THERMAL LOADING MODALITIES AND CUTANEOUS ACTIVE VASODILATION

by

MICHAEL A. FRANCISCO

A DISSERTATION

Presented to the Department of Human Physiology
And the Graduate School of the University of Oregon
In partial fulfillment of the requirements
For the degree of
Doctor of Philosophy

June 2020

DISSERTATION APPROVAL PAGE

Student: Michael A. Francisco

Title: Thermal Loading Modalities and Cutaneous Active Vasodilation

This dissertation has been accepted and approved in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Department of Human Physiology by:

Dr. Christopher Minson	Chairperson
Dr. John Halliwill	Core Member
Dr. Grant Simmons	Core Member
Dr. William Cresko	Institutional Representative

and

Kate Mondloch	Interim Vice Provost and Dean of the Graduate School

Original approval signatures are on file with the University of Oregon Graduate School.

Degree Awarded June 2020

DISSERTATION ABSTRACT

Michael Francisco

Doctor of Philosophy

Department of Human Physiology

June 2020

Title: Thermal Loading Modalities and Cutaneous Active Vasodilation

In humans, the primary response to increasing internal temperature is increased cutaneous blood flow along with sweating. These reflexes facilitate heat dissipation to the environment by expanding the thermal gradient between the core and the periphery of the body. The known mechanisms and transducers of cutaneous active vasodilation (CAVD) have been largely characterized by supine passive whole-body heating models and applied to our understanding of exercise heat loading. The goal of this dissertation was to assess the mechanisms of CAVD in response to varying thermal loading modalities and postures. First we sought to determine if posture could alter the nitric oxide component of CAVD. Second, we sought to confirm the cholinergic co-transmitter theory of active vasodilation during whole-body passive heating and expand these findings to exercise heat loading. Lastly, we sought to identify unknown vasoactive substances associated with cholinergic nerve co-transmission. Twenty four subjects were assigned to participated in one of two study protocols.

Protocol 1 assessed neuronal and endothelial nitric oxide synthase (nNOS and eNOS) isozyme contributions to the nitric oxide component of CAVD during passive whole-body heat loading in the seated and supine positions. We found that eNOS is the

primary mediator of NO during CAVD (Chapter IV). This finding conflicts with the findings of other studies, which have shown that the NO component of passive whole-body heat loading is mediated by nNOS.

Protocol 2 assessed the cholinergic co-transmitter theory of CAVD during exercise and passive whole-body heat loading. As discussed in Chapter V, we found that both thermal loading modalities result in CAVD through cholinergic nerve transmission, and that vascular transduction of active vasodilator nerve activity was similar between the two modalities.

The studies described herein provide evidence that CAVD is widely the same regardless of how thermal loading is accomplished. Despite exercise and passive whole-body heat loading having largely different hemodynamic and thermodynamic profiles, the afferent signals they share in common and those that delineate them do not appear to impact the mechanisms and transducers of CAVD as was originally thought.

This dissertation includes published and unpublished co-authored material.

CURRICULUM VITAE

NAME OF AUTHOR: Michael Francisco

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon, Eugene

DEGREES AWARDED:

Doctor of Philosophy, Human Physiology, 2020, University of Oregon
Master of Science, Human Physiology, 2016, University of Oregon
Bachelor of Science, Human Physiology, 2013, University of Oregon

AREAS OF SPECIAL INTEREST:

Thermal Physiology
Thermoregulation
Microcirculation
Heat Acclimation
Cardiometabolic Health
Post-Exercise and Post-Heat Stress Hypotension

PROFESSIONAL EXPERIENCE:

Graduate Research Fellow. University of Oregon, Human Physiology Department,
2018-Present

Instructor of Record. University of Oregon, Human Physiology Department,
2015, 2017, and 2019

Graduate Teaching Fellow. University of Oregon, Human Physiology Department,
2013-Present

GRANTS, AWARDS, AND HONORS:

The Eugene and Clarissa Evonuk Memorial Graduate Fellowship in Environmental or Stress Physiology "Thermal loading modalities and cutaneous active vasodilaton", University of Oregon, 2018

PUBLICATIONS

Cameron Colbert*, **Michael Francisco***, Emily Larson, Dylan Sieck, John R. Halliwill and Christopher T. Minson. Hemodynamics of post-exercise vs. post-hot-

water immersion recovery. (In review, 2019, *J Appl Physiology*) *co-first authorship

B.R. Ely, **M.A. Francisco**, J.R. Halliwill, S.D. Bryan, L.N. Comrada, E.A. Larson, V.E. Brunt, C.T. Minson. Heat therapy reduces sympathetic activity and improves cardiovascular risk profile in women who are obese with polycycstic ovary syndrome. *Am J Physiol Regul Inegr Comp Physiol.* 317(5): R630-R640, 2019.

V.E. Brunt, K.M Needham, L.N. Comrada, **M.A. Francisco**, T.M. Eymann, C.T. Minson. Serum from young, sedentary adults who underwent passive heat therapy improves endothelial cell angiogenesis via improved nitric oxide bioavailability. *Temperature.* 6(2):169-178, 2019.

Brett R. Ely, Laurie N. Blanchard, Jared Steele, **Michael A. Francisco**, Samuel N. Cheuvront, Christopher T Minson. Physiological Responses to Overdressing and Exercise-Heat Stress in Trained Runners. *Med Sci Sports Exerc*. 50(6): 1285-1296, 2018.

Michael A Francisco, Christopher T Minson. Cutaneous active vasodilation as a heat-loss thermoeffector. In: Thermoregulation: From Basic Neurosciences to Clinical Neurology. Handbook of Clinical Neurology.156:194-209, 2018

Michael A. Francisco, Vienna E. Brunt, Krista N. Jensen, Santiago Lorenzo, Christopher T. Minson. Ten days of repeated local forearm heating does not affect cutaneous vascular function. *J Appl Physiol*. 123(2):310-316, 2017.

Vienna E. Brunt, Taylor M. Eymann, **Michael A. Francisco**, Matthew J. Howard, Christopher T. Minson. Passive heat therapy improves cutaneous microvascular function in sedentary humans via improved nitric oxide-dependent dilation. *J Appl Physiol*. 121(3):716-723, 2016.

Vienna E. Brunt, Matthew J. Howard, **Michael A. Francisco**, Christopher T. Minson. Passive heat therapy improves endothelial function, arterial stiffness and blood pressure in sedentary humans. *J Physiol*. 594(23):7143-7144, 2016.

ACKNOWLEDGMENTS

I am thankful for the guidance and help of my advisor, Dr. Christopher Minson, and my dissertation committee, Dr. John Halliwill, Dr. Grant Simmons, and Dr. William Cresko. This research would not have been possible without your help. Thank you for exemplifying the type of scientist and mentor I strive to be.

I am also thankful of my lab mates, both past and present. Thank you Dr. Vienna Brunt for teaching me the ins and outs of our lab's skin blood flow research line. Thank you Dr. Brett Ely for being the best office mate, a wonderful idea sound board, and a true friend. Thank you Emily Larson, Brendan Kaiser, Brandon Gibson, and Dylan Sieck, for the countless hours you have spent helping me place fibers in the wee hours of any given Saturday morning. Thank you undergrads for being enthusiastic to help.

Thank you Karen Needham for your competence in everything and your patient demeanor. You are the guru of everything overlooked. Thank you Lee-Hwa Wang for volunteering your SAS programming expertise. I will always be grateful for your help.

To my friends, thank you for the encouraging words, always being there, and helping me throughout this process. To my parents, Dr. Carol Francisco and Dr. David Francisco, thank you for your unwavering support throughout my life. Mom, thank you for your statistical expertise and consultation on this project. Dad, thank you for marking up my writing with a lot of red and the words of encouragement. I recognize everything you have done for me as parents and I aspire to be like you two. To Jonathan, you are a true life mentor of mine, a best friend, and the best big brother I could ever have. To Amanda, thank you for being by my side throughout this, for your patience, for all the

small things that helped get me here, and for being the most wonderful person on the hardest of days.

I am grateful to my subjects, who volunteered their time and their physiology to this dissertation. You entrusted your health to me and without you there would be no human based research.

This investigation was partly funded by the Eugene & Clarissa Evonuk Memorial Fellowship and would not have been possible without this support.

TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

Background

Successful human temperature regulation is largely dependent on cutaneous vasomotor adjustments in nonglabrous (hairy) skin that act to expand or contract convective heat transfer between internal tissues and the periphery. Modulation of this thermal gradient ultimately determines heat loss and/or heat conservation between the periphery and the environment. Control of skin blood flow in nonglabrous skin is mediated by noradrenergic vasoconstrictor nerves and cholinergic active vasodilator nerves (46, 175, 176). Passive thermal loading in the supine position and the accompanying hyperthermia can increase skin perfusion from approximately 5-10% of cardiac output to approximately 50-70% of cardiac output (98, 178, 179, 181). The initial rise in skin blood flow during passive whole-body heating is due to vasoconstrictor withdrawal followed by increased vasodilator nerve activity known as cutaneous active vasodilation (CAVD) (46, 175). The active vasodilator nerves are responsible for approximately 80-95% of the abovementioned increase in skin blood flow during whole-body heat stress (98, 162). Many models of the structure and function of the CAVD system have been put forth. For example, it was previously thought that the skin blood flow response to whole-body heat stress was secondary to the sweating (sudomotor) response. Current convention holds that the CAVD system consists of a single set of nerves that release acetylcholine and other unknown substances (the co-transmission theory). This set of nerves is thought to be responsible for both sweating (sudomotor) and

CAVD activity during heat stress (78, 114). Alternatively, there is some evidence to suggest skin blood flow and sudomotor responses to heat stress are controlled by independent cholinergic nerves (38), however it is now thought that independent neural control is not likely (146). Due to the large role of the active vasodilator nerves in the skin blood flow response to whole-body heat stress, CAVD has been well studied in the supine human, but is still not entirely understood. For example, It is still unknown which vasodilator pathway(s) is responsible for the majority (approximately 50-70%) of the skin blood flow response to heat stress.

Currently nitric oxide (NO) is the principal identified vasodilator pathway contributing to CAVD (188, 189). It has been previously shown that pharmacological blockade (nonspecific) of NO synthase attenuates CAVD by approximately 30-45% in young healthy individuals during whole-body passive heating (148, 188, 189). Previously, the neuronal nitric oxide synthase (nNOS) isozyme has been implicated in the nitric oxide (NO) component of CAVD (117). Conversely, more recent work has demonstrated that the endothelial nitric oxide synthase (eNOS) isozyme is responsible for the NO component of CAVD during exercise (148). It was suggested that these disparate findings may be due to differences in active vasodilator nerve firing rates or increases in circumferential strain (associated with eNOS activity (138)) that are associated with exercise but not passive whole-body heat loading (148). These studies highlight distinct mechanistic differences in CAVD that may be dependent on the thermal loading modality used. Specifically, exercise and passive whole-body heating are largely different hemodynamic stressors that result in similar thermoregulatory reflexes. The afferent signals they share in common and those that delineate them may impact the mechanisms

and transducers of CAVD; yet these signals and their impact on CAVD are not well understood. For example, differences in the NOS isozyme contributions during upright exercise and supine whole-body passive heating may simply be due to differences in posture affecting the mechanisms of cutaneous vasodilation secondary to differences in afferent input (e.g. baroreception).

Nevertheless, previous convention still holds that the mechanisms of CAVD operate in a similar manner regardless of the mode by which an individual is thermally loaded (98). As such, the known mechanisms and transducers of CAVD have been largely characterized by supine passive whole-body heating models and applied to our understanding of exercise heat loading. The contradictory findings mentioned above suggest these preconceived notions to be incorrect. More research is needed to determine the similarities and differences of CAVD when thermal loading modalities are varied.

Statement of the problem

No studies have investigated the effects of posture on the NO component of CAVD. It is conceivable that posture (e.g. orthostasis) could explain differences in the NOS isozymes responsible for the NO component of CAVD during seated dynamic exercise and supine passive whole-body heating. It is also unknown if posture affects the relative contribution of NO to CAVD. Additionally, the most convincing evidence for the current co-transmission model of CAVD is derived from a singular study (114). This is problematic because these findings have not been independently corroborated, and the current model of co-transmission is based on a small sample size (observations in six individuals). It is unknown if this model extends to exercise heat loading or if cholinergic

co-transmission behavior is altered or absent from CAVD during exercise. Lastly, the substance(s) co-transmitted with acetylcholine are unknown.

Therefore, this dissertation had three aims. The first aim was to determine if postural effects could explain the observed differences in the NOS isozyme responsible for the NO component of CAVD during exercise and passive whole-body heating. The second aim was to confirm the cholinergic co-transmitter theory of active vasodilation during whole-body passive heating and expand these findings to exercise heat loading. The third aim was to identify unknown vasoactive substances associated with cholinergic nerve co-transmission.

In order to address these aims, we performed two separate studies. Study **Protocol 1** was designed to determine the contribution of nNOS and eNOS isozymes to cutaneous vasodilation during passive whole-body heating in the supine and seated positions (aim 1). We hypothesized that the NO component of CAVD during passive whole-body heating in the supine and seated positions would both be mediated by the nNOS isozyme. We also hypothesized that passive heating in the seated position would attenuate the overall skin blood flow response compared to passive heating in the supine position; but would not change the relative contribution of NO to reflex cutaneous vasodilation. Protocol 1 results are discussed in detail in Chapter IV.

Study Protocol 2 was designed to determine the extent to which cholinergic nerve activation mediates CAVD during passive whole-body heating and exercise heating (aim 2), and to identify unknown vasoactive substances associated with CAVD (aim 3). We hypothesized that the same cholinergic nerves are responsible for CAVD during passive whole-body heating and exercise heating. Secondarily, shotgun proteomics of

collected dialysate samples from the dermis was used in an attempt to identify unknown vasoactive substances that may be co-transmitted with ACh from active vasodilator nerves in the skin. Due to the 2020 Covid 19 pandemic, completion of dialysate proteomics analyses has been delayed indefinitely. Study Protocol 2 results are discussed in detail in Chapter V.

Significance

CAVD has been extensively studied in the supine human during passive whole-body heating. Very few studies have sought to characterize the mechanisms and transducers of CAVD unique to whole-body passive heating and delineate them from exercise heating. The research described in this dissertation was the first to 1) examine postural influences on the NO component of CAVD, 2) examine the cholinergic co-transmitter theory of CAVD during exercise heating, and 3) utilize shotgun proteomics in an attempt to identify unknown vasoactive substances associated with CAVD. While this line of research has no immediate clinical implications, chronic exercise and heat exposure have both been shown to improve cardiovascular health. Understanding the basic physiological similarities and differences underlying these stressors will improve our understanding of how they each elicit improvements in health. Additionally, this research has contributed to our understanding of the similarities and differences in human temperature regulation during exercise as well as passive whole-body heat loading.

Co-author Acknowledgement

The studies described in this dissertation were done with the help of several co-authors. Dr. Christopher T. Minson, Dr. John R. Halliwill, Karen M. Wiedenfeld-Needham, Brendan Kaiser, Emily Larson, and Brandon Gibson made substantial contributions to the work described in Chapters IV and V. Specific co-author and first author contributions are noted at the beginning of each chapter.

Acknowledgement of Previously Published Material

Chapter II of this dissertation contains substantial portions of material that have been previously published in the Handbook of Clinical Neurology: Thermoregulation: From Basic Neurosciences to Clinical Neurology, Chapter 12, 2018. This material was co-authored by myself and Dr. Christopher T. Minson as first author and corresponding author, respectively.

CHAPTER II

REVIEW OF THE LITERATURE

Acknowledgement of Previously Published Material

This chapter contains substantial portions of material that have been previously published in the Handbook of Clinical Neurology: Thermoregulation: From Basic Neurosciences to Clinical Neurology, Chapter 12, 2018. This material was co-authored by myself and Dr. Christopher T. Minson as first author and corresponding author, respectively.

Introduction

It is widely accepted that heat regulation in humans is modulated through cutaneous vasomotor adjustments, sweating, and thermogenesis (shivering). The importance of cutaneous vasomotor adjustments to thermal balance is unparalleled, as skin blood flow (SkBF) modulates convective heat transfer between the body core and skin surface where it then interfaces with the environment. The expansion or contraction of the thermal gradient from the core to the periphery via skin blood flow is therefore critical to increasing heat dissipation during environmental heat exposure or exercise, as well as reducing heat dissipation during exposure to cold environmental temperatures.

At rest, the skin receives approximately 5-10% of cardiac output; yet when maximally dilated (e.g. during passive heat stress in the supine position), the skin is estimated to receive up to approximately 6-8L/min, accounting for 50-70% of cardiac

output (CO) (98, 178, 179, 181). Of this profound increase in skin blood flow, cutaneous active vasodilation (CAVD) is responsible for approximately 80-95% (98, 180). The ability of the cutaneous vasculature to change from very low to very high blood flows is best understood as it pertains to thermoregulation. The cutaneous vascular bed is also involved in other cardiovascular adjustments by contributing to total peripheral resistance in a variety of situations, such as orthostasis and exercise. This is particularly true in a hot environment, as a competition exists between thermoregulatory and non-thermoregulatory reflexes (exercise, blood pressure regulation, etc.). While CAVD has been the independent focus of many studies during supine passive whole-body heating, relatively few studies have characterized the mechanisms and transducers of CAVD during orthostatic challenge (postural) or exercise. The complexity of the mechanisms involved in this system alone have made it difficult to understand CAVD in its entirety. The integration of multiple cardiovascular cues (e.g. exercise and posture) during thermal loading have also made it harder to understand CAVD.

This dissertation sought to:

1) examine the influences of posture on the nitric oxide component of CAVD
2) assess the contribution of sympathetic cholinergic nerve activation to the skin blood flow responses associated with CAVD during exercise and passive whole-body heat loading
3) identify unknown vasoactive substances associated with CAVD

In this chapter, the role of CAVD as a thermoregulatory process in humans will be reviewed. First, the importance of CAVD in modulating heat loss and the heat balance equation, thermo-sensing neurons that generate thermal cues (central & peripheral), and

the cutaneous neurovascular anatomy relevant to thermoregulation will be discussed. Second, we will present what was thought and what is now known about the mechanisms of CAVD in non-glabrous (hairy) skin, as this vasculature accounts for the majority of thermoregulatory heat loss via the CAVD system. Finally, we will summarize factors that can modify CAVD, such as sex hormones, posture, and exercise.

Thermal Regulation and the Heat Balance Equation

In humans, the skin surface is the boundary between the environment and the internal body. The thermal interaction between the environment and the body is described by the heat balance equation, defined as:

$$S = M - (\pm W) \pm (R + C) - E \qquad \textbf{(Eq 1)}$$

where ***S*** is the rate of storage of body heat, ***M*** is the rate of metabolic energy production, ***E*** is the rate of evaporative heat transfer, ***W*** is the rate of work, ***R*** is the rate of radiant heat exchange, and ***C*** is the rate of convective heat transfer ***(+ = gain, - = loss)*** (57)**.**

Blood flow from deep tissues to the skin surface transfers heat via convection. In the absence of sweat, elevations in SkBF will bring skin temperature closer to internal temperature, while reductions in skin blood flow will bring skin temperature closer to environmental temperature. In this way, the body is able to control sensible heat loss (via R and C) by controlling skin blood flow. In the presence of sweat, increases in skin temperature from increasing skin blood flow are reduced by the cooling effects of sweating, such that sensible heat loss (via R and C) is minimal. Instead, skin blood flow

is responsible for carrying heat to the skin where it can then be removed by sweat evaporation (via E). Therefore, sweating and CAVD are intrinsically linked and work in concert to effectively eliminate heat from the human body. The connection between sweating (sudomotor activity) and skin blood flow is discussed in more detail elsewhere (58).

Thermal Cues

While it is known that the reflex drive for increasing skin blood flow during thermal stress resides in the central nervous system, the specific thermal sensitive sites that detect internal and external temperature are not clearly defined. What is known is that the preoptic-anterior hypothalamus (PO/AH) in the brain is a primary site for thermoregulation with a lesser role for the spinal cord and peripheral thermoreceptors (12, 13). It is believed that this is where information about internal and surface temperatures are integrated and an appropriate efferent signal is generated.

The Preoptic Anterior Hypothalamus

The hypothalamus has been identified, through lesion and stimulation studies, as a participant in body temperature regulation since the late 1800's. It is now considered a critical site of central thermosensitivity (68), but other regions such as the spinal cord, splanchnic region, and skeletal muscle have been implicated in playing a role in temperature regulation (90, 130). The highly perfused hypothalamus is capable of rapidly responding to changes in blood temperature due to its low thermal inertia (perfusion per unit of organ mass) (72). Early animal studies have demonstrated that within the hypothalamus the PO/AH is the primary controller of thermoregulatory responses (69,

101). For example, warming the carotid blood or irrigating the third ventricle with warm saline has been shown to produce panting as well as cutaneous vasodilation (69, 101, 156). Within the hypothalamus there are 3 basic pools of neurons that have been characterized by their firing rates during local temperature changes. These include warm-sensitive neurons, cold-sensitive neurons, and temperature-insensitive neurons. Warm-sensitive neurons account for approximately 30% of the neuronal population and cold-sensitive neurons account for less than 5% of the neuronal population. Stimulation and lesion studies done by Kanosue et al. suggest that warm-sensitive neurons form the predominant thermoregulatory responses to all thermal stimuli (104, 105). Warm-sensitive neurons control heat loss, heat retention, and heat production outputs coming from the hypothalamus. More recent electro-physiologcal studies have shown that neurons in the PO/AH not only detect hypothalamic temperature but also receive and integrate input from thermoreceptors in the skin and spinal cord (**Fig 2.1**) (13). With this integrated information, the PO/AH can engage in thermoregulatory processes such as cutaneous vasomotor and sudomotor adjustments. In brief, the thermo-effector (sympathetic) signal for skin vascular control (158) leaves the PO/AH and inputs to the periaqueductal gray matter and ventral tegmental area of the midbrain. These midbrain areas directly feed onto the raphe/peripyramidal area of the medulla where the effector signal is then passed on to preganglionic neurons located in the intermediolateral column of the spinal cord. The effector signal is then passed on to postganglionic neurons in the sympathetic ganglia ultimately leading to the skin vasculature.

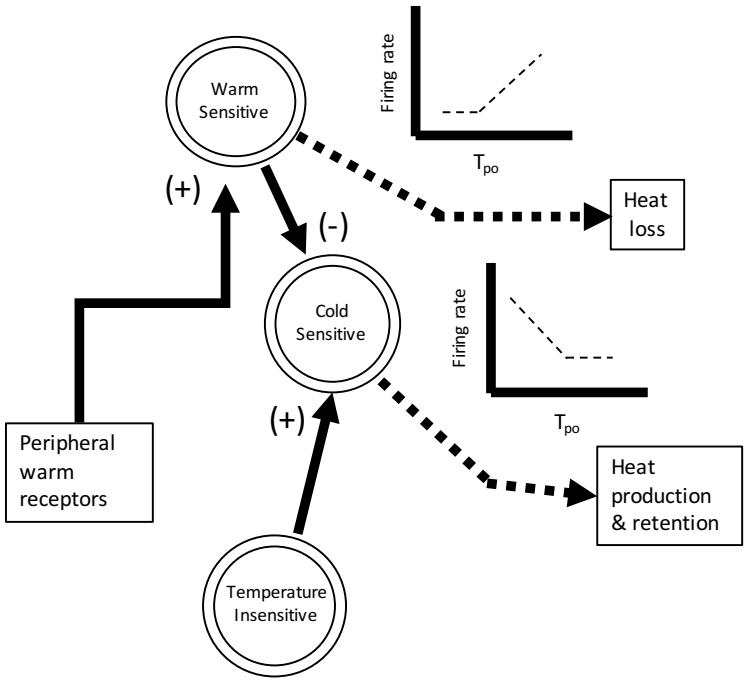

Figure 2.1. Adapted from Boulant (13). Model describing the 3 basic types of hypothalamic neurons on the basis of their firing rate responses to changes in preoptic temperature (T_{po}). Warm-sensitive neurons increase their firing rates during increases in preoptic temperature. Cold-sensitive neurons increase their firing rate during decreases in preoptic temperature. Temperature-insensitive neurons do not change their firing rates when T_{po} is changed. Warm-sensitive neurons have afferent synaptic input from skin and spinal thermoreceptors and are thought to be responsible for thermoregulatory responses. (+ = excitation, - = inhibition)

The Skin and Periphery

Environmental temperature is directly detected by sensory neurons via their projections in the skin where it is then relayed to the PO/AH for integration (Fig 2.1). Importantly, thermal cues in the cutaneous tissue are conveyed via non-myelinated C fibers and myelinated Aδ fibers. The actual detection of these thermal cues by the sensory neurons depends on the ion conductance of specific channels in a temperature-dependent manner. More recently, transient receptor potential vanilloid receptor (TRPV) channels 3 and 4 have been put forth as constitutive sensors of non-noxious warm stimuli. Interestingly, TRPV3 and TRPV4 channel expression is low in sensory neurons but is

abundant in epidermal and hair-follicle keratinocytes (32, 166, 193, 211). These observations have led to the hypothesis that temperature sensation is mediated by keratinocytes, which communicate with sensory neurons via the release of ATP and/or nitric oxide (NO) (143, 151, 155).

Cutaneous Anatomy

The cutaneous vasculature is arranged into a superficial and deep plexus that run parallel to the skin surface (15). The majority of the cutaneous blood vessels reside in the superficial plexus which is 1-2mm from the skin surface. Below the superficial plexus, in the lower dermal region, resides the deep plexus which consists of larger vessels that are formed from perforating vessels from deeper tissues. Ascending arterioles connect the deep and superficial plexuses as well as lateral vessels to sweat glands and hair follicles. The superficial plexus contains terminal arterioles, papillary loops (capillaries), and venules. The papillary loops provide a large surface area and are pivotal to heat exchange (15) and are also highly controlled by innervated arterioles. This basic vascular architecture is found in both glabrous and non-glabrous skin. Arteriovenous anastomoses (AVA) are found predominantly in glabrous skin (43, 89). AVAs are direct connections between the arterioles and venules that lie deep to papillary loops. AVAs allow blood to shunt the papillary loops and are therefore less efficient with heat transfer due to their small surface area and location in the dermis (15).

Four different sensory nerves have been identified in skin. These fiber types include myelinated Aα fibers, myelinated Aβ fibers, myelinated Aδ fibers, and unmyelinated C fibers. Type C and Aδ fibers are responsible for temperature sensing, but

respond to a variable range of stimuli (osmotic changes, mechanical stimulation, UV light, and chemical stimuli) and signal the central nervous system via the dorsal root ganglia and the spinal cord (177). Besides sensory neurons in the skin, autonomic fibers also exist (177).

Autonomic fibers innervate arterioles, AVAs, erector pili muscles, hair follicles, and both eccrine and apocrine sweat glands. In humans, the vasculature and sweat glands are innervated by both cholinergic and noradrenergic autonomic neurons. However, specific innervation varies by region. In nonglabrous skin, noradrenergic vasoconstrictor nerves as well as a cholinergic vasodilator system control skin blood flow to thermoregulatory challenges. The adrenergic vasoconstrictor system imparts a tonic vasoconstriction in the skin during thermoneutral conditions and can become active during cold stress. The cholinergic active vasodilator system is activated in response to elevations in internal temperature. In glabrous skin, adrenergic nerves innervate arterioles feeding papillary loops. Thermoregulatory activity in these areas is regulated by changes in adrenergic vasoconstrictor activity (28, 94, 106).

Responses to Hyperthermic Challenge

Regardless of the manner by which an individual becomes hyperthermic (via heat exposure and/or heat production), the same thermoregulatory reflexes attempt to attenuate and even reverse rising internal temperature. These reflexes involve increasing both skin blood flow and sweat rate in proportion to internal temperature (8) and can be modified by a variety of factors such as hydration status, circadian rhythm, and skin temperature. These modifiers will be discussed later in the chapter. However, the method

by which individuals are heat stressed in the laboratory can affect factors such as the timing, mechanisms, and effectiveness of the thermoregulatory reflexes. As such, a brief discussion of passive whole-body heat loading versus exercise heat loading and the basic cardiovascular adjustments is warranted.

When exposed to a hot environment, skin temperature (T_{sk}) begins to rise as the thermal gradient between the skin and the environment diminishes. This rise in T_{sk} reduces the gradient for heat transfer between the core tissues and the environment such that internal temperature will rise. The initial rise in core temperature causes an increase in skin blood flow that is mediated by vasoconstrictor withdrawal (46, 175) and the magnitude of this initial response is dependent on the environmental conditions (53). As internal temperature continues to rise, there is a marked increase in skin blood flow and sweating in non-glabrous skin that is the result of exceeding regulated body temperature to activate the CAVD system (94, 98, 176). In these areas, cutaneous active vasodilation can account for approximately 80-95% of the rise in skin blood flow that accompanies heat stress (98) (180).

If submerged in water (such as a hot tub or jacuzzi), the overall response is similar to that described above, but the thermodynamics and potential hemodynamics of the system have changed. For instance, the thermal conductivity of water is roughly 20 times larger than that of air (0.58 W/m/°C and 0.024 W/m/°C respectively) such that the rate at which internal temperature rises will be faster due to the increased rate of heat transfer and absence of evaporative cooling via sweating. In this system, loss of E combined with increasing skin blood flow will facilitate heat storage and inhibit heat dissipation.

The initial cutaneous vascular response to acute exercise involves a transient non-thermoregulatory reduction in skin blood flow that is mediated by increased sympathetic vasoconstrictor activity (11). As exercise progresses, the ensuing heat from cellular metabolism generates a thermal load that increases core temperature and initiates a thermoregulatory response similar to that described above. Differences in the mechanisms of CAVD and regulation of skin blood flow during passive and exercise heat stress are discussed in select sections below.

Transducers of CAVD

This section will review the history of CAVD research to provide a contextual framework for this dissertation. Briefly, research on the nerves associated with cutaneous vasodilation in response to thermal loading has been ongoing since the 1930's. There has been no further research on active vasodilator nerve activation in the last ~10 years and the current model of the cholinergic co-transmission theory of CAVD is now 25 years old.

Early Evidence for Reflex Cutaneous Vasodilation

The first evidence that skin blood flow was reflexively mediated came from Lewis & Pickering (140) and Gibbon & Landis (59) who observed that partial immersion of a limb or both forearms in warm water caused an increase in blood flow to the non-immersed limbs (legs or contralateral limb). It was posited by Lewis & Pickering that dual vasoconstrictor and vasodilator nerves may exist in the skin; however, conclusive evidence was not obtained until 1938 in a classic study by Grant & Holling (63). In this study, skin temperature was measured as a surrogate for skin blood flow in the forearm

and it was found that increasing forearm blood flow in response to whole body heating could be abolished by nerve blockade or sympathectomy (**Figure 2.2**). In addition, they noted that sympathectomy or nerve blockade caused a slight elevation in skin blood flow during normothermia.

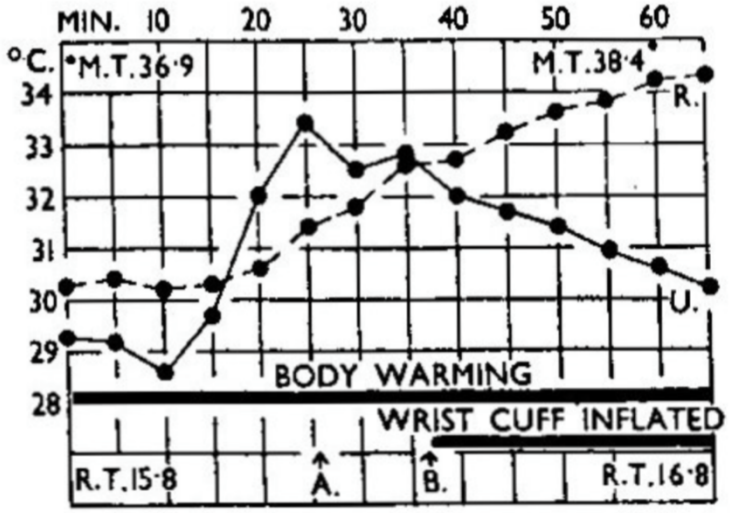

Figure 2.2. From Grant and Holling (63). Vascular effects of blocking internal cutaneous nerve on forearm skin temperature during body heating. Forearm temperatures were measured on Radial (R) and Ulnar (U) sides of the forearm. Procaine and adrenaline were injected around the cutaneous nerve above the elbow (Time point A) and resulted in anaethesia in the ulnar side of the arm.

Together, these findings demonstrated that cutaneous arterioles in non-glabrous skin had sympathetic vasodilator and vasoconstrictor nerves. These investigators also noted that sweating and the reflex increase in skin blood flow were initiated around the same time (or rise in body temperature) during heat stress, and proposed the notion that sudomotor activity was responsible for active dilation (this hypothesis is discussed in more detail later). Work by Edholm et al. (46) and Roddie et al. (175) confirmed the findings of Grant & Holling demonstrating that the initial rise in SkBF during whole body heating was due to vasoconstrictor withdrawal followed by vasodilator nerve

activity. Roddie et al. also demonstrated that administration of atropine (a muscarinic antagonist) abolished sweating but only reduced forearm vasodilation in response to body heating. This provided the first evidence that the sudomotor and active vasodilator systems may be mediated by a cholinergic mechanism. More recently, work by Kellogg et al. was able to verify and further characterize the dual efferent neural branches that innervate the cutaneous arterioles as a noradrenergic vasoconstrictor system and a non-adrenergic vasodilator system (109). These investigators found that application of bretylium tosylate (a prejunctional noradrenergic antagonist) via iontophoresis abolished vasoconstriction induced by cold stress but did not attenuate the vasodilator response induced by heat stress.

When the aforementioned studies are considered, a number of conclusions can be drawn. First, Lewis and Pickering demonstrated that increases in skin blood flow following heat stress are a neurally-mediated thermoregulatory response. Second, there is a noradrenergic vasoconstrictor system and a non-adrenergic vasodilator system. Third, cutaneous vasodilation and sweating are temporally linked and involve a sympathetic cholinergic mechanism.

