. skin temperature). Impairments in the skin blood flow response during local skin heating are interpreted as reductions in the initial peak and secondary plateau, which is predominantly mediated by nitric oxide (NO)-dependent mechanisms. Aging results in an attenuation of NO-dependent vasodilation during local skin heating. However, the effects of aging on the onset of local cutaneous vasodilation and rate of increase in skin blood flow as a function of skin temperature have not been examined. When the skin blood flow response is plotted against skin temperature there is an exponential relation. The following section proposes the use of exponential growth models to characterize the skin blood flow response to changes in local skin temperature as a novel way to assess cutaneous vasoreactivity. Further, we propose to inhibit nitric oxide synthase (NOS) with the non-specific NOS inhibitor (L-N^G-nitroarginine methyl ester; L-NAME) to examine age-related effects in NO-dependent vasodilation using exponential growth models.

Methods

Subjects

All 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 were voluntarily obtained from all subjects prior to participation. Fourteen healthy young and twelve healthy old men and women participated in the slow local heating protocol during this study. Of these participants, ten young and ten older subjects underwent functional *in vivo* assessment of cutaneous NO-dependent vasodilation during the slow local skin heating protocol which involved the use of intradermal microdialysis (MD) fibers. The remainder of the participants underwent the direct local skin heating protocol without

the use of MD fibers. All subjects were nonobese, nonsmokers, nondiabetic, and not currently taking cardiovascular medications and supplements including aspirin, vitamins, and antioxidants. Three of the young women were taking oral contraceptives and were tested in the low hormone phase of their medication. All women were tested in the follicular phase of their menstrual cycle (day 1-7) or were postmenopausal and not taking hormone replacement therapy. Subject characteristics are presented in Table A-1. By study design, there was a difference in age in the young and older groups ($P<0.001$). The older subjects had higher HbA1c values ($P<0.001$) and LDL cholesterol ($P<0.01$) compared to young. However, the older subjects HbA1c and LDL cholesterol values were within normal healthy ranges.

In Vivo Vasoreactivity Studies

Instrumentation. All protocols were performed in a thermoneutral laboratory (22.8±0.2°C) with the subject in a semisupine position and the experimental arm supported at heart level. In the subset of twenty participants who underwent *in vivo* assessment of cutaneous NO-dependent vasodilation, two MD fibers (10 mm, 30 kDa cutoff membrane, MD 2000, Bioanalytical Systems, West Lafayette, IN) were placed in the left ventral forearm skin by sterile technique as previously described (Holowatz and Kenney 2007b, Holowatz et al. 2006a). MD sites were placed at least 4 cm apart to ensure no cross-reactivity of pharmacological agents and to allow adequate space for placement of the local skin temperature controllers. The MD fibers were randomly assigned to deliver 1) lactated Ringer's solution to serve as a control and 2) 20 mM L-NAME (Calbiochem, San Diego, CA) to inhibit NOS. L-NAME was mixed just before usage, dissolved in lactated Ringer solution, and sterilized (Acrodisc, Pall, Ann Arbor, MI). The site-specific pharmacological agents (lactated Ringer's solution and L-NAME) were perfused for 60-90 minutes at a rate of 2 $\mu L \cdot min^{-1}$ (Bee Hive controller and Baby Bee microinfusion pumps, Bioanalytical Systems) before beginning slow local heating to allow adequate time for the initial insertion trauma to subside.

Cutaneous red blood cell flux was continuously measured directly over each MD site with an integrated laser-Doppler flowmetry probe (MoorLab, Moor Instruments, Devon, UK) to obtain an index of skin blood flow. Arterial blood pressure was measured via brachial auscultation every 5 min during the experiment and mean arterial pressure (MAP) was calculated as diastolic pressure plus one-third pulse pressure. Skin blood flow was expressed as both absolute cutaneous vascular conductance (CVC=laser-Doppler flux/MAP) and normalized as a

percentage of maximum (%CVC_{max}). Local skin temperature was controlled at each site using Peltier elements (Newport Temperature Controller 350B) with a laser-Doppler probe secured through the center of the device. Average local skin temperature ($\overline{T}_{sk}$) was assessed at each site using two copper-constantan thermocouples placed directly on the skin on opposite sides of the Peltier element.

Local Heating Protocol

Following the initial MD insertion hyperemia (or upon entry to the laboratory in those who did not have MD fibers), the Peltier elements were placed on the skin and clamped at 31°C for twenty minutes of baseline recordings. After baseline measurements, the Peltier elements temperature was increased at a rate of 0.6°C·min^{-1} in a ramp fashion to thermal tolerance (maximum was set at 42°C due for safety reasons). The Peltier element was clamped at the highest skin temperature achieved until 20 minutes of stable cutaneous blood flow was obtained. In the participants who had MD fibers, the site-specific pharmacological agents were perfused throughout baseline and local skin heating. Maximum skin blood flow was assessed as the skin blood flow plateau at the end of the local skin heating in those who did not have MD fibers, or by perfusion of 28 mM sodium nitroprusside (SNP) in those who had MD fibers. SNP did not significantly increase skin blood flow at the control site in those with MD fibers; therefore, we were able to bin our normalized data together for mathematical modeling purposes. The maximum skin temperature achieved in this study was 42°C because higher temperatures caused discomfort due to the large surface area and conductivity of the Peltier elements.

Mathematical Modeling

When skin blood flow is plotted against skin temperature during slow local heating there is an exponential relation. We used exponential growth models to characterize the cutaneous vasodilatory response to local skin heating for each subject to determine intraindividuals' differences in the lowest CVC obtained during thermoneutral skin temperatures, the rate of increase in skin blood flow (sensitivity), and the onset of rapid vasodilation to changes in local skin temperature. Further, we used the individual responses to create predictive models for the skin blood flow: local skin temperature relation. A multilevel growth curve was used to examine how aging alters the parameters of the skin blood flow: local skin temperature relation. All model fitting was performed using PROC NLMIXED in SAS 9.3 (Cary, NC, USA).

Statistical Analysis

CVC data were acquired at 40 Hz, digitized and stored on a personal computer until further analysis (WinDaq; Dataq Instruments, Akron, OH). CVC values were averaged over 1°C increments in $\overline{T}_{sk}$. Maximal CVC values were averaged over a stable plateau after maximally heating the skin in those who did not have MD fibers. In those who had MD fibers, maximal CVC values were averaged over a stable plateau in laser-Doppler flux during perfusion of 28 mM SNP. NO-dependent vasodilation was assessed by subtracting CVC from the L-NAME site from the CVC values in the control site for a given $\overline{T}_{sk}$.

Student's unpaired *t*-tests were used to determine significant differences between age groups for physical characteristics. Two-way mixed-model ANOVAs were used to detect age-related differences in CVC or calculated NO-dependent vasodilation (control site minus L-NAME site) over increasing $\overline{T}_{sk}$. In the subset of participants who underwent *in vivo* assessment of NO-dependent vasodilation, a three-way repeated-measure mixed-model ANOVA was used to detect age-related and local drug treatment differences in CVC responses over increasing $\overline{T}_{sk}$ (SAS version 9.3, Cary, NC, USA). Post hoc comparisons with Bonferroni corrections were performed when necessary to determine where differences between age and local drug treatments occurred. The level of significance was set at $\alpha = 0.05$ for main effects. Values are presented as means ± standard error (SE).

Preliminary Findings

Figure A-1 shows the combined skin blood flow responses during local skin heating in young and old subjects for the combined control or no MD sites. There was no difference in the absolute CVC (panel A) or $\%CVC_{max}$ (panel B) responses between groups.

NOS inhibition with L-NAME reduced the CVC (Figure A-2) and $\%CVC_{max}$ responses at higher $\overline{T}_{sk}$ versus the control site in the subset of participants who underwent *in vivo* assessment of NO-dependent vasodilation ($P<0.0001$). However, there was no difference in the control or L-NAME sites between groups. The $\%CVC_{max}$ data were omitted for clarity, but the vasodilation elicited from local skin heating was identical in direction and magnitude as the CVC responses. Figure A-3 shows the calculated NO-dependent vasodilation during local skin heating. NO-dependent vasodilation increased with local $\overline{T}_{sk}$. There was no difference in NO-dependent

vasodilation between the young and older subjects. Further, there was no difference in the direction or magnitude of the calculated NO-dependent vasodilation when assessed as $\%CVC_{max}$, with young and older subjects exhibiting a 31±7% and 35±9% reduction in $\%CVC_{max}$, respectively at a $\overline{T}_{sk}$ of 40°C.

Exponential models were used to characterize individual cutaneous vasodilatory responses elicited from local skin heating. Skin blood flow responses were assessed as CVC for a given change in $\overline{T}_{sk}$. A multilevel model was used to model between-group differences (young vs. old) assessed from within-subject changes in cutaneous vasoreactivity. The model is a two-phase exponential growth model, with Level 1 specified as:

$$CVC_{ti} = g0_{ti} + \exp(a_{ti}((\overline{T}_{sk})_{ti} - t0_{ti})).$$

Where CVC_{ti}, indicates an individual *i*'s CVC at skin temperature *t;* $g0_{ti}$ is the lowest CVC asymptote at thermoneutral skin temperatures, a_{ti} is the acceleration or rate of increase in skin blood flow for a given change in skin temperature, $(\overline{T}_{sk})_{ti}$ is the average local skin temperature, and $t0_{ti}$ is the left-to-right shift or onset of rapid vasodilation. When age-group was used as a predictor of the CVC response, the age-related changes in the slopes and intercepts from Level 1 were modeled at Level 2 as:

$$g0_n = \gamma_{0n} + B_{01} * n + u_{0n}$$

$$a_n = \gamma_{1n} + B_{11} * n + u_{1n}$$

$$t0_n = \gamma_{2n} + B_{21} * n + u_{2n}$$

In these models, n represents age group and is equal to 0 for young and 1 for old, γ represents the average group intercept, and u represents the group-specific deviations from the means. The first subscript 0, 1, or 2 represents the parameter (i.e. $g0$, a, or $t0$, respectively) that γ or u corresponds to. Therefore, the Level 1 equation can be used to predict young cutaneous vasodilatory responses and the Level 2 equations can be used to predict older cutaneous vasodilatory responses during local skin heating.

The parameter estimates and fit statistics for the *preliminary* models are presented in Table A-2. The rate of acceleration of skin blood flow was significantly different in the young

and older subjects (P<0.001). The regression coefficients are $B_{01} = -0.05107$, $B_{11} = -0.1670$, and $B_{21} = 0.07884$. The lowest CVC and the onset for rapid vasodilation were not significantly different between groups. Figure A-4 shows the individual and group predicted skin blood flow responses to changes in $\overline{T}_{sk}$.

We propose to repeat the exponential growth modeling for the subset of subjects who participated in the *in vivo* assessment of NO-dependent vasodilation to examine the control vs. L-NAME sites for both age-groups. Preliminary combined analysis of the young and older subjects shows that L-NAME significantly increases the *g0, a,* and *t0* compared to the control site (Figure A-5). This is surprising since younger subjects had a higher acceleration of skin blood flow vs. older subjects, suggesting that a higher acceleration of skin blood flow is a beneficial response. However, since all three parameters are different in the L-NAME site, shifting the curve to the right may necessitate a higher rate of acceleration in the L-NAME sites to compensate for the delayed onset of vasodilation. We still need to determine how age affects the L-NAME sites.

Figure Legends

Figure **A-1**. Group means ± SE vasodilatory response to increasing mean local skin temperature assessed as absolute cutaneous vascular conductance (CVC) (A) and normalized to maximal blood flow (%CVC_{max}) (B). There were no age-related differences in skin blood flow during slow local skin heating.

Figure **A-2**. Group means ± SE cutaneous vascular conductance (CVC) responses to increasing local skin temperature in the control and L-NAME site for (A) young and (B) old participants. L-NAME reduced cutaneous vasodilation at higher local skin temperatures. *P<0.001, **P<0.0001 Significant difference from the control site.

Figure **A-3**. Group means ± SE for the calculated NO-dependent vasodilation (cutaneous vascular conductance (CVC)) elicited from local skin heating. NO-dependent vasodilation was calculated as the control site minus the L-NAME site. NO-dependent vasodilation increased with increasing mean local skin temperature. There was no difference in NO-dependent vasodilation elicited from slow local heating in the young or older subjects.

Figure **A-4**. Individual and group predicted CVC responses to changes in mean skin temperature elicited from slow local skin heating. (Note: this is a preliminary graph because the older individual and age-predicted responses did not coinciding with the SAS output) **P<0.001 Significant difference in the rate of increase in skin blood flow for a change in skin temperature.

Figure **A-5**. Group predicted CVC responses to changes in mean skin temperature elicited from slow local skin heating in the control and the L-NAME sites in young and old men and women. (Note: this is a preliminary graph showing the combined (young + old) differences from the control and NOS inhibited site).

Table A-1. Subject Characteristics (mean±SE).

	Young	**Old**
Subjects (male, female)	(8,6)	(5,7)
Age (years)	24±1	73±2**
BMI	24.5±1.2	25.9±0.9
Systolic BP (mmHg)	119±3	122±4
Diastolic BP (mmHg)	75±2	75±2
MAP (mmHg)	90±2	91±2
Fasting Glucose ($mg \cdot dL^{-1}$)	88±2	89±8
HbA1c (%)	5.3±0.1	5.6±0.1**
HDL ($mg \cdot dL^{-1}$)	59±5	66±5
LDL ($mg \cdot dL^{-1}$)	83±8	118±6*
Total Cholesterol/HDL Ratio	3.2±0.2	2.8±0.2
Oral Temperature	36.5±0.1	36.6±0.1

**P<0.001,*P<0.01

Table A-2. Multilevel Exponential Growth Model for the Cutaneous Vascular Conductance Response to Local Skin Heating. **P<0.001, Significantly different vs. young.

	CVC	
	B	***SE***
Fixed Effect Estimate		
Intercept, γ_{01}	0.1634	0.0201
Rate of increase in CVC, γ_{11}	0.7353**	0.0117
Onset of rapid VD, γ_{21}	39.3857	0.3108
Random Effects Estimate		
Variance intercept, σ^2_{u0}	0.0071	0.0022
Variance in rate of increase in CVC, σ^2_{u1}	0.0992	0.054
Variance in onset of rapid VD, $\sigma^2_{u2}\,\sigma^2_{u2}$, σ^2_{u2}	1.3620	0.8536
Covariance, intercept X onset of rapid VD, $\sigma_{u0,u2}$	0.0008	0.0250
Covariance, intercept X rate of increase in CVC, $\sigma_{u0,u1}$	0.0157	0.0079
Covariance, rate of increase in CVC X onset of rapid VD $\sigma_{u1,u2}$	-0.1107	0.1486
Residual variance, σ^2_e	0.0194	0.0002

Figure **A-1**.