Continued use of pharmacological agents and new techniques such as intradermal microdialysis have shed light on the complexity and mechanistic redundancy present in CAVD, also highlighting the difficulty associated with its study.

The Bradykinin Hypothesis

Grant and Holling's observation that sweating and skin vasodilation appeared to be temporally linked (63) led to the hypothesis that the mechanism of CAVD was secondary to sudomotor activity. However, it was unknown if CAVD was caused by the

excitation of a specific nerve or by the release of vasoactive substances from the activated sweat glands (54, 63).

Fox and Hilton (54) believed that bradykinin, a polypeptide with vasodilator properties (52) and formed by the sweat gland, was the causative agent in CAVD similar to the vasodilation that follows salivary gland activation (76). To test this hypothesis, they measured markers of bradykinin in sweat and subdermal interstitial fluid during whole-body heat stress. These authors showed an increase in the bradykinin forming enzyme, kininogenase, in both the interstitium and in the sweat that coincided with CAVD during whole-body heating (54). More evidence supporting the bradykinin hypothesis was provided by the observation that individuals with anhidrotic ectodermal dysplasia, a rare syndrome in which sweat glands are congenitally absent, did not experience CAVD during whole-body heat stress (16). These studies provided indirect evidence that bradykinin is associated with CAVD, but did not demonstrate that bradykinin caused CAVD. This limitation was acknowledged by Fox and Edholm (53), who suggested that the final proof or disproof of the bradykinin hypothesis would come when a means to specifically block the bradykinin mechanism was found.

In 2002, Kellogg et al. administered HOE-140, a bradykinin $ß_2$ receptor antagonist, via intradermal microdialysis to directly assess the role of bradykinin in CAVD (112). These authors found that bradykinin $ß_2$ receptor blockade did not attenuate or alter the threshold of CAVD during whole-body heat stress. In addition, exogenous administration of bradykinin at bradykinin $ß_2$ receptor blocked sites did not result in vasodilation, confirming the absence of $ß_1$ receptors. From this observation it was

concluded that bradykinin formation from sweat gland activation is not responsible for CAVD.

Cholinergic Co-transmission Theory

Previous work by Roddie et al. showed that cutaneous vasodilation in response to heat stress contains a cholinergic component (175, 176). Infusions of intra-arterial atropine, a muscarinic antagonist, abolished sweating but only delayed the onset of CAVD and/or attenuated the magnitude of dilation, depending on the timing of atropine administration. Specifically, administration during hyperthermia did not attenuate CAVD but atropine administration prior to hyperthermia reduced the overall cutaneous vasodilation. These authors concluded that sudomotor activity is almost entirely mediated by cholinergic mechanisms whereas cholinergic mechanisms contribute but are not the primary mediators of CAVD.

An alternative to the bradykinin hypothesis was that CAVD is dependent on a co-transmitter system. Hokfelt et al. suggested that a single neuron was responsible for both sudomotor and CAVD responses to whole body heating (78). This co-transmitter system explained the observation that atropine abolished sweating but did not affect CAVD to a large extent. In 1995, Kellogg et al. provided the most convincing evidence of the co-transmitter theory (114) **(Fig 2.3).** Intradermal injection of botulinum toxin A (which blocks presynaptic cholinergic neurotransmitter release) as well as administration of atropine via iontophoresis during whole body heating led to two key findings. First, atropine administration abolished sweating and delayed the onset of cutaneous vasodilation but did not attenuate it. This confirmed the contribution of acetylcholine to sweating and minor contribution to cutaneous vasodilation. Second, botulinum toxin A

abolished both sweating and cutaneous active vasodilation during whole body heat stress, but not vasodilation to directly applied heat. This confirmed the release of an unknown neurotransmitter(s) from the cholinergic nerve terminal is the primary mediator of CAVD.

In a study by Crandall et al. (38), skin blood flow was found to decrease via active vasodilator withdrawal but sweat rate was found to increase when subjects performed isometric handgrip exercise during moderate hyperthermia. Follow up work (39) determined that the observed active vasodilator withdrawal was likely mediated by a metaboreflex, as skin blood flow in the exercised limb continued to decrease at the cessation of exercise and after the limb was occluded to trap metabolites. These findings suggested independent cholinergic nerve control of sweating and CAVD, or dual nerve theory. Interestingly, more recent work by McCord and Minson (146) demonstrated a non-neural mechanism may be responsible to for the previously observed reductions in skin blood flow during isometric handgrip exercise during hyperthermia. They found that this response could only occur when skin blood flow was significantly elevated prior to isometric handgrip exercise and was likely the result of myogenic autoregulation from increased perfusion pressures. These authors concluded that the separation of skin blood flow and sweating during isometric handgrip exercise during hyperthermia is not due to active vasodilator withdrawal. While these findings are hard to interpret, it is likely that there are not independent cholinergic nerves controlling skin blood flow and sudomotor activity.

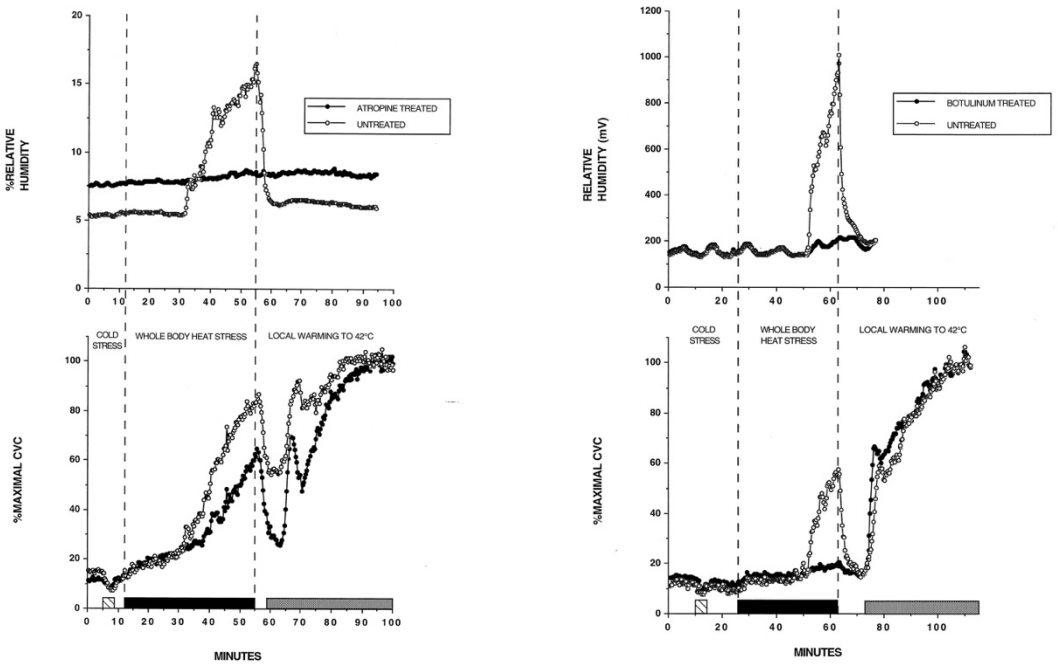

Figure 2.3. From Kellogg (114). (Right) The effect of botulinum toxin on cutaneous active vasodilation (CVC) and sweating (Relative humidity) during whole body heating and local warming. Open circles are untreated and closed circles are botulinum treated sites. (Left) The effect of atropine administration on cutaneous active vasodilation (CVC) and sweating (Relative humidity) during whole body heating and local warming. Open circles are untreated and closed circles are atropine treated sites.

The data from Kellogg et al., combined with the historic work of others, have established our current model of cutaneous active vasodilation: the cholinergic co-transmission theory. The exact identification of the unknown neurotransmitter(s) and the pathways involved in active vasodilation, have been targets of much research and debate. It should be noted that the aforementioned seminal work done by Kellogg et al. is now over 20 years old. Furthermore, the current understanding of the co-transmitter theory of CAVD is based on observations made in a limited sample size (N=6). To date, no one has corroborated these findings or expanded them to include different thermal loading modalities (e.g. exercise vs. passive whole-body heating). This is problematic, because

more recent research has suggested that there may be mechanistic differences in the skin blood flow responses to hyperthermic challenge that are dependent on how thermal loading is accomplished (see nitric oxide section below). In light of these observations, it is conceivable that CAVD may have different transduction pathways (e.g. different nerves). Therefore, we submit that the co-transmission theory of active vasodilation needs to be confirmed and expanded to include exercise heat loading.

Vasodilators Involved in CAVD

Multiple vasodilator pathways have been identified as having a role in CAVD. The synergistic nature of these pathways have made CAVD difficult to study. In vivo mechanistic studies have used pharmacological agents to inhibit or stimulate receptors such that our current understanding has been limited to the involvement of specific receptors and vasodilator pathways. Less is known about the particular vasodilator substances.

Nitric Oxide

Dietz et al. conducted the first study in humans to determine the role of NO in active vasodilation (45). In this study, intra-arterial infusions of the NO synthase inhibitor NG-monomethyl-L-arginine (L-NMMA) into the brachial artery during whole-body heating failed to attenuate a rise in forearm blood flow. It was concluded that NO was not involved in reflex vasodilation. Conversely, subsequent studies in humans have demonstrated that NO does in fact contribute to CAVD. In these studies, pharmacological blockade of NO synthase by either NG-nitro-L-arginine-methyl ester (L-NAME) via intradermal microdialysis or L-NMMA via intra-arterial infusion (188, 189) attenuated

cutaneous active vasodilation by approximately 30% in young healthy individuals during whole-body heating. Despite the observed attenuation of CAVD following NOS blockade, the precise role that NO played in CAVD was still unknown.

Previous research using the rabbit ear model had suggested that NO played a "permissive" role but was not an effector of active vasodilation (49, 50). To address this in humans, Crandall and MacLean (37) measured interstitial NO breakdown products during whole-body heating and found no measurable increase. This suggested that NO was indeed a permissive factor of CAVD; however, later work by Kellogg et al. (116) directly measuring NO using an amperometric probe found that bioavailable NO concentrations increased during heat stress. From this it was suggested that NO could be an effector of CAVD. To clarify these discordant findings, Wilkins et al. (218) administered L-NAME to block endogenous NO production while simultaneously delivering a low dose of sodium nitroprusside. The results from this study showed that NOS blockade attenuated CAVD and exogenous delivery of NO only partially restored active vasodilation (that is, NO plays a synergistic role in CAVD). These findings lend support to our current understanding that NO is a direct effector and not a permissive factor of CAVD in humans.

More recent evidence suggests that neuronal nitric oxide (nNOS) is the primary NOS isozyme that contributes to the NO component of CAVD during whole body heat stress. Administration of the nNOS inhibitor, 7-nitrioindazole (7-NI), via intradermal microdialysis significantly attenuated skin blood flow increases during whole-body heating, but had no effect on skin blood flow increases induced by local heating (**Fig 2.4**) (117). This finding, in conjunction with other work by Kellogg et al., determined that

nNOS contributes to the NO component of CAVD and endothelial nitric oxide synthase (eNOS) contributes to the NO component of vasodilation during local heating (117, 118, 120).

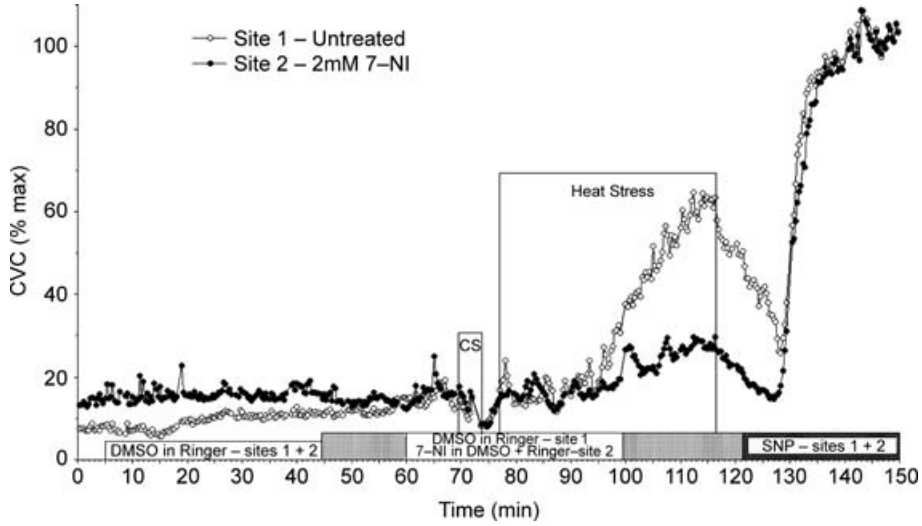

Figure 2.4. From Kellogg (117). The effect of nNOS inhibition with 7-NI on cutaneous active vasodilation. Open circles are untreated and closed circles are 7-NI treated sites.

Interestingly, work done by McNamara et al. has indicated that the eNOS isozyme contributes to CAVD during sustained dynamic exercise (148). These conflicting findings between active and passive heat stress highlight the possibility that the mechanisms of CAVD may vary depending on the source of the heat stress (dynamic exercise versus passive heating). These conflicting findings may also be the result of the posture in which subjects are heated as subjects were supine during passive heating and subjects were seated during exercise. McNamara et al., suggested that these differences could be due to differences in active vasodilator nerve firing rates (secondary to different afferent input) or due to increases in circumferential strain, which has been associated with eNOS activation (148). It had previously been demonstrated that an increase in shear

stress does not account for the rise in NO-dependent vasodilation in the skin (226). More recent work by Fujii et al. corroborated the findings by McNamara et al. (55). In their study, intradermal administration of the eNOS inhibitor, LNAA, attenuated cutaneous vascular conductance (CVC) in young healthy adults during two 30-minute bouts of cycling in the heat. It had previously been assumed that the mechanisms of CAVD were the same between exercise and passive heating. More recent research is suggesting that this assumption is incorrect. More work needs to be done to further dissect the underlying mechanistic differences between exercise and passive heating.

Regardless of the NOS isozyme, NO is a significant contributor to CAVD in both passive and active heat stress models. More importantly, the NO pathway can be activated by a number of receptors that have been implicated in CAVD; plus, NO blockade does not account for the entire response. This indicates the presence of other vasodilator pathways that work in concert with and/or compliment the NO pathway.

Vasoactive Intestinal Polypeptide

Vasoactive intestinal polypeptide (VIP) is a vasoactive peptide hormone that was first suggested to be the unknown co-transmitter involved in CAVD. This submission originated from Hokfelt et al. whose study of the cat paw led to the discovery of an atropine resistant co-transmitter mechanism that released both ACh and VIP (78). Additional immunohistochemical studies have indicated that VIP is co-localized with acetylcholine in nerves associated with both sudomotor and vasomotor activity in humans (71, 210). In light of the observed co-localization of VIP and ACh, it was hypothesized that a single branch of neurons could be the effectors for both sweating and active vasodilation; however, direct evidence of a role for VIP in CAVD is lacking.

The first experimental data testing the role of VIP in CAVD came from the study of cystic fibrosis (CF) patients, who have minimal levels of VIP in cutaneous nerves (73). Savage et al. (183) observed that CF patients maintained a normal CAVD response during heat stress which indicates that VIP is not involved in CAVD. Independent follow-up studies in CF patients by Kellogg et al. (107) and Wilkins et al. (219) tested whether enhanced ACh release or augmented NO production, respectively, were possible compensatory mechanisms of reduced VIP levels in CF patients. In both studies, no difference was found between control and CF subjects, arguing against the possibility of redundant mechanisms in the CAVD of CF patients (107, 219).

Pharmacological interventions to determine the effect of VIP on CAVD in healthy humans have also yielded mixed results. Bennet et al. delivered VIP_{10-28} to the skin (via intradermal microdialysis) to inhibit the VIP receptors, VPAC1 and VPAC2 (7). It was reported that VIP_{10-28} delivery attenuated the increase in skin blood flow during heat stress, indicating a role for VIP in CAVD. This finding was not confirmed in a subsequent study using VIP_{10-28} (220). Confounding both studies were the observations that in some subjects, concentrations of the VIP antagonist VIP_{10-28} also caused vasodilation, challenging the interpretation of the findings. These results were made even more unclear with the observation that VIP_{10-28} could augment the vasodilator response to exogenous VIP administration (via intradermal microdialysis) (217, 220). It was also demonstrated that the majority of vasodilation to VIP in the skin is through NO-dependent pathways. This suggests that even if VIP is an involved co-transmitter, it does not explain the full expression of CAVD.

Histamine

Histamine is another transmitter that has been implicated in CAVD. In a study by Wong et al., delivery of the H1 receptor antagonist pyrilamine and the H2 receptor antagonist cimetidine via microdialysis have indicated that only H1 receptors play a functional role in CAVD (227). Additionally, simultaneous administration of the NOS antagonist, L-NAME, with pyrilamine did not cause a further attenuation in CAVD **(Fig 2.5)**, suggesting that a portion of NO-mediated CAVD can be explained by H1 receptor activation. Lastly, exogenous VIP administration has been shown to have an H1 receptor component (217). Consideration of the aforementioned studies have led to the hypothesis that VIP release from the sympathetic cholinergic vasodilator nerves may cause some degree of H1 receptor activation (217, 227).

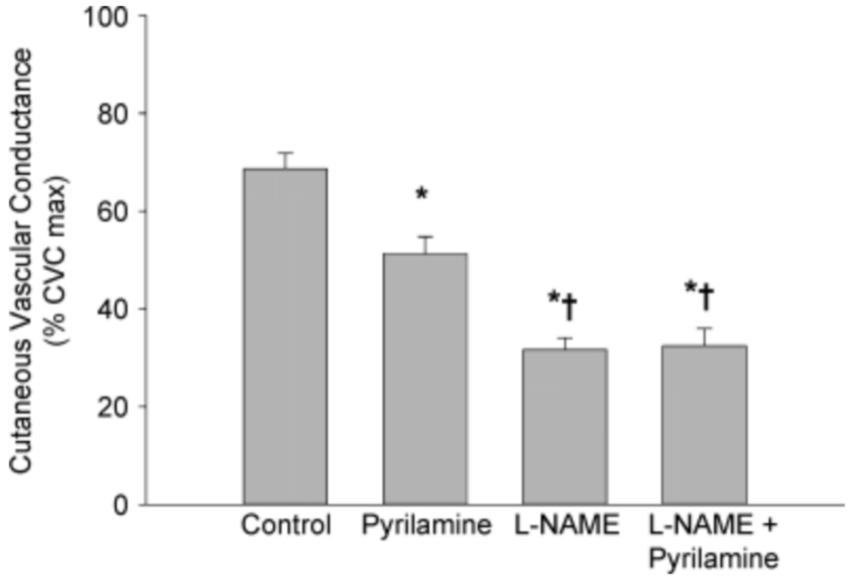

Figure 2.5. From Wong (227). Group mean values from plateau of skin blood flow (CVC) during whole body heating at control treated, Pyrilamine treated, L-NAME treated, and L-NAME+Pyrilamine treated sites. *P<0.05 versus control site; †P<0.05 versus Pyrilamine site.

Neurokinin Receptor-mediated Vasodilation

Substance P is a neurokinin localized in the skin (70, 87, 152) that causes a partially NO-mediated vasodilation (129, 225). In a study by Wong et al., repeated infusions of substance P to desensitize neurokinin-1 receptors (NK_1) (225) attenuated CAVD by approximately 30% (224) **(Fig 2.6)**. Interestingly, the addition of the NOS inhibitor L-NAME along with NK_1 desensitization attenuated CAVD by approximately 80% (224). Taken together, these data suggest that NK_1 receptor activation and NO contribute to CAVD in a largely independent fashion. Despite the aforementioned research, identification of a precise role for substance P in CAVD has been difficult for two reasons. First, it is unknown if substance P is what activates NK_1 receptors. Other peptides from the kinin family such as neurokinin A and neurokinin B could also be responsible for receptor activation during active vasodilation, although the NK_1 receptor has the highest affinity for substance P. Second, substance P has not been localized in active vasodilator nerves. Substance P is mainly found in sensory nerves and endothelial cells of the skin (70, 87, 152) making it unclear how sympathetic vasodilator nerves effect substance P release. Previous work by Charkoudian et al. found that both acute (to stimulate local sensory nerves) and chronic (to desensitize local sensory nerves) application of capsaicin to the skin did not affect CAVD in response to whole body heating, suggesting that substance P found in sensory neurons is not involved in active vasodilation (29). To date, the NK_1 receptor agonist and its origin of release remain unknown.

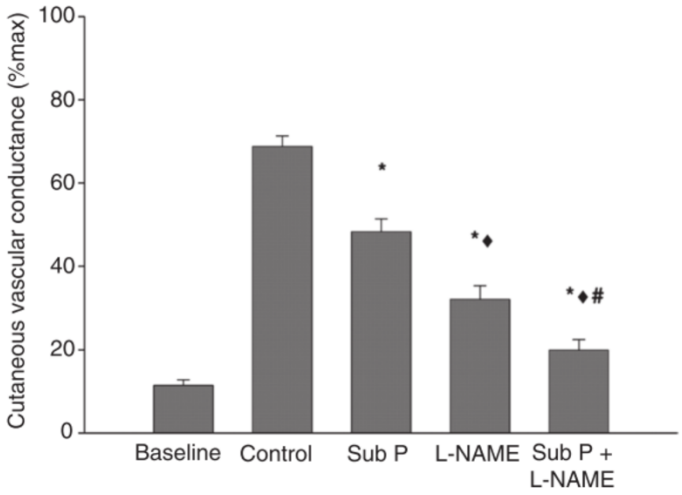

Figure 2.6. From Wong and Minson (224). Group mean values from plateau of skin blood flow (CVC) during whole body heating at control treated, Substance P treated, L-NAME treated, and L-NAME+Substance P treated sites. *P<0.05 versus control site; •P<0.05 versus Substance P site; #P<0.05 versus L-NAME site.

TRPV1 Receptors

As mentioned above, data from Charkoudian et al. suggest that sensory neurons are not involved in active vasodilation (29). More recent evidence from Wong and Fieger suggests that sensory neurons may actually play a role in CAVD (223). In this study, the role of TRPV1 channels in CAVD was studied. TRPV1 channels are predominantly found on sensory neurons and to a lesser extent on endothelial cells in skin. Pharmacological blockade of TRPV1 channels, by Wong and Fieger, using capsazepine delivered via microdialysis attenuated CAVD by approximately 25%. To further determine the role of TRPV1 in CAVD, the Wong also administered the NOS antagonist L-NAME with and without capsazepine (222). It was shown that there was no difference between NOS blockade and NOS + TRPV1 blockade, suggesting that TRPV1 channel activation may contribute to the NO-mediated component of CAVD. From these data, the author concluded that cutaneous sensory nerves contribute to CAVD; however, it is

unknown if TRPV1 channel activation on sensory neurons or endothelial cells is responsible for the TRPV1 component of CAVD. The aforementioned study by Charkoudian et al. suggests that it is endothelial TRPV1 channels that contribute to CAVD. This would also explain how TRPV1 channel activation contributes to the NO-mediated portion of CAVD.

Prostaglandins

A study by McCord et al. identified the prostanoid pathway as contributing to CAVD (145). In this study the authors inhibited the COX pathway using ketorolac delivered via intradermal microdialysis. COX blockade attenuated vasodilation by approximately 16% suggesting that prostanoids are involved in CAVD. Simultaneous NOS inhibition attenuated CAVD to a larger extent than independent NOS and COX inhibition, suggesting that COX mediates vasodilation independently of NO. A more recent study testing the effects of chronic aspirin therapy (COX inhibitor) demonstrated a blunted active vasodilator response to whole-body heating and subsequently confirmed the COX pathways involvement in CAVD (82).

Calcium-Activated Potassium Channels (Kca) and Endothelial Derived Hyperpolarizing Factors (EDHF)

Brunt et al. sought to determine the role that K_{Ca} channels and EDHFs contribute to active vasodilation (22). Use of tetraethylamonium (TEA), a K_{Ca} channel blocker and therefore an EDHF antagonist, with and without L-NAME highlighted a complicated role for EDHFs in CAVD. These authors found that EDHFs do not contribute to CAVD; however, simultaneous NOS inhibition augmented CAVD. When barium chloride, a K_{ATP} and K_{ir} channel blocker, was administered with both TEA and L-NAME, the

response was reduced to a level similar to L-NAME alone. These data suggest that EDHF is not obligatory to CAVD and that a complex interplay exists between the EDHF, NO, K_{ir}, and K_{ATP} pathways.

Neurotransmitter(s)

Neural control of the cutaneous vasculature, and more specifically CAVD, is of extreme importance to temperature regulation in humans. The development of new techniques and use of new pharmacological agents has introduced new insights and raised new questions about CAVD. Yet old questions remain unanswered. For instance, it is still unknown what substance(s) are co-transmitted alongside ACh from CAVD nerves. Pharmacological blockade of downstream receptors has given insights on what these substances might be but this is far from definitive evidence; since most pharmacological agents used to antagonize receptors and pathways likely have numerous physiological effects that are unknown to the researcher. Based on the mechanistic research described above, it is currently thought that VIP, histamine, and substance P may be substances that are co-transmitted by CAVD nerves or released by other cell types in response to CAVD nerve activation.

As previously stated, it is known that VIP is co-localized with acetylcholine in nerves associated with both sudomotor and vasomotor activity in humans (71, 210); it is also known that exogenous VIP administration has been shown to have an H1 receptor component (217). Taken together, VIP release from the sympathetic cholinergic vasodilator nerves may cause some degree of H1 receptor activation through secondary release of histamine from mast cells in tissue (**Figure 2.7**) (217, 227). Co-transmission of VIP could also have direct effects on the cutaneous vasculature. However,

pharmacological interventions to determine the effect of VIP on CAVD in healthy humans have yielded mixed results and the role of VIP in CAVD is inexact. Substance P has also been indirectly implicated as being responsible for approximately 30% of the CAVD response (224) **(Fig 2.6)**, but Substance P has not been localized in active vasodilator nerves. Substance P is mainly found in sensory nerves and endothelial cells of the skin (70, 87, 152) making it unclear how sympathetic vasodilator nerves effect Substance P release. What is clear is that a substance(s) is co-transmitted alongside acetylcholine from CAVD nerves. Currently it is unknown what this substance(s) is and if its mechanisms of action are primary or secondary in nature to the vasodilator response.

Summary

The studies discussed above on the mechanisms of CAVD have uncovered a highly complex system that involves the activation of multiple receptors and vasodilator pathways in a synergistic and sometimes redundant manner (**Fig 2.7**). It has become clear that CAVD is not just mediated by a single neurotransmitter such as ACh but is likely mediated by the co-transmission of numerous transmitters from cholinergic nerves. It is also conceivable that numerous types of sympathetic cholinergic nerves exist that innervate different organelles within the dermis of the skin. While our understanding remains murky at best, we do know different vasodilators are involved at different times (e.g. ACh) and some contributors may be secondary to the neurotransmitter released.

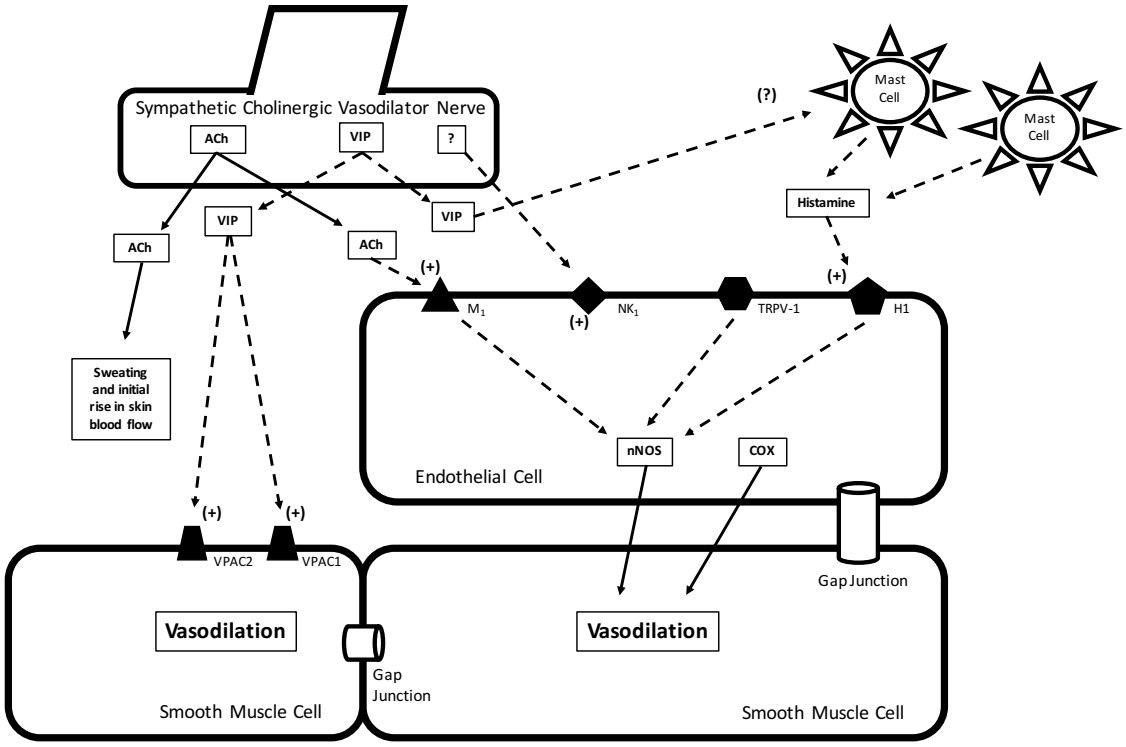

Figure 2.7. Summary of known and unknown mechanisms of cutaneous vasodilation. Acetylcholine (ACh) is released from sympathetic cholinergic vasodilator nerves and is responsible for sweating as well as the early rise in skin blood flow from CAVD. Possible co-transmitters include vasoactive intestinal peptide (VIP) as well as other unidentified substance(s). VIP will bind to vasoactive intestinal peptide receptor 1 and 2 (VPAC 1 and 2) to cause vasodilation. VIP may also cause mast cell degranulation and histamine release which may account for histamine receptor (H1) activation during CAVD. Lastly, activation of both neurokinin-1 receptors and transient receptor potential vanilloid receptor 1 (TRPV-1) have been implicated in CAVD. Within the endothelium, neuronal nitric oxide synthase (nNOS) and the cycloogenase (COX) pathway are thought to produce the nitric oxide and prostacyclin during CAVD.

Modifiers of CAVD

CAVD is the end result of a highly integrative process that relies on input from numerous signals. Many factors can modify the CAVD system. The final section of this chapter will briefly focus on acute and chronic modulators of CAVD, as well as CAVD in special populations. Johnson et al. published a detailed discussion of CAVD modulators (95).

Whole-Body Heating and Skin Temperature

It has been shown that skin temperature affects the internal temperature equilibrium point for active vasodilation and sweating (8, 96, 167, 168). At high skin temperatures there is a leftward shift (**Fig 2.8**) in the equilibrium point of CAVD resulting in an earlier onset of sweating and active vasodilation, as well as a greater level of skin blood flow and sweating at any internal temperature above the equilibrium point (96, 167, 168). The independent study of skin temperature and internal temperature on CAVD has been difficult because raising internal temperature typically involves raising skin temperature or activation of other reflex responses, such as those serving to maintain blood pressure during exercise.

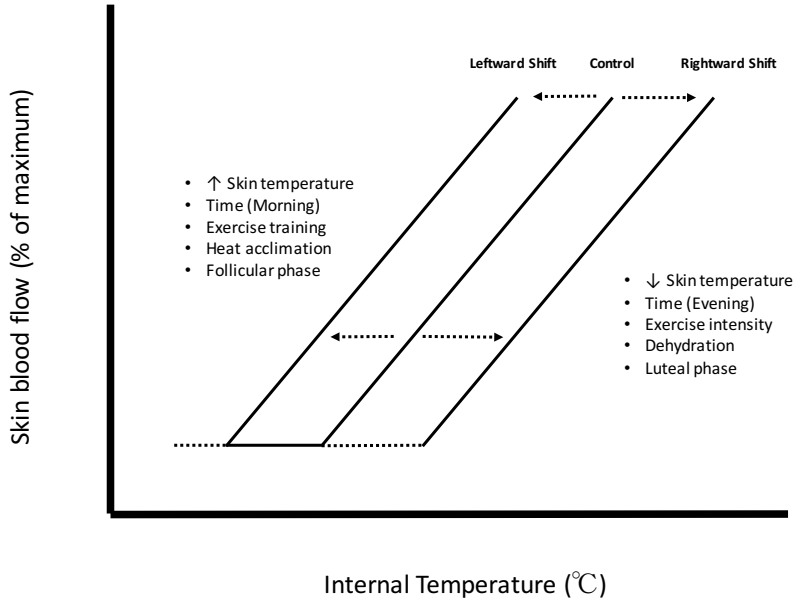

Figure 2.8. A schematic showing the relationship between skin blood flow and internal temperature as well as the internal temperature equilibrium point for active vasodilation. Factors that can shift the relationship left and right are listed.

Acute Modifiers: Circadian Rhythm

It is well known that there is a circadian rhythm in resting internal temperature in humans. This rhythm follows endogenous heat production such that internal temperature is lower in the morning and higher in the evening (4, 133). Cutaneous vascular responses to heat stress also show a circadian rhythm in the balance point for cutaneous vascular responses to hyperthermia (2, 200, 203, 214). For example, during passive heat stress the internal temperature equilibrium point shift to higher temperatures in the evening (**Fig 2.8**) and lower temperatures in the morning (2). Additionally, the gain of this response (internal temperature vs. skin blood flow) is lower in the morning than in the evening. A study by Aoki et al. using bretylium, administered via intradermal microdialysis, to block noradrenergic vasoconstriction demonstrated that the diurnal changes in reflex control of skin blood flow during whole body heating involves both the vasoconstrictor and vasodilator systems (3). Specifically, these authors found that the internal temperature equilibrium point for cutaneous vasodilation was higher in the evening at both control and bretylium treated sites, suggesting the shift in the balance point is dependent on the active vasodilator system. Furthermore, the gain of the response (internal temperature vs. skin blood flow) was reduced in the morning. Lastly, the gain was significantly lower at control sites when compared to bretylium treated sites, suggesting the sensitivity of cutaneous vasodilation is dependent on the vasoconstrictor system.

Acute Modifiers: Postural Influences

During orthostasis (upright posture), arterial baroreceptor activation results in an increase in total peripheral resistance (via peripheral vasoconstriction) to maintain an adequate perfusion pressure to the brain. This reflex vasoconstriction is mediated in part

by the cutaneous vasculature in both normothermic and hyperthermic conditions (36, 111). In prior work by Kellogg et al., they blocked adrenergic vasoconstrictor activity, using bretylium iontophoresis, and measured skin blood flow responses at breylium treated and control sites to -40 mmHg lower body negative pressure (LBNP) in both normothermic and hyperthermic conditions (111). These authors found that reductions in skin blood flow following LBNP during hyperthermia were due to active vasodilator withdrawal, whereas during normothermia, reductions in skin blood flow were due to enhanced vasoconstrictor activity. To determine which baroreceptor population was mediating this active vasodilator withdrawal, Crandall et al. used external neck pressure to unload carotid baroreceptors and low levels of LBNP to unload cardiopulmonary baroreceptors independently of each other (36). Again, skin blood flow responses were measured at bretylium treated and control sites and it was concluded that neither cardiopulmonary or carotid baroreceptor unloading could account for the attenuated active dilator response during orthostasis (36).

To date, the mechanisms of CAVD have principally only been explored in the supine position during passive whole-body heat loading. Mechanistic differences observed (eNOS vs. nNOS) during different thermal loading modalities may actually be due to differences in posture. We submit that, postural influences must be studied independently of differential thermal loading modalities to confirm the variable contribution of eNOS and nNOS during exercise and passive whole-body heat loading, respectively.

Acute Modifiers: Dynamic Exercise

The onset of acute dynamic exercise involves a reduction in skin blood flow due to increased cutaneous sympathetic vasoconstrictor outflow (10, 11, 229). As previously mentioned, dynamic exercise generates a thermal load that can initiate CAVD similar to passive heat stress, although perhaps through slightly different mechanisms. Probably the most notable modification between passive and exercise-induced heat stress is the rightward shift in the internal temperature equilibrium point (**Fig 2.8**) for active vasodilation (97, 110, 125, 204). The magnitude of this shift is dependent on exercise intensity, with greater shifts occurring at higher intensity exercises (206). Once internal temperature has surpassed the balance point during exercise, skin blood flow rises linearly with increasing internal temperature until an internal temperature of approximately 38°C is reached. At this point, skin blood flow plateaus at approximately 50% of max and any further increase in internal temperature does not result in a further increase in skin blood flow(17, 62, 98, 108, 125, 174). This plateau in skin blood flow is likely a limit imposed by competition for cardiac output between active muscle and skin blood flow (98).

Endurance exercise often results in dehydration which is associated with hyperosmolality, which leads to decreased skin blood flow and sweating responses to hyperthermia, even in the absence of hypovolemia (51, 157, 184, 185, 190). Specifically, Nadel et al. showed that individuals dehydrated by 3% body weight experienced rightward shifts in their internal temperature equilibrium point for CAVD (157). Conversely, hyperhydration does not seem to alter internal temperature equilibrium point

or modify the gain of the thermoregulatory responses when compared to euhydrated controls (136, 137, 157).

Chronic Modifiers: Exercise Training/Heat Acclimation

Both exercise training and heat acclimation have been shown to improve thermoregulatory responses to heat stress (47, 160, 174). This occurs by decreasing the internal temperature equilibrium point for active vasodilation and sweating **(Fig 2.8)** (47, 174). A component of exercise training is the repeated heat stress inherent with exercise and therefore some level of heat acclimation occurs with chronic exercise training (61). Because of the relationship with exercise and thermal loading, exercise training and heat acclimation are inextricably linked. Exercise in a hot environment has demonstrated larger shifts in balance point than just exercise alone (174). In the classic study by Roberts et al., 10 days of exercise in thermoneutral conditions shifted the equilibrium point for active vasodilation and sweating towards lower internal temperatures (174). When the same protocol was performed in a hot environment, the internal temperature equilibrium point shifted to a greater extent than just exercise alone. This is likely due to the increased thermal load associated with each training bout in the heat. The thermoregulatory benefits of exercise and therefore heat acclimation have been shown in both young and old individuals (207).

Chronic Modifiers: Sex Steroids

Sex hormones fluctuate with the menstrual cycle such that progesterone and estrogen are elevated during the midluteal phase and low during the early follicular phase. These sex hormones have been shown to alter internal temperature as well as thermoregulatory control of skin blood flow and sweating (75, 77, 131, 171, 200, 201,

205). For example, core temperature is reduced during the early follicular phase and is elevated during the midluteal phase when estrogen and progesterone are high. These fluctuations in internal temperature are also paralleled by fluctuations in the internal temperature equilibrium point for both active vasodilation and sweating. For instance, there is a leftward shift in the internal temperature equilibrium point for active vasodilation during the follicular phase and a rightward shift during the mid-luteal phase (**Fig 2.8**) (27, 75, 77, 149, 171, 200, 202, 205). These shifts in internal temperature and the balance point also occur with oral contraceptives that contain estrogens and progestins.

Interestingly, estrogen replacement therapy in women causes a decrease in equilibrium for CAVD and the addition of progesterone reverses this trend (18, 205). From these observations, it was concluded that estrogen seems to decrease internal temperature and thermoregulatory equilibrium points and progesterone promotes increases in body temperature.

Modifiers - Special Populations

Aging - The attenuated thermoregulatory responses to heat stress in aging populations are well documented (79, 83, 85, 86, 124, 128, 144, 154, 182). In particular, reductions in cutaneous active vasodilation are observed in the elderly during body heating and exercise (128, 170) and are associated with changes in the vascular responsiveness of the skin itself (79, 85, 86). Research by Holowatz et al. demonstrated that the decreasing vascular responsiveness of the skin is due in part to an attenuated NO-mediated vasodilation that is a result of decreased NO-bioavailability (79, 85, 86). Interestingly, these authors also found that older individuals rely more heavily on NO to

augment skin blood flow in response to hyperthermia (79); suggesting that aging may be affecting other vasoactive pathways involved in CAVD. Continued research has demonstrated that decreased NO bioavailability in aging populations is due to NO quenching via oxidative stress (85, 86) and NOS uncoupling via elevated arginase activity or reduced L-arginine levels, and reduced active tetrahydrobiopterin (BH4) levels (196, 197).

Hypertension & Type II Diabetes - In some but not all studies, individuals with essential hypertension have been shown to have a blunted active vasodilator response during both exercise and passive heating (80, 81, 113, 123, 126, 127). Work by Holowatz et al. showed that arginase inhibition (81) and antioxidant administration (80) both augmented active vasodilator responses in hypertensives through NO-dependent and independent mechanisms. In addition to the aforementioned mechanisms, maximal forearm skin blood flow has also been shown to be decreased in hypertensive subjects (24), suggesting that structural changes in the cutaneous vasculature are also occurring.

To date there are no studies that have directly addressed CAVD and type 1 diabetes. However, type 1 diabetes has been shown to negatively affect heat loss at high intensity exercise but not during more moderate bouts of exercise (26, 147, 199). Conversely, type 2 diabetics have been shown to have impaired active vasodilator responses (194, 216). These impairments manifest as a reduced magnitude of cutaneous vasodilation due to decreased responsiveness to NO (194) as well as a rightward shift in the internal temperature equilibrium point for active vasodilation (**Fig 2.8**) (216).

Heart Failure - Heart failure patients have impaired thermoregulatory responses to heat stress (42, 65). In a study by Cui et al., it was shown that the active vasodilator

response to whole body heating was reduced in individuals with heart failure (42). Similar to hypertensive individuals, maximal forearm skin vasodilation is impaired in heart failure patients, suggesting that cutaneous vascular remodeling contributes to the impaired active vasodilation (42). Subsequent work by Green et al. sought to determine if the NO-dependent component of CAVD could be attenuated and also contribute to the reduction in the cutaneous active vasodilator response (65). These authors determined that the NO-dependent component of CAVD was attenuated in heart failure patients.

Conclusions

The skin is the primary site where thermal interaction occurs between the human body and the environment. Modulation of skin blood flow allows humans to increase or decrease the convective heat transfer from internal tissues to the periphery where it can then engage in heat loss or heat retention with the environment. Therefore, neural control of the cutaneous vasculature, and more specifically CAVD, is of extreme importance to temperature regulation in humans. As has already been mentioned, the complexity and redundancy of this system has made its study difficult; however, the development of new techniques and use of new pharmacological agents has introduced new insights and raised new questions about CAVD. For instance, the potential mechanistic differences to CAVD during passive vs. active heating has changed the previously held notion that CAVD is mechanistically the same no matter the source of heat production. There are also old questions that have yet to be answered. Such as what vasodilator substance(s) mediate cutaneous active vasodilation in humans. To say we have only scratched the surface is an

understatement and continued research with new investigators and new methodologies is warranted.

CHAPTER III

METHODOLOGY

Outline

This dissertation sought to interrogate cutaneous active vasodilation (CAVD) in response to varying thermal loading modalities and postures using the techniques described in this chapter. Two studies were conducted in young, healthy, recreationally active subjects. Subjects were assigned to participate in one of the following study protocols.

Protocol 1: Postural Influences on the Nitric Oxide Component of CAVD

Protocol 1 (**Figure 3.1A**) was designed to determine the contribution of neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) isozymes to cutaneous vasodilation during passive whole-body heat loading in the supine and seated positions. Some subjects completed a second study day in the alternative posture (i.e., Study Day 1 = seated and Study Day 2 = supine or Study Day 1 = supine and Study Day 2 = seated). The specific methodology and results of this study are detailed in Chapter IV.

Protocol 2: Thermal Loading Modalities and CAVD Nerve Activation

Protocol 2 (**Figure 3.1B**) was designed to determine the extent to which cholinergic nerve activation mediates CAVD during exercise heat loading and passive whole-body heat loading by comparing skin blood flow responses at cholinergic nerve blocked and unblocked sites using a crossover study design. The specific methodology

and results of cholinergic nerve blockade on CAVD during exercise heat loading and passive whole-body heat loading are detailed in Chapter V.

Secondarily, this study sought to identify unknown vasoactive substances associated with CAVD. The specific methodology and the current status of the identification of unknown vasoactive substances associated with CAVD is also discussed in Chapter V.

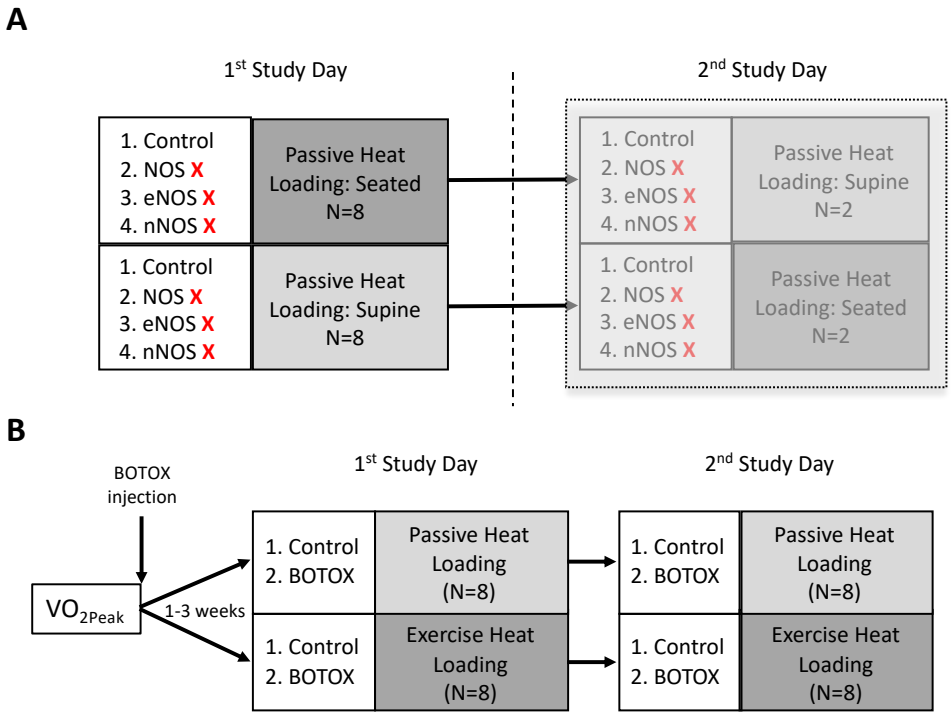

Figure 3.1. A schematic of Protocol 1 (A) and Protocol 2 (B). (A) Subjects were randomly assigned a posture for passive whole-body heat loading (N=16) and a subset of subjects completed a second study day in the alternate posture (N=4). X's represent enzyme blockade. (B) Subjects were randomly assigned an initial heating condition (N=8) and completed a second study day in the alternate heating condition 1-4 weeks later.

Human Subjects

All protocols in human subjects were approved by the Institutional Review Board at the University of Oregon (IRB #12102010.027, see Appendix page (128) for informed

consent documents). All subjects gave oral and written consent prior to participation in accordance with the *Declaration of Helsinki*.

33 individuals were assessed for qualification in this dissertation project. Subject inclusion criteria included age 18-30 years, recreationally-active (<150 minutes of intense exercise a week), no cigarette smoking, not taking prescription medications with the exception of hormonal contraceptives, not currently pregnant or breast-feeding, no history of allergic reactions to any drugs similar to those used in protocols 1 and 2, and no history of cardiovascular, metabolic, or other chronic diseases. Of these 33 individuals, sixteen (8 men, 8 women) individuals enrolled in Protocol 1 and eight (3 men, 5 women) individuals enrolled in Protocol 2. Subject demographics for both protocols are presented in Table 3.1.

Table 3.1. Demographics of subjects who completed Protocol 1 or Protocol 2. Data are mean ± S.E.

	Protocol 1 (N=16)	Protocol 2 (N=8)
# of women	8	5
Age (yrs)	23 ± 0.7	22 ± 1.1
Height (cm)	172 ± 3	173 ± 4
Weight (kg)	69 ± 5	68 ± 2
BMI (kg/m^2)	23.4 ± 1.6	22.7 ± 0.6

Prior to thermal loading study days, subjects abstained from food for 4 hours, alcohol and caffeine for 12 hours, and exercise and over-the-counter medications for 24 hours. All female subjects had a negative pregnancy test on the morning of each study day. For Protocol 1, women were studied during the low hormone phase of their menstrual cycle. Women taking hormonal contraceptives (N=6) were studied during the placebo phase and those naturally menstruating (N=2) were studied during the early

follicular phase of menstruation (self-reported). For Protocol 2, women taking hormonal contraceptives (N=4) were studied during the placebo phase and those naturally menstruating (N=1) were studied during the early follicular phase of menstruation (self-reported). In order to study females under consistent hormone levels for Protocol 2, females returned one full cycle later (i.e. 3-4 weeks apart) for their second study day visit. In Protocol 1, the single female that completed passive whole-body heating in both postures, returned 8 days after her first study day visit for her second visit.

Thermal Loading Study Days

As discussed in Chapter II, differences in thermal loading and/or posture, may result in an increased SkBF response by different mechanistic means. To explore these potential differences, this dissertation utilized both exercise heat loading and passive whole-body heat loading modalities to study the mechanisms and transducers of CAVD in young, healthy, recreationally active individuals.

General Instrumentation

Regardless of protocol or thermal loading modality, subjects reported to the Human Cardiovascular Control Laboratory in Esslinger Hall on the University of Oregon campus for their thermal loading study days. Upon arrival, subjects provided a urine sample to confirm euhydration via urine specific gravity <1.02 (Analog Regractometer; Atago, Tokyo, Japan). If urine specific gravity was >1.02, subjects drank 5 ml/kg of water prior to study. Dry nude body weight was measured before and after thermal loading to calculate mean whole body sweat rate. Subjects were instrumented with a sterile rectal thermistor probe (YSI Series 400; Yellow Spring Instruments, Yellow

Springs, OH) inserted approximately 10 cm past the anal sphincter to monitor internal temperature (T_c).

Skin surface thermistors (MSR145WD, MSR Electronics GmBH, Seuzach, Switzerland) were applied to standard locations on the chest, forearm, calf, and quadricep to continuously measure skin temperatures. Mean skin temperature (T_{sk}) was calculated using the 4-site weighting method previously developed by Ramanathan (172):

$$T_{sk} = 0.3(t_{chest} + t_{arm}) + 0.2(t_{thigh} + t_{calf}) \qquad (1)$$

Where

t_{chest} = skin temperature measured at the chest,

t_{arm} = skin temperature measured at the forearm,

t_{thigh} = skin temperature measured at the quadricep, and

t_{calf} = skin temperature measured at the calf.

Mean body temperature (T_b) was calculated using the internal temperature and mean skin temperature weighting method described by Gagge and Gonzalez (57):

$$T_b = 0.8(T_c) + 0.2(T_{sk}) \qquad (2)$$

Heart rate and ventilatory frequency were monitored using a 3-lead electrocardiogram (CardioCap; Datex Ohmeda, Louisville, CO). Baseline blood pressure was measured in triplicate following 20 minutes of rest and periodically throughout the studies via brachial oscillation (Datascope Accutorr Plus, Duluth, GA) and auscultation as described below.

Exercise Heat Loading

Exercise heat loading was accomplished by having subjects exercise on a stationary bicycle ergometer (Excalibur Sport, Lode, Groningen, Netherlands) at a constant workload that corresponded to 60% of their VO_{2peak} (VO_{2peak} test methodology) for 60 minutes (Figure 3.2.). This relative workload was based on McNamara et al. (148). During the first approximate 10 minutes of the 60-minute exercise interval, subjects were attached to a breath-by-breath metabolic measurement system (Parvomedics, Sandy, UT) to ensure they were exercising at 60% of their VO_{2peak}. Pulmonary gas exchange was then "spot" checked approximately every 20 minutes for the duration of exercise. During exercise heat loading study days, blood pressure in the brachial artery was measured using the auscultatory method (mercury sphygmomanometer and stethoscope) due to inaccuracy of oscillometric recorders during physical activity (164).

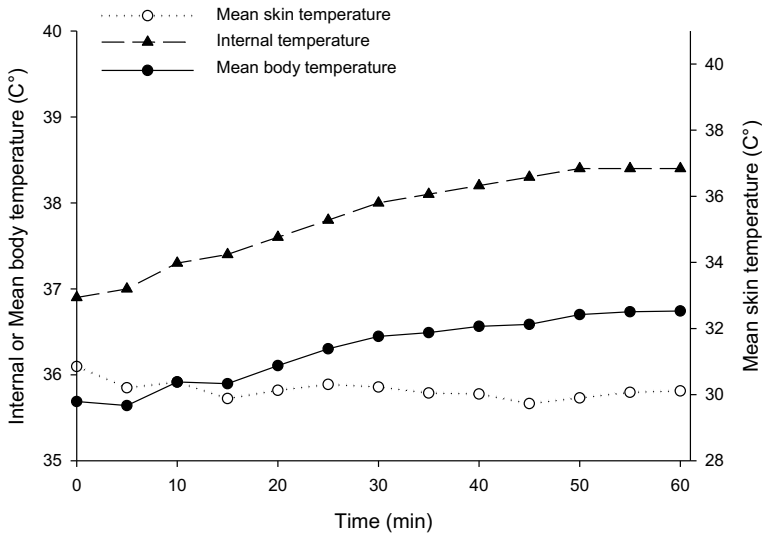

Figure 3.2. Representative temperature response to 60 minutes of exercise heat loading from one subject. Filled circles represent mean body temperature and filled triangles represent internal temperature (Left Y axis). Open circles represent mean skin temperature (Right Y axis).

Passive Whole-Body Heat Loading

Passive whole-body heat loading was accomplished by having subjects wear a two-piece nylon water-perfused suit (Med-Eng Systems, Ottawa, Canada), which covered all skin surfaces except the experimental arm, face, hands, and feet. In addition, subjects wore a water-impermeable plastic garment over the suit to mitigate evaporative heat loss. During thermal loading, mean skin temperature was maintained at approximately 39 °C for 60 minutes using a heated circulating water bath (HT CIRC 7L AD, Polyscience, Niles, Illinois) to perfuse hot water (50°C) through the suit (Figure 3.3). This water temperature was based on previous work from our lab and has been previously shown to raise internal temperature 0.7-1.0°C above baseline in approximately 60 minutes (22, 218, 224). During passive whole-body heat loading studies, blood pressure in the brachial artery was measured using the oscillometric method.

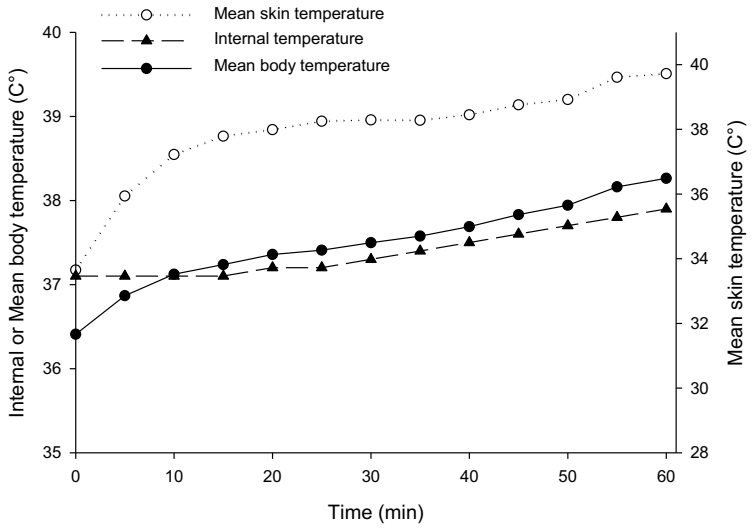

Figure 3.3. Representative temperature response to 60 minutes of passive whole-body heat loading from one subject. Filled circles represent mean body temperature, filled triangles represent internal temperature, and open circles represent mean skin temperature.

VO_{2peak} Test

VO_{2peak} was assessed using a graded cycling protocol on a stationary bicycle ergometer. The graded cycling protocol involved a 5-minute warm-up at 100 watts followed by 20 or 25 watt (Female and Male increases, respectively) increases in work load every minute until volitional fatigue. Heart rate was monitored via telemetry (Model RS400, Polar Electro, Lake Success, NY) and ratings of perceived exertion were noted at every work stage. VO_{2peak} was then validated by having subjects cycle at a supramaximal workload (115% VO_{2peak}) to ensure their VO_2 did not rise above their previously determined plateau.

Cutaneous Microvascular Assessment During Thermal Loading

As discussed in Chapter II, human temperature regulation is modulated through cutaneous vasomotor adjustments, sweating, and thermogenesis (shivering). The expansion of the thermal gradient from the core to the periphery via skin blood flow is critical to increasing heat dissipation during exercise heat loading or passive whole-body heat loading. The majority of recent research has used remote pharmacological interventions and local cutaneous microvascular imaging to study skin blood flow responses to hyperthermic challenge in humans. In this dissertation, intradermal microdialysis and laser-Doppler flowmetry were used to interrogate the CAVD system in humans during thermal loading.

Intradermal Microdialysis

Intradermal microdialysis, or skin microdialysis, consists of the placement of thin hollow fibers with tubular dialysis membranes into the dermis that are then perfused at a

low speed with perfusate. The presence of the dialysis membrane allows for the flow of tiny molecules between the perfusate inside the membrane and the interstitial space immediately surrounding the membrane (approximately 1 cm in diameter around the membrane). In this manner, intradermal microdialysis can facilitate the local delivery of substances to a small area of skin and the recovery of substances released from the same area of skin (Figure 3.4). Since intradermal microdialysis is relatively noninvasive and is well tolerated by human volunteers, it has been broadly used in the modeling of microvascular function (35, 84), drug discovery/ pharmacokinetics (21, 134, 161), and the study of normal/abnormal skin function (34, 150, 191) *in vivo*.

Human thermal physiology research also has employed this technique to "pharmaco-dissect" the vasodilator mechanisms of the SkBF response to hyperthermic challenge (4, 22, 117, 145, 148, 218–220, 222–225, 227). This is most commonly accomplished by delivering pharmacological agents dissolved in a vehicle (perfusate is most commonly Lactated Ringer's solution) to antagonize components of vasodilator pathways while simultaneously quantifying SkBF via laser-Doppler flowmetry in the volar forearm (Figure 3.4).

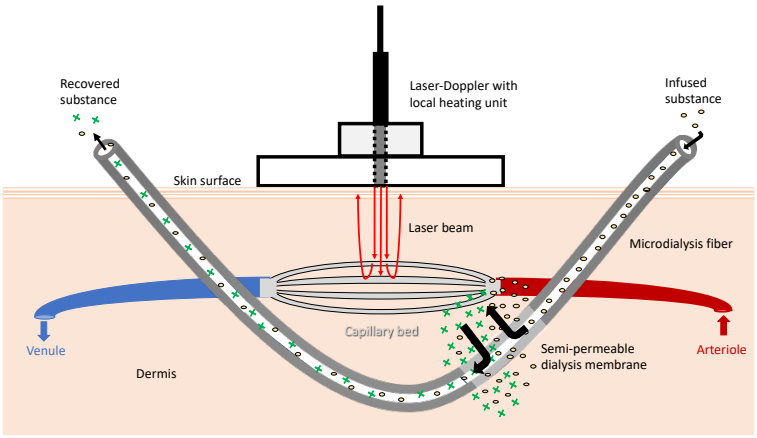

Figure 3.4. A schematic of the intradermal microdialysis technique used in conjunction with Laser-Doppler flowmetry and local thermal hyperemia.

Placement of microdialysis fibers in the dermis was performed using an aseptic technique. Linear microdialysis fibers (CMA 31 linear microdialysis, 10 mm membrane, 55Kda or 2000Kda CO; Harvard Bioscience, Holliston, MA) were placed in the dermal layer of the volar forearm of either the nondominant or dominant arm (randomized by subject), although other sites have been used to study regional variations in skin responses (64, 198). The volar forearm was chosen because most of the aforementioned thermal physiology research was conducted in the forearm and the volar surface is typically less sun damaged. Prior to fiber placement, a tourniquet was used to identify superficial veins to be avoided during placement and to also identify additional sites a minimum of 5 cm apart in cases where multiple fibers were to be placed. This ensured that fibers were placed in the microvasculature and prevented the interaction of pharmacological agents between fiber sites. Fibers were placed by first introducing a 25-gauge or 23-gauge needle approximately 1 mm below the surface of the skin, with entry and exit points 2-3 cm apart. Fibers were threaded through the lumen of the needle and the needle was removed leaving the dialysis membrane of the fiber centered between the needle entry and exit points (Figure 3.5).

A period of approximately ninety minutes was allowed to let needle insertion trauma and the resulting transient rise in skin blood flow resolve. Resolution of the skin blood flow rise was determined by the observed reduction and subsequent plateau of skin blood flow values. To maintain patency of the dialysis membrane during this time, fibers were perfused with Lactated Ringer's solution at a rate of 2.0 ul/min (CMA 102 Syringe

Pump; CMA Microdialysis AB, Solna, Sweden) until the start of study drug infusions or perfusate recovery.

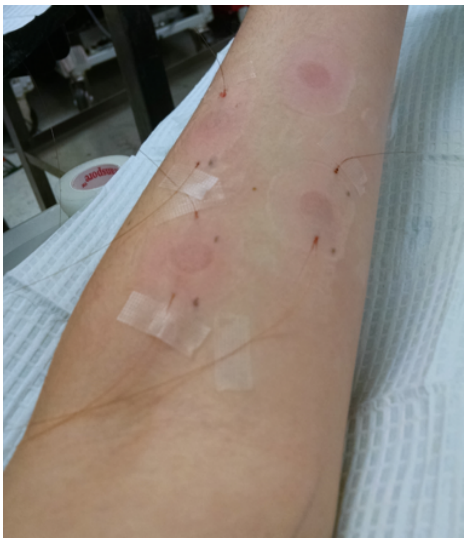

Figure 3.5. Three intradermal microdialysis fibers in the volar forearm with laser-Doppler flowmeters and local heating units removed. Photo by Michael Francisco.

Laser-Doppler Flowmetry

Most recent mechanistic studies of the SkBF response to hyperthermic challenge have quantified SkBF using laser-Doppler flowmeters. Laser-Doppler flowmeters, or probes, sit flush with and perpendicular to the skin surface (Figure 3.4) and can be mounted by themselves or seated in the center of local heating units on the skin surface (Figure 3.4 and Figure 3.6).

Laser-Doppler flowmetry is based on measuring the Doppler shift generated by the movement of red blood cells to the illuminating coherent light (139). Probes emit a beam of infrared light which penetrates the dermis and reflects off the dermal tissue and red blood cells; the reflected light is received by collecting fibers in the probe. Backscattered light carries information about red blood cell velocity (light frequency

shift) and concentration (fraction of total photons that are frequency shifted) (139). Red blood cell flux is then calculated as the product of red blood cell concentration and velocity.

In this dissertation, local heating units (SH02 Skin Heater/Temperature Monitor; Moor Instruments, Axminster, UK) were placed on the skin surface and integrated blunt needle laser-Doppler probes (MP12-V2, Moor Instruments) were seated in the local heating units to assess SkBF in real time (Figure 3.4 and Figure 3.6). Laser-Doppler probes were connected to a laser-Doppler server unit with three additional satellite units (moorLAB, Moor Instruments) allowing up to four laser-Doppler flowmeters to be used simultaneously.

Because blood vessel density across the skin is not uniform, red blood cell flux values are dependent on probe placement. To make inter-site and inter-subject comparisons using this technique, it is common to normalize site flux measures to a site's maximal flux. In the skin, maximal flux is achieved by locally heating the skin to >43°C and microdialysis infusion of the endothelium-independent nitric oxide donor, sodium nitroprusside. We achieved maximal flux at each site by using a combination of local heating to 44°C and microdialysis infusion of 56 mM sodium nitroprusside. Additionally, to account for changes in blood pressure, data were converted to cutaneous vascular conductance (CVC = flux / mean arterial pressure) and presented as a percentage of maximal CVC. By accounting for blood pressure and normalizing site flux measures to site maximal flux, the percent of maximal CVC values provide a strong indicator of a site's blood vessel tone and inter-site/inter-subject comparisons can be made.

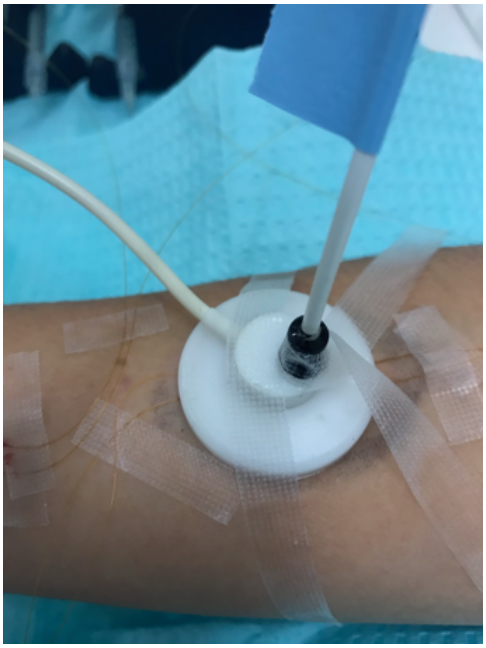

Figure 3.6. A laser-Doppler probe seated in a local heating unit positioned on the skin surface directly over a microdialysis fiber. Photo by Michael Francisco.

Pharmacological Interventions

Pharmacological agents in this dissertation were delivered via intradermal microdialysis or intradermal injection. Protocol 1 assessed the effects of posture on the NO component of CAVD. 4 microdialysis sites were randomly assigned to be perfused with:

1) Lactated Ringer's solution (sham site),

2) 5 mM N-propyl-L-arginine (NPLA),

3) 10mM N^5-(1-iminoethyl)-L-ornithine dihydrocholoride (L-NIO), or

4) 20mM N-nitro-L-arginine-methyl ester (L-NAME).

NPLA is an L-arginine analog that is a highly selective antagonist of nNOS based on in vitro and in vivo studies (1, 230). In addition, NPLA has been previously used to inhibit nNOS in human skin (20, 119, 148, 192). L-NIO is an L-arginine analog that has been

used to selectively block eNOS in vitro and in vivo (9, 44, 66, 91, 92, 148). It has also been previously used to inhibit eNOS in human skin (148). L-NAME is an analog of L-arginine, which non-specifically inhibits NO synthase and has been used extensively in human skin studies (23, 31, 56, 115, 129, 189, 197, 198, 225, 227).

Independent assessment of the SkBF responses at control, non-specific NOS blockade, nNOS specific blockade, and eNOS specific blockade sites, allows investigation of the relative contribution of NOS isoforms to the NO mediated portion of CAVD. Drug concentrations were selected as the minimum doses necessary for eliciting maximal inhibition based on previous skin studies (31, 148).

All drugs used were at the highest chemical purity available. Drugs were diluted to the appropriate concentrations by the primary investigator with sterile Lactated Ringer's solution. After dilution and before administration, perfusates were filtered through a 0.2μm filter needle. Investigational new drug approval was obtained through the U.S. Food & Drug Administration for the use of L-NIO and NPLA in these studies (IND# 141941; PI: Minson CT). Investigational new drug approval for the use of L-NAME was previously obtained. (IND# 124303; PI: Minson CT).

Protocol 2 assessed the SkBF responses to exercise and passive whole-body heat loading at cholinergic nerve blockade and control sites. nerves in the skin were blocked via intradermal injection of botulinum toxin type A (BOTOX®, onabotulinumtoxin A). Botulinum toxin binds irreversibly to presynaptic cholinergic nerve terminals, where it interacts with the soluble N-ethylemaleimide-sensitive fusion protein attachment protein receptor complex, to inhibit neurotransmitter release (6). This allowed study of the contribution of CAVD nerves (discussed in chapter II) to the SkBF response during

exercise and passive whole-body heat loading modalities. This model was used to recover perfusate at control and BOTOX® treated sites in an attempt to identify potential unknown neurotransmitters being co-released from CAVD nerves.