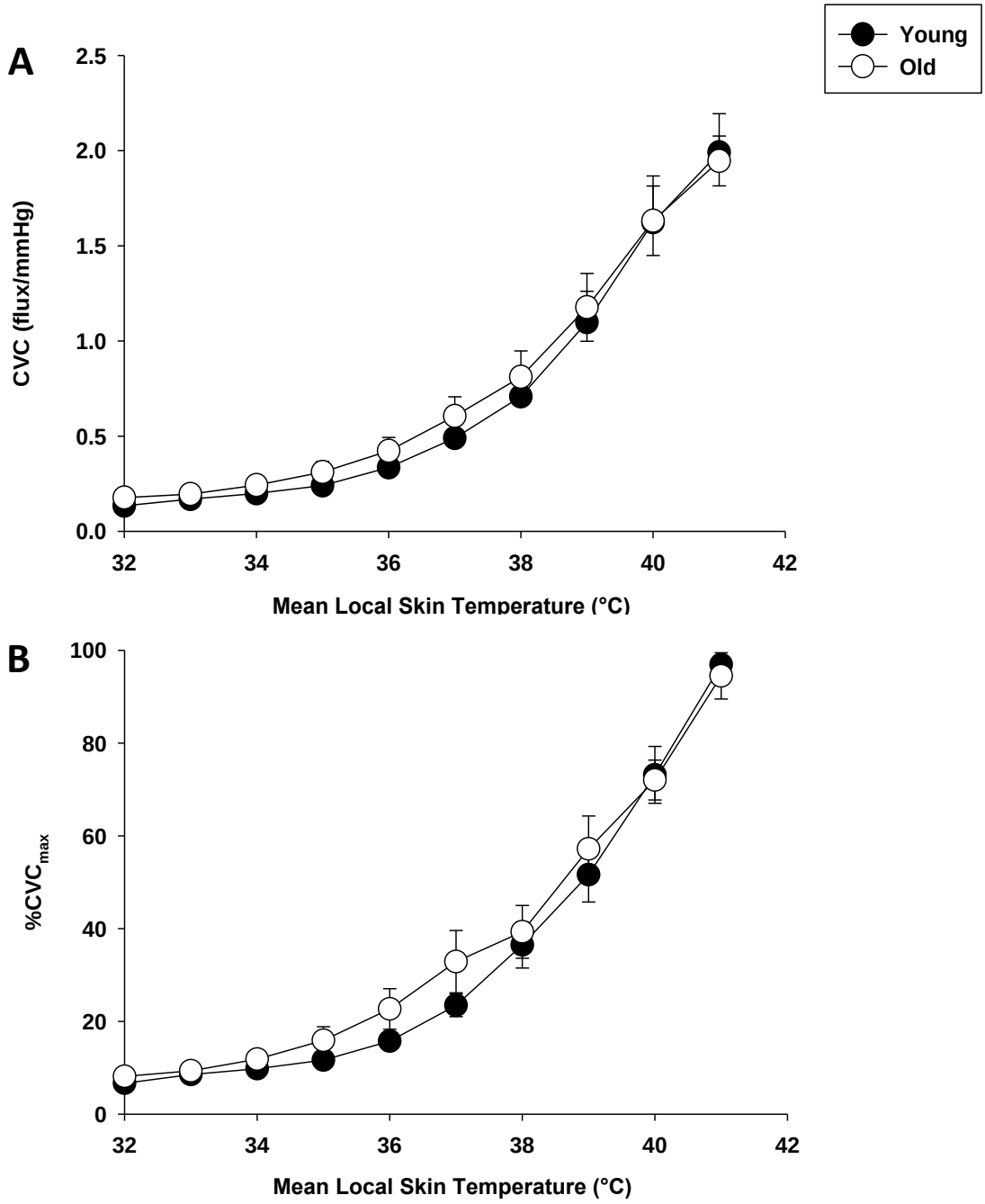

A
Young
Old
CVC (flux/mmHg)
2.5
2.0
1.5
1.0
0.5
0.0
32
34
36
38
40
42
Mean Local Skin Temperature (°C)
B
%CVCmax
100
80
60
40
20
0
32
34
36
38
40
42
Mean Local Skin Temperature (°C)

Figure **A-2**.

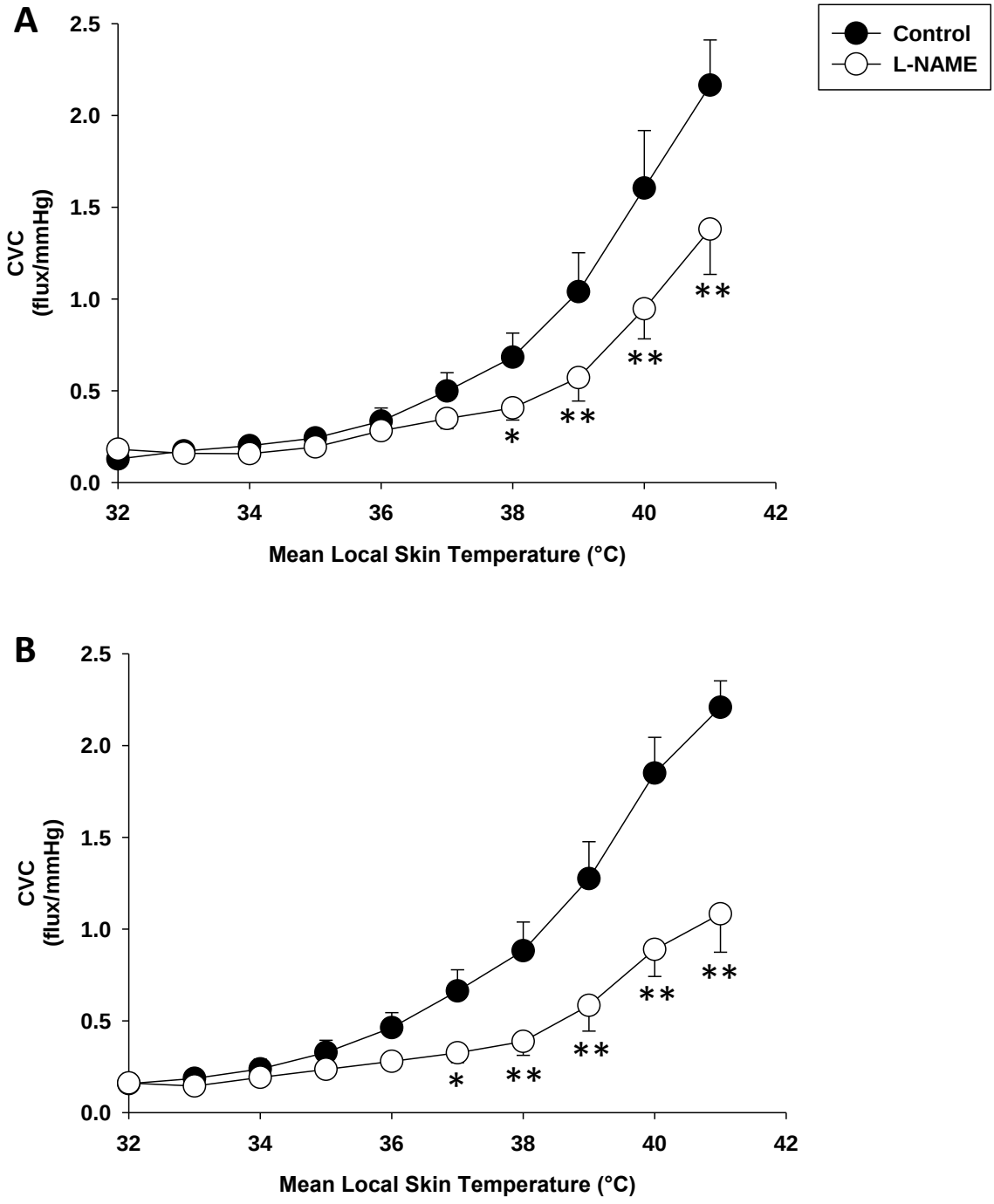

A
Control
L-NAME
CVC
(flux/mmHg)
2.5
2.0
1.5
1.0
0.5
0.0
32
34
36
38
40
42
Mean Local Skin Temperature (°C)
*
**
**
**
B
CVC
(flux/mmHg)
2.5
2.0
1.5
1.0
0.5
0.0
32
34
36
38
40
42
Mean Local Skin Temperature (°C)
*
**
**
**
**

Figure **A-3.**

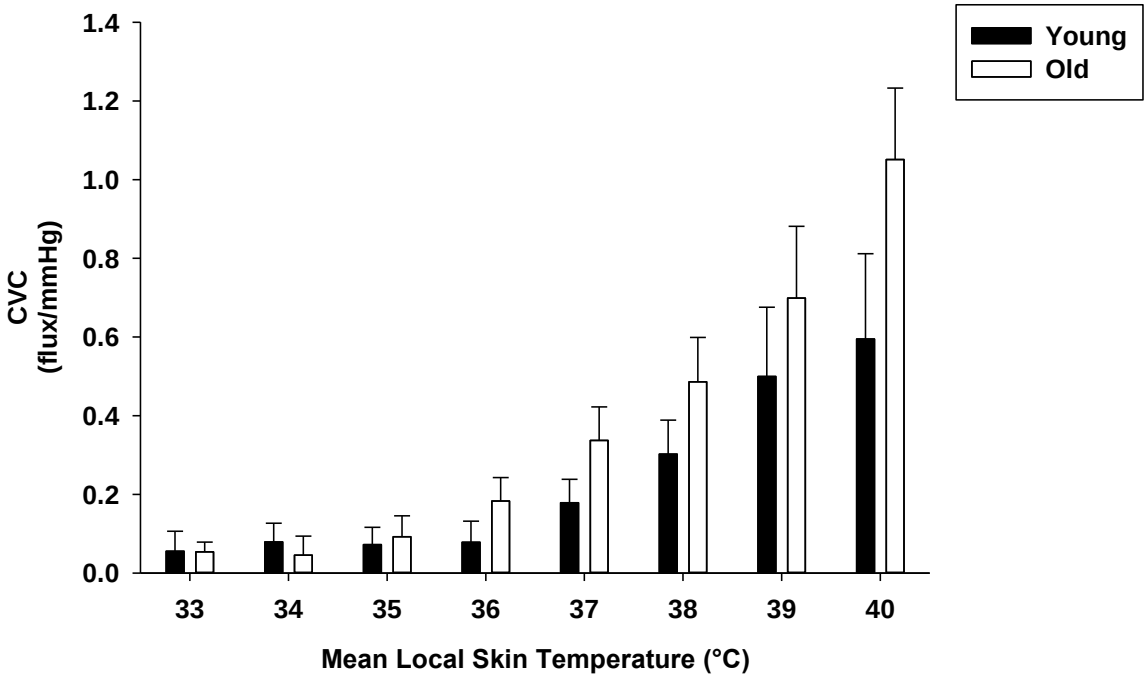


Figure **A-4. (*Preliminary results*)**

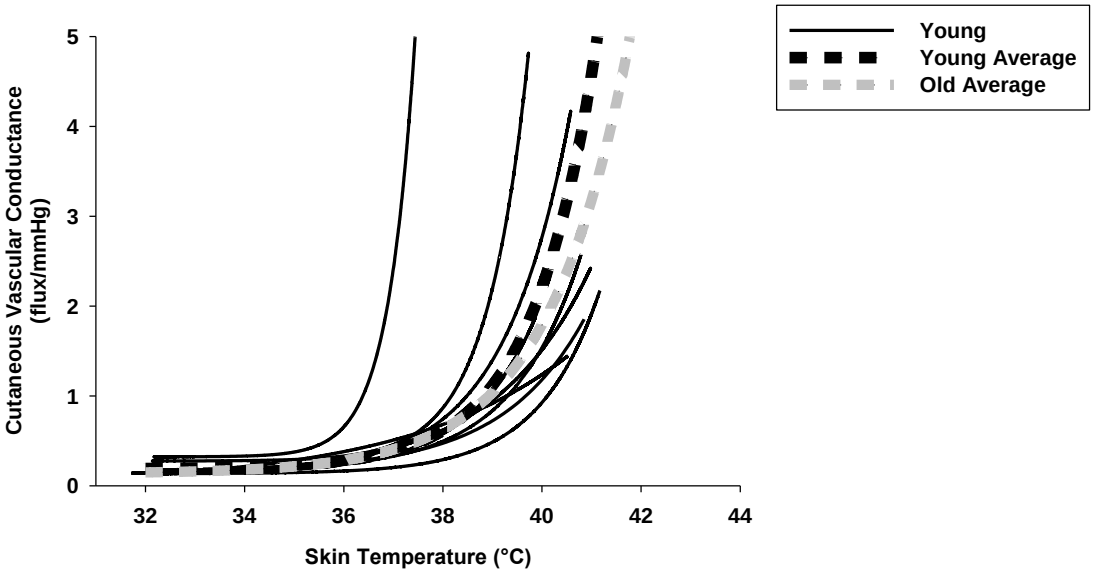
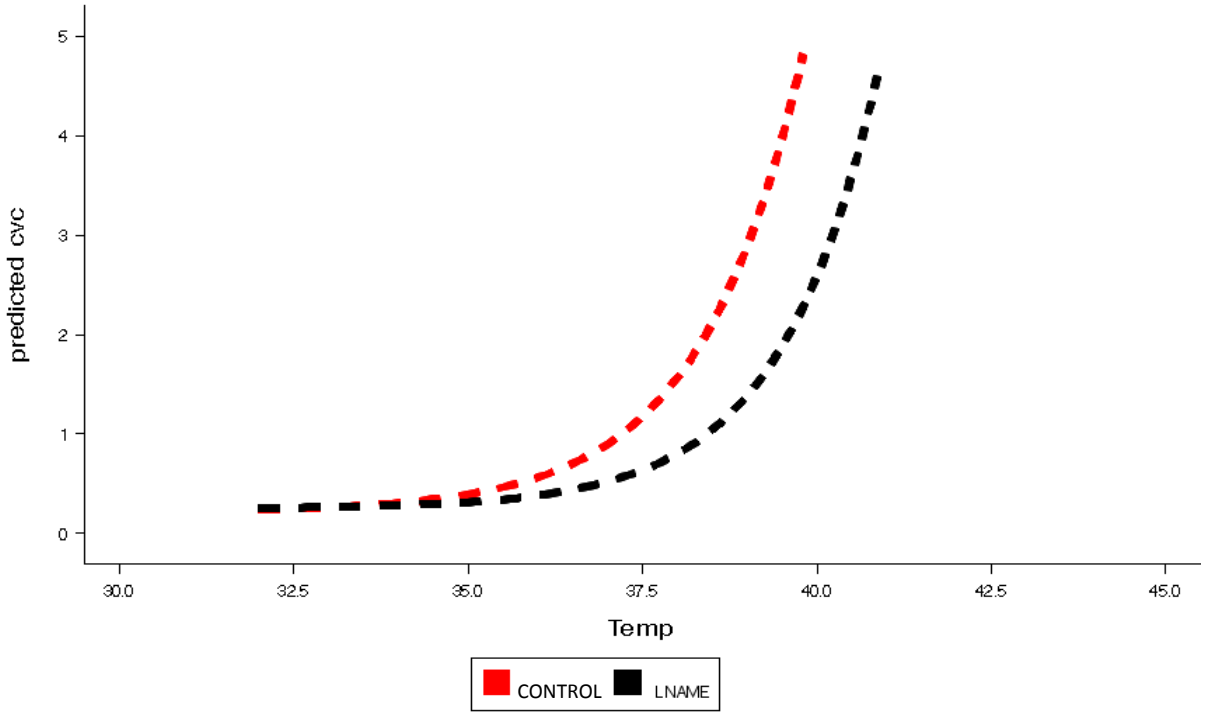