BOTOX® was procured as a lyophilized preparation (Allergan, Madison, NJ). Preparations were then reconstituted with preservative-free 0.9% Sodium Chloride Injection USP by the primary investigator and used within 24 hours of reconstitution. Standard insulin syringes (1/2 cc, 1/2", 31G) were used for intradermal injections. Subjects received three injections of approximately 5 units BOTOX® into the dermis (approximately 2 mm from the surface), with injection sites no farther than approximately 1.5 cm away from each other (Figure 3.7). Each injection site had a ring of effect that was approximately 2 cm in diameter. The "ring of effect" was identified by the primary investigator and drawn onto the skin surface using a permanent marker and photographs were taken to aid in re-identification of BOTOX® treated sites on thermal loading study days (Figure 3.7). Subjects were instructed to re-mark the "ring of effect" each day after showering or bathing. Subjects waited a period of at least 1 week after BOTOX® administration before participating in their first thermal loading study day.

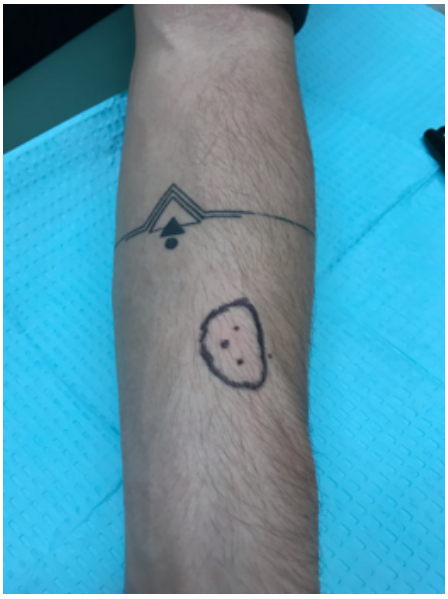

Figure 3.7: The ring of effect and three marked BOTOX® sites post-intradermal injection in the volar forearm in one subject. Photo by Michael Francisco.

Local Thermal Hyperemia

In the current dissertation, local heating was used in both Protocol 1 and Protocol 2 to test drug specificity (Figure 3.8) and the preservation of local vasodilator responses in cholinergic nerve blocked skin, respectively. Local skin heating and the resulting vasodilator response, known as "local thermal hyperemia", is one of the most characterized thermo-vascular responses in humans. Traditionally, local heating involves heating the skin to 42 °C at a rate of 0.1 °C/sec and results in a biphasic vasodilator response consisting of an initial peak and secondary plateau (Figure 3.8). The initial peak is mediated by a sensory nerve axon reflex that is likely the result of the release of calcitonin gene-related peptide and Substance P onto the cutaneous microvasculature (153, 213). The secondary plateau phase is approximately 60% dependent on eNOS generation of NO and 40% dependent on endothelial-derived hyperpolarizing factors (EDHFs) (23, 153).

Our lab recently developed a new local heating protocol in which the skin is heated to 39°C at a rate of 0.1°C/sec (31). Heating to this temperature results in a biphasic vasodilator response similar to heating to 42°C; however, the magnitude of the SkBF response is less (eg. the secondary plateau is approximately 40-50% of maximum vs. approximately 90-95% of maximum) and NO is an even larger contributor to the secondary plateau phase (approximately 80% eNOS mediated) (31, 153). For this dissertation we chose local heating to 39°C because of the large eNOS mediated vasodilation in the response and its relevance to Protocol 1 (discussed in Chapter IV).

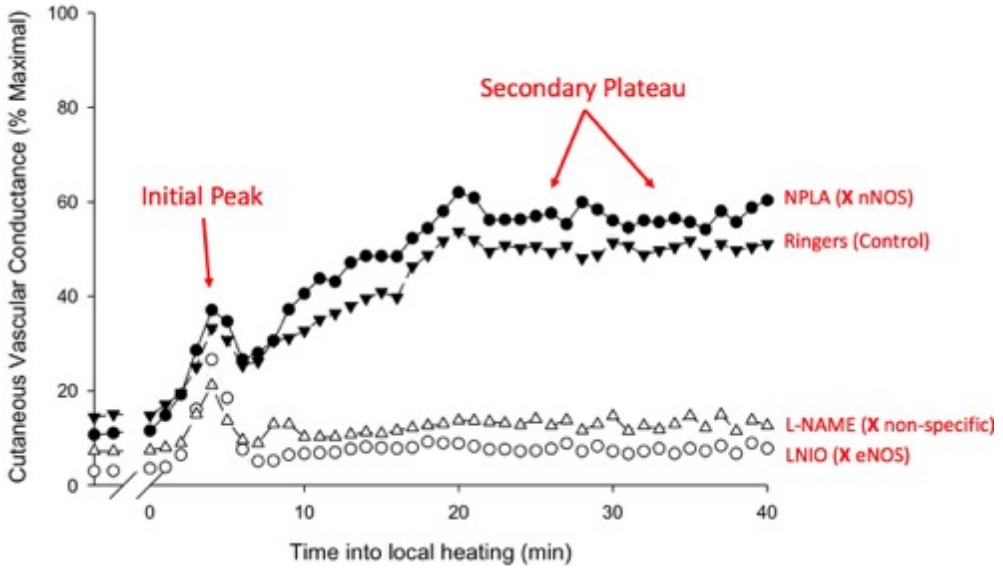

Figure 3.8. A representative tracing from one subject during local heating (39°C) of the skin at four microdialysis sites receiving: 1) Lactated Ringer's (control site), 2) 5mM N-propyl-L-arginine (NPLA), a neuronal nitric oxide synthase (nNOS) inhibitor, 3) 10mM N5-(1-iminoethyl)-L-ornithine dihydrocholoride (L-NIO), a endothelial nitric oxide synthase (eNOS) inhibitor, or 4) N-nitro-L-arginine-methyl ester (L-NAME), a non-specific nitric oxide synthase (NOS) inhibitor. For clarity, X's represent blockade of particular enzymes. Data are presented as a percentage of maximal cutaneous vascular conductance (% maximal), determined by infusion of 56 mM sodium nitroprusside and maximal heating to 44 °C.

Perfusate Recovery

The technique of microdialysis is commonly used in the sampling and collecting of small-molecular-weight substances from the interstitial space *in vivo* and *ex vivo*. For example, it is widely used in neuroscience to quantify neurotransmitters, peptides, and hormones in living animals and in the determination of neurotransmitter release from prepared tissues (30).

This dissertation sought to identify unknown vasoactive substances associated with CAVD using intradermal microdialysis to recover dialysate from cholinergic nerve blockade and control sites in the dermis of the volar forearm of human subjects. Lactated Ringer's was perfused at a rate of 2 ul/min (CMA 102 Syringe Pump; CMA Microdialysis AB, Solna, Sweden) through linear microdialysis fibers placed in the manner previously described. Dialysis membranes were polycarbonate-ether, 10 mm in length, and had either 55 Kda or 2000 Kda cutoffs (CMA 31 linear microdialysis, 10 mm membrane, 55 Kda or 2000 Kda CO; Harvard Bioscience, Holliston, MA). A peristaltic pump (Reglo ICC Digital Peristaltic Pump, IMSATEC, Wertheim, Germany) was attached to the distal "receiving" end of the fibers to reduce the build-up of back pressure and prevent ultrafiltration in the probe and improve analyte recovery efficiency (Figure 3.9). Thirty minute baseline dialysate samples were obtained at BOTOX® treated and untreated sites prior to thermal loading. Dialysate at BOTOX® treated and untreated sites was then collected for the entire duration (approximately 60 minutes) of exercise and passive whole-body heat loading. Dialysate samples were collected in 0.5 ml sterile cryotubes and were immediately frozen at -80 °C for future analysis.

In principle, once a microdialysis probe is inserted (described above) into tissue and perfusion begins, substances outside of the dialysis membrane diffuse through the membrane along their concentration gradient. In this manner the interstitial concentration of the analyte to be recovered can affect the overall amount of analyte found in the recovered perfusate. Importantly, the concentration of analyte collected will only represent a fraction of the extracellular fluid concentration. The diffusion of substances into the dialysis fiber and therefore recovery efficiency, are most affected by perfusate flow rate and composition, dialysis membrane properties, and both analyte properties and tissue properties;

Analyte recovery is inversely related to perfusate flow rate with near one hundred percent recovery efficiency at extremely low flow rates (approximately 0.1ul/min) and low recovery efficiency at high flow rates (100, 228). While extremely low flow rates are ideal for analyte recovery, these rates do not allow for sufficient time or volume resolutions to be practical. High flow rates produce considerable back pressure in the probe, resulting in ultrafiltration of the perfusate and reduced recovery efficiency (30). In addition, solutes contained in the perfusate may disturb the interstitial neurochemical milieu and alter basal levels of neurotransmitters. For example, high concentrations of calcium (2.4 vs. 1.2 mM) can result in stimulated neurotransmitter rather than basal neurotransmitter release (215). Lactated Ringer's and synthetic cerebral spinal fluid are most commonly used in dialysate recovery studies. For these reasons, we chose to perfuse Lactated Ringer's at a rate of 2 ul/min with the aid of a peristaltic pump.

Analyte recovery is directly proportional to membrane surface area (100). Greater membrane surface areas aid in the recovery of high molecular weight and/or "sticky"

compounds. Kendrick et al, observed a strong negative relationship between recovery efficiency and analytes with larger molecular weights or greater hydrophobic properties ("sticky") (121, 122). It is thought that differences in membrane material and cutoff size may also affect the recovery of large molecular weight and/or "sticky" analytes. However, differences by membrane material have only been shown *in vitro*; while no differences in recovery have been found *in vivo* (88). Because we were attempting to identify *unknown* substances, we chose to use fibers with 10 mm long membranes and larger cut-offs (55 Kda or 2000 Kda) to help aid in the recovery of any larger molecular weight and/or "sticky" analytes.

Consideration of tissue properties in dialysate recovery studies is also warranted when designing a dialysate recovery experiment. Analyte diffusion in tissue is typically slower than in an aqueous solution and this due to reduced fluid volume and tissue tortuosity (increased diffusional path) (159). Diffusion in tissue can be further slowed by analyte binding to cell surface receptors along the diffusional path. Because of this, it should be noted that tissue characteristics such as fluid volume, tortuosity, hindrance, and rate of analyte clearance, affect diffusional resistance and can therefore affect recovery efficiency. These factors can be experimentally problematic because they can reduce the dialysate levels of the analyte below the sensitivity of analytical detection, and any procedure (e.g. fiber placement) or treatment that affect the physiology of the tissue and its characteristics can lead to changes in probe recovery efficiency (30).

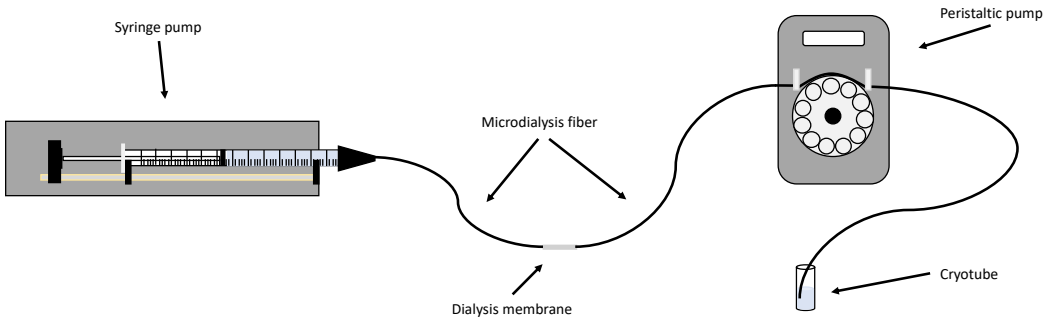

Figure 3.9. A schematic of the intradermal microdialysis set-up used to collect dialysate from BOTOX® treated and untreated sites in the dermis of the volar forearm.

Perfusate Analysis for Unknown Proteins

The Proteomics Shared Resource Facility at Oregon Health Sciences University was retained to run a shotgun proteomics analysis on the dialysate samples. Mass spectrometry-based proteomics has become an increasingly powerful tool that has allowed the detection and quantification of thousands of proteins. In particular, liquid chromatography-mass spectrometry (LC-MS) allows for a bottom-up approach that has been used extensively in the determination of proteins found in dialysates (14, 163, 165). The typical LC-MS work flow involves the digestion of proteins into peptides (via trypsin), peptide separation by LC, and ionization of peptide molecules before entering the MS where they can be detected or filtered based on their mass-to-charge values (165).

The general proteomic analysis workflow at Oregon Health Sciences University was provide and is as follows. The skin dialysate samples are ultra-filtrated using 10 kDa cutoff Amicon Ultra 0.5 ml ultrafiltration spin cartridges (Cat #UFC501024, Millipore-Sigma) to remove higher molecular weight proteins. Since the amount of recovered protein was below the limit of accurate detection by a Pierce BCA protein assay (< 0.1 µg/µl), the filtrate is divided into two equal portions, dried by vacuum centrifugation and

the entirety of each sample subsequently analyzed. One portion is analyzed by mass spectrometry without digestion, and the other portion was reduced using DTT, alkylated with iodoacetamide, and digested overnight with trypsin (0.9 µg per sample) in the presence of ProteaseMax™ detergent, as recommended by the manufacturer (ProMega Corporation). Following trypsin digestion, samples are filtered (0.22 um) and again dried by vacuum centrifugation.

Both dried trypsin digested and non-digested samples are dissolved in 22 µl of 5% formic acid and each analyzed by liquid chromatography-mass spectrometry (LC-MS). 20 µl of each sample is auto-injected and peptides separated using a Dionex NCS-3500RS UltiMate RSLCnano UPLC system, and mass spectrometric analysis is performed using an Orbitrap Fusion Tribrid instrument with an EasySpray nano source (Thermo Scientific). Peptides are desalted using an Acclaim PepMap 100 µm x 2 cm NanoViper C18, 5 µm trap column on a switching valve. After 5 min of loading and washing, the trap column is switched on-line to a PepMap RSLC C18, 2 µm, 75 µm x 25 cm EasySpray column (Thermo Scientific). Peptides are then separated using a 7.5–30% acetonitrile gradient over 60 min in a mobile phase containing 0.1% formic acid at a 300 nl/min flow rate. Survey MS scans are collected using the instrument's Orbitrap mass analyzer at a resolution of 120,000. Data-dependent MS/MS spectra were collected in the instrument's ion trap using higher-energy dissociation (HCD) following quadrupole isolation. The dynamic exclusion feature of the instrument and its top speed mode areused to increase the number of unique peptides that were identified.

RAW instrument files are processed using Proteome Discoverer version 1.4 (Thermo Scientific), SEQUEST HT software and a UniProt human database (Swiss

Bioinformatics Institute, Geneva, Switzerland). For trypsin digested samples, searches are configured with a static modification for alkylated cysteines (+57.021 Da), variable modifications for oxidation of methionine (+15.995 M residues), and trypsin specificity. Undigested samples are similarly searched, except without specifying cysteine alkylation and using no enzyme specification. A parent ion tolerance of 10 ppm, fragment ion tolerance of 1.0 Da, monoisotopic masses, and for trypsin cleaved samples, a maximum of 2 missed cleavages are allowed. Searches used a reversed sequence decoy strategy to control peptide false discovery using Percolator software (48, 102). Results are filtered to remove peptide identifications with q-scores > 0.05, to maintain false discovery rates below 5%. Differences in protein abundances across samples were estimated by comparison of numbers of assigned MS/MS spectra to each protein (135).

Final Remarks

This dissertation sought to interrogate the CAVD system in response to varying thermal loading modalities and postures using the techniques described in this Chapter. In Protocol 1, the contribution of nNOS and eNOS isozymes to cutaneous vasodilation during passive whole-body heat loading in the supine and seated positions was assessed. The specific methodology and results of this study are detailed below in Chapter IV. In Protocol 2, the extent to which cholinergic nerve activation mediates CAVD during exercise heat loading and passive whole-body heat loading was assessed. Identification of unknown vasoactive substances associated with CAVD was also attempted. The specific methodology and results of this study are detailed in Chapter V.

CHAPTER IV

POSTURAL INFLUENCES ON THE NITRIC OXIDE COMPONENT OF CUTANEOUS ACTIVE VASODILATION

Introduction

In humans, the primary response to increasing internal temperature (T_c) is increased cutaneous blood flow along with sweating. These reflexes facilitate heat dissipation to the environment by expanding the thermal gradient between the core and the periphery of the body. Noradrenergic vasoconstrictor nerves as well as a cholinergic vasodilator system control skin blood flow in human non-glabrous skin. During heat stress, the initial rise in internal temperature causes an increase in skin blood flow that is mediated by vasoconstrictor withdrawal (46, 175); the magnitude of this initial response is dependent on environmental conditions (53). As internal temperature continues to rise, there is a marked increase in skin blood flow and sweating in non-glabrous skin that is the result of exceeding the internal temperature threshold for activation of the cutaneous active vasodilator system (CAVD) (94, 98, 176). In non-glabrous skin, CAVD can account for approximately 80-95% of the rise in skin blood flow that accompanies heat stress (98, 180).

The robustness of the CAVD system to thermal challenge has made it the topic of much research. A number of vasodilator pathways have been identified as having a role in CAVD; yet the complexity of the mechanisms involved in this system make it difficult to understand. Nitric oxide (NO) has been shown to contribute approximately 30-45% to

CAVD in young healthy individuals during supine passive whole-body heating (79, 117, 119, 148, 188, 189). Previous research suggests that neuronal nitric oxide (nNOS) is the primary NOS isozyme that contributes to the NO component of CAVD during supine whole-body heat stress (117). Administration of the nNOS inhibitor, 7-nitrioindazole (7-NI), via intradermal microdialysis attenuated the skin blood flow response during passive whole-body heating, but had no effect on skin blood flow response to local heating (117). Further work by Kellogg et al., determined that nNOS contributes to the NO component of CAVD and endothelial nitric oxide synthase (eNOS) contributes to the NO component of vasodilation during local heating (117, 118, 120).

Interestingly, a study conducted by McNamara et al. indicated that the eNOS rather than the nNOS isozyme contributes to CAVD during sustained dynamic exercise (148). It was suggested that these disparate findings may be due to differences in active vasodilator nerve firing rates or increases in cyclic circumferential vessel wall strain (which has been shown to increase eNOS activity (138)) that are unique to exercise but not to passive whole-body heat loading (148). Taken together, these studies highlight distinct mechanistic differences in CAVD that may be dependent on the thermal loading modality used (e.g. semi-recumbent dynamic exercise vs. supine passive whole-body heat loading). It should be noted that exercise and passive-whole body heat loading are largely different stimuli that result in similar thermoregulatory reflexes. The afferent signals they share in common and those that delineate them may impact the mechanisms and transducers of CAVD; yet these signals and their impact on CAVD are not well understood. For example, differences in the NOS isozyme contributions during upright exercise and supine whole-body passive heating may simply be due to differences in skin

temperature or posture affecting the mechanisms of cutaneous vasodilation secondary to differences in afferent input (e.g. orthostatic and skin temperature inputs to the hypothalamus).

During orthostasis (upright posture), arterial baroreceptor activation results in an increase in heart rate and cardiac contractility and an increase in total peripheral resistance (via peripheral vasoconstriction and venoconstriction within the splanchnic circulation) to maintain mean arterial pressure or adequate perfusion pressure to the brain. This reflex vasoconstriction is mediated in part by the cutaneous vasculature in both normothermic and hyperthermic conditions (36, 111). Kellogg et al. blocked adrenergic vasoconstrictor activity, using bretylium iontophoresis and measured skin blood flow responses at bretylium treated and control sites to -40 mmHg lower body negative pressure (LBNP) in both normothermic and hyperthermic conditions (111). These authors found that reductions in skin blood flow following LBNP during hyperthermia were due to active vasodilator withdrawal, whereas during normothermia, reductions in skin blood flow were due to enhanced vasoconstrictor activity. This manifests as a rightward shift in the T_c-skin blood flow relationship (Chapter 2, Figure 2.8).

To determine which baroreceptor population is mediating the active vasodilator withdrawal, Crandall et al. used external neck pressure to unload carotid baroreceptors and low levels of LBNP to unload cardiopulmonary baroreceptors independently of each other (36). It was concluded that neither cardiopulmonary nor carotid baroreceptor unloading could account for the attenuated active dilator response during orthostasis (36). While it is unknown what modulates the rightward shift in the T_c-skin blood flow

relationship, it is well documented that baroreception and thermoregulatory reflexes produce an integrated cutaneous vascular response (41, 74). Thus, it is possible that posture (e.g. orthostasis and some variation in baroreception) is a requisite afferent input to change active vasodilator nerve behavior and result in variable NOS isozyme recruitment (148) To date, the NO component of CAVD has only been explored in the supine position during passive whole-body heat loading. The impact of posture on NO generation during CAVD is unknown.

Therefore, the purpose of this study (Protocol 1) was to examine the effect of posture on the NO component of CAVD during passive whole-body thermal loading. We hypothesized that the NO component of CAVD during passive whole-body heat loading in the supine and seated positions would both be mediated by the nNOS isozyme. We also hypothesized that passive whole-body heat loading in the seated position would attenuate the overall skin blood flow response compared to the supine position; but would not change the relative contribution of NO to reflex cutaneous vasodilation.

Methods

All protocols in human subjects were approved by the Institutional Review Board at the University of Oregon (IRB #12102010.027, see Appendix page (128) for informed consent documents). All subjects gave oral and written consent prior to participation in accordance with the *Declaration of Helsinki*.

Subjects

Sixteen young (18-30 years) recreationally-active (<150 minutes of intense exercise a week) individuals volunteered to participate in this study. All subjects were

non-smokers with no history of cardiovascular, metabolic, or other chronic diseases, not taking prescription medications with the exception of hormonal contraceptives, not currently pregnant or breast-feeding, and with no history of allergic reactions to any drugs used in this study. A summary of physical characteristics is listed in **Table 4.1**.

General Study Design and Setup

Subjects participated in a passive whole-body heat loading study in either the supine or seated position which was randomly assigned prior to participation. **Figure 4.1** illustrates the randomization of subjects by posture for the passive whole-body heat loading interval and study drug to a site on the forearm. Treatments (Control, NPLA, L-NIO, L-Name) were randomly assigned to sites on the forearm and are described below.

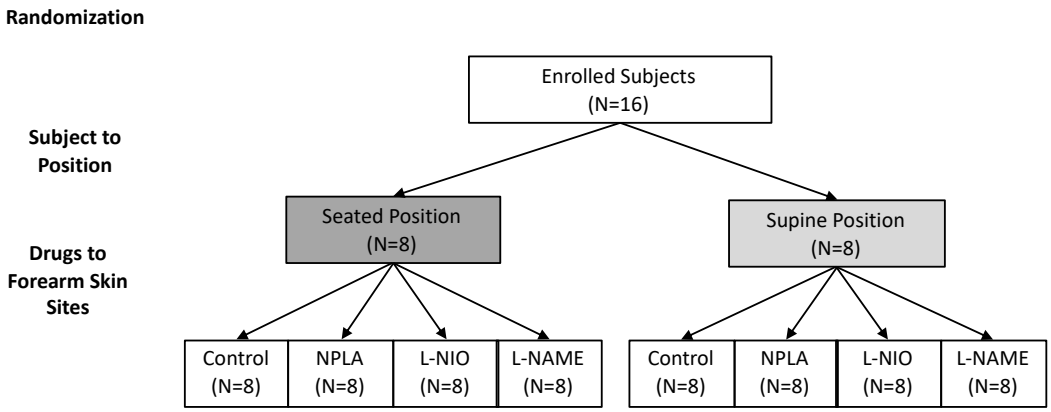

Figure 4.1. Randomization of subjects to a posture and study drug to a site on the forearm.

A subset of subjects (4 of the 16 subjects) completed thermal loading in both positions. For these individuals, thermal loading study sequences were randomized and a period of at least 1 week separated the two thermal loading treatments. The subset of four

subjects underwent the thermal loading paradigm in both the seated and supine positions. After their initial study day, the four subjects crossed over and underwent thermal loading using the alternative position. Figure 4.2 shows the thermal loading positions for the four crossover subjects. Study drugs were administered to the same forearm during each of the two study periods. Four naïve drug treatment sites were identified on the subjects' forearms during the second study period, and drug treatment was again randomly assigned to a forearm site.

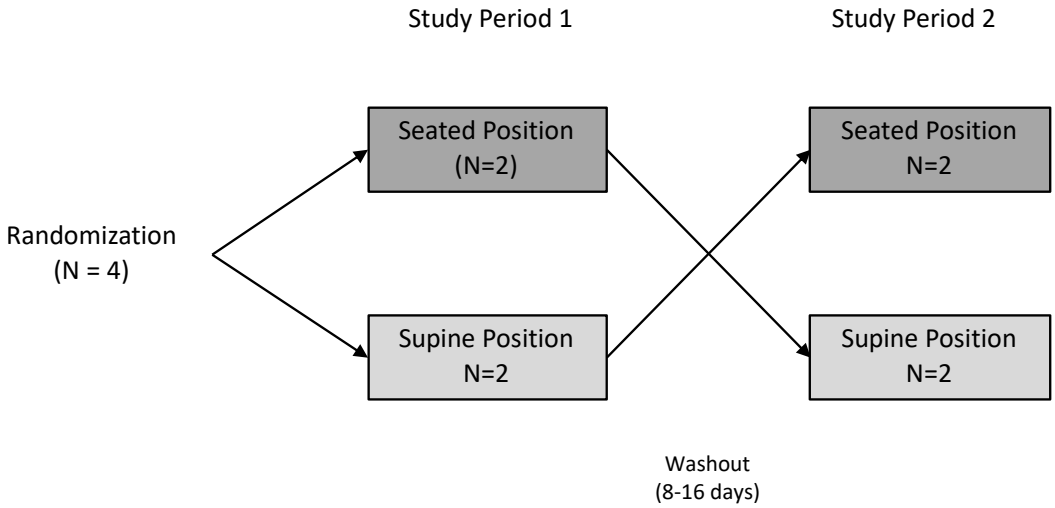

Figure 4.2. Two period crossover study design for four subjects undergoing passive whole-body heat loading in both the seated and supine positions.

Prior to thermal loading study days, subjects abstained from food for 4 hours, alcohol and caffeine for 12 hours, and exercise and over-the-counter medications for 24 hours. All female subjects had a negative pregnancy test on the morning of each study day. Women were studied during the low hormone phase of their menstrual cycle. Women taking hormonal contraceptives (N=6) were studied during the placebo phase and those naturally menstruating (N=2) were studied during the early follicular phase of menstruation (self-reported). The single female that completed passive whole-body

heating in both postures was unable to return one full cycle later; she returned 8 days after her first study day visit for her second visit.

On thermal loading study days, subjects reported to the Human Cardiovascular Control Laboratory in Esslinger Hall on the University of Oregon campus. Upon arrival, subjects provided a urine sample to confirm euhydration via urine specific gravity <1.02 (Analog Regractometer; Atago, Tokyo, Japan). If urine specific gravity was >1.02, subjects drank 5 ml/kg of water prior to study. Dry nude body weight was measured before and after thermal loading to calculate mean whole body sweat rate. Subjects were instrumented with a sterile rectal thermistor probe (YSI Series 400; Yellow Spring Instruments, Yellow Springs, OH) inserted approximately 10 cm past the anal sphincter to monitor internal temperature (T_c).

Skin surface thermistors (MSR145WD, MSR Electronics GmBH, Seuzach, Switzerland) were applied to standard locations on the chest, forearm, calf, and quadricep to continuously measure skin temperatures. Mean skin temperature (T_{sk}) was calculated using the 4-site weighting method previously developed by Ramanathan (172):

$$T_{sk} = 0.3(t_{chest} + t_{arm}) + 0.2(t_{thigh} + t_{calf}) \qquad (1)$$

Where

t_{chest} = skin temperature measured at the chest,

t_{arm} = skin temperature measured at the forearm,

t_{thigh} = skin temperature measured at the quadricep, and

t_{calf} = skin temperature measured at the calf.

Mean body temperature (T_b) was calculated using the internal temperature and mean skin temperature weighting method described by Gagge and Gonzalez. (57):

$$T_b = 0.8(T_c) + 0.2(T_{sk}) \qquad (2)$$

Heart rate and ventilatory frequency were monitored using a 3-lead electrocardiogram (CardioCap; Datex Ohmeda, Louisville, CO). Baseline blood pressure was measured in triplicate following 20 minutes of rest and every 5 minutes throughout thermal loading via brachial oscillation (Datascope Accutorr Plus, Duluth, GA).

Passive Whole-Body Heat Loading

Passive whole-body thermal loading was accomplished by having subjects wear a two-piece nylon water-perfused suit (Med-Eng Systems, Ottawa, Canada), which covered all skin surfaces except the experimental arm, face, hands, and feet. In addition, subjects wore a water-impermeable plastic garment over the suit to mitigate evaporative heat loss. During thermal loading, mean skin temperature was maintained at approximately 39°C for 60 minutes using a heated circulating water bath (HT CIRC 7L AD, Polyscience, Niles, Illinois) to perfuse hot water (50°C) through the suit. This water temperature was based on previous work from our lab and has been shown to raise internal temperature 0.7-1.0°C above baseline in approximately 60 minutes (22, 218, 224).

NOS Blockade

Intradermal microdialysis was used to deliver nitric oxide synthase antagonists prior to and throughout thermal loading. Under aseptic conditions, all subjects had four microdialysis fibers (CMA 31 linear microdialysis, 10 mm membrane, 55Kda CO; Harvard Bioscience, Holliston, MA) placed in the dermal layer of the skin of either the

dominant or nondominant volar forearm. Fibers were placed by first introducing a 25-gauge needle approximately 1 mm below the surface of the skin, with entry and exit points 2-3 cm apart. Fibers were threaded through the lumen of the needle and the needle was removed leaving the dialysis membrane of the fiber centered between the needle entry and exit points. Fibers were at least 5 cm apart to prevent the interaction of pharmacological agents between sites. A period of approximately 90 minutes was allowed to let needle insertion trauma and the resulting transient rise in skin blood flow resolve. This was confirmed by observing a plateau in laser-Doppler (described below) flux values. To maintain patency of the dialysis membrane during this time, fibers were perfused with Lactated Ringer's solution at a rate of 2.0 ul/min (CMA 102 Syringe Pump; CMA Microdialysis AB, Solna, Sweden) until the start of study drug infusions.

Forty-five minutes prior to passive whole-body heat loading, microdialysis fibers were randomly assigned to be perfused at 2ul/min with 1) Lactated Ringer's solution (sham site), 2) 5 mM N-propyl-L-arginine (NPLA), 3) 10mM N^5-(1-iminoethyl)-L-ornithine dihydrocholoride (L-NIO), or 4) N-nitro-L-arginine-methyl ester (L-NAME). NPLA is an L-arginine analog that is a highly selective antagonist of nNOS based on in vitro and in vivo studies (1, 230). In addition, NPLA has been previously used to inhibit nNOS in human skin (20, 119, 148, 192). L-NIO is an L-arginine analog that has been used to selectively block eNOS in vitro and in vivo (9, 44, 66, 91, 92, 148). It has also been used to inhibit eNOS in human skin (148). L-NAME is an analog of L-arginine, which non-specifically inhibits NO synthase and has been used extensively in human skin studies (23, 31, 56, 115, 129, 189, 197, 198, 225, 227).

All drugs used were at the highest chemical purity available and drug concentrations were selected as the minimum doses necessary for eliciting maximal inhibition based on previous skin studies (31, 148). Drugs were diluted to the appropriate concentrations by the primary investigator with sterile Lactated Ringer's solution and were perfused for the entire duration of the study.

Skin Blood Flow

Skin blood flow at the four microdialysis sites was quantified throughout the study using laser-Doppler flowmetry. Local heating units (SH02 Skin Heater/Temperature Monitor; Moor Instruments, Axminster, UK) were placed on the skin surface directly above the previously placed microdialysis fibers and integrated blunt needle end laser-Doppler probes (MP12-V2, Moor Instruments) were seated in the local heating units to measure red blood cell flux, an index of skin blood flow. Laser-Doppler flux was continuously recorded during a 2-minute baseline period and throughout the approximate 60-minute thermal loading period at all 4 drug sites.

After completion of passive whole-body thermal loading and when skin red blood cell flux had returned to baseline levels at all 4 sites, a local heating protocol was used to ensure that L-NIO effectively inhibited and NPLA had no effect on NO-dependent vasodilation independent of passive whole-body heat stress (31). Local heating results in a biphasic vasodilator response consisting of an initial peak and secondary plateau (17, 34). The initial peak is mediated by a sensory nerve axon reflex that is likely the result of the release of calcitonin gene-related peptide and substance P onto the cutaneous microvasculature (153, 213). The secondary plateau, is approximately 40-50% of the maximum skin blood flow response and is approximately 80% eNOS mediated.

Briefly, the local heaters at all sites were set to 33°C and a five minute baseline was recorded. All sites were then locally heated to 39°C at a rate of 0.1°C/sec. Maximal laser-Doppler flux was then obtained at each site by locally heating the skin to 44°C as well as infusing 56 mM sodium nitroprusside at each site. Timing of a thermal loading study day is summarized in **Figure 4.3**.

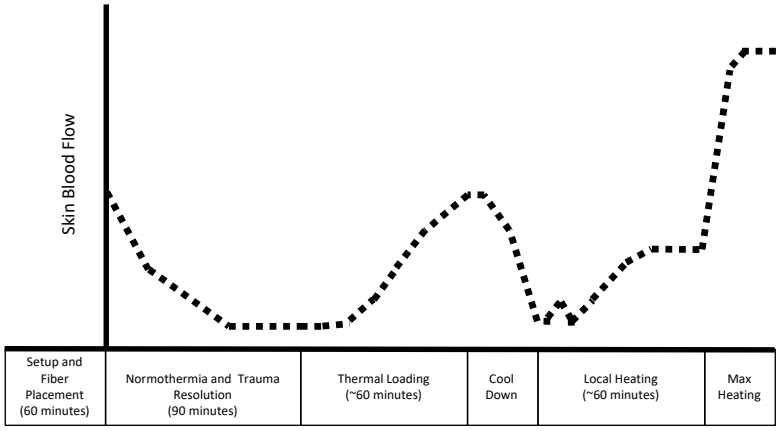

Figure 4.3. A representative tracing of skin blood flow at a control site throughout the thermal loading study day.

Data Analysis

Skin red blood cell flux (RBCflux) data were digitized and recorded to a computer using data acquisition software (Windaq; Dataq Instruments, Akron, OH). The RBCflux measurements at each site during the 2-minute baseline period were averaged. Starting at the beginning of thermal loading, RBCflux measurements were averaged over each sequential one-minute interval during the approximate 60-minute thermal loading period. The red blood cell flux values were converted to cutaneous vascular conductance (CVC) and expressed as a percent of maximum CVC (%CVCmax). %CVCmax was derived as follows:

$$CVC = RBCflux / MAP$$

$$\%CVCmax = [CVC / CVCmax] \times 100 ,$$

where MAP is mean arterial pressure and was calculated as

$$MAP = \frac{1}{3}(\text{systolic blood pressure}) + \frac{2}{3}(\text{diastolic blood pressure}).$$

Skin blood flow responses at each site associated with increases in internal temperature were characterized using the %CVCmax value immediately after each 0.1°C increase in internal temperature.

Statistics of Hemodynamics and Skin Blood Flow Results

Statistical analyses of hemodynamic and temperature data were conducted using SigmaPlot 11.0 (Systat Software, San Jose, CA). Because there were no discernable differences between men and women, data from the two groups were combined for analysis. Hemodynamic and temperature data before and at the end of thermal loading were compared using a 2-way repeated measures analysis of variance (Intervention vs. Time).

Statistical analyses of skin blood flow data were conducted using SAS 9.4 (SAS, Cary, NC). %CVCmax (observed values and change from baseline values) as a function of temperature increase and at the maximal temperature increase common to both the seated and supine positions (0.7 °C). The primary analysis included the naïve study day of all 16 subjects (i.e., the second study day for the 4 cross-over subjects was excluded).

%CVCmax in the seated and supine positions as internal temperature increased was compared using a mixed model repeated measures analysis variance. The analysis

included position (seated, supine), site on the forearm (Control, NPLA, L-NIO, L-NAME), temperature increase (0.1° C increments from 0.1 to 0.7 °C), site-by-temperature increase interaction, and position-by-site-by-temperature increase interaction as fixed effects. A first-order autoregressive covariance structure was used to model the correlations among temperature increases within site.

%CVCmax in the seated and supine positions at 0.7 °C was compared using a mixed model repeated measures analysis of variance. The analysis included position, site on the forearm, and position-by-site interaction. A compound symmetry covariance structure was used to model the correlations among sites.

%CVCmax differences were examined in detail at the maximum internal temperature increase common to both positions (0.7 °C). Because the %CVCmax results from the seated and supine passive heating intervals identify the same NO synthase isozyme (eNOS or nNOS) as contributing to CAVD, %CVCmax differences between the seated and supine positions were explored further by comparing the least squares mean (LS mean) differences both between sites within position and between sites across the seated and supine positions.

In the subset of four crossover subjects, potential position effects of NO synthase isozyme (eNOS or nNOS) contributions to CAVD, were summarized and examined graphically. at the maximum internal temperature common to both positions. Results from the crossover subjects were consistent with those from the main study. Due to the small sample size, no further analyses were conducted. .

Results

Subject Demographics

Subject demographics are presented as means ± S.E. in **Table 4.1.** Eight women participated in this study.

Table 4.1. Demographics and baseline characteristics of subjects who completed passive whole-body heat loading (N=16). Data are mean ± S.E. A subset of subjects completed whole-body heat loading in both postures (4 of the 16).

	Supine (N=8)	Seated (N=8)
# of women	3	5
Age (years)	23 ± 1	23 ± 1
Height (cm)	170 ± 3	168 ± 3
Weight (kg)	65 ± 7	58 ± 4
BMI (kg/m^2)	22 ± 2	20 ± 1

cm, centimeters; kg, kilograms; BMI, body mass index.

Hemodynamics and Thermal Loading

Group mean hemodynamic data for passive whole-body heat loading in the seated and supine positions are presented in **Table 4.2** below. Baseline heart rate, systolic blood pressure, diastolic blood pressure, mean arterial pressure, and pulse pressure were similar for the two postures. Baseline internal temperature (T_c) and mean body temperature (T_b) were also not different between postures (**Figure 4.4 and Table 4.2, respectively**). Heart rate increased equally during passive whole-body heat loading in each position [$P<0.001$ for each position; Seated: 54 ± 2 (baseline) vs. 102 ± 3 (end of thermal loading); Supine: 53 ± 3 (baseline) vs. 104 ± 4 (end of thermal loading) beats min^{-1}, respectively]. Similarly, systolic blood pressure and pulse pressure increased to a similar extent relative to baseline by the end of passive whole-body heat loading in the seated and supine

positions (**Table 4.2**). Diastolic blood pressure and mean arterial pressure remained unchanged from baseline. Both postures resulted in similar increases in internal temperature and mean body temperature during passive whole-body heat loading (**Figure 4.4 and Table 4.2, respectively**). There were no hemodynamic or body temperature differences between the two postures during passive whole-body heating. Additionally, there were no differences between the two postures for the subset of individuals studied in both positions.

Table 4.2. Group mean hemodynamic and mean body temperature data at baseline and end of thermal loading in the seated and supine positions (N=16) for naïve subject study days. Data are mean ± S.E.

	HR (beats min^{-1})	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	PP (mmHg)	T_b (C°)
Seated (N=8)						
Baseline	54 ± 2	113 ± 3	70 ± 3	85 ± 2	43 ± 3	36.2 ± 0.1
Thermal loading	102 ± 4*	129 ± 4*	66 ± 2	87 ± 3	64 ± 4*	38.0 ± 0.2*
Supine (N=8)						
Baseline	53 ± 3	116 ± 4	71 ± 2	86 ± 2	45 ± 3	36.1 ± 0.1
Thermal loading	101 ± 3*	130 ± 6*	68 ± 3	89 ± 4	61 ± 4*	38.2 ± 0.2*

HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure; T_b, mean body temperature; S.E., standard error. *P < 0.05 vs. baseline.

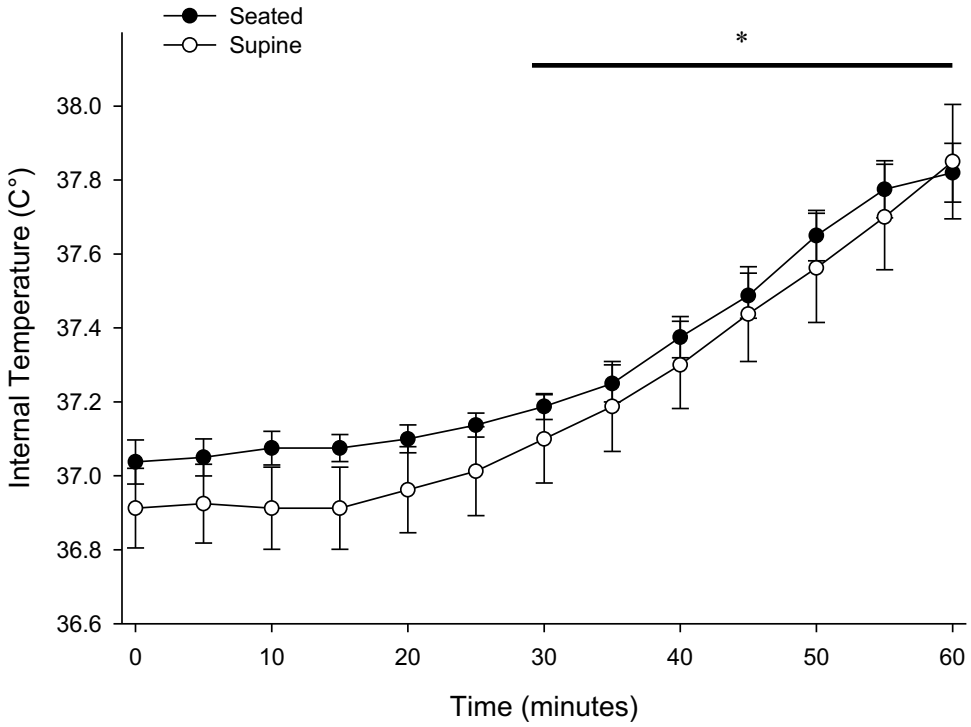

Figure 4.4. Internal temperature during 60 minutes of passive whole-body heat loading in the seated (closed circles; N=8) and supine (open circles; N=8) positions. Data are mean ± S.E. *P < 0.05 vs. baseline (time 0).

Skin Blood Flow

%CVCmax measurements associated with 0.1 °C internal temperature increases during passive whole-body thermal loading in the seated and supine positions at each treatment site are summarized in **Figure 4.5** and **Figure 4.6**, respectively. Increases in %CVCmax elicited following a 0.7 °C increase in internal temperature at each site during thermal loading in both positions are summarized in **Figure 4.7**. Baseline skin blood flow values were not different by site or by posture (P>0.050). Skin blood flow responses to increasing internal temperature were similar for the two postures (position differences, P = 0.811; position-by-site-by-temperature increase interaction, P=0.184) but differed by drug site (site-by-temperature increase interaction, P = 0.008).

During thermal loading in the seated position, skin blood flow values at the control site were elevated relative to baseline starting at a 0.1 °C ($P < 0.001$) increase in internal temperature and reached 51 ± 5 %CVCmax by the end of thermal loading (**Figure 4.5**). In the supine position, skin blood flow values at the control site were elevated relative to baseline starting at a 0.1 °C ($P < 0.050$) increase in internal temperature and reached 50 ± 8 %CVCmax by the end of thermal loading (**Figure 4.6**)

Increases in skin blood flow at the NPLA sites as internal temperature increased did not differ from the control sites during thermal loading for both the seated and supine positions (all $P > 0.072$). At the maximum thermal load (0.7 °C internal temperature increase) for the seated and supine positions, the NPLA sites reached 55 ± 5 and 41 ± 6 %CVCmax, respectively, and there were no postural differences (position*site interaction, $P = 0.275$; **Figure 4.7**).

At L-NAME sites, skin blood flow was significantly increased ($P < 0.050$) relative to baseline by internal temperature increases of 0.4 and 0.3 °C in the seated and supine positions, respectively. Attenuation of the skin blood flow response relative to the control sites was evident starting at a 0.2 °C increase in internal temperature for both the seated ($P < 0.001$) and supine ($P = 0.013$) positions (**Figure 4.5** and **Figure 4.6**). At the end of thermal loading for the seated and supine positions, L-NAME sites had reached 16 ± 3 and 20 ± 7 %CVCmax, respectively and were significantly different from their Control sites ($P < 0.001$ and $P < 0.001$, respectively). There were no differences by posture ($P = 0.635$; **Figure 4.7**).

Skin blood flow at L-NIO sites was increased relative to baseline starting at 0.3°C increase in internal temperature for the seated position and at 0.2°C for the supine

position (P < 0.050). Evidence of an attenuated response relative to the Control was present starting at a 0.2°C and a 0.3°C internal temperature increase for the seated and supine positions, respectively (P < 0.050). By the end of thermal loading in the seated and supine positions (0.7°C increase in internal temperature), L-NIO sites had reached 27 ± 4 and 27 ± 5 %CVCmax, respectively, and were significantly different from their Control sites (P = 0.002 and P = 0.003, respectively) (**Figure 4.7**). The difference between the seated and supine L-NIO sites at a 0.7°C increase in internal temperature was not significant (P = 0.972).

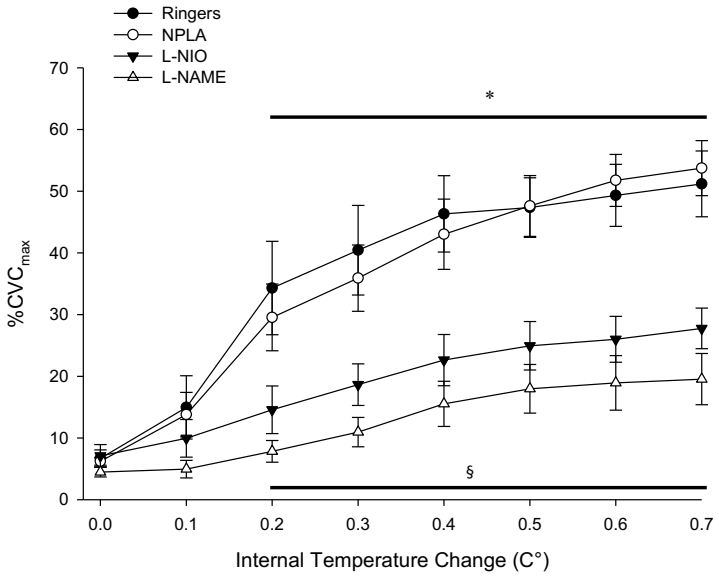

Figure 4.5. Seated Position. Percentage of maximal cutaneous vascular conductance (%CVCmax) at Control (Ringers; filled circles), nNOS blockade (NPLA; open circles), eNOS blockade (L-NIO; closed inverted triangles), and nonspecific NOS blockade (L-NAME; open triangles) sites is shown for each 0.1 °C increase in internal temperature from baseline during passive whole-body heat loading in the seated position (N=8). Data are mean ± S.E. *P < 0.05 for Control and NPLA sites vs. baseline. §P < 0.05 for L-NIO and L-NAME vs. Control.

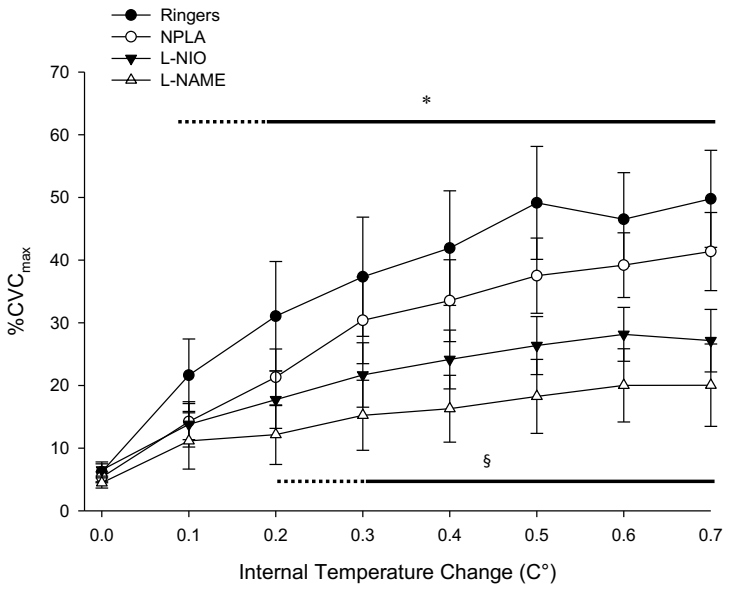

Figure 4.6. Supine Position. Percentage of maximal cutaneous vascular conductance (%CVCmax) at Control (Ringers; filled circles), nNOS blockade (NPLA; open circles), eNOS blockade (L-NIO; closed inverted triangles), and nonspecific NOS blockade (L-NAME; open triangles) sites is shown for each 0.1 °C internal temperature increase from baseline during passive whole-body heat loading in the supine position (N=8). Data are mean ± S.E. *P < 0.05 Control and NPLA sites vs. baseline (the solid line represents Control and NPLA; the dotted line represents Control). §P < 0.05 for L-NIO and L-NAME vs. Control (the solid line represents L-NAME and L-NIO; the dotted line represents L-NAME).

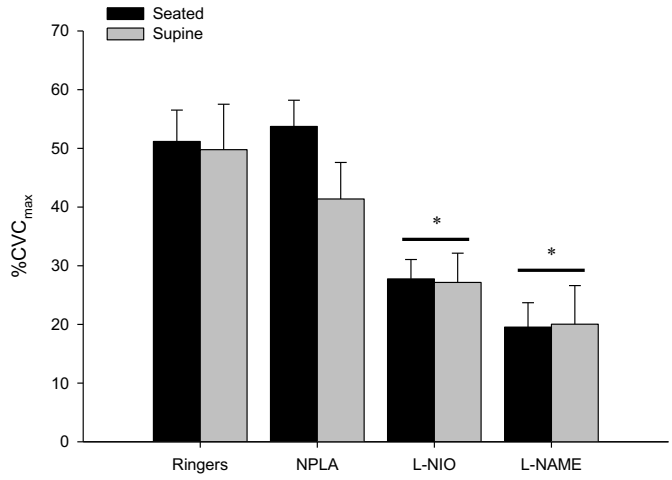

Figure 4.7. Percentage of maximal cutaneous vascular conductance (%CVCmax) at Control (Ringers), nNOS blockade (NPLA), eNOS blockade (L-NIO), and nonspecific NOS blockade (L-NAME) sites at a 0.7C° increase in internal temperature during passive whole-body heat loading in the seated (black bars; N=8) and supine (gray bars; N=8) positions. Data are mean ± S.E. There were no differences in posture. *P < 0.05 vs. Control site in the same posture. L-NIO and L-NAME were not different.

Discussion

This study investigated the effect of posture on the NO component of CAVD during passive whole-body heat loading. The main findings of the current study are 1) the NO component of CAVD during thermal loading in the seated and supine positions is eNOS rather than nNOS mediated and 2) differences in posture did not change the overall skin blood flow response or the relative contribution of NO to reflex cutaneous vasodilation. The finding that eNOS mediates the NO component of CAVD during thermal loading in the seated positions is contrary to our hypothesis which supposed the nNOS isozyme mediated the NO component of CAVD during passive whole-body heat loading. The finding that posture did not affect the overall skin blood flow response is also contrary to our hypothesis which supposed that the skin blood flow response in the seated position would be blunted when compared to the supine position. These suppositions were based on the observations that pharmacological blockade of nNOS (117, 118, 120) attenuated the entire NO component of CAVD and that orthostasis results in a rightward shift in the T_c-skin blood flow relationship via active vasodilator withdrawal during hyperthermia (111).

nNOS vs. eNOS

Nitric oxide (NO) has been shown to contribute approximately 30% to CAVD in young healthy individuals during supine passive whole-body heating (79, 117, 119, 148, 188, 189). Previous research suggested that neuronal nitric oxide (nNOS) is the primary NOS isozyme that contributes to the NO component of CAVD during supine whole body heat stress (117). These findings, in conjunction with other work by Kellogg et al., determined that nNOS contributes to the NO component of CAVD and endothelial nitric

oxide synthase (eNOS) contributes to the NO component of vasodilation during local heating (117, 118, 120). A study conducted by McNamara et al. indicated that the eNOS rather than the nNOS isozyme contributes to CAVD during sustained dynamic exercise (148). More recent work by Fujii et al. corroborated the findings by McNamara et al (55). In their study, intradermal administration of the eNOS inhibitor, LNAA, attenuated CVC in young healthy adults during two 30-minute bouts of cycling in the heat. McNamara et al., suggested that these differences in NOS isozyme contribution could be due to variable active vasodilator nerve activation (secondary to different afferent input) or due to increases in circumferential strain, which has been associated with eNOS activation (148). While these findings currently reflect differences between exercise and passive heating, it is possible that differences in the afferent inputs due to skin temperature or posture (e.g. orthostasis), rather than exercise, may alter the mechanisms of reflex skin vasodilation.

In the present study, no differences in the skin blood flow responses during passive whole-body heat loading were found when posture was varied (**Figure 4.5, 4.6, and 4.7**). Furthermore, our data suggest NO directly contributes approximately 60% to the increase in skin blood flow during whole body passive heating regardless of posture, which is higher than what most other studies that have shown (i.e., 30-45%) (79, 117, 119, 148, 188, 189) but is within previously reported ranges (22). In conflict with the findings of others (117, 118, 120, 148), our data suggest that eNOS rather than nNOS is the primary isoform involved in reflex cutaneous vasodilation during passive heating (**Figure 4.7**). There are at least two possible explanations for these conflicting findings.

First, it is possible that NO generation in the cutaneous vasculature during thermal stress involves both nNOS and eNOS isoforms. It is possible that nNOS is a minor contributor to the initial cutaneous vasodilation whereas eNOS is the primary NO mediator and is responsible for sustained cutaneous vasodilation. In support of this, ACh has been shown to contribute to the onset of cutaneous vasodilation but is not the primary mediator of cutaneous vasodilation (114, 175, 176). This is notable because the nNOS isozyme is found at nerve terminals and is involved in the modulation of ACh release from cholinergic neurons (173). While not reported and not analyzed statistically, the present study observed what appears to be a roughly 3-5 minute delay in the skin blood flow rise at nNOS blocked sites relative to control sites as a function of time into heating. This is illustrated in **Figure 4.8** which shows %CVCmax as a function of time post start of thermal loading in the seated position. Results for the supine position showed a similar delay. Therefore, it is possible that nNOS activation contributes to the initial rise in skin blood flow via modulation of ACh release from active vasodilator nerves.

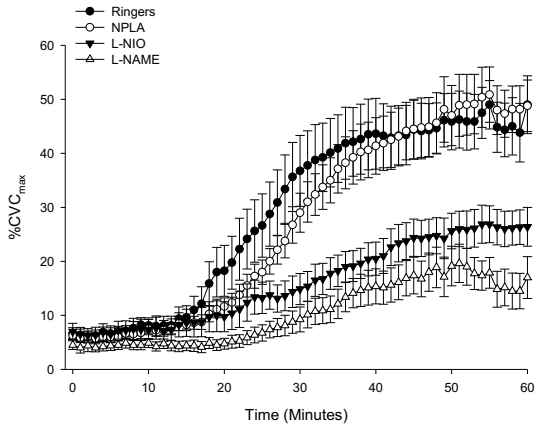

Figure 4.8.The increase in percentage of maximal cutaneous vascular conductance (%CVCmax) at Control (Ringers; filled circles), nNOS blockade (NPLA; open circles), eNOS blockade (L-NIO; closed inverted triangles), and nonspecific NOS blockade (L-NAME; open triangles) sites is shown over time into passive whole-body heat loading in the seated position (N=8). Data are mean ± S.E.

How eNOS mediates sustained cutaneous vasodilation in this paradigm is less clear. One possibility is that eNOS could be directly activated by temperature increases from blood; however, studies with isolated NOS isoforms have shown that eNOS activity is unaltered by physiological (5-45°C) temperatures alone (212). Another possibility is that temperature is mediating processes secondary to eNOS activation. For example, temperature-dependent ATP release from human erythrocytes has been previously demonstrated (103); and ATP is a modest endothelial-dependent vasodilator that is capable of activating eNOS (40, 209). Transient potential villanoid receptor 4 channels (TRPV4) found on the endothelium can also activate eNOS through a temperature-dependent mechanism (67, 195). Because heat is convectively carried to the periphery by blood, it is conceivable that ATP release from erythrocytes and temperature-dependent TRPV4 activation may represent primary mediators of initial and sustained eNOS activation during heat stress. This activation may then be modulated with other stimuli such as the increased blood flow itself (and presumably shear stress) (138) although shear stress does not appear to result in NO-dependent vasodilation in the skin (226).

Second, it is possible that use of pharmacological agents in the present study, and other studies conducted in humans have led to misinterpretations of the underlying physiological processes involved in CAVD. Studies in humans are limited to using pharmacological agents that can be safely administered, and more specific blockers are often times unsuitable for humans. For example, diphenyleneiodonium chloride is a potent eNOS inhibitor (half maximal inhibitory concentration (IC_{50}) = 180 nM) when compared to L-NIO (IC_{50} = 500 nM) but has only been used in rats and is not FDA approved for human use due to its carcinogenic and teratogenic properties.

Additionally, we are not able to determine exactly which pathways are being affected by our interventions. Most NOS antagonists effect all the NOS isozymes to a varying degree and their relative specificity can be heavily dose specific. Because these drugs are being administered intradermally via microdialysis, it is not possible to determine the exact concentration of drug once it reaches the interstitium. This is further cofounded by the over generalization of drug specificity claims by drug manufacturers. Some vendors advertise L-NIO as an eNOS specific antagonist while others advertise it as a nonspecific NOS antagonist. This is best illustrated by the manufacturer SigmaAldrich, which changed their L-NIO description from eNOS specific to NOS nonspecific midway through the present study, then changed the description back to eNOS specific during the writing of this dissertation. SigmaAldrich currently advertises L-NIO as 5 times more potent as an inhibitor of eNOS than L-NAME despite them both having an IC_{50} = 500nM. In an attempt to circumnavigate these issues during pilot work, we used L-NIO and NPLA purchased from SigmaAldrich, Cayman Chemical, and TOCRIS; however, we observed no differences in the skin blood flow responses by drug vendor.

It should be noted the present study utilized the same drugs and concentrations as used by McNamara et al. (148) and found the eNOS isozyme is the primary mediator of the NO component of CAVD during passive whole-body heating, which is in agreement with what they found during exercise but is in conflict with what they found during passive whole-body heating.

Conclusion, Perspectives, and Bridge

In this study, we found that eNOS rather than nNOS, is the primary NOS isoform responsible for the NO component of CAVD during passive whole-body thermal loading,

regardless of posture. This finding is in contrast to previous data demonstrating nNOS the primary mediator of the NO component of CAVD during passive whole body heat stress. While still unclear, we believe that nNOS may modulate the initial rise in skin blood flow and eNOS may be the primary contributor to sustained NO-mediated vasodilation during passive whole body heat stress. We submit that the differences in the present study may be due to pharmacological interactions unknown to the researcher. What is clear however, is the need to open a fieldwide dialogue about the use of pharmacological agents in the determination of mechanistic pathways *in vivo*, their interpretation, and the re-evaluation of what we know about the CAVD system.

While we sought to determine if posture could explain mechanistic differences in the NO component of CAVD between exercise and passive whole body heating in the present study, our understanding of the CAVD system during exercise and passive heating is murky at best. In the next chapter, we sought to confirm the cholinergic co-transmitter theory of CAVD during whole body passive heating and determine if exercise alters cholinergic co-transmission behavior or results in the recruitment of different nerves entirely.

CHAPTER V

THERMAL LOADING MODALITIES AND CUTANEOUS ACTIVE VASODILATOR NERVE ACTIVATION

Introduction

The cutaneous vasculature plays a key role in thermal balance. Skin blood flow (SkBF) modulates convective heat transfer between the body core and the skin surface. At rest, the skin receives approximately 5-10% of cardiac output; yet when maximally dilated, such as during whole body heating, the skin is estimated to receive up to approximately 6-8 L/min, accounting for 50-70% of cardiac output (98, 154, 178, 179, 181). Cutaneous vasomotor adjustments in non-glabrous skin are modulated by noradrenergic and cholinergic sympathetic nerves. These nerves innervate arterioles that feed papillary loops in the cutaneous superficial plexus and are critical to heat dissipation or retention; papillary loops provide a large surface area and also demarcate the thermal interface between the human body and the environment (15).

During hyperthermic challenge, the initial rise in internal temperature causes an increase in skin blood flow that is mediated by noradrenergic vasoconstrictor withdrawal (46, 175). As internal temperature continues to rise, further increases in skin blood flow are mediated by the engagement of the cholinergic vasodilator nerves. This is known as cutaneous active vasodilation (CAVD). CAVD has been the focus of much research and is responsible for approximately 80-95% of the rise in skin blood flow that accompanies heat stress (98, 180).

Previous work by Roddie et al. was the first to show that cutaneous vasodilation in response to heat stress contains a cholinergic component (175, 176). Infusion of intra-arterial atropine, a muscarinic antagonist, abolished sweating but only delayed the onset of cutaneous vasodilation and/or attenuated the magnitude of dilation, depending on the timing of atropine administration. These authors concluded that sudomotor activity is almost entirely mediated by cholinergic mechanisms whereas acetylcholine (ACh) contributes but is not the primary mediator of CAVD.

A seminal study by Kellogg et al. expanded the findings of Roddie et al. and established the co-transmitter theory of CAVD (114). In this study, intradermal injection of botulinum toxin A (BOTOX®, which blocks presynaptic cholinergic neurotransmitter release) as well as administration of atropine via iontophoresis during passive whole-body heat loading in six individuals led to two key findings. First, atropine administration abolished sweating and delayed the onset of cutaneous vasodilation but did not attenuate it. This confirmed the contribution of ACh to sweating and a minor contribution to cutaneous vasodilation. Second, BOTOX® abolished both sweating and cutaneous active vasodilation during whole body heat stress, but not vasodilation to directly applied heat. This suggested the release of an unknown neurotransmitter(s) from the cholinergic nerve terminals is the primary mediator of CAVD.

Immunohistochemical detection studies have identified vasoactive intestinal peptide (VIP) (7, 183, 217, 220) and calcitonin gene related peptide in active vasodilator nerves (71, 187, 210); and Substance P (183, 224) and histamine (227) have been found in dermal sensory nerves, endothelial cells, and/or mast cells (70, 87, 152). The

localization of these substances in the dermis make them likely co-transmitter(s) however, definitive proof of co-transmission during hyperthermic challenge is lacking.

Identification of the unknown neurotransmitters is further confounded by the observation that different thermal loading modalities result in different mechanisms of NO mediated cutaneous vasodilation. Specifically, blockade of endothelial nitric oxide synthase (eNOS) was found to block the NO component of CAVD during exercise heat loading whereas neuronal nitric oxide synthase (nNOS) blockade was found to block the NO component of CAVD during passive whole-body heat loading (117, 148). These findings demonstrate variable roles for eNOS and nNOS that are dependent on the thermal loading modality employed. It was suggested that these differences could be due to differential active vasodilator nerve firing rates (secondary to different afferent input) resulting in the release of vasoactive substances unique to the mode of thermal loading. These findings highlight the possibility that CAVD nerve transduction may vary depending on the source of the heat stress (exercise heat loading vs. passive whole-body heat loading). This is problematic because it had previously been assumed that the mechanisms and transducers of CAVD were the same regardless of how thermal loading was accomplished. It is possible that differences in thermal loading modalities and the accompanying inputs that modulate active vasodilator outflow (e.g. motor commands, skin temperature, metaboreflexes etc.), may alter cholinergic co-transmission behavior (142) or result in the recruitment of different nerves entirely.

There has been no further research on vasodilator nerve activation in approximately the last 10 years and the current model of the cholinergic co-transmission theory of CAVD is largely based on a singular study that is now 25 years old. The

findings by Kellogg et al. have not been independently corroborated and the current model of co-transmission is based on observations in six individuals (114). Second, it is unknown if the cholinergic co-transmitter theory of CAVD applies to other thermal loading modalities such as exercise heat loading. It is conceivable that exercise may alter cholinergic nerve transduction or achieve cutaneous vasodilation by an entirely different mechanism. Therefore, this study sought to confirm the cholinergic co-transmitter theory of CAVD during passive whole-body heat loading and expand these findings to exercise heat loading using a cross-over study design. It was hypothesized that the same cholinergic nerves are responsible for CAVD during exercise and passive whole-body heat loading.

Secondarily, this study sought to identify the vasoactive substances co-transmitted with ACh from active vasodilator nerves in the skin using shotgun proteomics. By taking an agnostic approach to determine potential neurotransmitter(s) involved in CAVD, we hoped to not only identify the unknown co-transmitter(s) but to also determine if different thermal loading modalities could result in the variable co-transmission of these substances.

Methods

All protocols in human subjects were approved by the Institutional Review Board at the University of Oregon (IRB #12102010.027, see Appendix page (128) for informed consent documents). All subjects gave oral and written consent prior to participation in accordance with the *Declaration of Helsinki*.

Subjects

Eight young (18-30 years) recreationally-active (<150 minutes of intense exercise a week) individuals volunteered to participate in this study. All subjects were non-smokers with no history of cardiovascular, metabolic, or other chronic diseases, not taking prescription medications with the exception of hormonal contraceptives, not currently pregnant or breast-feeding, and had no history of allergic reactions to any drugs used in this study. A summary of physical characteristics is listed in **Table 5.1**.

Aerobic Fitness Testing

Upon enrollment in the study and prior to thermal loading study days, each subject reported to the Evonuk Environmental Physiology Core in Esslinger Hall on the University of Oregon campus to complete a VO_{2peak} test. VO_{2peak} was assessed using a graded cycling protocol on a stationary bicycle ergometer (Excalibur Sport, Lode, Groningen, Netherlands). The graded cycling protocol involved a 5-minute warm-up at 100 watts followed by 20 or 25 watt (Female and Male increases, respectively) increases in work load every minute until volitional fatigue. Subjects were attached to a breath-by-breath metabolic measurement system (Parvomedics, Sandy, UT) to measure oxygen uptake. Heart rate was monitored via telemetry (Model RS400, Polar Electro, Lake Success, NY) and ratings of perceived exertion were noted at every work stage. VO_{2peak} was then validated by having subjects cycle at a supramaximal workload (115% VO_{2peak}) to ensure their VO_2 did not rise above their previously determined plateau.

Cholinergic Nerve Blockade

Cholinergic nerve blockade was achieved through intradermal botulinum toxin type A (BOTOX®) administration. After VO_{2peak} testing and prior to thermal loading

study days, subjects received three intradermal injections of approximately 5 units of BOTOX® into the volar forearm (approximately 2 mm from the surface), with injection sites no farther than approximately 1.5 cm away from each other. The "ring of effect" was identified by the primary investigator and drawn onto the skin surface using a permanent marker and photographs were taken to aid in re-identification of BOTOX® treated sites on thermal loading study days. Subjects were instructed to re-mark the "ring of effect" each day after showering or bathing and waited a period of at least 1 week after BOTOX® administration before participating in thermal loading study days.