Figure **A-5**. **(*Preliminary results from combined young and old sites*)**



Appendix B: EXPANDED METHODS AND THEORY FOR SPECTRAL ANALYSIS

Rationale

The human cutaneous circulation is a model circulation to examine more global microvascular dysfunction because it is an easily *accessible* vascular bed in which non- to minimally invasive stimuli provide a means to assess endothelium-dependent and independent resistance vessel function (Holowatz et al. 2008, Minson 2010b, Cracowski and Roustit 2010, Holowatz et al. 2005, Thompson, Holowatz and Kenney 2005). One commonly used method to assess skin blood flow is laser Doppler flowmetry (LDF). Laser Doppler flowmetry allows for semiquantitative assessment of cutaneous blood flow, corresponding to the number of red blood cells and their mean velocity (Weidenhagen et al. 1996, Salerud and Nilsson 1986). Periodic oscillations occur within LDF signals. These oscillations correspond with vasodilation and vasoconstriction of the cutaneous arteriole's vascular smooth muscle (vasomotion). The LDF recordings can be decomposed so that the periodic oscillations can be separated into their different frequency intervals using spectral analysis, providing insight into the physiological mechanisms mediating skin flowmotion. The five frequency intervals that have been associated with central hemodynamic and peripheral regulation of skin flowmotion are:

1.) Heart beat: 1Hz (0.4-2.0Hz)
2.) Respiration: 0.4Hz (0.15-0.4)
3.) Intrinsic myogenic activity of vascular smooth muscle: 0.1Hz (0.5-0.15Hz)
4.) Neurogenic activity of vessel wall: 0.04Hz (0.2-0.5Hz)
5.) Endothelial signaling: 0.01Hz (0.008-0.2Hz)

Laser-Doppler Flowmetry Pre-Signal Processing

Data Collection and Acquisition

LDF (moorLAB™, Moor Instruments Ltd, UK) was used to assess cutaneous blood flow and measured in arbitrary units (AU) for spectral analysis. The moorLAB™'s lasers have a 785±10nm wavelength. The raw signal is processed by moorLAB™ allowing for low frequency signals to be derived. The LDF signal is band-pass filtered within the moorLAB™ in the range from 20Hz to 15kHz. The moorLAB™ lasers used do not need electrical contact to the optical probe so leakage currents are zero (FDA Class 1 laser standards). The signal from the moorLAB™ is then sampled at 40Hz by A/D conversion (WinDaq; Dataq Instruments, Akron, OH) and stored in a digital format on a computer for further signal processing.

Sampling Rate of Laser-Doppler Flowmetry Signal

The sampling rate is important to consider during the A/D conversion of the LDF signal. To prevent a loss of information or aliasing (when oscillations within the signal move and can be shown at different frequencies within the spectrum), the digital signal must comply with the sampling theorem. The sampling theorem states that the sampling rate must be more than twice the maximum frequency that is present in the signal. The moorLAB™ has a low pass filter at 20Hz, which means that the maximum frequency in the signal is 20Hz. Therefore, our sampling rate must be at least 40Hz to comply with this theorem.

Signal Processing

Fast Fourier Transformation

The LDF signal is comprised of a sum of oscillations occurring at different frequencies. Initially, the input LDF signal is in the time domain and expressed as Laser Doppler flux (in arbitrary units). The LDF signal can undergo spectral analysis such as fast Fourier transformations (FFT) to transform the data from the time domain into the characteristic frequencies that make up the periodic oscillations. A characteristic, or basic, frequency is the frequencies contained in the LDF signal that have amplitudes that are significantly greater than

other frequencies within the signal. Microvascular blood flow has several characteristic frequencies with complicated oscillations in the time domain with different times of repetition. The characteristic frequencies continuously change, similar to what you see with heart rate variability, due to naturally occurring perturbations.

The assumption with spectral analysis is that the LDF signal is stationary and ergodic. A signal is stationary if its statistical properties, such as mean and variance, do not change with time and whose probability distribution is constant throughout the signal. A signal is ergodic if the recorded signal is representative of the entire process. This is an important concept when analyzing cutaneous vasoreactivity because in physiological systems these are only approximate assumptions. The variance in laser Doppler flux increases with increasing blood flow. Therefore, the data must be analyzed during plateaus in skin blood flow to meet these assumptions. In the present thesis the LDF signal is processed during the following periods of the local skin heating response: baseline, local skin heating plateau, during local skin heating after a plateau occurs with perfusion of a nitric oxide synthase) inhibitor (L-N^G-Nitroarginine Methyl Ester; L-NAME) and during maximal vasodilation elicited from perfusion a nitric oxide (NO) donor (sodium nitroprusside; SNP).

Estimation of Power Spectral Density Using Fast Fourier Transformations

The power spectral density (PSD) is the distribution of the signal power of the characteristic frequency intervals contained within the LDF signal. Because the LDF signal is a random, *in vivo* measurement of a portion of the skin blood flow response to local skin heating, the PSD within the studied tracings can only be an estimate of the true spectrum. One way to estimate the PSD is through use of periodograms computed from the finite-length of LDF tracings that underwent FFT. A periodogram is the square of the FFT, however, it contains spectral bias due to the large portion of random error that arises from sharp truncations of the FFT of the LDF signal. To reduce this error window functions are used, which truncates the sequence gradually rather than abruptly. The random error in the PSD is dependent on the standard deviation between the estimates, which can be reduced by increasing the number of windows (Avery et al. 2009). The bias error depends on the frequency resolution of the estimate, which is the sampling rate divided by the window length M (Equation 1). Increasing the length of the windows

improves the frequency resolution and reduces the bias error. A balance between the number of windows and their length needs to be achieved because increasing the number of windows reduces random error at the cost of reducing window length, which increases the bias error. In the study presented in this thesis we based our window number and length off of the analysis by Avery *et al.* (Avery et al. 2009).

$$\mathbf{fr = fs/M}$$

Equation 1-Calculation of the frequency resolution of the signal, where fr is the frequency resolution, fs is the sampling rate, and M is the window length.

The window function used in the present study was the non-parametric Welch method. This window function is one of the most commonly used methods and was chosen due to the non-normality of the data and because it reduces the noise in estimated power spectra with imperfect and finite data. The Welch method splits the data into overlapping segments of digitized data points (N) and divides them into L windows of data with window length (M), which overlap by D points (Equation 2). Periodograms are calculated for each window. The average of all the periodograms is then used as an estimate of PSD, with the power of the signal (AU^2) equal to the area under the PSD curve and represented as AU^2/Hz. Different window types within the Welch method can be used to truncate FFT sequences in a symmetrical manner. The study in this dissertation used the Hanning window. All signal processing was performed in MATLAB statistical software (version 2013a, Natick MA) using MATLAB program provided by Professor Geraldine Clough and technical assistance from Andrew Geronimo (See MATLAB Program).

$$\mathbf{L = (2N/M)+1}$$

Equation 2-Calculation of the number of windows used in the Welch method of estimating PSD, where L is the number of windows, N is the number of data points, and M is the window length.

Methods for Power Spectral Density Estimation

In the present study the LDF signal was sampled between 40-44Hz and saved in a digitized format in WinDAQ. The WinDAQ file was then cut into 600 second (24001 data points) sections from baseline, local skin heating plateau, during perfusion of L-NAME, and during SNP perfusion (See Appendix C). The excel files were brought into MATLAB and

detrended to remove the mean value from the signal, making it equal to zero so that the mean of the flux would not appear as a power frequency at 0 Hz, distorting the low frequency intervals of interest. We detrended our data to a power of 10 based on previous calculations performed by Avery *et al* (Avery et al. 2009).

The PSD of the LDF signal in our essential hypertensive and age-matched normotensive men and women was estimated using the Welch method. MATLAB extracted 500 seconds of data or 20001 data points (N) into window lengths of 250 seconds (10001 data points) (M) that overlapped by 50% and was truncated using a Hanning window. The frequency resolution of the signal is 0.004Hz, which is sufficient to measure the power of the low frequencies around 0.01Hz (Equation 1)(Avery et al. 2009). A detailed list of instructions for converting WinDAQ data files into CSV files and the MATLAB program are described in further detail in the section entitled MATLAB Program.

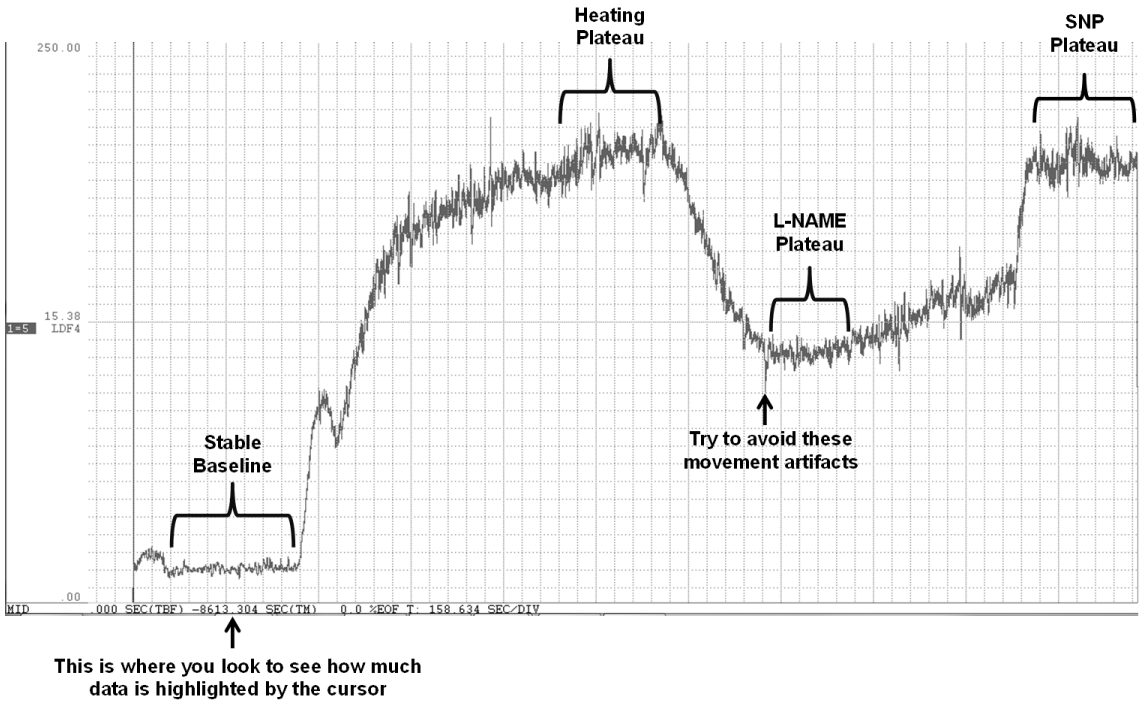
Step-by-Step WinDAQ and Excel File Preparation

Converting WinDAQ Data Files into a .CSV File

1) Open the WinDAQ file with the waveform browser
2) Press F8 to get the notes from that experiment
3) Look to see what channel the microdialysis site of interest was recorded on
4) Go to view→format screen→check one waveform
5) Open the site to be analyzed in the channel on the screen
    a. To do this make sure the WinDAQ Browser screen is active
    b. Type 1 = [channel number the site to analyze was on] (**NOTE:** This will be 1 plus the number the site was recorded on because the first channel is used to record the notes taken during the experiment. For example, if the Control site was recorded as site number 3 then you would type 1= 4). These numbers must be highlighted (left side).
6) Press F7 to maximally compress the data to visualize the skin blood flow response. It is ***extremely*** important to select an area of data that does not have movement artifacts because this will throw off the analysis (Figure B-1). The flatter the data the better.

7) The four parts of the local heating response analyzed in the current study are the baseline, heating plateau, L-NAME plateau, and SNP plateau.
8) Click on the bottom of the waveform screen to move the cursor. Drag the cursor to the beginning of the STABLE data to be analyzed.
9) Press F4 to clear the cursor timer. It will say OFF. The area where it will say OFF is highlighted in Figure B-1 (the arrow underneath the stable baseline).

Figure **B-1**. Representative local heating data viewed with in the WinDAQ Browser.

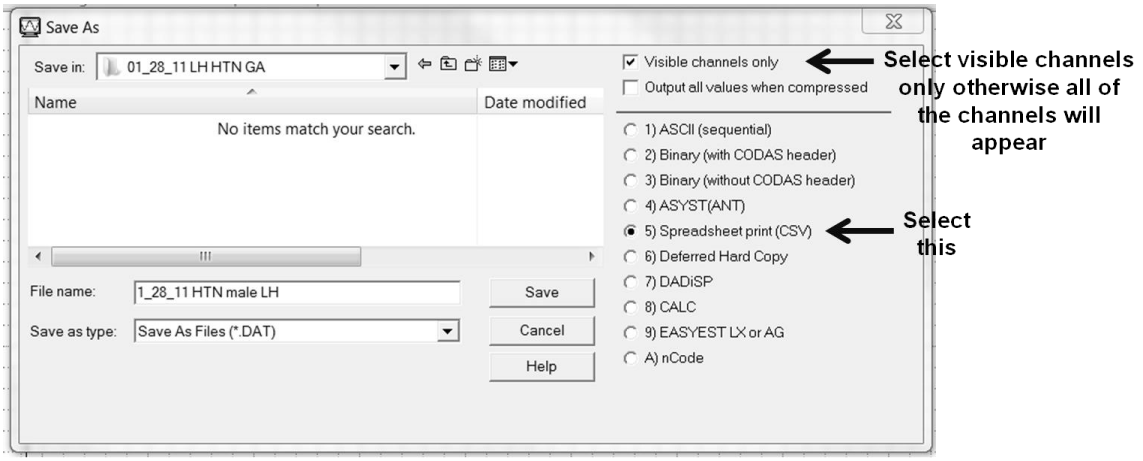


10) Press F4 again to mark the beginning of the area to be analyzed. Now the cursor timer will say .000 SEC.
11) Drag the cursor to the end of the area to be analyzed. In the study contained in this thesis this was about 545 seconds because the data files were saved at 44Hz. In future studies, the files should be saved at 40Hz (and so the area should be around 599 seconds). It is OK to make the file longer than 545 seconds to ensure the file is long enough. You can delete the extra data points when editing the excel file.
12) Once the area of interest is selected, press F7 to decompress the data for analysis. In the SEC/DIV box (Figure B-2) type in 0.025. Make sure that the MID bullet is checked. Then press OK.

Figure **B-2**. Setting the compression of the data file for future analysis.

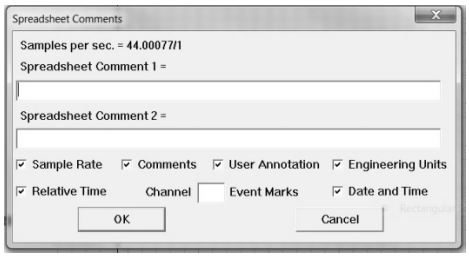
13) Next go to File→Save As. Make sure that the visible channels only box is checked and that the spreadsheet print (CSV) bullet is highlighted (Figure B-3). Then click Save.

Figure **B-3**-Schematic illustrating how to save the WinDAQ file as an Excel .CSV file.



14) A spreadsheet comments menu will come up. Make sure the boxes in Figure B-4 are ticked.

Figure **B-4**. Schematic illustrating the spreadsheet comments window.



MATLAB Program	Function
clear all	*Clearing all previous commands*
close all	
SUBTYPE = {'Hypertensive Subjects', 'Normotensive Subjects' }	*Dividing the data into 2 groups*
for subjtype = 1:length(SUBTYPE)	
disp(['type = ' SUBTYPE{subjtype}])	
cd(['E:\Becky\Data Files\Matlab Files\' SUBTYPE{subjtype}])	*Mapping the address of the folders containing the data files*
folders = dir;	
folders = folders(arrayfun(@(x) x.name(1), folders) ~= '.');	
for subjects = 1:length(folders)	
disp(['subject = ' num2str(subjects)])	
cd(['E:\Becky\Data Files\Matlab Files\' SUBTYPE{subjtype}... '\' eval(['folders(' num2str(subjects) ').name']) '\Excel Files for MATLAB'])	*Labeling files by blood pressure classification (subjtype), folders, and phase of local heating (condition)*
conddir = dir;	
conddir = conddir(arrayfun(@(x) x.name(1), conddir) ~= '.');	
for condition = 1:length(conddir)	
disp(['condition = ' num2str(condition)])	
[numbers, strings] = xlsread(['E:\Becky\Data Files\Matlab Files\'...	*Reading the Excel files in MATLAB*
SUBTYPE{subjtype} '\' eval(['folders(' num2str(subjects) ').name']) ...	

```
            '\Excel Files for MATLAB\'
eval(['conddir(' num2str(condition)
').name'])]);
if ~isempty(numbers)
            newData1.data = numbers;
end
          if ~isempty(strings)
            newData1.textdata = strings;
end
vars = fieldnames(newData1);
for i = 1:length(vars)                          Creating new variables in the base
            assignin('base', vars{i},           workspace from those fields
newData1.(vars{i}));
end
D=data;                                         Insert name of file and names it D
t=D(:,1);                                       This denotes that t (time) is in the first
y=D(:,2);                                       column of D
fs=1/mean(diff(t));                             This denotes that y (signal for processing) is
order=10;                                       in column 2
          N=length(y);                          Calculates the sampling frequency and
          y=y(:);                               names it fs
          x=[0:N-1]'/N;                         Detrend Program
          X=ones(N,1);
          for n=1:order
                    X=[X,x.^n];
          end
          a_est=inv(X'*X)*(X'*y);
          y1_detrend=y-X*a_est;
          T = X;
          y1_est=T*a_est;
          figure                                Creating figure of detrended data for each
          plot(t,y,t,y1_est,'r');               individual file
xlabel('time from start of
```

```
heating(seconds)');
ylabel('skin blood flux(AU)');
figure
plot(t,y1_detrend)
xlabel('time from start of
heating(seconds)');
ylabel('detrended skin blood flux(AU)');
Tw=250;                                  Power Spectral Density Estimation Program
overlap=0.5;                             Specifying window length
w=hanning(round(Tw*fs));                 Percent overlap between windows
x = y1_detrend;                          Selection of hanning windows
window = w;                              Estimate the PSD for the signal x, using
function [P,f]=spec1(x,window,overlap,fs)
Nfft=length(window);                     Spectrogram function
N=length(x);                             FFTs of length (window), and the % overlap
x=reshape(x,N,1);                        (0-1)
window=reshape(window,Nfft,1);
P=nan;
f=nan;
shift=round((1-overlap)*Nfft);
if(shift>0)&(Nfft<N)
            y=[];
            weight=[];
            weight0=sum(window.^2);
  weight1=sum(window.^2);
            i_start=1;
            i_end=Nfft;
            no_windows=0;
                    while i_end<=N
         x_temp=x(i_start:i_end).*window;
                    weight=[weight,weight0];
          y=[y,x_temp];
no_windows=no_windows+1;
```

```
i_start=i_start+shift;
        i_end=i_end+shift;
end
Y=fft(y)';
Y2=abs(Y).^2;
if no_windows>1
        P=mean(Y2);
else
        P=Y2;
end
        P=P/weight0/fs;
if mod(Nfft,2)==0
P(2:Nfft/2+1)=P(2:Nfft/2+1)+P(Nfft:-
1:Nfft/2+1);
       P=P(1:Nfft/2+1);
else
        P(2:(Nfft-
1)/2+1)=P(2:(Nfft+1)/2)+P(Nfft:-
1:(Nfft+1)/2+1);
        P=P(1:(Nfft+1)/2);
end
         P(1)=2*P(1);                    Naming the groups so you can extract the
         f=[0:length(P)-1]/Nfft*fs;      filename (blood pressure, subject, phase of
end                                      local heating response)
Filenamesa1{subjtype,subjects,condition}
= [SUBTYPE{subjtype} ' Type, '...        Naming the group so you can extract the
        folders(subjects).name ' Subject, '  power for a given frequency into excel
conddir(condition).name ' Condition'];
Pa1{subjtype,subjects,condition} = P;    Naming the group so you can extract the
fa1{subjtype,subjects,condition} = f;    frequency
                close all end end end    Ending program
```

Appendix C: INFORMED CONSENT FORMS

NOS Isoform and Spectral Analysis Studies

INFORMED CONSENT FORM FOR CLINICAL RESEARCH STUDY
The Pennsylvania State University

Title of Project: **The NOS isoforms mediating local heating-induced cutaneous vasodilation, and NOS gene-expression and activity in humans with essential hypertension**

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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.

1. <u>Purpose of the study:</u> About one quarter to one third of adults in the United States are being treated for high blood pressure. If you add that some people have yet to find out they have high blood pressure, it is easy to see that high blood pressure is a common problem. High blood pressure increases the risk for diseases of the heart and blood vessels. The diseases impair the ability of the heart and blood vessels to function properly. People with high blood pressure have changes that affect how well their blood vessels dilate (get bigger) and constrict (get smaller) to control blood flow. Some of these changes occur in the small vessels of the skin before they occur in the larger blood vessels throughout the body. Therefore, we can use the skin to explore these changes. Our research has explored substances the body makes that help to control blood flow. One of those substances, "nitric oxide" (NO), works to dilate blood vessels. People with high blood pressure make less NO. This research explores how high blood pressure changes the body's control of NO production. We may include up to 60 people in the study.

2. Overview: The screening for this study involves a blood draw. The study has 3 parts. You do not have to do all parts. Part 1 uses a technique called "microdialysis." Part 2 involves a "biopsy" in which we take two very small samples of skin from your arm. We test the samples for substances that your body makes to dilate your blood vessels. Part 3 also uses microdialysis. You can do the biopsy on the same day as microdialysis.

Microdialysis (MD) 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 fluids like those that are found in your body's tissues through the tubing. This fluid is called "lactated Ringer's solution." 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 fluids in your skin. During the experiment, we will add substances to the fluid in the tubing. The substances can only reach a 2.5 cm^2 (0.4 $inch^2$), nickel-sized area of skin at each tube. These substances are like some of the natural chemicals found in your body. The test substances are:

1. L-NAME (*N^G-nitro-L-arginine methyl ester*) – like a natural protein found in your cells. It stops chemical reactions that involve that protein.
2. SNP (*sodium nitroprusside*) causes your blood vessels to get as large as they can.
3. NPLA *(N^{ω}-Propyl-L-arginine)*– blocks one of the proteins that helps to make NO
4. 1400W *(N-[3-(aminomethyl)benzyl] acetamidine)* – blocks one of the proteins that helps to make NO
5. Acetylcholine–a natural substance made by nerves in your body. It causes muscles to contract.

3. 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 some procedures.***

You could be asked to repeat a trial, procedure, or test. This could happen for many reasons such as equipment failure, power outage, inconclusive test results, etc. You do not have to repeat a trial, procedure, and/or test if you do not wish to do so.

_____ initial Visits 1-2: Blood Pressure Visits If we think that you 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 make these readings on 2 separate visits to the Noll Lab then during your screening visit. Also, you wear a monitor to record your blood pressure for 24 hours. The monitor has a cuff that goes around your arm. A control unit hangs on a strap around your waist or on your shoulder.

_____ initial Visit 3: Screening You do not eat or drink after midnight during the night before your exam. You report to the Noll Lab for your appointment. When you arrive, we measure your blood pressure. The research and/or the Clinical Research Center (CRC) staff perform the screening procedures. The staff draws 15 ml (1 Tbsp) from a vein in your arm. If you take thyroid medicine, we draw 3.5 ml (0.2 Tbsp) more blood for a thyroid test. We send the blood sample to a lab to see if the proteins, blood cells, electrolytes, etc. are within normal levels. We may test the blood for other substances of interest. We do not perform genetic tests on the blood. We do not test the blood for the presence of disease (e.g. HIV). After the blood draw, we give you juice and a snack bar. The staff performs records a medical history, heart rate, blood pressure, height, weight, waist circumference, and 12-lead ECG. If you are a woman of

childbearing-age, you submit a urine sample for a pregnancy test. We may measure the thickness of folds of skin at several places on your body to determine your percent body fat.

_____ **initial** **Visit 4: Microdialysis (MD) Experiment** You do not drink fluids that contain caffeine (i.e. coffee, tea, Coca Cola, etc.) for 12 hours before the experiment. You may eat your typical breakfast. When you arrive at the laboratory, you wash your forearm and pat it dry. If you are a woman of childbearing-age, you submit a urine sample for a pregnancy test. You recline on a bed. Then we prepare the MD sites on your arm. We insert 4 MD tubes into the skin on your arm.

Inserting Microdialysis Tubing: We place a tight band around your upper arm so we can easily see your veins. For each MD site, we make pairs of pen-marks on your arm 2.5 cm (1 inch) apart and away from veins. We remove the tight band. The MD tubing will enter and exit your skin at the marks. 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. We thread the tubing through the needle. Next, we withdraw the needle leaving the tubing in your skin. Any redness of your skin subsides in about 60 – 120 minutes. The treatments at the four MD sites are:

1. Lactated Ringer's
2. Lactated Ringer's + L-NAME
3. Lactated Ringer's + NPLA
4. Lactated Ringer's + 1400W.

We tape a thin probe and its holder over each site where there is MD tubing in your skin. The thin probe measures skin blood flow with a weak laser light. We can control the temperature of the holders. The holders will start at 33°C (91.4°F). During the experiment, we measure blood pressure with a cuff that inflates on your upper arm while the researcher listens with a stethoscope at the inside of your elbow. We place 3 sticky tabs on your chest to which we attach the wires of an ECG machine that measures your heart rate. Throughout the experiment, we measure skin blood flow and skin temperature at the MD sites.

Experiment: When the redness from placing the MD tubing in your skin is gone, the experiment begins. The temperature of the heater on your skin is 33°C (91°F). We start the Lactated Ringer's flowing through the tubing in your skin. After 10 minutes, we add the test-substances to the Ringer's (see above) for about one hour. Then we collect data for about 20-minutes. Then we increase the temperature of all sites to 42°C (108°F). After about 50 minutes, we replace the lactated Ringer's, NPLA, and 1400W with L-NAME so that all sites receive lactated Ringer's + L-NAME. After about 30 minutes, we replace the lactated Ringer's + L-NAME at all sites with lactated Ringers + SNP and increase the local heat to 43°C (109°F) for about 40 minutes to produce the maximal skin blood flow.

You may have us perform the skin biopsy right after the MD experiment or wait until another day.

_____ **initial** **Visits 4 (or 5) : Biopsy Experiment** If you are a woman of childbearing-age, you submit a urine sample for a pregnancy test. The researcher takes two small pieces of skin from your forearm (skin biopsy) using the following method. First, you wash your arm with soap and warm water. Then you sit or lie on a bed. The researcher cleans the top of the lidocaine vial

with alcohol. An approved clinician wipes your skin with alcohol and injects lidocaine into the skin of your arm at the biopsy sites to numb them. The researcher will wait a couple of minutes after injecting the lidocaine to give the drug time to work. The researcher will clean the biopsy site 3 times with an orange cleanser (betadine) and an alcohol pad. If you are allergic to betadine, we will use only alcohol. The researcher will gently touch the site with the tip of a needle to see if you can feel anything. You may feel the slight pain of the pin-prick or only pressure. If you can feel pain, the researcher will wait a little longer or the approved clinician will add more lidocaine into the skin. When the site is numb, the researcher will place a sterile drape over your arm. The biopsy sites are located in an opening in the middle of the drape. The researcher uses a punch-tool that looks like a screwdriver that has a round, hollow tip. The tip is 3mm (0.12 in) in diameter. The hollow tip acts like a cookie cutter. The researcher places the tip of the punch against the skin at the biopsy site and applies mild pressure. You will feel the pressure. The tip of the punch will go about 3 mm (0.12 in) into your skin. The punch collects a small piece of skin about 3mm x 2mm (0.12 in x 0.08 in). The researcher holds sterile gauze on the site to stop any bleeding. The researcher may also apply pressure with sterile dressing to the biopsy site to stop bleeding, if necessary. The researcher places the piece of skin into a small container. The researcher uses the punch to remove the second piece of skin in the same way. A sterile bandage will also be applied to your arm. The researcher will give you instructions about how to take care of the biopsy site. You may be asked to come back to the laboratory 14 days after the initial biopsy was taken to have the skin site checked by staff.

_____ **initial** **Visits 5 (or 6) : MD Experiment - Acetylcholine (ACh) Dose Response**
You do not drink fluids that contain caffeine (i.e. coffee, tea, Coca Cola, etc.) for 12 hours before the experiment. You may eat your typical breakfast. When you arrive at the laboratory, you wash your forearm and pat it dry. If you are a woman of childbearing-age, you submit a urine sample for a pregnancy test. You recline on a bed. Then we prepare the MD sites on your arm. We insert 4 MD tubes into the skin on your arm in the same manner as that described for visit 4. The treatments for the four MD sites are:

1. Lactated Ringer's
2. Lactated Ringer's + L-NAME
3. Lactated Ringer's + NPLA
4. Lactated Ringer's + 1400W

After 20 minutes, we switch between fluids containing ACh (15 minutes) and those with no ACH (30 minutes) at all MD sites. The amount of ACh changes with each switch. We use up to 5 ACh amounts. At the end, we replace the fluid at all sites with lactated Ringers + SNP and increase the local heat to 43°C (109°F) for about 40 minutes to produce the maximal skin blood flow.

4. 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, 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 be nervous about needles. If so, your blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick to your stomach, 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. In this case, 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, but has never occurred in our lab or others that we know of. 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 cm^2 (0.4 $inch^2$) area of skin at each tubing site. The amount that enters the skin is very small. However, there is a chance of your having a bad reaction to the substances. This reaction could produce redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, blood pressure change and/or fainting. 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, NPLA , 1400W, Acetylcholine, and SNP: Only minute amounts of these substances enter the nickel-sized area of skin around the MD tubing. We and/or other researchers have used these substances in human skin. There have been no reports of bad reactions.