General Study Design and Setup

Each subject participated in both an exercise heat loading study day and a passive whole-body heat loading study day. Thermal loading study sequences were randomized and a period of at least 1 week separated the two thermal loading studies. All female subjects had a negative pregnancy test on the morning of each study day. Women were studied during the low hormone phase of their menstrual cycle. Women taking hormonal contraceptives (N=4) were studied during the placebo phase and those naturally menstruating (N=1) were studied during the early follicular phase of menstruation (self-reported). In order to study females under consistent hormone levels, females returned one full cycle later (i.e. 3-4 weeks apart) for their second study day.

Prior to thermal loading study days, subjects abstained from food for 4 hours, alcohol and caffeine for 12 hours, and exercise and over-the-counter medications for 24 hours. On thermal loading study days, subjects reported to the Human Cardiovascular Control Laboratory in Esslinger Hall on the University of Oregon campus. Upon arrival, subjects provided a urine sample to confirm euhydration via urine specific gravity <1.02

(Analog Regractometer; Atago, Tokyo, Japan). If urine specific gravity was >1.02, subjects drank 5 ml/kg of water prior to study. Dry nude body weight was measured before and after thermal loading to calculate mean whole body sweat rate. Subjects were instrumented with a sterile rectal thermistor probe (YSI Series 400; Yellow Spring Instruments, Yellow Springs, OH) inserted approximately 10 cm past the anal sphincter to monitor internal temperature (T_c).

Skin surface thermistors (MSR145WD, MSR Electronics GmBH, Seuzach, Switzerland) were applied to standard locations on the chest, forearm, calf, and quadricep to continuously measure skin temperatures. Mean skin temperature (T_{sk}) was calculated using the 4-site weighting method previously developed by Ramanathan (172):

$$T_{sk} = 0.3(t_{chest} + t_{arm}) + 0.2(t_{thigh} + t_{calf}) \qquad (1)$$

Where

t_{chest} = skin temperature measured at the chest,

t_{arm} = skin temperature measured at the forearm,

t_{thigh} = skin temperature measured at the quadricep, and

t_{calf} = skin temperature measured at the calf.

Mean body temperature (T_b) was calculated using the internal temperature and mean skin temperature weighting method described by Gagge and Gonzalez (57):

$$T_b = 0.8(T_c) + 0.2(T_{sk}) \qquad (2)$$

Heart rate and ventilatory frequency were monitored using a 3-lead electrocardiogram (CardioCap; Datex Ohmeda, Louisville, CO). Baseline blood pressure

was measured in triplicate following 20 minutes of rest and every 5-10 minutes throughout the thermal loading via brachial oscillation (Datascope Accutorr Plus, Duluth, GA) or auscultation as described below.

Exercise Heat Loading

Exercise heat loading was accomplished by having subjects exercise in the seated position on a stationary bicycle ergometer (Excalibur Sport, Lode) at a constant workload that corresponded to 60% of their VO_{2peak} for 60 minutes. This relative workload was based on McNamara et al. (148). During the first approximate 10 minutes of the 60-minute exercise interval, subjects were attached to a breath-by-breath metabolic measurement system (Parvomedics, Sandy, UT) to ensure they were exercising at 60% if their VO_{2peak}. Pulmonary gas exchange was then "spot" checked approximately every 20 minutes for the duration of exercise. During exercise heat loading study days, blood pressure in the brachial artery was measured using the auscultatory method (mercury sphygmomanometer and stethoscope) due to inaccuracy of oscillometry during physical activity (164).

Passive Whole-body Heat Loading

Passive whole-body heat loading was accomplished by having subjects wear a two-piece nylon water-perfused suit (Med-Eng Systems, Ottawa, Canada) which covered all skin surfaces except the experimental arm, face, hands, and feet. In addition, subjects wore a water-impermeable plastic garment over the suit to mitigate evaporative heat loss. During thermal loading, mean skin temperature was maintained at approximately 39°C for 60 minutes using a heated circulating water bath (HT CIRC 7L AD, Polyscience, Niles, Illinois) to perfuse hot water (50°C) through the suit. This water temperature was

based on previous work from our lab which demonstrated an increase in internal temperature of 0.7-1.0 °C above baseline in approximately 60 minutes (22, 218, 224). During passive whole-body heat loading studies, blood pressure in the brachial artery was measured using the osillometric method.

Dialysate Recovery

During thermal loading study days, intradermal microdialysis was used to sample the interstitial space at baseline and throughout thermal loading. Placement of microdialysis fibers in the volar dermis of the forearm was performed using aseptic technique. Two linear microdialysis fibers (CMA 31 linear microdialysis, 10 mm membrane, 55Kda or 2000Kda CO; Harvard Bioscience, Holliston, MA) were placed in parallel at the BOTOX® treated site and two more fibers were similarly placed at an untreated site. Fibers were placed by first introducing a 25-gauge or 23-gauge needle approximately 1 mm below the surface of the skin, with entry and exit points 2-3 cm apart. Fibers were threaded through the lumen of the needle and the needle was removed leaving the dialysis membrane of the fiber centered between the needle entry and exit points. A period of 90 minutes was allowed to let needle insertion trauma and the resulting transient rise in skin blood flow resolve. This was confirmed by observing a plateau in laser-Doppler (described below) flux values. Fibers were continuously perfused with Lactated Ringer's solution at a rate of 2.0 ul/min (CMA 102 Syringe Pump; CMA Microdialysis AB, Solna, Sweden). A peristaltic pump (Reglo ICC Digital Peristaltic Pump, IMSATEC, Wertheim, Germany) was attached to the distal "receiving" end of the fibers to reduce the build-up of back pressure and prevent ultrafiltration and improve analyte recovery efficiency (**Chapter III, Figure 3.9**). Baseline dialysate from

the BOTOX® treated and untreated site was collected in 0.5 ml sterile cryotubes for a period of approximately 30 minutes. Dialysate at BOTOX® treated and untreated sites was then collected for the entire duration (approximately 60 minutes) of thermal loading. All samples were immediately frozen at -80 °C for future analysis.

Skin Blood Flow

Skin blood flow at the BOTOX® treated and untreated sites was quantified using laser-Doppler flowmetry. Local heating units (SH02 Skin Heater/Temperature Monitor; Moor Instruments, Axminster, UK) were placed on the skin surface directly above the previously placed microdialysis fibers and integrated blunt needle laser-Doppler probes (MP12-V2, Moor Instruments) were seated in the local heating units. Laser-Doppler flux was continuously recorded at baseline and throughout the study. After either exercise heat loading or passive whole-body heat loading, the BOTOX® treated and untreated sites were locally heated to 39°C at a rate of 0.1°C/sec. Local heating results in a biphasic vasodilator response consisting of an initial peak and secondary plateau that is independent of cholinergic/noradrenergic nerve activation. This was done to confirm the preservation of each site's local vasodilator response. Maximal laser-Doppler flux at each site was then obtained by locally heating the skin to 44°C as well as infusing 56mM sodium nitroprusside.

Data Analysis

Skin red blood cell flux (RBCflux) data were digitized and recorded to a computer using data acquisition software (Windaq; Dataq Instruments, Akron, OH). The RBCflux measurements within each site during the 2-minute baseline period were averaged. Starting at the beginning of thermal loading, RBCflux measurements were

averaged over each sequential one-minute interval during the approximate 60-minute thermal loading period. The RBCflux average values were converted to cutaneous vascular conductance (CVC) and expressed as a percent of maximum CVC (%CVCmax). %CVCmax was derived as follows:

$$CVC = RBCflux / MAP$$

$$\%CVCmax = [CVC / CVCmax] \times 100 ,$$

where MAP is mean arterial pressure and was calculated as MAP = $\frac{1}{3}$(systolic blood pressure) + $\frac{2}{3}$(diastolic blood pressure).

Skin blood flow responses at each site associated with increases in internal temperature were characterized using the %CVCmax minute average immediately after each 0.1°C increase in internal temperature. Similarly, responses at each site associated with 0.1 °C increases in mean body temperature were characterized using the %CVCmax minute average immediately after the 0.1 °C increase in mean body temperature.

Statistics

Statistical analyses of hemodynamic and temperature data were conducted using SigmaPlot 11.0 (Systat Software, San Jose, CA). Because there were no discernable differences between men and women, data from the two groups were combined for analysis. Hemodynamic and temperature data before and at the end of thermal loading were compared using a 2-way repeated measures analysis of variance (Intervention vs. Time).

Statistical analyses of skin blood flow data were conducted using SAS 9.4 (SAS, Cary, NC). %CVCmax as a function of temperature increase during exercise heat

loading and passive whole-body heat loading were compared using a correlated errors model at the maximum observed temperature increase common to both heat modalities. The analysis included treatment sequence, thermal loading modality (passive heating, exercise), study period (1, 2), site on the forearm (BOTOX®, Control), and heat modality-by-site interaction as fixed effects. Within-subject correlation between forearm sites was modeled using an unstructured covariance matrix.

Within each heat modality, %CVCmax (observed values and change from baseline) as a function of internal temperature increase was analyzed using a longitudinal repeated measures model. The experimental unit for this analysis was subject, and there were two within-subject effects for each subject: site and internal temperature increase. The model included site on the forearm (BOTOX® vs. Control), temperature increase (increments of 0.1 °C), and site-by-temperature increase interaction as fixed effects. Temperature-increase correlations for the two sites were modeled using a first-order autoregressive covariance structure [AR(1)].

To determine the smallest internal temperature increase at which a difference between BOTOX® and Control sites was demonstrated for each heat modality, a sequential testing procedure that started at the maximum observed temperature increase (i.e., 0.7 °C for internal temperature increase) and compared BOTOX® to Control at incrementally smaller temperature increases was conducted. Sequential testing proceeded until a failure to reject the null hypothesis of no difference between the BOTOX® and Control sites occurred at the 2-sided 5% level of significance. No significant differences were claimed after the first failure to reject the null hypothesis occurred.

A similar sequential testing procedure was followed to determine the smallest internal temperature increase at which a %CVCmax change from baseline was demonstrated at the BOTOX® and Control sites for each heat modality. The sequential testing procedure within each site within heat modality compared the %CVCmax results starting at 0.7 °C and compared %CVCmax to baseline (two-sided paired t-test) at incrementally smaller temperature increases.

Dialysate Analysis

The Proteomic Shared Resource Facility at Oregon Health Sciences University was retained to run a shotgun proteomics analysis on dialysate samples. Due to the COVID-19 pandemic (2020), analysis of the samples sent to the proteomics core laboratory has been delayed. However, the proteomics core laboratory provided a description of the workflow they are using to analyze the samples obtained. The workflow is as follows. The skin dialysate samples are ultra-filtrated using 10 kDa cutoff Amicon Ultra 0.5 ml ultrafiltration spin cartridges (Cat #UFC501024, Millipore-Sigma) to remove higher molecular weight proteins. Since the amount of recovered protein is below the limit of accurate detection by a Pierce BCA protein assay (< 0.1 µg/µl), the filtrate is divided into two equal portions, dried by vacuum centrifugation and the entirety of each sample subsequently analyzed. One portion is analyzed by mass spectrometry without digestion, and the other portion is reduced using DTT, alkylated with iodoacetamide, and digested overnight with trypsin (0.9 µg per sample) in the presence of ProteaseMax™ detergent, as recommended by the manufacturer (ProMega Corporation). Following trypsin digestion, samples are filtered (0.22 um) and again dried by vacuum centrifugation.

Both dried trypsin digested and non-digested samples are dissolved in 22 µl of 5% formic acid and each analyzed by liquid chromatography-mass spectrometry (LC-MS). 20 µl of each sample is auto-injected and peptides separated using a Dionex NCS-3500RS UltiMate RSLCnano UPLC system, and mass spectrometric analysis is performed using an Orbitrap Fusion Tribrid instrument with an EasySpray nano source (Thermo Scientific). Peptides are desalted using an Acclaim PepMap 100 µm x 2 cm NanoViper C18, 5 µm trap column on a switching valve. After 5 min of loading and washing, the trap column is switched on-line to a PepMap RSLC C18, 2 µm, 75 µm x 25 cm EasySpray column (Thermo Scientific). Peptides are then separated using a 7.5–30% acetonitrile gradient over 60 min in a mobile phase containing 0.1% formic acid at a 300 nl/min flow rate. Survey MS scans are collected using the instrument's Orbitrap mass analyzer at a resolution of 120,000. Data-dependent MS/MS spectra are collected in the instrument's ion trap using higher-energy dissociation (HCD) following quadrupole isolation. The dynamic exclusion feature of the instrument and its top speed mode are used to increase the number of unique peptides that are identified.

RAW instrument files are processed using Proteome Discoverer version 1.4 (Thermo Scientific), SEQUEST HT software and a UniProt human database (Swiss Bioinformatics Institute, Geneva, Switzerland). For trypsin digested samples, searches are configured with a static modification for alkylated cysteines (+57.021 Da), variable modifications for oxidation of methionine (+15.995 M residues), and trypsin specificity. Undigested samples are similarly searched, except without specifying cysteine alkylation and using no enzyme specification. A parent ion tolerance of 10 ppm, fragment ion tolerance of 1.0 Da, monoisotopic masses, and for trypsin cleaved samples, a maximum

of 2 missed cleavages is allowed. Searches use a reversed sequence decoy strategy to control peptide false discovery using Percolator software (48, 102). Results are filtered to remove peptide identifications with q-scores > 0.05, to maintain false discovery rates below 5%. Differences in protein abundances across samples are estimated by comparison of numbers of assigned MS/MS spectra to each protein (135).

Results

Subject Demographics and Oxygen Uptake

Subject demographics and exercise data are presented as means ± S.E. in **Table 5.1**. Five women and three men participated in this study. Subjects were 22 ± 1 years old, with a mean height and weight of 173 ± 4 cm and 66 ± 2 kg, respectively. Subjects' average relative oxygen uptake was 43.11 ± 4.13 $ml^{-1}kg^{-1}min$ and peak power achieved during the VO_{2peak} test averaged 246 ± 27 W. Subjects' power during exercise heat loading averaged 140 ± 24 W.

Table 5.1. Demographics of subjects who completed Protocol 2. Data are mean ± S.E.

	Protocol 2 (N=8)
# of women	5
Age (years)	22 ± 1
Height (cm)	173 ± 4
Weight (kg)	66 ± 2
BMI (kg/m^2)	22.7 ± 0.6
VO_{2Peak} (ml/kg/min)	43.11 ± 4.13
VO_{2Peak} peak power (W)	246 ± 27
Power during exercise (W)	140 ± 24

cm, centimeters; kg, kilograms; BMI, body mass index; S.E., standard error VO_{2Peak}, maximal oxygen consumption; W, watts.

Hemodynamics and Thermal Loading

Mean hemodynamic results for both thermal loading modalities are presented in **Table 5.2**. Baseline heart rate, systolic blood pressure, diastolic blood pressure, mean arterial pressure, and pulse pressure were similar between thermal loading modalities. Baseline internal temperature was also not different between thermal loading modalities (**Figure 5.1**). However, baseline mean body temperature (T_b) was significantly higher for passive whole-body heat loading than for exercise heat loading ($P = 0.004$; 36.2 ± 0.1 vs. 35.6 ± 0.1 C°, respectively). By the end of both thermal loading modalities, heart rate was elevated relative to baseline ($P < 0.050$) but there was a greater rise during exercise heat loading than during passive whole-body heating ($P < 0.050$; 146 ± 4 vs. 100 ± 6 beats min^{-1}, respectively). Pulse pressure was elevated relative to baseline ($P < 0.050$) at the end of both thermal loading modalities but was also greater at the end of exercise than the end of passive whole-body heating ($P < 0.050$; 99 ± 5 vs. 63 ± 3 mmHg, respectively). Mean body temperature increased relative to baseline ($P < 0.050$) during both thermal loading modalities but increased to a greater extent during passive whole-body heating than during exercise ($P < 0.05$; 38.0 ± 0.1 vs. 36.4 ± 0.2 C°, respectively). Internal temperature increased more rapidly during exercise but was not significantly different ($P = 0.167$) from passive whole-body heating by the end of thermal loading (**Figure 5.1**; 37.8 ± 0.1 vs. 38.0 ± 0.1, respectively).

Table 5.2. Mean hemodynamic and mean body temperature at baseline and end of the thermal loading periods (N=8). Data are mean ± S.E.

	HR (beats min^{-1})	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	PP (mmHg)	T_b (C°)
Exercise						
Baseline	62 ± 3	115 ± 3	63 ± 4	80 ± 2	52 ± 4	35.6 ± 0.1$^{\#}$
Thermal loading	146 ± 4*$^{\#}$	153 ± 3*$^{\#}$	53 ± 3*$^{\#}$	86 ± 2*	99 ± 5*$^{\#}$	36.4 ± 0.2*$^{\#}$
Passive						
Baseline	59 ± 2	117 ± 2	66 ± 2	79 ± 4	51 ± 1	36.1 ± 0.1
Thermal loading	100 ± 6*	132 ± 3*	62 ± 2	89 ± 2*	63 ± 3*	38.0 ± 0.1*

HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure; T_b, mean body temperature; S.E., standard error. *P < 0.050 vs. baseline. $^{\#}$P < 0.050 vs. passive whole-body heat loading at the same time point.

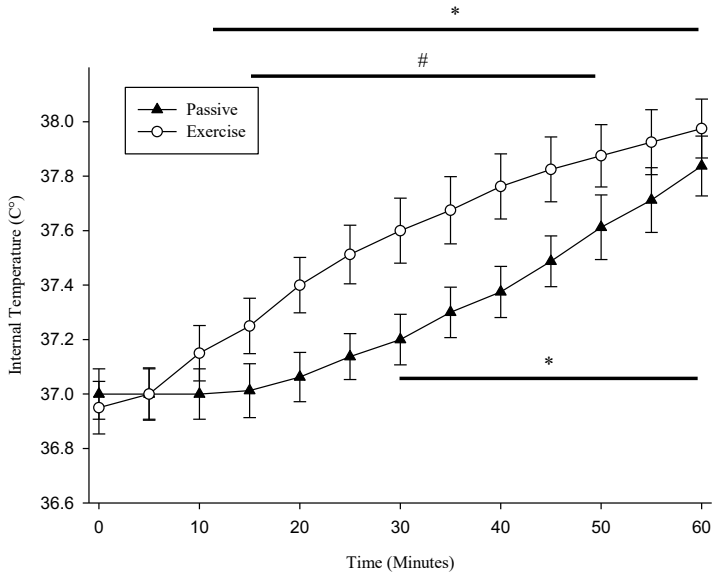

Figure 5.1. Internal temperature during 60 minutes of exercise (open circles; N=8) and passive whole-body heating (closed triangles; N=8). Data are mean ± S.E. *P < 0.050 vs. baseline (time 0). #P < 0.050 vs. passive whole-body heating at the same time point.

Skin Blood Flow

%CVCmax increase as a function of increasing internal temperature from baseline to the end of both thermal loading modalities in 0.1 °C increments is presented below in **Figure 5.2**. There were no differences in %CVCmax between sites at baseline (Exercise; BOTOX®: 6 ± 1 vs. Control: 6 ± 1 %CVCmax, Passive whole-body heating; BOTOX®:

11 ± 3 vs. Control: 9 ± 2 %CVCmax). %CVCmax was significantly (P<0.001) elevated at Control sites by an internal temperature increase of 0.1 °C for exercise heating and by 0.2 °C for passive whole body heating when compared to baseline (Exercise: 14 ± 1 at 0.1 °C, Passive whole-body heating: 21 ± 6 %CVCmax at °C) and continued to increase throughout thermal loading.

In contrast, %CVCmax remained relatively constant at BOTOX® treated sites throughout both thermal loading modalities. During exercise, %CVCmax at the BOTOX® site was significantly greater than baseline by a 0.1 C° increase in internal temperature ($P < 0.050$) but remained relatively constant thereafter. During passive whole-body heating, %CVCmax at the BOTOX® site showed no increase relative to baseline at any time point ($P > 0.050$).

Differences in %CVCmax at the Control sites and their adjacent BOTOX® sites occurred beginning at a 0.4 °C increase in internal temperature during both exercise ($P < 0.050$ 0.032; 21 ± 5 vs. 13 ± 4 %, respectively) and passive whole-body heating ($P < 0.050$; 33 ± 5 vs. 15 ± 3, respectively).

Detection of a difference between BOTOX® and Control for passive whole-body heating most likely delayed until the 0.4 °C increase in internal temperature due primarily to one subject. Subject 21 appeared to have an attenuated but not blocked skin blood flow response to passive whole body heating at the BOTOX® treated site during the second study day. This may have been due to inconsistent remarking of the "ring" of effect by the subject, which may have resulted in skin blood flow being assessed at the boundary of the "ring" of effect rather than in the middle of the BOTOX® treated skin. When Subject 21 was removed from the passive whole body heating %CVCmax

analysis, differences between the BOTOX® and Control sites were detected beginning at a 0.2 °C internal temperature increase (P<0.050).

Cholinergic nerve blockade attenuated the skin blood flow response to a 0.7 °C increase in internal temperature for both thermal loading modalities (**Figure 5.3**). At the end of thermal loading, there were no differences between thermal loading modalities (modality, P = 0.909; modality-by-microdialysis site interaction, P=0.230). %CVCmax at the BOTOX® treated sites (Exercise: 19 ± 2 and Passive whole-body heating: 15 ± 5 %CVCmax), was significantly less (P<0.001) than at the control sites (Exercise: 35 ± 4 and Passive whole-body heating: 38 ± 2 %CVCmax).

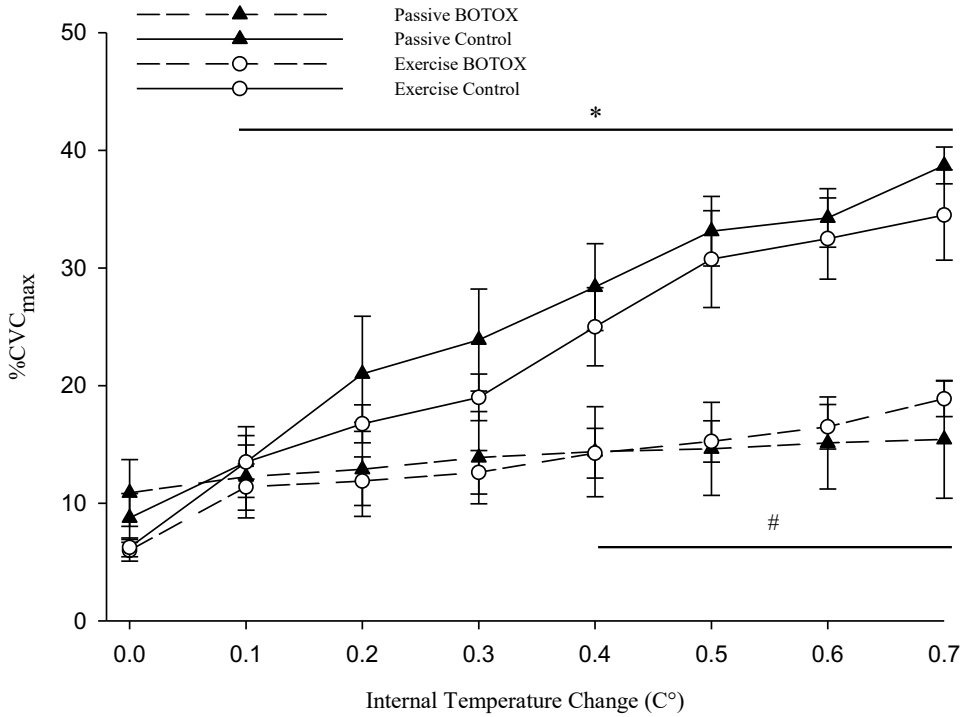

Figure 5.2. The percentage of maximal cutaneous vascular conductance (%CVCmax) at BOTOX® treated (dotted lines) and untreated (solid lines) is shown for each 0.1 °C increase in core temperature from rest during exercise (open circles; N=8) and passive whole-body (closed triangles; N=8)) heat loading. Data are mean ± S.E. *P < 0.05 vs. baseline for both Exercise and Passive Control sites and for the Exercise BOTOX site. The Passive BOTOX site was not significantly different from baseline at any time point. #P < 0.05 for BOTOX® vs. Control within heat modality.

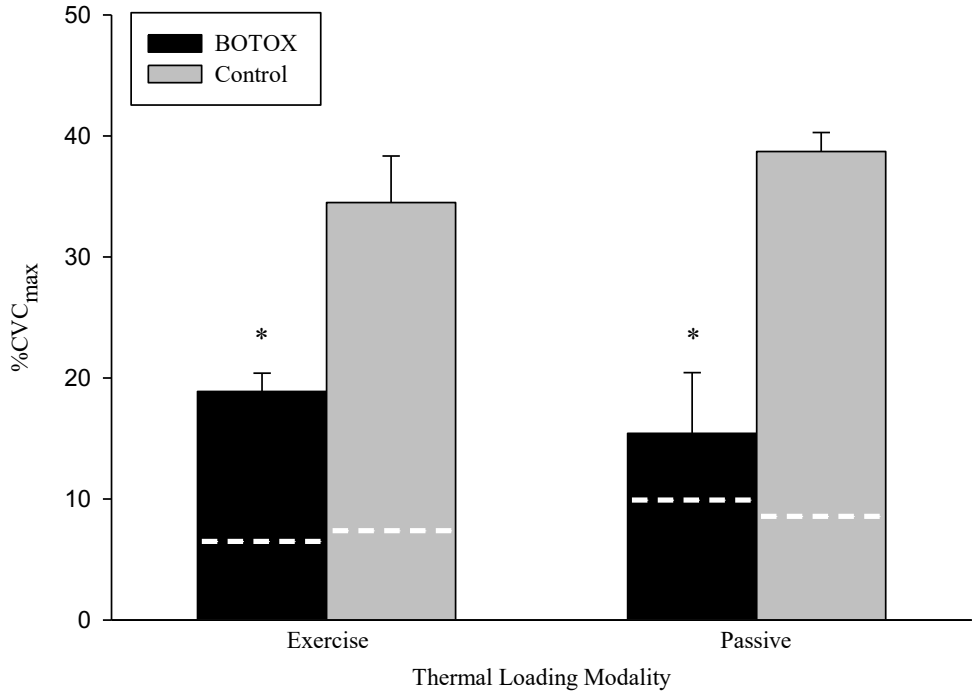

Figure 5.3. Percentage of maximal cutaneous vascular conductance (%CVCmax) at BOTOX® treated (black bars) and untreated (gray bars) sites in response to a 0.7 °C increase in internal temperature during exercise (N=8) and passive whole-body heat loading (N=8). Dotted white lines represent mean site baselines. Data are mean ± S.E. There were no differences by thermal loading modality. *P < 0.05 vs. control site in the same thermal loading modality.

Dialysate Proteomics

Prior to the Proteomic Shared Resource Facility at Oregon Health Sciences University, samples from one pilot subject who underwent whole-body heating (dialsate sampled in untreated dermis) and one pilot subject who underwent whole-body heating with BOTOX® treatment (dialysate sampled at BOTOX® treated and untreated sites) were analyzed. Within non-trypsin digested samples, 146 protein groups were identified. This included syntaxin-4, keratin type I and II, and dermicidin. Trypsin digested samples identified even more protein groups. These included calcium-dependent secretion activator 1, calmodulin 1, muscarinic acetylcholine receptor M2 and M3, nitric oxide

synthase, SNARE-associated protein snapin, synaptic vesicle membrane protein VAT-1, syntaxin-binding protein 3, and vesicle transport protein SFT21. A table of the peptide spectral matches (psm), which is the total number of identified peptide spectra matched for a given protein, by subject condition and sample are provided in **Table 5.3**. While identified, these substances were not present in any discernable way that would suggest cholinergic nerve activation or blockade. We are hopeful that continued analysis of samples will provide more clarity and allow us to conduct a more in depth analysis of the proteomics data.

Table 5.3. Peptide spectrum numbers for proteins found in dialysate in two pilot subjects at baseline (BL) and whole-body heat loading (WBH). Subject 15 had dialysate collected at an untreated site only. Subject 16 had dialysate collected at both botulinum toxin type A treated (BOTOX®) and untreated (Control) sites.

Protein	s015		s016			
	BL	WBH	Control BL	Control WBH	BOTOX® BL	BOTOX® WBH
Keratin type I	31	1	2	7	4	5
Keratin type II	14	-	1	4	3	4
Dermicidin	8	-	3	13	-	4
Syntaxin-4	-	-	1	-	-	-
Syntaxin-binding protein	-	1	-	-	-	-
SNARE-associated protein snapin	-	-	-	1	-	-
Synaptic vesicle membrane protein VAT-1	-	-	1	-	-	-
Muscarinic ACh receptor M2	-	-	1	-	-	-
Muscarinic ACh receptor M3	-	-	1	-	-	-
Nitric oxide synthase	-	1	-	-	-	-
Calmodulin 1	-	-	1	-	2	-

Discussion

This is the first study to investigate cutaneous active vasodilator nerve activation in response to different thermal loading modalities. The main findings of the present study are 1) CAVD in response to exercise heat loading and passive whole-body heat loading are both mediated by cholinergic nerve transmission and 2) differing thermal loading modalities did not change cholinergic nerve transduction and the resulting skin blood flow response. These conclusions come from the following observations: selective

presynaptic cholinergic nerve blockade attenuated the skin blood flow response to exercise heat loading and passive whole-body heat loading modalities at the maximum temperature increase similarly, and the internal temperature-skin blood flow relationship at BOTOX® treated and untreated sites was comparable between thermal loading modalities.

Previous work by Roddie et al. provided the first evidence that the active vasodilator system may be mediated by a cholinergic mechanism (175, 176). Continued work by Kellogg et al. confirmed that the active vasodilator nerves were indeed cholinergic and established the co-transmitter theory of CAVD (114). Their study suggested the release of unknown neurotransmitter(s) from the cholinergic nerve terminals is the primary mediator of CAVD although this has never been independently confirmed and was done in a limited sample size (N=6). Previous convention has held that the mechanisms of CAVD operate in a similar manner regardless of the mode by which an individual is thermally loaded (98). Hence, the known mechanisms of CAVD have been largely characterized by passive whole-body heat loading models and generalized to exercise heat loading. It is unknown if the theory of cholinergic co-transmission theory of CAVD extends to exercise heat loading. Additionally, the substance(s) co-transmitted with ACh is still unknown.

Exercise and Passive Whole-body Heat Loading

Increased cardiac output during both exercise as well as thermal stress is well documented (5, 25, 93, 132, 221). Increases in cardiac output are largely in service of increased blood flow to active muscle and to the skin circulation, respectively. Previous research has determined that the observed increase in cardiac output during supine

passive whole-body heat loading and exercise is primarily driven by an increased heart rate (14, 40, Francisco publication in review). In the present study, heart rate increased to approximately 146 beats min^{-1} by the end of exercise and approximately 100 beats min^{-1} by the end of passive whole-body heat loading which is within previously documented ranges (14, 18, 40, Francisco publication in review). Increases in cardiac output act to increase systolic blood pressure and the magnitude of the heart rate increases during both thermal loading modalities reflect the observed increases in systolic blood pressure (**Table 5.2**). Pulse pressure increased during exercise which is similar to what others have shown during 1 hour of dynamic exercise (28, Francisco et al. in review). The present study also showed a significant increase in pulse pressure during passive whole-body heat loading which is contrary to what others have shown (148). Consideration of this is important because pulse pressure represents one of the largest hemodynamic differences between the two thermal loading modalities. It has been suggested as a potential mechanism to explain previously observed differences in CAVD during exercise and passive whole-body heating (27).

For both thermal loading modalities, internal temperature increased to similar levels upon completing thermal loading (**Figure 5.1)**. However, exercise resulted in a quicker internal temperature rise that began to plateau while passive whole-body heat loading resulted in a delayed internal temperature rise followed by rapid increase. These differences may reflect differences in the thermal loading modalities themselves. During exercise heat loading, the ensuing heat from cellular metabolism (i.e., active muscle) generates a thermal load that increases internal temperature and initiates a thermoregulatory response. When passively whole-body heat loaded, skin temperature

begins to rise as the thermal gradient between the skin and the environment diminishes. This rise in skin temperature reduces the gradient for heat transfer between the core tissues and the environment such that internal temperature will rise. The observed Time vs. Internal temperature relationships in the present study (**Figure 5.1**), suggest that exercise resulted in faster thermal loading; whereas passive whole-body heat loading was delayed due to the slow reversal of the aforementioned thermal gradients.

Mean body temperature was higher at the end of passive whole-body heat loading than at the end of exercise heat loading (**Table 5.2**). This may be because subjects were able to engage in evaporative heat loss during exercise which reduced mean skin temperature (**Chapter 3; Figure 3.2**) and subsequently reduced mean body temperature. During passive whole-body heat loading, evaporative heat loss was prevented by the addition of a water-impermeable plastic garment. This prevented reductions in mean skin temperature and subsequently mean body temperature (**Chapter 3; Figure 3.3)**.

Cutaneous Active Vasodilator Nerve Activation During Passive Whole-Body Heat Loading

It has been previously shown that cholinergic nerve blockade with BOTOX® locally abolished the CAVD response to passive whole-body heat loading in humans (114). In the study mentioned above, whole body heating for 35-45 minutes with concurrent skin blood flow assessment via laser-Doppler flowmetry was done in six individuals who had previously received a 5 unit intradermal injection of BOTOX®. Skin blood flow in these subjects rose to 52 ± 9% of the maximal level at untreated sites and 17 ± 2% of the maximal level at BOTOX® treated sites by the end of passive whole-body heating. In the present study, an hour of passive whole-body loading (an

approximate 0.7 C° increase in internal temperature) increased skin blood flow to 38 ± 2 %CVCmax at untreated sites and 15 ± 5 %CVCmax at BOTOX® treated sites (**Figure 5.3**).

The similar skin blood flow responses at the BOTOX® treated sites suggest that we were successful in abolishing the active vasodilator response. The differences in the absolute skin blood flow values at the untreated sites between these two studies could be due to differences in posture or in the magnitude of thermal loading achieved. The aforementioned work by Kellogg et al. reported neither (114); although, similar studies from Kellogg et al. suggest that subjects were likely supine while passively heated (116, 117). Conversely, passive whole-body heat loading in the present study was done in the seated position. It is well known that orthostasis causes a rightward shift in the T_c-skin blood flow relationship (**Chapter II, Figure 2.8**) via active vasodilator withdrawal. Therefore, differences in posture during heat loading may explain these dissimilar findings. It is also possible that subjects in the present study had a smaller rise in internal temperature. This would result in a smaller skin blood flow response and also explain the differences in the absolute skin blood flow responses at the untreated sites.

The finding that cholinergic nerve blockade abolished CAVD during passive whole-body heat loading in the present study is not novel. However, our understanding of the cholinergic active vasodilator nerves involved in thermoregulation was largely based on a limited sample size (N=6) and had never been confirmed. Our data corroborate the conclusions of Kellogg et al. that CAVD is mediated by cholinergic nerve activation (114).

Cutaneous Active Vasodilator Nerve Activation During Exercise Heat Loading

It had previously been assumed that the mechanisms and transducers of CAVD were the same regardless of how thermal loading is accomplished. More recent research has suggested that this assumption is incorrect and active vasodilator nerve transduction and/or the mechanisms of cutaneous vasodilation may vary depending on the source of the heat stress (e.g. exercise vs. passive whole-body heat loading) (148). In the present study, cholinergic nerve blockade attenuated the skin blood flow response to exercise heat loading to a similar extent as during passive whole-body heat loading (**Figure 5.3**).

When the differences in the thermal loading modalities and their previously proposed impact on CAVD are considered, this is striking for two reasons. First, it had been suggested that cyclic circumferential cutaneous vascular wall strain (due to pulse pressure) which is likely higher during exercise than passive heating, may result in a different local milieu that could affect active vasodilator nerve function at the synaptic cleft. Second, it had also been suggested that differences in afferent input to the hypothalamic regions regulating efferent active vasodilator nerve activity (e.g. skin temperature, blood pressure, etc) could alter vasodilator nerve activity (148). We found that differences in pulse pressure and mean body temperature between the two thermal loading modalities (**Table 5.2**) did not change vascular transduction of active vasodilator nerve activity (assessed by the T_c-skin blood flow relationships in **Figure 5.2)**.

There does appear to be a rightward shift (non-significant) in the T_c-skin blood flow relationship (**Figure 5.2**) during exercise which is expected, as exercise is known to cause a rightward shift in the internal temperature equilibrium point (**Fig 2.8**) for active vasodilation (97, 110, 125, 204). Despite the shift, the gain and magnitude of the skin

blood flow responses at BOTOX® treated and untreated sites for both thermal loading modalities was similar. Our data offer conclusive evidence that the same cholinergic nerves are responsible for CAVD during exercise heat loading as during passive whole-body heat loading; and that different modes of thermal loading, does not change cholinergic nerve transmission behavior.

Co-transmitter(s)

The current study sought to identify unknown vasoactive substances associated with CAVD. Putative cholinergic nerve co-transmitters include substance P (224), vasoactive intestinal peptide (VIP) (7, 183, 217, 220), calcitonin gene related peptide (183), and histamine (227).

Analysis of dialysate samples has been delayed due to unprecedented circumstances (COVID-19 pandemic of 2020). Results from two subjects have yielded nothing definitive. We have identified the syntaxin protein family and the SNARE-associated protein snapin which are both associated with contact formation between the synaptic vesicle and the plasma membrane at the terminal end of the neuron (19). Similarly, synaptic vesicle membrane protein VAT-1 is associated with cholinergic synaptic vesicles and is thought to be involved in vesicular transport and exocytosis. Detection of proteins associated with neurotransmitter release is a positive sign that we are able to recover substances from the synaptic cleft. We have identified receptors and enzymes associated with CAVD such as muscarinic receptors and eNOS but no substances that activate these receptors or enzymes. Interestingly, we detected dermicidin which is an anti-microbial peptide secreted by human sweat glands (186).We are successfully detecting the receptors and enzymes associated with CAVD and substances

secreted from sweat glands yet the substances being released from the active vasodilator nerves remain elusive.

Continued analysis will shed more light on these issues yet more work and new methods must be employed to determine the unknown neurotransmitter(s) associated with cholinergic active vasodilator nerves. For instance, artificial stimulation of these nerves independent of thermal loading may result in larger concentrations of analyte release and provide a more stable environment for dialysate recovery studies.

It has been shown that altering Ca^{2+}, Na^{+}, and K^{+} concentrations in the perfusate can result in quantification of stimulated rather than basal neurotransmitter release (215). Of these electrolytes, changing the concentration of K^{+} in the perfusate has been used to induce artificial neurotransmitter release in the brain (208). Perhaps a similar technique in conjunction with dialysate recovery at BOTOX® treated and untreated sites in the skin could be a new approach to addressing an old question. What is being co-transmitted with ACh from these active vasodilator nerves?

Conclusions and Perspectives

This is the first study to confirm the cholinergic co-transmitter theory of active vasodilation during passive whole-body heat loading and investigate this phenomenon during exercise heat loading using a crossover study design. We have demonstrated that CAVD in response to exercise and passive whole-body heat loading are both mediated by cholinergic nerve transmission; and differing thermal loading modalities did not change the vascular transduction of active vasodilator nerve activity. Proteomics analysis of the collected dialysate samples is ongoing. We are hopeful that this approach will help

identify potential neurotransmitter(s) involved in CAVD and provide a more global picture of the similarities and differences of CAVD during exercise and passive whole-body heat loading.

CHAPTER VI

CONCLUSIONS

It was previously assumed that the mechanisms of cutaneous active vasodilation (CAVD) operate in a similar manner regardless of the mode by which an individual is thermally loaded (98). The known mechanisms and transducers of CAVD have been largely characterized using supine passive whole-body heating models and applied to our understanding of exercise heat loading. More recent studies have highlighted the possibility that CAVD may be unique to the thermal loading modality used (55, 148). Specifically, heat introduced endogenously (exercise) versus exogenously (passive whole-body heating) are largely different stimuli that result in similar thermoregulatory reflexes. The afferent signals they share in common and those that delineate them may impact the mechanisms and transducers of CAVD; yet these signals and their impact on CAVD have been largely unexplored until now. The overall purpose of this dissertation was to assess cutaneous active vasodilation (CAVD) in response to varying thermal loading modalities and postures. To this end, two studies were conducted in young, healthy, recreationally active subjects.

In Chapter IV we sought to determine the relative contribution of neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) isozymes to cutaneous vasodilation during passive whole-body heat loading in the supine and seated positions. We found no postural differences in the overall skin blood flow response or relative contribution of NO to CAVD. Interestingly, we found that the NO component of CAVD was eNOS rather than nNOS mediated. This is in direct conflict with others, who

have shown that nNOS is the primary isoform involved in CAVD during passive whole-body heating (117, 118, 120, 148).

It is possible that NO generation in the cutaneous vasculature during thermal stress involves both nNOS and eNOS isoforms, but that nNOS contributes to the initial cutaneous vasodilation whereas eNOS is responsible for the overall sustained cutaneous vasodilation. This may explain why some studies have observed nNOS and others have observed eNOS contributions to the NO component of CAVD (55, 117, 118, 120, 148). It is also possible that these variable findings represent an often overlooked reality of mechanistic research, drugs are not as specific as we think and most likely have diffuse effects that are unknown to the investigator. How these drugs, or even individual "batches"of drug, change the interstitial milieu and the overall dynamics of the tissue is unknown. It is possible that the effects of the drugs themselves are preferentially selecting the NOS isozyme utilized in CAVD.

There is new evidence emerging that suggests fiber insertion results in tissue trauma and a sustained proinflammatory response. For example, it has been shown that some inflammatory mediators remain elevated up to 4 days after fiber insertion (33). Newly emerging intradermal dialysate recovery studies are also demonstrating a bias towards proteins relevant to the injury process within dialysate approximately 3 hours after fiber placement (33, 60). Microdialysis fiber placement techniques often vary by lab. It is possible that post fiber placement wait times, the use of ice before or after fiber placement, and needle size used to place microdialysis fibers could affect the magnitude and duration of tissue trauma. It is possible that the inflammation inherent with fiber

placement could be affecting the NOS isozyme utilized in CAVD; and that different research groups are studying variably inflamed tissue.

With these things considered, it is clear that we are studying skin blood flow responses to thermal challenge in an inflamed tissue with drugs that may not be as selective as we think. What is less clear is how these interact with each other and independently of each other within the tissue. Do they change physiological function? Future research should aim to address these issues in depth. Despite these limitations, our study utilized the same drugs and concentrations as used in previous research and still found differences in the NOS isozymes contributing to the NO component of CAVD during passive whole-body heating. We are the first to show eNOS mediation during passive whole-body heating and this is similar to what others have shown during exercise (55, 148). We believe that eNOS is the primary mediator of the NO component of CAVD with a lesser potential role for nNOS regardless of differences in thermal loading or posture. We also believe a fieldwide dialogue about the use of pharmacological agents in the determination of mechanistic pathways *in vivo* and the re-evaluation of what we know about the CAVD system is necessary. Perhaps standardization of the intradermal microdialysis technique and pharmacological agents used would be a good start to a conflicted field.

In Chapter V, we sought to determine the extent to which cholinergic nerve activation mediates CAVD during exercise heat loading and passive whole-body heat loading. Secondarily, we sought to identify unknown vasoactive substances associated with CAVD. We found that CAVD in response to exercise and passive whole-body heat loading are both mediated by cholinergic nerve transmission. Different thermal loading

modalities (exercise vs. passive whole-body heat loading) did not change the vascular transduction of active vasodilator nerve activity. These are perhaps the most important findings of this dissertation; because the cholinergic co-transmitter theory of CAVD had never been independently confirmed and its translation to exercise heat loading was unknown. We are the first to corroborate the cholinergic co-transmitter theory of CAVD during passive whole-body heat loading and extend it to exercise heat loading. We believe that cutaneous vasodilator nerves are responsible for similar skin blood flow responses regardless of how thermal loading is accomplished. However, we cannot definitively say that these nerves are similarly activated or release similar substances. Proteomics analysis of the collected dialysate samples is ongoing. We are hopeful that this approach will help identify potential neurotransmitter(s) involved in CAVD and provide a more global picture of the similarities and differences of CAVD during exercise and passive whole-body heat loading.

It is also possible that we will be unsuccessful in the identification of neurotransmitter(s) released from active vasodilator nerves. This possibility is derived from previous research demonstrating the effects of the "trauma" layer surrounding microdialysis probes in brain dialysate recovery studies (141, 169). It has been shown that neurotransmitter release sites adjacent to probes are damaged and neurotransmitter release is suppressed in these regions. The aforementioned authors believe that nerve disruption associated with probe implantation may extend 1mm from the probe. This may minimally impact studies in which the probe is imbedded in purely neural tissue. This may have a major impact on neurotransmitter recovery in tissues where nerves are fewer and farther between, such as dermal tissue. When this is considered, along with the

duration of the inflammatory response mentioned above, it is possible that we may be further exacerbating a potential problem acknowledged in Chapter V. Recovered analyte concentration (e.g., neurotransmitter(s) associated with CAVD may not be great enough to detect.

Heat introduced endogenously (exercise) verses exogenously (passive whole-body heating) have largely different hemodynamic profiles. Despite this, different modes of thermal loading result in similar thermoregulatory reflexes. The afferent signals they share in common and those that delineate them do not appear to impact the mechanisms and transducers of CAVD as was originally thought. eNOS appears to regulate the NO component of CAVD regardless of posture; and this is similar rather than different with exercise. Additionally, CAVD during exercise and passive whole-body heating are both mediated by cholinergic nerves and to similar extents. Because of the discordant findings presented in this dissertation with past research, more research should be done to elucidate the mechanisms of NO generation in CAVD (i.e. nNOS versus eNOS. Lastly, future research should develop new approaches to address an old question. What is being co-transmitted with ACh from these active vasodilator nerves?

Artificial stimulation of these cholinergic active vasodilator nerves independent of thermal loading may result in larger concentrations of analyte release and a more controlled environment in which to conduct dialysate recovery studies. For example, changing the concentration of K^+ in the perfusate has been used to induce artificial neurotransmitter release in the brain (208). Additionally, stimulation of these nerves independent of thermal loading could improve analyte recovery by removing the burden of time. Because of subject comfort, it is not feasible to induce hyperthermia in a subject

for periods longer than an hour. Analyte recovery efficiency could be improved by reducing the perfusate flow rates. Slower flow rates would be feasible if dialysate recovery was not constrained to approximately 1 hour. It is also possible that the unknown co-transmitter(s) is a low molecular weight compound that is the by-product of an unknown enzymatic reaction rather than a peptide capable of being detected by proteomics. Pending the results of the proteomics analysis currently delayed at OHSU, metabolomics analysis of dialysate samples may yield much more informative findings.

APPENDIX

INFORMED CONSENT DOCUMENT

TITLE: "Mechanisms of Cutaneous Vasodilation in Humans" PROTOCOL #4
INVESTIGATOR: Dr. C.T. Minson and Colleagues
APPROVED BY INSTITUTIONAL REVIEW BOARD: August, 2019.
(12102010.027)

This is an important form. Please read it carefully. It tells you what you need to know about this study. If you agree to take part in this research study, you need to sign this form. Your signature means that you have been told about the study and what the risks are. Your signature on this form also means that you want to take part in this study.

Why is this study being done?

During sustained exercise and/or conditions of high environmental temperatures, the body temperature in humans will increase. If body temperature increases too much, we are not able to function properly. This may lead to heat-related illnesses (such as heat stroke) and even death. The primary way that humans can combat a big increase in core body temperature is to increase skin blood flow to the skin and to sweat. The increase in skin blood flow brings warm blood from our deep body core to the surface where the heat can be released by the evaporation of sweat. Unfortunately, we still do not fully understand how skin blood flow is controlled in humans. In addition, it is unknown if the control of skin blood flow is different during passive whole-body heating and active (exercise) heating. The purpose of this study is to gain a better understanding of the mechanisms that allow humans to increase skin blood flow during heat stress. This knowledge will help us to understand the changes in skin blood flow that occur as people age and in certain disease states, such as diabetes. As people age or as diabetes progresses, the ability to increase skin blood flow, to shift warm blood from the core of the body to the skin surface (to release the heat) is less compared to younger, healthy people. This decrease in skin blood flow is the primary reason older people and diabetics are at greater risk for heat-related illnesses during climatic heat waves. We will further our understanding of these problems by addressing one or more of following questions in this protocol:

- Does the mode by which an individual is heated (exercise vs. passive whole-body heating) effect the mechanisms by which skin blood flow increases during heat stress?

What will happen in the study? (You will be asked to participate in sections of the protocol that have been circled below)

1. If you are interested in participating in the study, we will schedule an appointment with you to meet with one of the investigators of the study to discuss the project, to see the laboratory, and to read this form. If we have scheduled this appointment, it means you meet all initial subject criteria (based on initial phone and/or email conversations). Additionally, during this initial session, you will fill out a health history form so that we can ensure you are healthy enough to participate in the study and that you meet all subject inclusion/exclusion criteria. This form will also be used to provide information to medical personnel in the event of an emergency. Investigators will then schedule an aerobic fitness test session. This visit should last about 60 minutes.

2. For the aerobic fitness test session, you will report to in the Evonuk Environmental Physiology Core (EEPCore) in Esslinger Hall (Room 151). In total, you will spend about one hour in the lab on the test (VO_{2peak} test) date. You will need to wear a t-shirt, shorts, running shoes, and refrain from eating at least two hours prior to arrival. In addition, you will need to refrain from consuming caffeine (for example, coffee, tea, red bull, coke, etc.) or medications (except oral contraceptives) for 12 hours prior to the study and abstain from alcohol or exercise for 24 hours prior to the study. If you use oral contraceptives, you should take this when you normally do so.

3. If you are a woman who can still become pregnant, you will be asked to undergo a pregnancy test during the exercise test visit (and again during the study visits). For this test, you will be asked to collect a sample of urine in the women's restroom near the physiology lab. If the test is "positive," indicating that you are pregnant, you will not be allowed to participate and will be advised to see your physician or the University of Oregon Health Center.

4. <u>Aerobic Fitness Test:</u> You will pedal on an exercise bicycle while wearing a mouth piece, nose clip, and electrocardiogram electrodes (heart rhythm monitor). If you are a woman, the adhesive patches used for this test will be placed on you by one of the women in the lab. After a 5-minute warm-up, you will be asked to maintain a selected pedaling rate as pedaling resistance (work) is increased every minute until you reach your maximum exercise capacity. This is to measure your $VO2_{peak}$ which is a measure of your overall aerobic fitness level. It normally takes 10 to 15 minutes for people to reach their maximum effort. This session will establish your maximal exercise tolerance on a bike and will be used to establish the appropriate workload for the exercise session on the study visit.

5. At the end of the fitness test, we may administer an intradermal injection of a small dose of Botulinum toxin type A (BOTOX) to a $0.5cm^2$ area of skin on the ventral side of your forearm. The site of BOTOX administration will then be marked with a sharpie marker. You will be sent home with this sharpie marker and asked to re-mark the treated site daily (after showering or washing etc.). This is done so that we can relocate the treated site on the subsequent visit.

6. You should notify the investigator immediately if you feel any significant discomfort or concern about your well-being at any time during the initial visit. Some examples of discomfort include fatigue and muscle soreness.

7. On the study day, you will arrive at Dr. Minson's laboratory in Esslinger Hall at the University of Oregon. This testing will take approximately 6 hours. Prior to arrival, you will be asked to refrain from all over-the-counter medications for 24 hours, alcohol and caffeine for 12 hours, and food for 4 hours. Your height and weight and resting blood pressure will be measured. You will be asked to provide a urine sample, which will be tested by an investigator for urine specific gravity (USG) to ensure that you are hydrated. If you are dehydrated, you will be asked to drink 5mL/kg body weight of fluids prior to heating. Female subjects will also be asked to take a urine pregnancy test. You cannot participate in the study if the pregnancy test is positive, as the study procedures could be harmful to an unborn child. Additionally, female subjects will be studied during menses, or during the placebo phase, if taking birth control. You will be randomly assigned to a treatment sequence (Exercise first and passive Whole-body heating second; or passive Whole-body heating first and Exercise second) which are described below. Note: You will both Exercise and be passively Whole-body heated during the study.

8. Your nude body weight will be measured by a member of the same sex prior to study. You will stand behind a privacy screen while this measurement is taken. Your nude body weight will also be measured at the end of the study. This will be done so that we can quantify the volume of sweat you lose during the study.

9. To measure your body core temperature, we will give you a rectal probe in a sterilized packet labeled with your subject number. It is made of a thin rubber (flexible) material that is inserted 10 centimeters (approximately 4 inches) past the anal sphincter. The probe will remain in place throughout the entire study session (up to 6 hours). The probe has a "tail" that will be connected to an external apparatus. The procedure may be a little uncomfortable at first (during insertion) but it should not be painful at any time. You will be instructed how to self-insert the rectal probe, as well as how to remove it and clean it. If you need assistance, a lab researcher of the same sex will help you. Once in place, you may not even feel the probe at all. This technique is widely used and it's considered the "gold standard" procedure for measuring body ("core") temperature.

10. You will have up to 6 small tubes (these are called "microdialysis fibers", and are smaller than the lead of a pencil) placed in the skin of your forearm. (Note: if you previously received an intradermal injection of BOTOX, two of these fibers will be placed at this site.) Microdialysis fiber placement: A small needle will be placed just under the surface of your skin and will exit back out about 1½ inches from where it entered your skin. The small tubes will be threaded through the needle, and the needle will be withdrawn, leaving the small tubes under your skin. These will remain in your skin throughout the rest of the study.

11. We will also put some very small doses of drugs through the small tubes in your skin. These will be infused before heat stress and for the duration of the heating period. These drugs will cause the vessels of your skin next to the small tubes to either dilate or become narrow. You should not feel anything when the drugs are going into your skin. However, it is possible you may feel a slight tingling in the skin where the probe is. All of the drugs will be infused into the small area of skin and will not circulate through your body.

<u>You will receive the following drugs in your skin (only the ones circled):</u>

a. L-NAME: this stops nitric oxide from being produced and causes the skin vessels to narrow
b. Sodium nitroprusside: this is a substance that is used to lower blood pressure in patients and causes the skin vessels to open
c. Toradol (Ketorolac): this is a substance that may cause your blood vessels to narrow.
d.Tetraethylammonium (TEA): this is a substance that may cause your blood vessels to narrow.
e. Sulfaphenazole: This is a substance that may cause your blood vessels to narrow.
f. Acetylcholine: This is a substance that may cause your blood vessels to open.
g. Atropine: This is a substance that may cause your blood vessels to narrow.
h.Glibenclamide (Glyburide): This is a substance that may cause your blood vessels to narrow.
i. Ebselen: This is a substance that may cause your blood vessels to narrow.
j. Tempol: This is a substance that may cause your blood vessels to open.
k. Estradiol: This is a hormone that may alter blood flow.
l. Progesterone: This is a hormone that may alter blood flow.
m. Testosterone: This is a hormone that may alter blood flow.
n. L-NNA: this stops nitric oxide from being produced and causes the skin vessels to narrow.
o. Barium chloride: This is a substance that may cause your blood vessels to narrow.
p. Potassium chloride: This is a substance that may cause your blood vessels to dilate.
q. Insulin Aspart: This is a substance that may cause your blood vessels to dilate.
r. L-NIO: This stops nitric oxide from being produced and causes the skin vessels to narrow.
t. NPLA: This stops nitric oxide from being produced and causes the skin vessels to narrow.

12. In addition, we may collect some samples of fluid from the ends of the tubes in your skin. The sample is being collected so that we can identify substances being released in the skin when people are heat-stressed. The vials will be coded such that the investigators can only determine that the samples came from the same person and the time each sample was taken. They will not be able to determine your identity from the sample. Once the study is completed and all samples are analyzed, any remaining or extra sample will be destroyed.

13. We need to wait about 1-2 hours after the small tubes are placed in your skin to let the insertion trauma (redness of your skin around the small tubes) go away.

During this time, you will be prepared for either exercise or whole-body heating. In addition, a small probe (laser-Doppler probe) and local heater will be placed over each area of skin where the small tubes are so that we can measure skin blood flow over the small tube.

14. We will place up to 7 wires (called "thermocouples") over your body in order to measure skin temperature. These will be placed directly on your skin at the following sites (Circled): upper back, lower back, chest, abdomen, thigh, calf, and forearm. They will be held in place with tape. A member of the same sex will place these.

15. Whole-Body Heating: You will wear a two-piece nylon suit that has small tubes sewn into the inside of the suit for the duration of the passive Whole-body heating portion of the study. We will infuse water through these tubes during the experiment to control your skin and body temperature. We do not want you to get too hot so we will ask you from time-to-time if you are too hot. If you do become too hot you need to tell the investigator and we will lower the temperature of the water going through the suit. The suit will cover your whole body, except for your feet, hands, and head. You will wear a thin t-shirt and shorts under the suit. You can bring your own t-shirt and shorts, or we can provide a set for you to wear if you prefer. You will also wear a plastic suit over the nylon suit. This will help to keep the heat in during heat stress. We will warm your body by running warm water through the suit until your body temperature is increased from about 98.6°F (normal temperature) to about 101°F. It will take about 40-70 minutes for your body temperature to reach 101°F. During the heating, we will measure your heart rhythm (ECG) from the sticky electrodes on your chest and abdomen and your body temperature. We will ask you to rate your perceived exertion, your thermal comfort, and how wet your skin feels. After heating, we will immediately cool your body by running cool water through the suit until your body temperature is back to normal.

16. During the study, we will periodically inflate a small cuff that is placed on your middle and ring fingers of one of your hands to measure your blood pressure (portapres device). We will only inflate this cuff on one finger for about 10 minutes at a time. If the cuff becomes uncomfortable, let the investigator know and they will turn it off for a few minutes.

17. Blood pressure will also be measured periodically throughout the study using an inflation cuff on your upper arm. We may also stop (occlude) blood flow to the experimental arm by inflating a blood pressure cuff on the upper arm to 250 mmHg. We will stop blood flow to the arm three times for a period of 3-10 minutes with a 20-minute recovery period between each blood flow occlusion.

18. We may measure cardiac output non-invasively using the acetylene uptake method in which the subject will breathe in a mix of safe gases that we will analyze. You will breathe on a mouthpiece for 8-10 breaths during which time you will be breathing in a gas mixture containing 0.6 % acetylene, 9.0 % helium, 20.9 % oxygen, and the rest nitrogen. At the same time, we will collect expired

gas through the mouthpiece and analyze the concentrations of acetylene and helium you exhale. There are no risks associated with breathing acetylene or helium, particularly in such low concentrations. We will measure cardiac output three times at baseline, and every 20 minutes while being whole-body heated or exercised on the bike.

19. Between treatment's (Exercise vs. passive Whole-body heating) you will be seated in a phlebotomy chair and further cooled until your body temperature has returned to resting level. At this point, you will begin the treatment not previously done.

20. Exercise: For the exercise component of this study, you will exercise on a recumbent bicycle for one hour at a moderate intensity. The intensity will be set by the result of the aerobic fitness test (see item 4 on this list). The workload will be set so that you are exercising at 60 percent of your $VO2_{peak}$ score. We will ensure that you are working at the appropriate workload by having you breathe into a mouthpiece (similar to that described at item 4) for the first 15 minutes of exercise so that we can collect your expired air. We will analyze the levels of oxygen and carbon dioxide in your expired air in order to calculate oxygen consumption and your exercise workload will be adjusted to meet the desired 60 percent of $VO2_{peak}$ workload. We will make these measurements (only 2-3 minute durations after the first 15 minutes) every 20 minutes while exercising. During exercise, we will be taking the exact same measures as are taken during passive Whole-body heating and outlined above. This means we will be collecting skin blood flow measures, skin temperatures, body core temperature, and blood pressure data from your arms while you are pedaling. You will be provided 500 milliliters of water to drink during exercise.

21. Prior to de-instrumentation, we will heat each of laser-doppler sites with a small heater up to 44□□Celsius (107 degrees Fahrenheit) to maximally open the vessels in the skin. This is below the temperature where heating becomes painful (about 110 degrees Fahrenheit) and well below the temperature that may burn the skin (about 113 degrees Fahrenheit). If the you think the heater is becoming painful, we will lower the temperature

22. After the study, we will remove the small tubes in your skin and a bandage will be placed over the area of skin where the tubes were placed.

23. Although you will not be allowed outside food or beverages during the study, you will be given a light snack and fluids to drink before you are sent home.

How long will I be in the study?

You will come in for an initial visit to learn about the study (20-30 minutes). If you qualify for the study, you will be in the study on only one day. The body heating experiment will take approximately 5 hours.

What are the risks of the study?

Skin Microdialysis: There may be some discomfort during the insertion of the small fibers in your skin. Once the needle is in place, the pain should subside. There is also a risk of syncope (fainting) during needle placement. You will be sitting in a reclining chair during the study, which reduces this risk, and you will be asked to inform the investigator if you feel light-headed, nauseous, dizzy, etc. during needle placement. If you do experience any of these symptoms, we will discontinue placing the needles and ensure the symptoms subside. Infusions through the fibers should not be painful, and there should only be minor swelling at the site. At the end of the study, the fibers will be withdrawn and a sterile dressing will be applied. Any swelling or redness after the study should be gone a few hours after completion of the study. Although the small tubes are sterile, there is a slight risk of infection and/or allergic reaction at the sites where the small tubes were placed in your skin. You will be instructed how to keep the area clean for a day or two following the study. If you see any signs of infection (redness, swelling, and/or pain around the sites) or experience some other abnormal reaction at the insertion site following the study, please contact us immediately. We will show you photos of what normal and abnormal healing looks like at the sites. There is a possibility the fibers may break while in your skin of while they are being removed. We remove the fibers in a way such that we can still remove the entire fiber, even if it does break. However, there is still a slight risk a small part of the fiber could remain in your skin. If this occurs, the piece should be able to work its way out of the skin within a few days (similar to a splinter), and we will follow-up with you to ensure this has happened. If the piece does not work its way out, or if a site seems infected, we will evaluate the site(s) and, if necessary, recommend you seek medical treatment with a healthcare provider.
Please note, all medical costs will be your responsibility.

Whole Body Heating: The heat exposure can be physically demanding and can cause light- headedness, low blood pressure, fatigue, nausea, or cramps. Therefore, investigators will check in with subjects and will instruct subjects not to attempt to prolong the heating experiment if they do not feel well. Skin temperature, core temperature, heart rate (via ECG), and blood pressure will all be continually monitored closely the procedure. Subjects will be given Gatorade® and salty snacks to counteract the large amount of sweating they may experience during the protocol. Additionally, we will measure body weight before and after heat stress in order to ensure all fluids lost due to sweating has been replaced with fluids.

VO_2Peak testing: There is potential risk associated with graded exercise testing that is categorized as More than Minimal Risk. The risks associated with graded exercise testing include light-headedness, fatigue, shortness of breath, risk of myocardial infarction and death. According to the American College of Sports Medicine, these general statements can be made regarding the safety of maximal graded exercise testing: The risk of death during or immediately after an exercise test is <0.01%. The risk of myocardial infarction during or immediately after an exercise test is <0.04%. The risk of complication requiring hospitalization (including myocardial infarction) is approximately 0.1%. The magnitude of severity for risks of light-headedness, fatigue, shortness of breath from the VO_2Peak testing is very low, but the probability of these

risks occurring is moderate. The magnitude of severity for risks of myocardial infarction and death from the VO_2Peak testing is very, very high, but the probability of these risks occurring is very, very low.

Aerobic Exercise: Like the graded exercise testing, there is potential risk associated with high intensity aerobic cycling exercise that is categorized as *More than Minimal Risk*. The risks associated with this are similar to graded exercise testing. However, ECG and blood pressure will be constantly monitored during testing. Procedures will be terminated immediately if any subject feels light-headed, nauseated, or experiences any other adverse signs or symptoms. In the event of an emergency, we will directly call 911. The magnitude of severity for risks of light-headedness, fatigue, shortness of breath from the aerobic exercise is very low, but the probability of these risks occurring is moderate. The magnitude of severity for risks of myocardial infarction and death from the aerobic exercise is very, very high, but the probability of these risks occurring is very, very low.

Rectal temperature probes: The use of rectal probes to measure core temperature carries minimal risk. The primary risk is of damage to the lining of the rectum; however, this risk is very slight as we use a flexible thermistor that is designed for this purpose. We will exclude any subjects who have had recent rectal, anal, vaginal, or prostate surgery. In addition, we will exclude any subjects who have a personal history of heart disease, as the use of a rectal thermometer can cause a vagal reaction, increasing the potential for arrhythmias and fainting. There is also the risk of infection, either by the subject not washing their hands properly or exposure by others to a poorly cleaned probe. Each probe is cleaned by the subject after use, and sterilized by the investigators before reuse. The risk of infection is similar to that of having a bowel movement, and is considered minimal (similar to daily experience). There is also the risk of embarrassment. The approach is typically well tolerated by subjects, and the investigative team is professional in regard to their treatment of the subjects.

Blood Flow Occlusion: The inflation of the blood pressure cuff to stop blood flow may cause a slight tingling sensation and may cause slight bruising. The sensations with prolonged blood flow occlusion greater than 10 minutes are similar to those when a limb has “fallen asleep.” During certain surgical procedures, blood flow is often stopped for 2 hours without any significant risk to the patient. If, at any time, subjects experience any discomfort, they may request that the blood pressure cuff be either loosened or removed.

Local Skin Heating: The local skin heaters may cause some minor skin discomfort. The goal is to warm the area of skin to a temperature that has been determined to be below the threshold for pain. There is a slight risk of burning the skin at this sight, so subjects will be instructed to tell the investigators of any pain they are feeling. If the local heating becomes painful, the temperature of the local heater will be lowered. The heating device may be removed at any time if the subjects experience any discomfort.

Study Drugs: We will be infusing or placing on the skin (in the cream) very small doses of each drug (well below the dosages determined to be safe by the FDA), and

only into a very small area of the skin. The drugs are only infused into the small area of skin where the microdialysis tubes are placed. Therefore, these drugs will not directly cause any systemic (whole body) changes in blood pressure. However, as with any infusions or medications, there is the possibility that you are allergic to the drug and may have an allergic reaction to the drug including changes in blood pressure and difficulty breathing.

Laser-Doppler Probes: These probes send a small light into the skin. You will not feel anything except the probe touching the skin. The only risk associated with this procedure is that there might be some slight skin irritation (redness) to the adhesive tape used to hold the probe on the skin. There are no major risks associated with this device.

Finger Blood Pressure: In some people, this blood pressure cuff causes their finger to become red and uncomfortable after a long period (over 40 minutes). We will only inflate the cuff for 10 minutes at a time and then give your finger a rest. If your finger becomes uncomfortable during the 10 minutes the cuff is inflated, the investigator will turn it off for a few minutes. There are no major risks associated with this device.

May I participate if I am pregnant or breast-feeding?

This study may be harmful to an unborn or breast-fed child. There is not enough medical information to know what the risks might be to a breast-fed infant or to an unborn child in a woman who takes part in this study. Breast-feeding mothers are not able to take part in this study. Women who can still become pregnant must have a negative pregnancy test no more than 24 hours before taking part in this study. If the pregnancy test is positive (meaning that you are pregnant), you will not be able to take part in the study.

Are there benefits to taking part in this study? This study will not make your health better.

What other choices do I have if I don't take part in this study?

This study is only being done to gather information. You may choose not to take part in this study.

What are the costs of tests and procedures?

You will not need to pay for any tests or procedures that are done just for this research study.

You will get **$60** for participating in this study. This money is for the inconvenience and time you spent in this study. If you start the study but stop before the study has ended, you will get part of this money. The partial amount will be calculated as $10 per hour participated in the study.

Please note, compensation from participation in Human Subjects Research studies is taxable income. If your compensation totals $600 or more in a calendar year, the University is required to report the income to the IRS. The University requires its departments to track participant compensation and may contact you to complete a Form W-9 for tax reporting purposes. Because of the federal and University tracking requirements, your name will be associated with participation in research. Department and University administrators will have access to this information, but will not have access to research data.

Who can answer my questions?