Skin Biopsy: You may stop the procedure at any time. Trained staff performs the biopsy. You may lie on a bed during the biopsy, if you wish. The researcher will make sure that you are informed and ready. You may still be nervous about needles or the procedure. If so, your blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick to your stomach, or may faint. The lidocaine will numb the site so that you feel very little or no pain during the biopsy. You will feel the pressure of the biopsy tool on your skin. As with any event that breaks the skin, you could get an infection. Trained staff use sterile techniques to keep the risk of infection very small. The skin biopsy may cause some pain, swelling, bleeding, and bruising. Gauze pressed onto the site stops bleeding. The researcher closes the wound with a steri-strip and places a sterile bandage on the site. We give you instructions about caring for the biopsy site. The biopsy will likely leave a small scar. The skin of some people overreacts to injury. If you are one of these, your skin may produce a scar that is larger and easier to see. There may be some minor pain for a couple of days when the lidocaine wears off. The pain would be like that felt after some blood draws.

Betadine: Hospitals and researchers use this orange-colored fluid to clean and sterilize the skin. You could have a bad reaction to Betadine if you are allergic to iodine. You will inform us if you have this allergy 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 blood pressure change and/or fainting.

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.

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

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 be nervous about needles. If so, your blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick to your stomach, or may faint. To keep the chance of infection minimal, the staff uses the same techniques used in hospitals. Do not exercise hard for 24 hours before a blood draw.

Medical Screening: You may feel shy about giving health information. The staff collects the information in a private and professional manner. You may feel shy about being measured. You may request someone of the same sex to conduct the screening.

Phone screening form: Only the researcher uses this form. We use the form to help decide whether you are a good candidate for the study. You may feel shy about answering questions. You may request someone of the same sex to ask you the questions. We collect the information in a private and professional manner. The completed form is kept confidential and secure.

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.

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.

Skin Fold Measurements: Your percent body fat is measured using a tool that looks like tongs. The tongs gently measure the thickness of skin folds at several places on your body. There are no risks to this measure, but you may feel shy about having it performed. You may have a person of the same gender perform the measure.

Blood Pressure (manual and/or Cardiocap 5): The researchers measure blood pressure using the method common in a doctor's office or with a machine. A cuff inflates on your upper arm. As the cuff slowly deflates, the researchers listen with a stethoscope at the bend in your elbow or the machine takes a reading. During the short time the researchers inflate the cuff, your arm may feel numb or tingly.

Blood Pressure Monitor: You wear the device for 24 hours to collect blood pressure. The device uses AA or rechargeable batteries for power. We place a cuff on your upper arm. We attach the control unit to the cuff. We hang the unit from strap on your shoulder or around your waist. The controller takes a measure once every hour by making the cuff inflate, taking a reading, and deflating the cuff. You need to stay as still as possible and relax your arm on a flat surface. You need to keep the system dry and properly attached for the whole 24-hours. This could be a bother to you. The unit may interfere with daily routines such as showering and sleeping. The control unit prevents the cuff from inflating too high (more than 300 mmHg) or too long (more than 180 seconds). Disconnect the rubber hose from the control unit to leave the air out of the cuff if the batteries should fail while the cuff is inflated. You could have an allergic reaction in the area of

the cuff caused by the cuff's fabric. The reaction could include itching, rash, and/or swelling. The pressure of the cuff on your skin could cause one or several small reddish or purplish spots to form in the skin. Usually these are harmless and will disappear in a few days. However, the spots could lead to a sudden, longer lasting bruising or inflammation of a vein. The pressure of the cuff could cause bruising. You will tell us whether you bruise easily. If you bruise easily, you will not use the monitor. We will give you verbal and written instructions.

5a. Benefits to you: You will receive a medical screening that could inform you about your health. You will learn your blood pressure. This is important knowledge. High blood pressure contributes to many serious health problems. If you have high blood pressure, we will advise you to work with a health care provider to keep your blood pressure controlled.

b. Potential benefits to society: As many as one quarter to one third of adults in the United States are currently being treated for high blood pressure. People with high blood pressure have a greater risk for heart disease and death. They can also have a decreased quality of life. This research could lead to a better understanding of high blood pressure causes the decreased response of blood vessels. The knowledge gained could help to prevent and treat the impaired responses of blood vessels due to high blood pressure. Also, the project helps to provide important experience, education, and degree-work for students of Penn State.

6. 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.

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

_____ initial	Visit 1-2	Blood Pressure	15 mins (30 min total)
_____ initial		Blood Pressure Monitor	24 hours
_____ initial	Visit 3	Screening	1 hour
_____ initial	Visit 4	MD Experiment	5 hours
_____ initial	Visit 4 (or 5)	Biopsy Experiment	1 hour

(In 7-10 days, you will return for an extra 15-min visit for stitch-removal if you had a stitch)

_____ initial	Visit 5	ACh Experiment	5 hours

Total: **12.5 hours**

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

9. Right to ask questions: Please contact Lacy Alexander (W: 814-867-1781, H: 814-880-9217) or Jane Pierzga (W: 814-865-1236, H: 814-692-4720) with questions, complaints, or concerns about this research. You can also call this number if you feel this study has harmed you.

If you have any questions, concerns, or problems about your rights as a research participant or would like to offer input, please contact Penn State University's Office for Research Protections (ORP) at (814) 865-1775. The ORP cannot answer questions about research procedures. Questions about research procedures can be answered by the research team.

10. Payment for Participation:
MD Experiment: You will receive $10.00 for each of the MD probes inserted in your arm (maximum $40.00). You will receive $20.00 more for completing the MD experiment.
Biopsy Experiment: You will receive $50.00 for each of the biopsies (maximum $100.00).
MD Experiment: You will receive $10.00 for each of the MD probes inserted in your arm (maximum $40.00). You will receive $20.00 more for completing the MD experiment.
Total = $220.00
In addition, you may choose one of the following: lab T-shirt, bag, or other item we may offer.

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 an MD trial you will be paid for each probe that was inserted plus $10.00 for that trial. This is because $10.00 is one half of $20.00. 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.

11. Injury Clause: In the unlikely event you become injured as a result of your participation in this study, medical care is available. Please call Lacy Alexander (W: 814-863-2948, 814-880-9217), Susan Slimak (W: 814-863-8556, H: 814-237-4618), or Jane Pierzga (W: 814-865-1236, H: 814-692-4720). It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

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

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

If your blood pressure is above normal, we will advise you to inform a health care provider. High blood pressure is a condition that can develop over many years, and you have may had this condition for a long time. You will need health care for this condition. You may wish to talk

with a medical provider of your choice before being in our study. We will give the results of our measurements to you or, upon your request, send them to your doctor.

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

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

_____

Volunteer **Date**

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

_____

Investigator **Date**

Mathematical Modeling of Local Cutaneous Vasodilatory Responses with Aging Study

INFORMED CONSENT FORM FOR CLINICAL RESEARCH STUDY
The Pennsylvania State University

Title of Project: **Analysis of the skin blood flow response to local thermal provocation as a means to assess microvascular function.**

Principal Investigator: **Rebecca Bruning**
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Advisor: **Lacy Alexander, Ph.D.**
113 Noll Laboratory
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Assistants: **Jane Pierzga, M.S.** **Phone: 814-865-1237** **Email: jmp141@psu.edu**
Susan Slimak, RN **Phone: 814-863-8556** **Email: sks31@psu.edu**

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. Lacy Alexander Holowatz.

1. <u>Purpose of the study:</u>

The human body controls the amount of blood flowing through a healthy vascular bed by constricting or dilating the vessels to change their size. Also, the body changes the pressure pushing blood through the vessels. The mechanisms the body uses to control blood flow can differ depending upon the stimulus (e.g. temperature, stoppage of blood flow, emotional stress, etc.). Problems with the function of the very small blood vessels of the body are one of the earliest markers of vascular disease.

The vascular bed in the skin is a web of small blood vessels that is easy to access and study. The health of the skin's blood vessels can mirror the health of the body's other blood vessels. Therefore, exploring blood flow control in the skin's blood vessels helps us to learn about the onset, effects, and treatment of vascular disease throughout the body. For over ten years, our lab has studied the mechanisms that control skin blood flow in young and older humans, and in those with high blood pressure (hypertension) and high cholesterol. We have seen that aging and disease can impair that control as well. Much of our ongoing research investigates the reasons for that impairment.

We and other researchers have shown that direct changes in local skin temperature are a reliable way to detect impairments in vascular function in older and hypertensive people. For example, aging and hypertension result in less dilation of the skin's vessels with warming. Your blood vessels make a substance called "nitric oxide" (NO). NO helps blood vessels to dilate. The blood vessels in the skin of older men and women, and those with certain diseases make less NO.

We use a special technique called "microdialysis" (MD) in our study. MD involves placing very thin

plastic tubing between the layers of your skin on your arm. The largest part of the tubing is about 6 times the diameter of a human hair. We pump fluid like that found in your body's tissues (Lactated Ringer's solution) through the thin tubing. The thin tubing acts like the very small blood vessels in your skin by allowing the exchange of substances between the fluid and the tissue around the tubing. The substances can only reach a 2.5 cm^2 (0.4 $inch^2$), nickel-sized area of skin. MD allows us to deliver a substance that blocks the blood vessels from making NO at the site of the tubing.

In this project we use a system that allows us to heat or cool small areas of skin on your forearm. We measure skin blood flow under the heated/cooled areas of skin using laser Doppler flowmetry. Laser Doppler flowmetry works by shining a weak laser light into the skin.

The first goal of our project is to study the skin blood flow response from full vessel constriction to full vessel dilation (Protocols 1-3). The second goal is to see how the skin blood flow response changes when the skin blood vessels are not able to make NO (Protocol 2). Protocol 4 uses a new type of laser blood flow monitor (FLPI) to examine the dynamics of the control of blood flow during local heating and hyperemia. "Hyperemia" is the increased blood flow that occurs in tissue when blood flow is restored after a short stoppage. This knowledge could help us create tests that could detect people who may develop cardiovascular disease and monitor drug treatments that are designed to improve blood vessel function.

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.***

You could be asked to repeat a trial, procedure, or test. This could happen for many reasons such as equipment failure, power outage, inconclusive test results, etc. You do not have to repeat a trial, procedure, and/or test if you do not wish to do so.

_____ initial Day 1: Screening You do not eat or drink for 10-11 hours (10 pm) prior to your screening. You will report to Noll Lab for your appointment. The research staff performs the screening. The staff measures your blood pressure, height, weight, waist circumference, and heart rate. The staff reviews your medical history. The staff draws 21 ml (1 Tbsp) of blood from a vein in your arm. We send some of the blood sample to a lab to see if the proteins, blood cells, electrolytes, etc. are within normal levels. We perform some of the tests at Noll Lab. If you are a woman of childbearing age, you will submit a urine sample for a pregnancy test. If you do not have thyroid test results, the staff will draw 3.5 ml (0.2 Tbsp) of blood from a vein in your arm. We send the blood sample to a lab that tests it for thyroid levels. The lab destroys the sample after testing.

If we think that you have high blood pressure, we will measure your blood pressure three times. This will make sure that you belong in the high blood pressure group. We make these readings during your screening visit and on a separate visit to the Noll Lab. Also, you will wear a monitor to record your blood pressure for 24 hours. The monitor has a cuff that goes around your arm. A control unit hangs on a strap around your waist or on your shoulder.

_____ initial Day 2: Blood Pressure Visit (potential hypertensive participants only) You will report to Noll Lab. You will return the blood pressure monitor, and we will measure your blood pressure.

_____ initial Day 3: Experiment: You will be asked to participate in *one to two* of four protocols (see below). You will not eat or drink anything containing caffeine (ex. coffee, tea, Coca Cola, chocolate) for 12 hours before the experiment. In addition, we will ask you to refrain

from strenuous exercise for at least 12 hours prior to the experiment. When you arrive at the laboratory, we measure your heart rate, blood pressure, and oral temperature. You recline on a bed. Then we prepare for the experiment.

_____ **initial <u>Protocol 1: Local Heating/Cooling (No MD)</u>:**

The experimental sites will include:

1. Heated site
2. Cooled site
3. Thermoneutral site

A. <u>Instrumentation</u>: We mark where we will place the temperature controllers with pen. We place three temperature control units (9 cm^2 (1.4 in^2)) on the ventral surface of your forearm with double-sided sticky disks. The units are separated by at least 2.54 cm (1.0 in). We connect a thin laser probe to each unit. The probe uses a weak laser light to assess changes in skin blood flow. We do not turn on one control unit so that its temperature can change with that of your skin. The temperature at the heated site begins at 31°C (88°F), whereas that at the cooled site begins at 34°C (93°F). During the experiment, we measure blood pressure. We use a cuff that inflates on your upper arm while we listen with a stethoscope at the inside of your elbow. Also, we measure blood pressure using an automated blood pressure cuff that inflates every 5 minutes. We place 3 sticky tabs on your chest to which we attach the wires of an ECG machine that measures your heart rate. Throughout the experiment, we measure skin blood flow, heart rate, and blood pressure. After we place the control units and lasers on the skin, we will begin the experiment.
B. <u>Baseline</u>: We will collect 10 minutes of baseline measurements at the starting temperatures (see above).
C. <u>Heating/Cooling of the Skin</u>: Then we will heat one control unit from 31°C to 42°C (108°F) while we cool the other unit from 34°C to 15°C (59°F). The heating/cooling rate will be 0.6°C/min (1.1°F/min) for the local heating/cooling protocol. The protocol ends when skin blood flow stabilizes after the heating/cooling sites have reached the highest and lowest temperatures (about 30 minutes).
D. <u>De-instrumentation</u>: We may use adhesive remover when taking the sticky disks off your skin.

_____ **initial <u>Protocol 2- Local Heating/Cooling + 1 MD Site</u>**:

The experimental sites will include:

1. Heated site
2. Heated site + 1 MD site (L-NAME)
3. Cooled site
4. Thermoneutral site

A. _____ **initial <u>Inserting MD Tubing</u>:** We mark where we will place the temperature controllers with pen. We then mark one site for MD and make two dots 2.5 cm (1 in) apart to mark the entrance and exit points for the MD tubing. We clean your arm with

betadine 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. 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. Any redness of your skin subsides in about 60 – 120 minutes.

B. Hyperemia (increase in blood flow from needle insertion): We wait until the redness from MD insertion has resolved before we begin data collection. Lactated Ringer's solution will flow through the tubing in your skin.
C. Instrumentation: The procedure is the same as that for Protocol 1 (see "instrumentation" above) except we added a temperature controller over the MD site (site 2). During instrumentation, the fluid perfusing the microdialysis site is changed from lactated Ringer's solution to 10 mM L-NAME.
D. Baseline: The procedure is the same as that for Protocol 1.
E. Heating/cooling of the skin: The heating/cooling procedure is the same as in Protocol 1, except there are two heated sites instead of one (described above). The MD site will be perfused with 10 mM L-NAME during heating/cooling of the skin.
F. Maximal vasodilation: After skin blood flow has leveled off in all sites, sodium nitroprusside (SNP) will flow through the tubing to make vessels maximally dilate.
G. De-instrumentation: We may use adhesive remover to assist in lifting the tape from your skin. We clean the places where the tubing enters and exits your skin with alcohol, and pull the tubing from your skin. We place a sterile bandage over the sites where the tubing was in your skin. We may place a bag of ice on your arm for 10 minutes to reduce any bruising that may occur. The staff measures your blood pressure and heart rate before you leave.

_____ **initial Protocol 3- Local Heating/Cooling + 2 MD Sites (L-NAME and Control)** :

The experimental sites will include:

1. Heated site + MD (lactated Ringer's solution; control)
2. Heated site + MD (L-NAME; NOS inhibited***)***
3. Cooled site
4. Thermoneutral site

We use the same procedures as Protocol 2 with a second MD site. Lactated Ringer's solution will flow through the added MD site (site 1) during the hyperemia and heating/cooling portion of the protocol. Near the end of the protocol, SNP will flow through the tubing to cause maximal dilation of the blood vessels.

_____ initial Protocol 4- FLPI Experiment: Local Heating / Reactive Hyperemia (LH/RH):

The "FLPI" is a type of laser blood flowmeter that records skin blood flow over a larger area and displays results in real time.

Reactive Hyperemia: You gently wash your arm with soap and water. You lie on a bed or recliner. The researchers place two ring-shaped local heaters 35mm diameter (about 1.5 inches)

on your forearm with sticky rings. The researchers label each site with a marker pen. They fill the inner well of each heater with 2 ml (about 1/2 teaspoon) of water. They place a clear cover over the well. They set the temperature of the local heaters to a comfortable 34°C (93°F). The researchers position and focus the FLPI camera 8-10 inches above your arm. They place a blood pressure cuff on each of your arms. They record blood pressure every 5-7 minutes on the arm that does not have the local heaters. They record baseline data for about 20 minutes with the FLPI. The researchers inflate the cuff on the arm sporting the local heaters for 5 minutes so that blood does not enter or exit the arm (occlusion). Then they rapidly deflate the cuff and blood flows back into your arm while they record data with the FLPI. They wait at least 20 minutes and repeat the occlusion. They wait at least 20 more minutes and repeat the occlusion a third time. The researchers will record data until the skin blood flow becomes stable (about 10 minutes).

Local Heating: The researchers continue to record blood pressure every 5-7 minutes on the arm that does not have the local heaters. They record skin blood flow with the FLPI. They collect baseline date for about 20 minutes. They increase the temperature to 42°C (107°F) and wait 40-50 minutes for the skin blood flow to become stable. Then they raise the temperature to 43°C (109°F). The researchers collect data for about 30 more minutes. Then they remove the local heaters and wipe the water from your arm. You wash your arm with soap and water. Your blood pressure and heart rate are measured before you leave.

3. Discomforts and risks:

Microdialysis (MD): 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 be nervous about needles. If so, your blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick to your stomach, 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. In this case, 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, but has never occurred in our lab or others that we know of. 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 cm^2 (0.4 $inch^2$) area of skin at each tubing site. The amount that enters the skin is very small. However, there is a chance of your having a bad reaction to the substances. This reaction could produce redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, blood pressure change and/or fainting. 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: This drug is similar to the natural amino acid, L-arginine; and it inhibits production of NO. We and/or others have used this drug in humans in research. Only minute amounts of L-NAME enters the nickel-sized area of skin around the MD tubing. This drug affects only that small area around the MD tubing. L-NAME has been used with MD, and there have been no bad reactions. Although unlikely, it is possible that you could have a bad reaction.

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.

Betadine: Hospitals and researchers use this orange-colored fluid to clean the skin. You could have a bad reaction to Betadine if you are allergic to iodine. You will inform us if you have this allergy 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 blood pressure change and/or fainting.

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 placed into their holders on the skin.

FLPI: The FLPI is a special camera that shines a low-energy laser light on the surface of the skin to measure blood flow. The FLPI makes photos and movies of skin blood flow. You may be able to see a harmless red light your skin. The FLPI presents no risk as used in this study.

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. In addition, during the experiment we will use an automated cuff that will inflate every 5 minutes during the experimental protocol. During the short time the cuff is inflated, your arm may feel numb or tingly.

Occlusion of Blood Flow to the Arm: Your arm will likely feel numb or tingly when the researchers inflate the cuff. This feeling is akin to your arm falling asleep. While the cuff is inflated, the color of your arm may become lighter, grayed, or bluish. When they deflate the cuff, your arm may appear reddened for a few minutes. The cuff may cause mild bruising. The technique is unlikely to produce lasting ill effects

Screening 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 be nervous about needles. If so, your blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick to your stomach, or may faint. To keep the chance of infection minimal, the staff uses the same techniques used in hospitals. Do not exercise hard for 24 hours before a blood

draw.

Local cooling/heating: We measure the temperature of your skin under the holders. The cooling is very mild and goes no lower than a brisk autumn day (about 18ºC, 64.4ºF). During heating, the skin will feel very warm but will not hurt. The heating will make the skin on your arm under the holder 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. In addition, we will monitor skin temperature and reduce the temperature of the local heating unit if skin temperature reaches 42°C.

Tape and adhesive disks: The tape or adhesive disks could cause a rash on your skin. During screening, you will tell us if you are sensitive to tape. If a disk sticks very strongly, removing the disk could cause an abrasion like a rug-burn on your skin. An abrasion can feel tender or slightly painful. If you are sensitive to tape, you may have an increased chance for abrasion. An abrasion has occurred only once during the years that the disks have been used in this and similar studies in our lab. If a disk sticks strongly to your skin or if you know that you are sensitive to tape, we use an adhesive remover like that used in a doctor's office to remove the disks. If you get an abrasion a nurse checks the site. Antibiotic ointment and a sterile bandage are applied. We tell you how to take care of the site. You could have an allergic reaction to the adhesive remover. The reaction could include rash, itching, fever, or breathing problems. Also, it could include changes in pulse, and/or blood pressure, convulsions, shock, and/or loss of consciousness. If a bad reaction should occur, medical help will be summoned.

Medical Screening: You may feel shy about giving health information. The staff collects the information in a private and professional manner. You may feel shy about being measured. You may request someone of the same sex to conduct the screening.

Phone screening form: Only the researcher uses this form. We use the form to help decide whether you are a good candidate for the study. You may feel shy about answering questions. You may request someone of the same sex to ask you the questions. We collect the information in a private and professional manner. The completed form is kept confidential and secure.

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

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 Pressure Monitor: You wear the device for 24 hours to collect blood pressure. The device uses AA or rechargeable batteries for power. We place a cuff on your upper arm. We attach the control unit to the cuff. We hang the unit from strap on your shoulder or around your waist. The controller takes a measure once every hour by making the cuff inflate, taking a reading, and deflating the cuff. You need to stay as still as possible and hold your arm slightly away from your body while the cuff is inflated. You need to keep the system dry and properly attached for the whole 24-hours. This could be a bother to you. The unit may interfere with daily routines

such as showering and sleeping. The control unit prevents the cuff from inflating too high (more than 300 mmHg) or too long (more than 180 seconds). If the cuff fails to deflate within 3 minutes, disconnect the rubber hose from the control unit to deflate the cuff. You could have an allergic reaction in the area of the cuff caused by the cuff's fabric. The reaction could include itching, rash, and/or swelling. The pressure of the cuff on your skin could cause one or several small reddish or purplish spots to form in the skin. Usually these are harmless and will disappear in a few days. However, the spots could lead to a sudden, longer lasting bruising or inflammation of a vein. The pressure of the cuff could cause bruising. You will tell us whether you bruise easily. If you bruise easily, you will not use the monitor. We will give you verbal and written instructions.

4a. Benefits to you: You will receive a medical screening that could inform you about your health. You will learn your blood pressure and blood cholesterol levels. This is important knowledge. High blood pressure and blood cholesterol contribute to many serious health problems. If you have high blood pressure or blood cholesterol, we will advise you to work with a health care provider to keep your blood pressure controlled.

b. Potential benefits to society: The 2012 American Heart Association Statistical Update estimated that 82.6 million American adults (>1 in 3) have 1 or more types of cardiovascular disease. Cardiovascular disease decreases the quality of life and is the leading cause of death in the United States. This research could lead to new ways to detect vascular problems and lead to earlier treatment. Also, the project helps to provide important experience, education, and degree-work for students of Penn State.

5. Alternative procedures that could be utilized: These procedures are used in research around the world. These procedures are the best ways to explore the questions and fulfill the goals of this project.

6. Time duration of the procedures and study: ***The circled statements apply to you. Please read the circled statements. Then write your initials by the circled statements.***

	_____ **initial** Day 1 (Screening & BP Monitor Pick Up)	¾hr, no longer than 1¼hrs
1.	_____ **initial** Blood Pressure Monitor	24hrs
	_____ **initial** Day 2 Blood Pressure Monitor Drop Off	¼hr
	_____ **initial** Day 3 (Heating/Cooling Protocol)	
	Protocol 1	2½hrs, no longer than 3 ½hrs
	Protocol 2	3½hrs, no longer than 4 ½hrs
	Protocol 3	3½hrs, no longer than 4 ½hrs
	Protocol 4	4 hrs
	Total Time without Blood Pressure Monitor	2-3 visits, (totaling 3¼-9¾ hrs)
	Total Time with Blood Pressure Monitor	3-4 visits, (totaling 27½-34 hrs)

7. Statement of confidentiality: Volunteers are coded by an identification number for statistical analyses. All records are kept in a secure location. All records associated with your participation in the study will be subject to the usual confidentiality standards applicable to medical records (e.g., such as records maintained by physicians, hospitals, etc.), and in the event of any

publication resulting from the research no personally identifiable information will be disclosed. The Office of Human Research Protections in the U.S. Department of Health and Human Services, the U.S. Food and Drug Administration (FDA), The Pennsylvania State University Office for Research Protections (ORP) and The Pennsylvania State University Institutional Review Board may review records related to this project.

8. Right to ask questions: Please contact Rebecca Bruning. (W: 814-863-8556, M: 814-441-9942), Lacy Alexander Holowatz, Ph.D. (W: 814-867-1781, M: 814-880-9217), Susan Slimak, RN (W: 814-863-8556, H: 814-237-4618), or Jane Pierzga (W: 814-865-1236, H: 814-692-4720) with questions, complaints, or concerns about this research. You can also call these numbers if you feel this study has harmed you. If you have any questions, concerns, or problems about your rights as a research participant or would like to offer input, please contact Penn State University's Office for Research Protections (ORP) at (814) 865-1775. The ORP cannot answer questions about research procedures. Questions about research procedures can be answered by the research team. If there are findings during the research that could relate to you wanting to help with the study, you will be told of the findings.

9. Compensation:

Heating/Cooling Protocols: You will receive $30.00 for completing the heating/cooling portion of the experiment and $15.00 for each MD fiber.

Total - Protocol 1-$30
Protocol 2-$45
Protocol 3-$60
Protocol 4-$30

In addition, you may choose one of the following: lab T-shirt, bag, or other item we may offer.

For each trial, you are paid an amount of money equal to the part of the experiment that you complete. For instance, if you complete only half of Protocol 1 you will be paid $15.00 for that trial. This is because $15.00 is one-half of $30.00. In Protocols 2 and 3 you will be paid the amount of money equal to the part of the experiment that you complete plus $15 per MD fiber. You may be asked to repeat an experiment. If you agree to repeat an experiment, you will be paid for the repeated experiment as stated above.

10. Injury Clause: In the unlikely event you become injured as a result of your participation in this study, medical care is available. Please call Rebecca Bruning. (W: 814-863-8556, M: 814-441-9942), Lacy Alexander Holowatz, Ph.D. (W: 814-867-1781, M: 814-880-9217), Susan Slimak, RN (W: 814-863-8556, H: 814-237-4618), or Jane Pierzga (W: 814-865-1236, H: 814-692-4720). It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

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

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

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

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

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**

Platelet Inhibition and Exercise Study

INFORMED CONSENT FORM FOR CLINICAL RESEARCH STUDY
The Pennsylvania State University

Title of Project: **Low-dose Aspirin and Human Skin Blood Flow**

Principal Investigator: **Lacy Alexander, PhD.**
Address: 113 Noll Laboratory
Phone: 814-867-1781
Email: lma191@psu.edu

Assistants: **Jane Pierzga, MS, Research Assistant**
Phone: 814-865-1236, e-mail: jmp141@psu.edu
Susan Slimak, RN
Phone: 814-863-8556, e-mail: sks31@psu.edu
Rebecca Bruning, Graduate Assistant
Phone: 814-863-2948, e-mail: rsb227@psu.edu

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.

1. <u>Purpose of the study:</u> When you are exposed to heat, nerves in your skin make natural chemicals that cause the skin's blood vessels to get bigger. This increases blood flow through those vessels, helping to cool your body. If something prevents the blood flow increase, you could become prone to heat illness. The researchers observed that taking daily low-dose aspirin or Plavix ® (a similar drug) reduced the increase in skin blood flow (SkBF) in people exposed to whole-body heating. Regular, small doses of aspirin or Plavix ® have "anti-clotting" effects that help to prevent strokes, blood clots, and heart attacks. The positive effects of aspirin and Plavix ® treatment are clear; their effects on how the body responds to heat stress are not. By exploring the actions of aspirin and Plavix ®, the researchers can learn about the effects of these drugs on SkBF. Also, they can explore whether platelets (substances in blood that cause blood to clot) have a role in widening blood vessels to cool your body. The researchers look at the effect of aspirin and Plavix ® on the blood flow in your skin under several conditions.

2. <u>Some of the procedures used in this study:</u> As many as 30 people may screen for this study.

- This study involves blood draws for the screening and experiments.
- There are two types of experiments: resting and exercise. You repeat each type 3-4 times. You do not have to be in both types. The resting experiment that has two parts: Local Heating (LH) and Reactive Hyperemia (RH). The researchers call the resting experiment "LH/RH."
- You will take aspirin, Plavix®, or a placebo (fake drug) as pre-treatments for experiments. The order of the drugs is random and unknown to you and the researcher.
- You take Furosemide (Lasix®) on one day to increase urine output for one LH/RH

experiment.

- LH/RH uses a technique called "Microdialysis" (MD). MD 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. The tubing acts like small blood vessels. The tubing allows substances to pass between the fluid in the tubing and the fluids in your skin. The researchers add test-substances to the fluid that are like some of the natural chemicals found in your body. The substances only reach a nickel-sized area of skin at each tube. The test substances are:
  1. L-NAME (*N^G-nitro-L-arginine methyl ester*) –can prevent your blood vessels from dilating.
  2. SNP (*sodium nitroprusside*) - causes your blood vessels to get as large as they can.
- In another part of the LH/RH experiment, the researchers restrict blood flow to one of your arms for a short time.
- The Exercise experiment involves biking in a warm room. The researchers measure your body's inner temperature with a probe that well-trained personnel place in your esophagus or "food tube."

3. <u>Procedures:</u> *You may request personnel of the same gender to perform procedures.*
Note: Do not exercise hard for 24 hours before a scheduled blood draw (i.e. Screening Day 1 and Experiments).

<u>Note on tests, procedures, and experiments:</u> A test, procedure (e.g. blood draws), or experiment may have to be repeated for various reasons. Examples of these reasons include problems with a blood sample, uncertain test-results, or power outage during an experiment. In such cases, the researchers will ask you to repeat the test, procedure, or experiment. You may decline.

_____ initial <u>Screening Day 1:</u> Do not eat or drink 10 hours before your exam. Report to Noll Lab for your appointment. Research and/or Clinical Research Center (CRC) staff conduct the screening. Well-trained personnel draw 18 ml (4 tsp) of blood from a vein in your arm. The researchers analyze the blood for clotting ability, blood cells, fats in the blood, and blood chemistry. If you take thyroid hormone, well-trained personnel draw an extra 4 ml (1 tsp) to check the level of thyroid hormone. You have a physical exam including blood pressure, height, weight, and 12-lead ECG. Women of childbearing-age submit urine samples for pregnancy tests. The researchers may measure thicknesses of skin-folds on your body to determine your percent body fat.

_____ initial <u>Screening Day 2</u>: Report to Noll Lab for a medical history and graded exercise test (GXT). Bring clothes in which you can exercise. For the GXT, you pedal a bike to measure your fitness level. You wear a nose clip and breathe into a tube to measure exhaled oxygen and carbon dioxide. Well-trained personnel measure blood pressure and monitor ECG. The bike becomes a little harder to pedal every 2 minutes. You rate how hard you work (RPE) by using a numbered scale matched to short phrases. The test is most accurate if you pedal as long as you can. However, you may stop at any time. The test is 10-20 minutes long. After a 20-30 minute break, you pedal 5-10 minutes while the researchers collect your expired air. They record the bike's workload-setting that matches 60%-70% of your greatest effort in the GXT.

Visits 3 – 9: Experiments

Schedule Overview (Treatments are low-dose aspirin, Plavix®, or placebo.):

a. Pre-treatment 1 – 7-10 days (in your own home)
b. LH/RH and Exercise Experiments (These occur on two separate days that are at least one day apart and sometime during days 7 - 10 of pretreatment 1)
c. 2-week washout period (at least)
d. Pre-treatment 2 – 7-10 days (in your own home)
e. LH/RH and Exercise Experiments (These occur on two separate days that are at least one day apart and sometime during days 7 - 10 of pretreatment 2)
f. 2-week washout period (at least)
g. Pre-treatment 3 – 7-10 days (in your own home)
h. LH/RH and Exercise Experiments (These occur on two separate days that are at least one day apart and sometime during days 7 - 10 of pretreatment 3)
i. 2-week washout period (at least)
j. Furosemide (Lasix®) Treatment and LH/RH Experiment (may be at beginning of schedule instead)

   You arrive at Noll Lab around 7 AM. You take 40mg – 80 mg of Furosemide in pill-form. The drug increases urine output for several hours. You lose about 3% of your body's weight in water. About 2 hours later, the researchers collect urine to track weight loss. You ingest sports drink to maintain weight loss at 3%. The researchers measure blood pressure every 15 minutes. Five hours after furosemide, well-trained staff draws blood (about 13 ml, 3 tsp). Then LH/RH starts. You do not drink during LH/RH. After LH/RH, you may have more sports drink.

_____ initial Preparation For All Experiments: You complete the scheduled pre-treatment and/or washout periods.

One day before experiment: Please drink at least 6 glasses of fluid (water, juice, milk, etc.). Do not drink fluids that contain caffeine (i.e. coffee, tea, Coca Cola, etc.) for 12 hours before the experiment.

Day of experiment: Eat your typical breakfast. Bring shorts and short-sleeved shirt. For the exercise experiment, bring comfortable shoes, too.

When you arrive: The researchers measure heart rate, blood pressure, and weight. You wash your forearms and pat them dry. You drink 10 ml of sports drink for every kg of body-weight (or 2 tsp / 2 lbs). For example, someone weighing 72 kg (160 lbs) drinks 720 ml (3 cups). You give a urine sample. Well-trained personnel draw a baseline blood sample (16 ml or 3 tsp).

_____ initial A. Local Heating / Reactive Hyperemia Experiment (LH/RH)

This experiment has two parts: "Local Heating" (LH) and "Reactive Hyperemia" (RH). The researchers conduct the two parts of this experiment at the same time, but on different arms. LH usually uses the left arm, and RH uses the right. The researchers tell you the times during the experiment when you may take a break.

_____ initial a. Local Heating: The researchers place a tight band around your upper arm so they can see your veins. The researchers mark four MD sites on your forearm devoid of veins. The researchers connect a battery-powered controller to a small chamber (9.5 mm, 0.4 inch diameter) that contains bretylium tosylate and to an electrode. At two sites, the researchers attach a chamber and electrode nearby. The researchers apply a weak current (200 μamp) for 20 minutes. This moves bretylium into skin without needles (iontophoresis). Bretylium does not allow your skin's blood vessels to constrict (get smaller) when your skin is cold. The researchers remove the chambers and electrodes.

You dress in shorts. Women wear a sports bra, also. You don a suit lined with tubing. You lie on a bed. Water (34-36°C, 93–97°F) flows through the suit's tubing. The researchers tape laser probes and their holders onto your skin at the bretylium-sites. About one hour after iontophoresis, the researchers pump ice water through the suit's tubing for 3 minutes. Then they switch to warm water. If the bretylium works, the experiment proceeds. If the block does not work, you will not do the local heating experiment this day. You may come back another day to try again.

Inserting Microdialysis (MD) Tubing: For each MD site, the researchers make pairs of pen-marks on your arm 2.5 cm (1 inch) apart. MD tubing enters and exits your skin at the marks. The researchers clean your arm with an iodine-based fluid and then alcohol. An ice-bag on your arm for 5 minutes numbs your skin. They insert a thin needle into your skin near an entry mark. The needle's tip travels between the layers of skin for 2.5 cm (1 inch) and leaves your skin near the matching exit mark. The researchers thread the tubing through the needle. They withdraw the needle leaving the tubing in your skin. They insert 4 MD probes. Any redness of your skin subsides in 60 – 120 minutes. They tape a laser probe and holder over each MD site.

Experimental procedure: When the redness from inserting the MD tubing is gone, the experiment begins. The researchers start Lactated Ringer's flowing through the MD tubing. The holders start at about 33°C (91.4°F). After the SkBF is stable for at least 10 minutes, the researchers add L-NAME to the fluid flowing through two sets of MD tubing for the rest of the experiment:

MD Tubing 1: Lactated Ringer's
MD Tubing 2: Lactated Ringer's + L-NAME
MD Tubing 3: Lactated Ringer's (Bretylium pre-treated site)
MD Tubing 4: Lactated Ringer's + L-NAME (Bretylium pre-treated site)

The researchers obtain a second baseline for 20 minutes. They increase the temperatures at all sites to about 42.0°C (107.6°F). After SkBF is stable (about 40 minutes), Lactated Ringer's + L-NAME flows through all MD tubing. The SkBF becomes stable again (about 40 minutes). The researchers increase the temperature to about 43°C (109.4°F) for about 30 minutes. At the same time, they switch the fluid to Lactated Ringer's + SNP. This increases SkBF at all sites to the highest level possible. The experiment ends. The researchers clean where the tubing enters and exits your skin with alcohol. They pull the tubing from your skin and place a sterile bandage over the sites. They may place a bag of ice on your arm for 10 minutes to reduce any bruising.

_____ initial b. Reactive Hyperemia Experiment: The researchers tape two laser probes and their holders on your other forearm. They place a cuff on your upper arm. They inflate the cuff for 5 minutes. During this time, blood does not enter or exit your arm. The researchers deflate the cuff so that blood flow returns to your arm. They wait at least 20 minutes and then repeat 2 more times. Then they increase the temperature to about 43°C (109.4°F) for about 30 minutes. This increases the SkBF at the sites to the highest level possible. Then the experiment ends.

LH/RH Measurements: Throughout both experiments, the researchers measure SkBF with the laser probes. They also measure skin temperatures with the probe-holders. They measure heart rate and blood pressure about every 5-7 minutes with cuffs on your upper arm and/or finger.

LH/RH Experiment Ends: The researchers re-test the Bretylium site (see above). Well-trained personnel draw a blood sample (about 13 ml, 3 tsp). The researchers measure your blood pressure and heart rate before you leave.

_____ initial B. Exercise Experiment

Well-trained personnel place a tube into a vein in your arm so that they can draw blood. ***If the tube should stop working, the well-trained personnel remove it. You may give permission to insert a new tube, stop the experiment and repeat it another day, or decline to be in the experiment. If the tube stops working near the end of the experiment, you may choose to have the last couple of blood draws performed with a regular needle-stick for each draw. The well-trained personnel use normal saline to clear blood from the tube after each blood draw. You may choose to have numbing cream (LMX) applied to the site at which the well-trained personnel will insert the tube. After the personnel apply cream, they wait about 30 minutes prior to inserting the tube in your vein. The cream cannot be used as effectively for replacement tubes inserted into veins once the experiment begins.***

Body temperature: The researchers seal a temperature probe in a tube that looks like a strand of spaghetti. Well-trained personnel coat the tube with Lidocaine gel to make the tube slippery and numb tissue touching the tube. They can use water instead of gel. They insert the tube through your nose and into your esophagus or "food tube." You drink water or sports drink through a straw while the well-trained personnel guide the probe into place. The inserted length of tube is equal to ¼ of your height. The probe in the end of the tube rests in your esophagus at heart level. When the tube is in place, they anchor the free end into a headband. Also, they use tape on your face to keep the tube still. You do not drink or eat, but you can talk with the probe in place. The temperature-reading is more stable if you limit swallowing.

You enter a warm room (about 30°C/86°F and 40% relative humidity). You sit on a stationary, semi-recumbent bike. The researchers tape six wires to your skin (back, calf, thigh, abdomen, chest, and arm) to measure temperature. They tape 2 laser probes and their holders on the skin of your right forearm to measure SkBF. They strap a Polar heart rate monitor around your chest.

Plethysmography: Plethysmography measures the blood flowing into your forearm. The researchers place a cuff around your left wrist and upper arm. They place a strain gauge that looks like a rubber band around your left forearm between the cuffs. During the experiment, the researchers perform a series of 4 readings every 10 minutes. Each series lasts about 1 minute.

For each series, the wrist-cuff inflates to stop blood flow to your hand. The upper arm-cuff inflates allowing blood flow into your arm, but blocking flow out. This causes a slight increase in the size of your forearm detected by the gauge. For each series, the wrist cuff remains inflated while the upper arm cuff switches 4 times between inflation (10 seconds) and deflation (5 seconds). Then the researchers deflate both cuffs.

Experimental procedure: You rest for 15 minutes during baseline readings. When the readings are stable, you begin to pedal the bike, and well-trained personnel draw a second blood sample (about 9 ml, 1 tsp). You pedal for one to two hours. The researchers measure your heart rate, blood pressure (every 5-7 minutes), SkBF, forearm blood flow, skin temperature, and your body's temperature throughout exercise. You use charts to rate how hard you are working (RPE) and how warm you feel. Numbers on the charts refer to descriptions of effort or warmth. Qualified personnel draw blood (about 9 ml, 1 tsp) when your body's temperature rises about ½ °C (about 1°F) above your baseline temperature. They draw another blood sample (about 9 ml, 1 tsp) when your temperature rises 1°C (about 2°F) above baseline. When your temperature reaches 39°C (102 °F) or you have pedaled for 2 hours (whichever comes first), they draw a blood sample (about 9 ml, 1 tsp). Then exercise ends. However, you may stop pedaling sooner if you wish. The researchers cool the room to 25°C (77°F) and reduce the humidity to 30%. You rest on the bike for 45 minutes. As you rest, the researchers heat the laser probe-holders to 43°C (109.4°F) for about 30 minutes. This increases the SkBF at all sites to the highest level possible. Well-trained personnel draw a last blood sample (about 8.5 ml, 0.7 Tbsp), remove the tube, and apply a bandage. Well-trained personnel remove the probe from your throat. The researchers measure your weight and take a urine sample. Then the experiment ends.

4. <u>Discomforts and risks:</u> Note: Before the researchers perform any procedure, they verbally inform you of the training and/or experience of the researcher performing the procedure. You may stop a procedure at any time. During screenings and experiments, medical assistance is readily available. If a problem arises that requires medical attention during an experiment, a researcher stays with you. Another researcher summons assistance from the emergency medical system by calling 911 and meets the responders at the door of Noll Lab. All of the researchers have current basic life support training. In addition, some of the researchers have current first aid training. If a problem arises while you are at the Clinical Research Center, the problem is handled according to their standard procedures.

<u>Microdialysis (MD):</u> The risks are less than that for a blood draw because microdialysis uses only a small, local area of skin. In contrast, a blood draw involves large blood vessels and blood also. MD can cause some pain and bruising like that from a blood draw. However, the researchers use ice to numb your arm and small needles to reduce pain when they insert the tubing. You are not likely to feel pain from MD tubing once it is in place. You may feel a little pain when the researchers remove the tubing. You may be nervous about needles. If so, your blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick to your stomach, or may faint. If the tubing breaks during removal, they remove the tubing by pulling on the other end. This presents no extra risk to you. The tubing could break leaving a small piece under your skin. This has not occurred in any of our studies. In this case, they may have to cut the thin layer of skin over the tubing to remove the tubing. Mild pressure with sterile gauze stops any slight bleeding. Infection is possible, but has never occurred in our or any other

lab of which the researchers know. Sterile techniques and supplies keep the risk minimal. The researchers apply a sterile bandage afterward. They tell you how to care for the sites.

Fluid flowing through the tubing: The substances flowing through the tubing only go to a 2.5 cm^2 (0.4 $inch^2$) area of skin at each tubing-site. The amount entering the skin is very small. There is a remote 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, blood pressure change and/or fainting.

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 and SNP: Only minute amounts of these substances enter the nickel-sized area of skin around the MD tubing. The researchers and/or other researchers have used these substances in human skin. There have been no reports of bad reactions.

Low-dose Aspirin (81 mg daily): When taking aspirin you may notice that it takes longer to stop bleeding. You may also bleed or bruise more easily. Minor side effects are upset stomach; heartburn; tiredness; or headache. Other minor side effects are diarrhea or constipation, mild nausea, vomiting, and stomach gas. Serious side effects are black, bloody, or tarry stools; coughing up blood or vomit that looks like coffee grounds; severe nausea, vomiting, or stomach pain; and tinnitus (ringing or other noise in your ears). You could have confusion, general ill feeling or flu-like symptoms, pain on swallowing, redness, blistering, peeling or loosening of the skin, including inside the mouth or nose, trouble passing urine or change in the amount of urine, feeling unusually weak or tired, or yellowing of the eyes or skin. You could have an allergic reaction. This could include a rash, itching, and difficulty breathing, tightness in the chest, and/or swelling of the mouth, face, lips, or tongue. You will receive instructions and more information for aspirin on a separate paper.

Clopidogril (Plavix ®, 25 mg daily): When taking Plavix ® you may notice that it takes longer to stop bleeding. You may also bleed or bruise more easily. Serious side effects are severe allergic reactions (rash; itching; difficulty breathing; tightness in the chest; swelling of the mouth, face, lips, or tongue); bleeding in the eye; change in vision; change in the amount of urine; chest pain; dark or bloody urine; black, tarry stools, unusual or severe bleeding (e.g., increased menstrual bleeding, unexplained vaginal bleeding, unusual bleeding from the gums when brushing); loss of appetite; pale skin; seizures; severe, persistent headache; sore throat or fever; speech problems; unusual bruising; weakness; unexplained weight loss; yellowing of skin or eyes. A rare but serious condition called Thrombotic thrombocytopenic purpura (TTP) may develop while taking Plavix ®. This condition requires urgent treatment and could be fatal. TTP includes kidney problems, broken red blood cells, anemia, problems with the nervous system, fever, and reduced blood platelets. Symptoms of TTP include fever, weakness, fatigue, pallor, shortness of breath on exertion , heart rate over 100 beats per minute, purplish spots in the skin produced by small bleeding vessels near the surface of the skin (purpura), bleeding into the skin or mucus membranes, headache, confusion, speech changes, changes in consciousness, yellowish color to the skin. The FDA warns that some people being treated for cardiovascular disease with Plavix

cannot break down the Plavix. This is because they have a low level of a certain enzyme in the liver. The drug does not work very well in these people. Also, Plavix increases their risk of heart attack and stroke when they suddenly stop taking the drug. This lack of ability to break down Plavix occurs in 2% of whites, 4% of blacks, and 14% of Chinese (3% of the population as a whole). A costly genetic test can tell you if you are one of those people at risk; however, the researchers will not be performing this test. These side effects also occurs in people taking certain drugs that reduce stomach acid such as Prilosec, Zegerid (omeprazole), Prevacid (lansoprazole), Protonix (pantoprazole), Aciphex (rabeprazole), and Nexium. These drugs reduce the same enzyme in the liver. You will not be in the study if you are taking these drugs. You will not start taking these drugs while you are in the study. You will receive instructions and more information for Plavix ® on a separate paper.

Furosemide (Lasix ®, 40 mg): Furosemide increases urine output for several hours. Doctors use Furosemide to treat fluid-buildup in people with congestive heart failure, hypertension, liver disease, or some kidney disorders. The researchers use Furosemide to decrease your body's water on one day of LH/RH experiments. This alters your blood's resistance to flow. You are likely to feel thirsty after taking Furosemide. The researchers give you water or sports drink when the experiment ends. You take 40 – 80 mg in pill-form that one day. Most side effects result from long-term use of Furosemide. Mild side effects of Furosemide include diarrhea, constipation, or stomach pain; headache; numbness, burning, pain, or tingly feeling; dizziness; or blurred vision. More serious side effects are dry mouth, thirst, nausea, vomiting; feeling weak, drowsy, restless, or light-headed; fast or uneven heartbeat; muscle pain or weakness; urinating less than usual or not at all; easy bruising or bleeding, unusual weakness; a red, blistering, peeling skin rash; hearing loss; or nausea, stomach pain, low fever, loss of appetite, dark urine, clay-colored stools, jaundice (yellowing of the skin or eyes). An allergic reaction can include hives; difficulty breathing; and/or swelling of the face, lips, tongue, or throat.

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 be nervous about needles. If so, your blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick to your stomach, or may faint. To keep the chance of infection minimal, the staff uses the same supplies and techniques used in hospitals. Well-trained personnel use a needle to withdraw blood for the screening and the LH/RH experiment. They take out the needle as soon as they obtain the blood sample. Well-trained personnel insert a catheter (a small plastic tube) into a vein for blood samples in the Exercise experiment. They remove the catheter when they withdraw the last blood sample of the Exercise experiment.

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

Medical Screening: You may feel shy about giving health information. The researchers collect information in a private and professional manner. You may feel shy about being measured. You may request someone of the same sex to conduct the screening.

Phone screening form: The form helps us to decide whether you are a good candidate for the study. You may feel shy about answering questions. The researchers collect information in a private and professional manner. Some people fill out forms at our secured website. The website encrypts submitted forms. The researchers keep completed forms confidential and secure.

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

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

Skin temperatures: The researchers tape wires to 6 sites on your skin. The tape may irritate your skin.

Skin Fold Measurements: The researchers measure your percent body fat using a tool that looks like tongs. The tongs gently measure the thickness of skin folds at several places on your body. There are no risks to this measure, but you may feel shy. The researchers make the measures privately and professionally.

Blood Pressure (manual and/or Cardiocap 5): The researchers measure blood pressure using the method common in a doctor's office or with a machine. A cuff inflates on your upper arm. As the cuff slowly deflates, the researchers listen with a stethoscope at the bend in your elbow or the machine takes a reading. During the short time the researchers inflate the cuff, your arm may feel numb or tingly. The cuff could cause mild bruising.

Blood Pressure (Finapres): The small cuff on your finger pulses with your blood pressure. In time, your finger may feel numb or tingly. The researchers can move the cuff to another finger or stop the reading to rest your finger.

Metabolic Measurements: On Screening Day 2, well-trained personnel collect your expired air to measure O_2, CO_2, and volume. There are no risks to the collection of expired air.

Ratings of Perceived Exertion (RPE) and Thermal Sensation Scales: The only correct answers are those that truly describe what you are feeling.

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. The researchers watch you closely.

Forearm Blood Flow: Your arm and/or wrist may feel numb or tingly when the researchers inflate the cuffs. The cuffs may cause mild bruising. The tape may irritate. The mild pressure of the strain gauge may leave a mark on your skin that remains for a short while after the researchers remove the gauge. The technique is unlikely to produce lasting ill effects.

Exercise in the heat: You will feel very warm and will sweat. You will likely have an increase in blood pressure, heart rate, and breathing rate. Exercise in the heat can cause fatigue, cramps, quick shallow breathing, an unsteady breathing pattern, and/or lightheadedness. Although unlikely, you could faint or have nausea. Severe problems like unsteady heart rate, chest pain or heart attack are rare. The researchers watch you closely.

Stationary Bike: It is possible for you to stumble or fall getting on or off the bike leading to cuts, scrapes, dislocations, broken bones, head injury, or abnormal heart rhythms. The researchers assist you on and off the bike. The researchers will tell you the safe use of the bike and watch you closely while you are on the bike.

Core Temperature (esophageal probe): Inserting the probe may tickle your nose. You might sneeze. Although rare, the probe could irritate or scratch soft tissue in your nose and throat. A thin coat of Lidocaine gel on the probe helps to prevent this. You may have us use water instead of gel. You drink as well-trained personnel insert the probe. While drinking, you could choke or get fluid in your windpipe. You may gag. Gagging or coughing when the tip of the probe is at the back of your upper throat could push the probe toward your mouth. In this case, they remove the probe by gently pulling it back through your nose. They reinsert the probe when you are ready. When in place, the probe may feel like a vitamin pill that is stuck in the back of your throat. This feeling is common, usually fades in time, but normally does not go away while the probe is in place. Likely, you will notice the feeling more when you swallow, talk, or turn your head. Although rare, vomiting and nausea can occur. The probe's wires are fragile. Once the probe is in place, the researchers make sure it still works correctly. If not, the well-trained personnel remove the probe and request to insert another. You may decline and not be in the exercise experiment. Although unlikely, it is possible to place the probe into your windpipe. The researchers have never misplaced a probe into the windpipe in any of our many projects. Placing the probe in the windpipe could cause coughing, difficulty in speaking, and/or could irritate or damage the vocal cords. If the probe enters the windpipe, the well-trained personnel gently remove it.

Lidocaine gel: The gel tastes unpleasant to some people. Therefore, the well-trained personnel put very little gel on the probe. If the taste is strong, it could make you gag. There is a very small chance of an allergic reaction that could include rash, swelling, itching, redness, difficulty in swallowing, and/or heart or breathing trouble.

Bretylium Iontophoresis: Iontophoresis lasts 20 minutes. The controller does not allow the current to run longer than one hour. This technique can cause chemical burns if allowed to run for well beyond an hour. You may feel a slight itchy or tingly feeling while the current is on. Your skin may redden slightly. The redness ends before the experiment begins. The sticky disks may irritate your skin. The bretylium affects the nerves only at the site of iontophoresis (0.64 cm2, 0.1 in^2) and has no whole-body effects. The researchers and other researchers have used

iontophoresis of bretylium in humans for many years. There have been no adverse effects of bretylium used in this fashion. Although unlikely, an allergic reaction could produce redness, itching, rash, and/or swelling. A severe allergic reaction could cause fever, difficulty in breathing, changes in pulse, convulsions, and/or fainting.

Cold Stress: The researchers pump ice water through the suit for 3 minutes, but you may have us re-warm you at any time. You may shiver when you skin grows cold. You may find the cold unpleasant. Your heart rate and blood pressure may temporarily increase a little. There are no lasting bad effects.

Providone Iodine: Hospitals and researchers use this orange-colored fluid to clean and sterilize the skin. You could have a bad reaction if you are allergic to iodine. 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 blood pressure change and/or fainting. The researchers can use alcohol instead.

Latex: Some gloves and medical materials are made of latex rubber. If you are allergic to latex, you will not participate in the study.

LMX Cream: LMX (lidocaine) is a numbing cream often used for anal or rectal problems. The researchers will not use LMX if you are allergic to lidocaine. The most common side effects are irritation, numbness, and redness. Other side effects are headache, feeling lightheaded, feeling nervous, and twitching. You could also become confused, vomit, have blurred vision, or feel chest pain. You could become dizzy, feel drowsy, and have an abnormal heart rhythm. Severe allergic reactions include rash, hives, itching, problems breathing, and tightness in the chest, swelling of the mouth, face, lips, or tongue. LMX could cause the skin to become more sensitive. The numbing effects of the LMX are likely to have stopped by the time you leave the lab; nonetheless there is a chance that you could have an accidental injury to the area due to the loss of feeling. Most of the side effects are related to someone being exposed to a large amount of lidocaine. The researchers apply very little LMX to the small area of skin at the site of the tube-insertion. In the case of severe reactions to the cream, the researchers call 911. They can handle minor problems (e.g. skin-rash) at the lab.

5a. Benefits to you: 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: Heart disease is the number one cause of death of men and women in America. The benefit of daily low-dose aspirin as one way to help prevent heart disease has been well established. The study explores a new side effect of daily aspirin that could put people at greater risk of heat illness. This could prompt a change in the way doctors use aspirin in their patients someday thereby improving the safety and well-being of people taking low-dose aspirin. Also, this study explores the possible means by which aspirin exerts its effects on blood vessels in the skin. This could further our knowledge of how the body controls SkBF during heat stress.

6. <u>Time duration of the procedures and study</u>: You will need to visit the Noll Lab for the following:

_____ initial Visit 1	Screening	1/2 hour
_____ initial Visit 2	Screening	1 hour
_____ initial Visits 3-9	Experiment Days:	

LH/RH Experiment (4): 6 hours each
Exercise Experiment (3): 5 hours each
Total: 40.5 hours

7. <u>Alternative Procedures:</u> The researchers could measure your temperature with a probe inserted into your rectum, under your tongue, or in your ear. You could also swallow a pill that measures temperature. These techniques are less accurate for the purposes of this project, and are not used in this study. The other techniques in the study are used in research worldwide. They are the best means by which to meet the goals of this study with minimal discomfort and risk to you.

8. <u>Statement of confidentiality</u>: The researchers code volunteers by an identification number for statistical analyses. They keep all records in a secure location. All records associated with your participation in the study are subject to the usual confidentiality standards applicable to medical records (e.g., such as records maintained by physicians, hospitals, etc.). In the event of any publication resulting from the research, the researchers do not disclose personally identifiable information. The Office of Human Research Protections in the U.S. Department of Health and Human Services, The U.S. Food and Drug Administration (FDA), The Penn State University Office for Research Protections (ORP) and The Penn State University Institutional Review Board may review records related to this project.

9. <u>Right to ask questions:</u> Please contact Lacy Holowatz (W: 814-867-1781, M: 814-880-9217), Rebecca Bruning (W: 814-863-2948, M: 989-351-9080), Susan Slimak (W: 814-863-8556, H: 814-237-4618), or Jane Pierzga (W: 814-865-1236, H: 814-692-4720) with questions, complaints, or concerns about the research. You can also call this number if you feel this study has harmed you. If you have any questions, concerns, problems about your rights as a research participant or would like to offer input, please contact The Pennsylvania State University's Office for Research Protections (ORP) at (814) 865-1775. The ORP cannot answer questions about research procedures. Questions about research procedures can be answered by the research team.

10. <u>Payment for Participation:</u>

<u>LH/RH Experiment:</u> You receive $15.00 for each MD tube inserted in your arm and $40.00 more for each completed experiment. ($100.00 for each experiment x 4 experiments = $400.00 total for RH/LH experiments)
For each incomplete experiment, the researchers pay you an amount equal to the part of the experiment that you complete. For instance, if you complete only half of an LH/RH experiment, the researchers pay you for each probe that the researchers inserted plus $20.00 for that trial. This is because $20.00 is one half of $40.00.

Exercise Experiment: You receive $100.00 for each completed experiment. ($100.00 for each experiment x 3 experiments = $300.00 total for Exercise experiments).
For incomplete experiments, the researchers pay you a portion of the $100.00 equivalent to the percent of the experiment that you completed.

Total for the project: $700.00 In addition, you may have a lab T-shirt, bag, or sport bottle.

The researchers may ask you to repeat a trial. If you agree to repeat a trial, they pay you 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.

11. Injury Clause: In the unlikely event you become injured as a result of your participation in this study, medical care is available. Please call Lacy Holowatz (W: 814-867-1781, M: 814-880-9217), Rebecca Bruning (W: 814-863-2948, M: 989-351-9080), Susan Slimak (W: 814-863-8556, H: 814-237-4618), or Jane Pierzga (W: 814-865-1236, H: 814-692-4720). It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

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

14. Abnormal Test Results: In the event that the researchers obtain abnormal test results, they will apprise you of the results immediately and advise you to contact a health care provider for follow-up.

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

The researchers will give you a copy of this signed and dated consent form for your records.

_____

Volunteer **Date**

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

_____

Investigator **Date**

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Graduate Teaching and Research Assistant, Dept. of Kinesiology, The Pennsylvania State University	2009-2013

Selected Awards

EOPIG Jack Borgenicht Ph.D. Research Award	2012
American Physiological Society, Caroline tum Suden Frances A. Hellebrandt Professional Development Opportunity Award	2011
Graduation with Honors, Summa Cum Laude, Alma College	2009
President's Cup Award (4.0), Alma College	2006-2008

Peer Reviewed Publications

1. Smith CJ, Santhanam L, **Bruning RS**, Stanhewicz AE, Berkowitz DE, Holowatz LA. Upregulation of inducible nitric oxide synthase contributes to attenuated cutaneous vasodilation in essential hypertensive humans. *Hypertension*. 58(5):935-42, 2011.
2. Stanhewicz AE, **Bruning RS**, Smith CJ, Kenney WL, Holowatz LA. Local tetrahydrobiopterin administration augments reflex cutaneous vasodilation through nitric oxide-dependent mechanisms in aged human skin. *J Appl Physiol*. 112(5):791-97, 2012.
3. **Bruning RS**, Santhanam L, Stanhewicz AE, Smith CJ, Berkowitz DE, Kenney WL, Holowatz LA. Endothelial nitric oxide synthase mediates cutaneous vasodilation during local heating and is attenuated in middle-aged human skin. *J Appl Physiol.*112(12): 2019-26, 2012.
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5. Kenney WL, Stanhewicz AE, **Bruning RS**, Alexander LM. Blood pressure regulation III: What happens when one system must serve two masters: temperature and pressure regulation? *Eur J Appl Physiol*. (Epub ahead of print), May 1, 2013.
6. *Dahmus JD, ***Bruning RS**, Kenney WL, Alexander LM. Oral clopidogrel improves cutaneous microvascular function through EDHF-dependent mechanisms in middle-age humans. *Am J Physiol Regul Integr Comp Physiol*. (Epub ahead of print), June 26, 2013.
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