You may talk to Dr. Christopher Minson or one of the Co-Investigators at any time about any question you have on this study. You may contact Dr. Minson by calling (541) 346-4105 or on his cell phone (541) 953-2231, and the Co-investigators at (541) 346-4507.

What are my rights if I take part in this study?

Taking part in this research study is your decision. You do not have to take part in this study, but if you do, you can stop at any time. Your decision whether or not to participate will not affect your relationship with The University of Oregon.

You do not waive any liability rights for personal injury by signing this form. All forms of medical diagnosis and treatment whether routine or experimental, involve some risk of injury. In spite of all precautions, you might develop medical complications from participating in this study.

The investigators may stop you from taking part in this study at any time if it is in your best interest, if you do not follow the study rules, or if the study is stopped. You will be told of important new findings or any changes in the study or procedures that may happen.

If you experience harm because of the project, you can ask the State of Oregon to pay you. A law called the Oregon Tort Claims Act limits the amount of money you can receive from the State of Oregon if you are harmed. If you have been harmed, there are two University representatives you need to contact. Here are their addresses and phone numbers:

General Counsel	Research
Compliance Services	Office of the President
	University of
Oregon University of Oregon	Eugene, OR 97403
Eugene, OR 97403	(541) 346-2510
(541) 346-3082	

What about confidentiality?

Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission. Subject identities will be kept confidential by assigning you a "subject identification number". The names associated with each subject identification number will be kept in a locked file cabinet in Dr. Minson's office.

I have had an opportunity to have my questions answered. I have been given a copy of this form. I agree to take part in this study.

If you have questions regarding your rights as a research subject, contact Research Compliance Services, 5219 University of Oregon, Eugene, OR 97403, 541/346-2510.

Your signature indicates that you have read and understand the information provided above, that you willingly agree to participate, that you may withdraw your consent at any time and discontinue participation without penalty, that you will receive a copy of this form, and that you are not waiving any legal claims, rights or remedies.

_____ _____

(Date) (Signature of Participant)

_____

(Printed Name of Participant)

_____ _____

(Date) (Signature of Individual Obtaining Consent)

REFERENCES CITED

1. **Alderton WK**, **Cooper CE**, **Knowles RG**. Nitric oxide synthases: structure, function and inhibition. *Biochem J* 357: 593–615, 2001.

2. **Aoki K**, **Kondo N**, **Shibasaki M**, **Takano S**, **Katsuura T**. Circadian variation in skin blood flow responses to passive heat stress. *Physiol Behav* 63: 1–5, 1997.

3. **Aoki K**, **Stephens DP**, **Johnson JM**. Diurnal variation in cutaneous vasodilator and vasoconstrictor systems during heat stress. *Am J Physiol - Regul Integr Comp Physiol* 281: R591-5, 2001.

4. **Aschoff J**. Circadian control of body temperature. *J Therm Biol* 8: 143–147, 1983.
5. **Astrand PO**, **Cuddy TE**, **Saltin B**, **Stenberg J**. Cardiac Output During Submaximal and Maximal Work. *J Appl Physiol* 19: 268–274, 1964.

6. **Barnes M**. Botulinum toxin - Mechanisms of action and clinical use in spasticity. *J Rehabil Med Suppl* : 56–59, 2003.

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

8. **Benzinger TH**. Heat regulation: homeostasis of central temperature in man. *Physiol Rev* 49: 671–759, 1969.
9. **Bergeron LH**, **Willcox JM**, **Alibhai FJ**, **Connell BJ**, **Saleh TM**, **Wilson BC**, **Summerlee AJS**. Relaxin peptide hormones are protective during the early stages of ischemic stroke in male rats. *Endocrinology* 156: 638–46, 2015.

10. **Bevegård BS**, **Shepherd JT**. Reaction in man of resistance and capacity vessels in forearm and hand to leg exercise. *J Appl Physiol* 21: 123–132, 1966.

11. **Blair DA**, **Glover WE**, **Roddie JC**. Vasomotor Responses in the Human Arm During Leg Exercise. *Circ Res* 9: 264–274, 1961.

12. **Boulant JA**. Hypothalamic Neurons Regulating Body Temperature. In: *Handbook of Physiology, Environmental Physiology*. Hoboken, NJ, USA: John Wiley & Sons, Inc., 1996.
13. **Boulant JA**. Role of the preoptic-anterior hypothalamus in thermoregulation and fever. *Clin Infect Dis* 31 Suppl 5: S157-61, 2000.

14. **Bourcier S**, **Benoist J-F**, **Clerc F**, **Rigal O**, **Taghi M**, **Hoppilliard Y**. Detection of 28 neurotransmitters and related compounds in biological fluids by liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* 20: 1405–21, 2006.

15. **Braverman IM**. The cutaneous microcirculation: ultrastructure and microanatomical organization. *Microcirculation* 4: 329–340, 1997.

16. **Brengelmann GL**, **Freund PR**, **Rowell LB**, **Olerud JE**, **Kraning KK**. Absence of active cutaneous vasodilation associated with congenital absence of sweat glands in humans. *Am J Physiol* 240: H571-5, 1981.

17. **Brengelmann GL**, **Johnson JM**, **Hermansen L**, **Rowell LB**. Altered control of skin blood flow during exercise at high internal temperatures. *J Appl Physiol* 43: 790–4, 1977.

18. **Brooks EM**, **Morgan AL**, **Pierzga JM**, **Wladkowski SL**, **O'Gorman JT**, **Derr JA**, **Kenney WL**. Chronic hormone replacement therapy alters thermoregulatory and vasomotor function in postmenopausal women. *J Appl Physiol* 83: 477–84, 1997.

19. **Brunger AT**, **Choi UB**, **Lai Y**, **Leitz J**, **Zhou Q**. Molecular Mechanisms of Fast Neurotransmitter Release. *Annu Rev Biophys* 47: 469–497, 2018.

20. **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: 2019–2026, 2012.

21. **Brunner M**, **Langer O**. Microdialysis versus other techniques for the clinical assessment of in vivo tissue drug distribution. *AAPS J* 8: E263–E271, 2006.

22. **Brunt VE**, **Fujii N**, **Minson CT**. No independent, but an interactive, role of calcium-activated potassium channels in human cutaneous active vasodilation. *J Appl Physiol* 115: 1290–1296, 2013.

23. **Brunt VE**, **Minson CT**. KCa channels and epoxyeicosatrienoic acids: major contributors to thermal hyperaemia in human skin. *J Physiol* 590: 3523–34, 2012.

24. **Carberry PA**, **Shepherd AM**, **Johnson JM**. Resting and maximal forearm skin blood flows are reduced in hypertension. *Hypertension* 20: 349–55, 1992.

25. **Carroll JD**, **Hess OM**, **Hirzel HO**, **Krayenbuehl HP**. Dynamics of left ventricular filling at rest and during exercise. *Circulation* 68: 59–67, 1983.

26. **Carter MR**, **Mcginn R**, **Barrerra-Ramirez J**, **Sigal RJ**, **Kenny GP**, **Barrera-Ramirez J**, **Sigal RJ**, **Kenny GP**. Impairments in Local Heat Loss in Type 1 Diabetes during Exercise in the Heat. *Med Sci Sport Exerc* 46: 2224–2233, 2014.

27. **Charkoudian N**. Influences of female reproductive hormones on sympathetic control of the circulation in humans. *Clin Auton Res* 11: 295–301, 2001.

28. **Charkoudian N**. Skin blood flow in adult human thermoregulation: how it works, when it does not, and why. *Mayo Clin Proc* 78: 603–12, 2003.

29. **Charkoudian N**, **Fromy B**, **Saumet JL**. Reflex control of the cutaneous circulation after acute and chronic local capsaicin. *J Appl Physiol* 90: 1860–4, 2001.

30. **Chefer VI**, **Thompson AC**, **Zapata A**, **Shippenberg TS**. Overview of Brain Microdialysis. *Curr Protoco Neurosci* , 2009.

31. **Choi PPJ**, **Brunt VEV**, **Fujii N**, **Minson CTC**. New approach to measure cutaneous microvascular function: an improved test of NO-mediated vasodilation by thermal hyperemia. *J Appl Physiol* 117: 277–283, 2014.

32. **Chung MK**, **Lee H**, **Caterina MJ**. Warm temperatures activate TRPV4 in mouse 308 keratinocytes. *J Biol Chem* 278: 32037–32046, 2003.

33. **Clausen TS**, **Kaastrup P**, **Stallknecht B**. Proinflammatory tissue response and recovery of adipokines during 4 days of subcutaneous large-pore microdialysis. *J Pharmacol Toxicol Methods* 60: 281–287, 2009.

34. **Clough GF**, **Jackson CL**, **Lee JJP**, **Jamal SC**, **Church MK**. What can microdialysis tell us about the temporal and spatial generation of cytokines in allergen-induced responses in human skin in vivo? *J Invest Dermatol* 127: 2799–2806, 2007.

35. **Cracowski J-L**, **Minson CT**, **Salvat-Melis M**, **Halliwill JR**. Methodological issues in the assessment of skin microvascular endothelial function in humans. *Trends Pharmacol Sci* 27: 503–508, 2006.

36. **Crandall CG**, **Johnson JM**, **Kosiba WA**, **Kellogg DL**. Baroreceptor control of the cutaneous active vasodilator system. *J Appl Physiol* 81: 2192–8, 1996.

37. **Crandall CG**, **MacLean DA**. Cutaneous interstitial nitric oxide concentration does not increase during heat stress in humans. *J Appl Physiol* 90: 1020–4, 2001.

38. **Crandall CG**, **Musick J**, **Hatch JP**, **Kellogg DL**, **Johnson JM**. Cutaneous vascular and sudomotor responses to isometric exercise in humans. *J Appl Physiol* 79: 1946–1950, 1995.

39. **Crandall CG**, **Stephens DP**, **Johnson JM**. Muscle metaboreceptor modulation of cutaneous active vasodilation. *Med Sci Sports Exerc* 30: 490–6, 1998.

40. **Crecelius AR**, **Kirby BS**, **Richards JC**, **Garcia LJ**, **Voyles WF**, **Larson DG**, **Luckasen GJ**, **Dinenno FA**. Mechanisms of ATP-mediated vasodilation in humans: modest role for nitric oxide and vasodilating prostaglandins. *Am J Physiol Heart Circ Physiol* 301: H1302-10, 2011.

41. **Crossley RJ**, **Greenfield ADM**, **Plassaras GC**, **Stephens D**. The interrelation of thermoregulatory and baroreceptor reflexes in the control of the blood vessels in the human forearm. *J Physiol* 183: 628–636, 1966.

42. **Cui J**, **Arbab-Zadeh A**, **Prasad A**, **Durand S**, **Levine BD**, **Crandall CG**. Effects of heat stress on thermoregulatory responses in congestive heart failure patients. *Circulation* 112: 2286–2292, 2005.

43. **Daanen HAM**. Finger cold-induced vasodilation: a review. *Eur J Appl Physiol* 89: 411–426, 2003.

44. **Díaz-Troya S**, **Najib S**, **Sánchez-Margalet V**. eNOS, nNOS, cGMP and protein kinase G mediate the inhibitory effect of pancreastatin, a chromogranin A-derived peptide, on growth and proliferation of hepatoma cells. *Regul Pept* 125: 41–46, 2005.

45. **Dietz NM**, **Rivera JM**, **Warner DO**, **Joyner MJ**. Is nitric oxide involved in cutaneous vasodilation during body heating in humans? *J Appl Physiol* 76: 2047–53, 1994.

46. **Edholm OG**, **Fox RH**, **Macpherson RK**. Vasomotor control of the cutaneous blood vessels in the human forearm. *J Physiol* 139: 455–65, 1957.

47. **Eichna LW**, **Park CR**, **Nelson N**, **Horvath SM**, **Palmes ED**. Thermal regulation during acclimatization in a hot, dry (desert type) environment. *Am. J. Physiol.* 163: 585–597, 1950.

48. **Elias JE**, **Gygi SP**. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat Methods* 4: 207–214, 2007.

49. **Farrell DM**, **Bishop VS**. Permissive role for nitric oxide in active thermoregulatory vasodilation in rabbit ear. *Am J Physiol* 269: H1613-8, 1995.

50. **Farrell DM**, **Bishop VS**. The roles of cGMP and cAMP in active thermoregulatory vasodilation. *Am J Physiol* 272: R975-81, 1997.

51. **Fortney SM**, **Wenger CB**, **Bove JR**, **Nadel ER**. Effect of hyperosmolality on control of blood flow and sweating. *J Appl Physiol* 57: 1688–95, 1984.

52. **Fox R**, **Goldsmith R**, **Kidd D**, **Lewis G**. Bradykinin as a vasodilator in man. 157: 589–602, 1961.

53. **Fox RH**, **Edholm OG**. Nervous control of the cutaneous circulation. *Br Med Bull* 19: 110–4, 1963.

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

55. **Fujii N**, **Meade RD**, **Alexander LM**, **Akbari P**, **Foudil-bey I**, **Louie JC**, **Boulay P**, **Kenny GP**. iNOS-dependent sweating and eNOS-dependent cutaneous vasodilation are evident in younger adults, but are diminished in older adults exercising in the heat. *J Appl Physiol* 120: 318–327, 2016.

56. **Fujii N**, **Reinke MC**, **Brunt VE**, **Minson CT**. Impaired acetylcholine-induced cutaneous vasodilation in young smokers: roles of nitric oxide and prostanoids. *Am J Physiol Heart Circ Physiol* 304: H667-73, 2013.

57. **Gagge AP**, **Gonzalez RR**. Mechanisms of heat exchange: biophysics and physiology. In: *Handbook of Physiology - Environmental Physiology*. 1996, p. 45–83.

58. **Gagnon D**, **Crandall CG**. Sweating as a heat loss thermoeffector. *Handb Clin Neurol* 156: 211–232, 2018.

59. **Gibbon J**, **Landis E**. Vasodilation in the lower extremities in response to immersing the forearms in water. *J Clin Invest* 11: 1019–1036, 1932.

60. **Gill C**, **Parkinson E**, **Church MK**, **Skipp P**, **Scott D**, **White AJ**, **O'Connor CD**, **Clough GF**, **O'Connor CD**, **Clough GF**. A qualitative and quantitative proteomic study of human microdialysate and the cutaneous response to injury. *AAPS J* 13: 309–317, 2011.

61. **Gisolfi C**, **Robinson S**. Relations between physical training, acclimatization, and heat tolerance. *J Appl Physiol* 26: 530–4, 1969.

62. **González-Alonso J**, **Teller C**, **Andersen SL**, **Jensen FB**, **Hyldig T**, **Nielsen B**. Influence of body temperature on the development of fatigue during prolonged exercise in the heat. *J Appl Physiol* 86: 1032–9, 1999.

63. **Grant RT**, **Holling HE**. Further observations on the vascular responses of the human limb to body warming; evidence for sympathetic vasodilator nerves in the normal subject. *Clin Sci* 3: 273–285, 1938.

64. **Greaney JL**, **Stanhewicz AE**, **Kenney WL**, **Alexander LM**. Lack of limb or sex differences in the cutaneous vascular responses to exogenous norepinephrine. *J Appl Physiol* 117: 1417–1423, 2014.

65. **Green DJ**, **Maiorana AJ**, **Siong JHJ**, **Burke V**, **Erickson M**, **Minson CT**, **Bilsborough W**, **O'Driscoll G**. Impaired skin blood flow response to environmental heating in chronic heart failure. *Eur Heart J* 27: 338–343, 2005.

66. **Gross GJ**, **Hsu A**, **Pfeiffer AW**, **Nithipatikom K**. Roles of endothelial nitric oxide synthase (eNOS) and mitochondrial permeability transition pore (MPTP) in epoxyeicosatrienoic acid (EET)-induced cardioprotection against infarction in intact rat hearts. *J Mol Cell Cardiol* 59: 20–9, 2013.

67. **Güler AD**, **Lee H**, **Iida T**, **Shimizu I**, **Tominaga M**, **Caterina M**. Heat-evoked activation of the ion channel, TRPV4. *J Neurosci* 22: 6408–6414, 2002.

68. **Hammel HT**, **Pierce JB**. Regulation of Internal Body Temperature. *Annu Rev Physiol* 30: 641–710, 1968.

69. **Hammouda M**. The central and the reflex mechanism of panting. *J Physiol* 77: 319–36, 1933.

70. **Harrison S**, **Geppetti P**. Substance p. *Int J Biochem Cell Biol* 33: 555–76, 2001.

71. **Hartschuh W**, **Reinecke M**, **Weihe E**, **Yanaihara N**. VIP-immunoreactivity in the skin of various mammals: immunohistochemical, radioimmunological and experimental evidence for a dual localization in cutaneous nerves and merkel cells. *Peptides* 5: 239–45, 1984.

72. **Hayward JN**, **Baker MA**. A comparative study of the role of the cerebral arterial blood in the regulation of brain temperature in five mammals. *Brain Res* 16: 417–40, 1969.

73. **Heinz-Erian P**, **Dey RD**, **Flux M**, **Said SI**. Deficient vasoactive intestinal peptide innervation in the sweat glands of cystic fibrosis patients. *Science* 229: 1407–8, 1985.

74. **Heistad DD**, **Abboud FM**, **Mark AL**, **Schmid PG**. Interaction of thermal and baroreceptor reflexes in man. *J Appl Physiol* 35: 581–586, 1973.

75. **Hessemer V**, **Brück K**. Influence of menstrual cycle on shivering, skin blood flow, and sweating responses measured at night. *J Appl Physiol* 59: 1902–10, 1985.

76. **Hilton SM**, **Lewis GP**. The mechanism of the functional hyperaemia in the submandibular salivary gland. *J Physiol* 129: 253–71, 1955.

77. **Hirata K**, **Nagasaka T**, **Hirai A**, **Hirashita M**, **Takahata T**, **Nunomura T**. Effects of human menstrual cycle on thermoregulatory vasodilation during exercise. *Eur J Appl Physiol Occup Physiol* 54: 559–65, 1986.

78. **Hökfelt T**, **Johansson O**, **Ljungdahl Å**, **Lundberg JM**, **Schultzberg M**. Peptidergic neurones. *Nature* 284: 515–521, 1980.

79. **Holowatz LA**, **Houghton BL**, **Wong BJ**, **Wilkins BW**, **Harding AW**, **Kenney WL**, **Minson CT**. Nitric oxide and attenuated reflex cutaneous vasodilation in aged skin. *Am J Physiol - Hear Circ Physiol* 284: 1662–1667, 2003.

80. **Holowatz LA**, **Kenney WL**. Local ascorbate administration augments NO- and non-NO-dependent reflex cutaneous vasodilation in hypertensive humans. *AJP Hear Circ Physiol* 293: H1090–H1096, 2007.

81. **Holowatz LA**, **Kenney WL**. Up-regulation of arginase activity contributes to attenuated reflex cutaneous vasodilatation in hypertensive humans. *J Physiol* 581: 863–72, 2007.

82. **Holowatz LA**, **Kenney WL**. Chronic low-dose aspirin therapy attenuates reflex cutaneous vasodilation in middle-aged humans. *J Appl Physiol* 106: 500–505, 2008.

83. **Holowatz LA**, **Kenney WL**. Peripheral mechanisms of thermoregulatory control of skin blood flow in aged humans. *J Appl Physiol* 109: 1538–44, 2010.

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

85. **Holowatz LA**, **Thompson CS**, **Kenney WL**. L-Arginine supplementation or arginase inhibition augments reflex cutaneous vasodilatation in aged human skin. *J Physiol* 574: 573–81, 2006.

86. **Holowatz LA**, **Thompson CS**, **Kenney WL**. Acute ascorbate supplementation alone or combined with arginase inhibition augments reflex cutaneous vasodilation in aged human skin. *AJP Hear Circ Physiol* 291: H2965–H2970, 2006.

87. **Holzer P**. Neurogenic vasodilatation and plasma leakage in the skin. *Gen Pharmacol* 30: 5–11, 1998.

88. **Hsiao JK**, **Ball BA**, **Morrison PF**, **Mefford IN**, **Bungay PM**. Effects of different semipermeable membranes on in vitro and in vivo performance of microdialysis probes. *J Neurochem* 54: 1449–52, 1990.

89. **Hurley HJ**, **Mescon H**. Cholinergic innervation of the digital arteriovenous anastomoses of human skin; a histochemical localization of cholinesterase. *J Appl Physiol* 9: 82–4, 1956.

90. **Jessen C**, **Ludwig O**. Spinal cord and hypothalamus as core sensors of temperature in the conscious dog. *Pflügers Arch - Eur J Physiol* 324: 205–216, 1971.

91. **Jiang MH**, **Kaku T**, **Hada J**, **Hayashi Y**. Different effects of eNOS and nNOS inhibition on transient forebrain ischemia. *Brain Res* 946: 139–147, 2002.

92. **Jiang Z**, **Li C**, **Arrick DM**, **Yang S**, **Baluna AE**, **Sun H**. Role of nitric oxide synthases in early blood-brain barrier disruption following transient focal cerebral ischemia. *PLoS One* 9: e93134, 2014.

93. **Johnson BD**, **Beck KC**, **Proctor DN**, **Miller J**, **Dietz NM**, **Joyner MJ**. Cardiac output during exercise by the open circuit acetylene washin method: Comparison with direct Fick. *J Appl Physiol* 88: 1650–1658, 2000.

94. **Johnson JM**, **Kellogg DL**. Thermoregulatory and thermal control in the human cutaneous circulation. *Front Biosci (Schol Ed)* 2: 825–53, 2010.

95. **Johnson JM**, **Minson CT**, **Kellogg DL**. Cutaneous vasodilator and vasoconstrictor mechanisms in temperature regulation. *Compr Physiol* 4: 33–89, 2014.

96. **Johnson JM**, **Park MK**. Reflex control of skin blood flow by skin temperature: role of core temperature. *J Appl Physiol* 47: 1188–93, 1979.

97. **Johnson JM**, **Park MK**. Effect of upright exercise on threshold for cutaneous vasodilation and sweating. *J Appl Physiol* 50: 814–8, 1981.

98. **Johnson JM**, **Proppe DW**. Cardiovascular adjustments to heat stress. In: *Handbook of Physiology, Environmental Physiology*, edited by Fregley M, Blatteis C. Oxford University Press, 1996.

99. **Johnson JM**, **Proppe DW**. Cardiovascular Adjustments to Heat Stress. In: *Comprehensive Physiology*. Hoboken, NJ, USA: John Wiley & Sons, Inc., 2011, p. 215–243.

100. **Johnson RD**, **Justice JB**. Model studies for brain dialysis. *Brain Res Bull* 10: 567–571, 1983.

101. **Kahn RH**. Ueber die Erwärmung des Carotidenblutes. *Arch. Anat. Phys*. .

102. **Käll L**, **Canterbury JD**, **Weston J**, **Noble WS**, **MacCoss MJ**. Semi-supervised learning for peptide identification from shotgun proteomics datasets. *Nat Methods* 4: 923–5, 2007.

103. **Kalsi KK**, **González-Alonso J**. Temperature-dependent release of ATP from human erythrocytes: mechanism for the control of local tissue perfusion. *Exp Physiol* 97: 419–32, 2012.

104. **Kanosue K**, **Yanase-Fujiwara M**, **Hosono T**. Hypothalamic network for thermoregulatory vasomotor control. *Am J Physiol* 267: R283-8, 1994.

105. **Kanosue K**, **Zhang YH**, **Yanase-Fujiwara M**, **Hosono T**. Hypothalamic network for thermoregulatory shivering. *Am J Physiol* 267: R275-82, 1994.

106. **Kellogg DL**. In vivo mechanisms of cutaneous vasodilation and vasoconstriction in humans during thermoregulatory challenges. *J Appl Physiol* 100: 1709–1718, 2006.

107. **Kellogg DL**, **Hodges GJ**, **Orozco CR**, **Phillips TM**, **Zhao JL**, **Johnson JM**. Cholinergic mechanisms of cutaneous active vasodilation during heat stress in cystic fibrosis. *J Appl Physiol* 78229: 963–968, 2007.

108. **Kellogg DL**, **Johnson JM**, **Kenney WL**, **Pérgola PE**, **Kosiba WA**. Mechanisms of control of skin blood flow during prolonged exercise in humans. *Am J Physiol* 265: H562-8, 1993.

109. **Kellogg DL**, **Johnson JM**, **Kosiba WA**. Selective abolition of adrenergic vasoconstrictor responses in skin by local iontophoresis of bretylium. *Am J Physiol* 257: H1599-606, 1989.

110. **Kellogg DL**, **Johnson JM**, **Kosiba WA**. Control of internal temperature threshold for active cutaneous vasodilation by dynamic exercise. *J Appl Physiol* 71: 2476–82, 1991.

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

112. **Kellogg DL**, **Liu Y**, **McAllister K**, **Friel C**, **Pérgola PE**. Bradykinin does not mediate cutaneous active vasodilation during heat stress in humans. *J Appl Physiol* 93: 1215–1221, 2002.

113. **Kellogg DL**, **Morris SR**, **Rodriguez SB**, **Liu Y**, **Grossmann M**, **Stagni G**, **Shepherd AM**. Thermoregulatory reflexes and cutaneous active vasodilation during heat stress in hypertensive humans. *J Appl Physiol* 85: 175–80, 1998.

114. **Kellogg DL**, **Pérgola PE**, **Piest KL**, **Kosiba WA**, **Crandall CG**, **Grossmann M**, **Johnson JM**. Cutaneous active vasodilation in humans is mediated by cholinergic nerve cotransmission. *Circ Res* 77: 1222–1228, 1995.

115. **Kellogg DL**, **Zhao JL**, **Coey U**, **Green J V**. Acetylcholine-induced vasodilation is mediated by nitric oxide and prostaglandins in human skin. *J Appl Physiol* 98: 629–632, 2005.

116. **Kellogg DL**, **Zhao JL**, **Friel C**, **Roman LJ**. Nitric oxide concentration increases in the cutaneous interstitial space during heat stress in humans. *J Appl Physiol* 94: 1971–1977, 2003.

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

118. **Kellogg DL**, **Zhao JL**, **Wu Y**. Endothelial nitric oxide synthase control mechanisms in the cutaneous vasculature of humans in vivo. *Am J Physiol Heart Circ Physiol* 295: H123–H129, 2008.

119. **Kellogg DL**, **Zhao JL**, **Wu Y**. Roles of nitric oxide synthase isoforms in cutaneous vasodilation induced by local warming of the skin and whole body heat stress in humans. *J Appl Physiol* 107: 1438–1444, 2009.

120. **Kellogg DL**, **Zhao JL**, **Wu Y**, **Wu Y**. Roles of nitric oxide synthase isoforms in cutaneous vasodilation induced by local warming of the skin and whole body heat stress in humans. *J Appl Physiol* 107: 1438–44, 2009.

121. **Kendrick KM**. Use of microdialysis in neuroendocrinology. *Methods Enzymol* 168: 182–205, 1989.

122. **Kendrick KM**, **De La Riva C**, **Hinton M**, **Baldwin BA**. Microdialysis measurement of monoamine and amino acid release from the medial preoptic region of the sheep in response to heat exposure. *Brain Res Bull* 22: 541–544, 1989.

123. **Kenney WL**. Decreased core-to-skin heat transfer in mild essential hypertensives exercising in the heat. *Clin Exp Hypertens A* 7: 1165–72, 1985.

124. **Kenney WL**. Control of heat-induced cutaneous vasodilatation in relation to age. *Eur J Appl Physiol Occup Physiol* 57: 120–5, 1988.

125. **Kenney WL**, **Johnson JM**. Control of skin blood flow during exercise. *Med Sci Sports Exerc* 24: 303–12, 1992.

126. **Kenney WL**, **Kamon E**. Comparative physiological responses of normotensive and essentially hypertensive men to exercise in the heat. *Eur J Appl Physiol Occup Physiol* 52: 196–201, 1984.

127. **Kenney WL**, **Kamon E**, **Buskirk ER**. Effect of mild essential hypertension on control of forearm blood flow during exercise in the heat. *J Appl Physiol* 56: 930–5, 1984.

128. **Kenney WL**, **Morgan AL**, **Farquhar WB**, **Brooks EM**, **Pierzga JM**, **Derr JA**. Decreased active vasodilator sensitivity in aged skin. *Am J Physiol* 272: H1609-14, 1997.

129. **Klede M**, **Clough G**, **Lischetzki G**, **Schmelz M**. The effect of the nitric oxide synthase inhibitor N-nitro-L-arginine-methyl ester on neuropeptide-induced vasodilation and protein extravasation in human skin. *J Vasc Res* 40: 105–114, 2003.

130. **Kniffeki KD**, **Mense S**, **Schmidt RF**. Muscle receptors with fine afferent fibers which may evoke circulatory reflexes. *Circ Res* 48: I25-31, 1981.

131. **Kolka MA**, **Stephenson LA**. Control of sweating during the human menstrual cycle. *Eur J Appl Physiol Occup Physiol* 58: 890–5, 1989.

132. **Koroxendis GT**, **Shepherd JT**, **Marshall RJ**. Cardiovascular response to acute heat stress. *J Appl Physiol* 16: 869–872, 1961.

133. **Kräuchi K**, **Wirz-Justice A**. Circadian rhythm of heat production, heart rate, and skin and core temperature under unmasking conditions in men. *Am J Physiol* 267: R819-29, 1994.

134. **Kreilgaard M**. Assessment of cutaneous drug delivery using microdialysis. *Adv Drug Deliv Rev* 54 Suppl 1: S99-121, 2002.

135. **Krey JF**, **Wilmarth PA**, **Shin J-B**, **Klimek J**, **Sherman NE**, **Jeffery ED**, **Choi D**, **David LL**, **Barr-Gillespie PG**. Accurate label-free protein quantitation with high- and low-resolution mass spectrometers. *J Proteome Res* 13: 1034–1044, 2014.

136. **Latzka WA**, **Sawka MN**. Hyperhydration and glycerol: thermoregulatory effects during exercise in hot climates. *Can J Appl Physiol* 25: 536–45, 2000.

137. **Latzka WA**, **Sawka MN**, **Montain SJ**, **Skrinar GS**, **Fielding RA**, **Matott RP**, **Pandolf KB**. Hyperhydration: thermoregulatory effects during compensable exercise-heat stress. *J Appl Physiol* 83: 860–6, 1997.

138. **Laughlin MH**, **Newcomer SC**, **Bender SB**. Importance of hemodynamic forces as signals for exercise-induced changes in endothelial cell phenotype. *J Appl Physiol* 104: 588–600, 2008.

139. **Leahy MJ**, **de Mul FFM**, **Nilsson GE**, **Maniewski R**. Principles and practice of the laser-Doppler perfusion technique. *Technol Heal Care* 7: 143–162, 1999.

140. **Lewis T**, **Pickering G**. Vasodilation in the limbs in response to warming the body, with evidence for sympathetic vasodilator nerves in man. *Heart* 16: 33–51, 1931.

141. **Lu Y**, **Peters JL**, **Michael AC**. Direct Comparison of the Response of Voltammetry and Microdialysis to Electrically Evoked Release of Striatal Dopamine. *J Neurochem* 70: 584–593, 2002.

142. **Lundberg JM**, **Rudehill A**, **Sollevi A**, **Fried G**, **Wallin G**. Co-release of neuropeptide Y and noradrenaline from pig spleen in vivo: importance of subcellular storage, nerve impulse frequency and pattern, feedback regulation and resupply by axonal transport. *Neuroscience* 28: 475–86, 1989.

143. **Mandadi S**, **Sokabe T**, **Shibasaki K**, **Katanosaka K**, **Mizuno A**, **Moqrich A**, **Patapoutian A**, **Fukumi-Tominaga T**, **Mizumura K**, **Tominaga M**. TRPV3 in keratinocytes transmits temperature information to sensory neurons via ATP. *Pflügers Arch - Eur J Physiol* 458: 1093–1102, 2009.

144. **Martin HL**, **Loomis JL**, **Kenney WL**. Maximal skin vascular conductance in subjects aged 5-85 yr. *J Appl Physiol* 79: 297–301, 1995.

145. **McCord GR**, **Cracowski J-L**, **Minson CT**. Prostanoids contribute to cutaneous active vasodilation in humans. *AJP Regul Integr Comp Physiol* 291: R596–R602, 2006.

146. **McCord GR**, **Minson CT**. Cutaneous vascular responses to isometric handgrip exercise during local heating and hyperthermia. *J Appl Physiol* 98: 2011–2018, 2005.

147. **McGinn R**, **Carter MR**, **Barrera-Ramirez J**, **Sigal RJ**, **Flouris AD**, **Kenny GP**. Does type 1 diabetes alter post-exercise thermoregulatory and cardiovascular function in young adults? *Scand J Med Sci Sports* 25: e504–e514, 2015.

148. **Mcnamara TC**, **Keen JT**, **Simmons GH**, **Alexander LM**, **Wong BJ**. Endothelial nitric oxide synthase mediates the nitric oxide component of reflex cutaneous vasodilatation during dynamic exercise in humans. *J Physiol* 592: 5317–5326, 2014.

149. **Meendering JR**, **Torgrimson BN**, **Houghton BL**, **Halliwill JR**, **Minson CT**. Menstrual cycle and sex affect hemodynamic responses to combined orthostatic and heat stress. *Am J Physiol Circ Physiol* 289: H631–H642, 2005.

150. **Melgaard L**, **Hersini KJ**, **Gazerani P**, **Petersen LJ**. Retrodialysis: A review of experimental and clinical applications of reverse microdialysis in the skin. *Skin Pharmacol. Physiol.* 26 Karger Publishers: 160–174, 2013.

151. **Mihara H**, **Boudaka A**, **Sugiyama T**, **Moriyama Y**, **Tominaga M**. Transient receptor potential vanilloid 4 (TRPV4)-dependent calcium influx and ATP release in mouse oesophageal keratinocytes. *J Physiol* 589: 3471–3482, 2011.

152. **Milner P**, **Bodin P**, **Guiducci S**, **Del Rosso A**, **Kahaleh MB**, **Matucci-Cerinic M**, **Burnstock G**. Regulation of substance P mRNA expression in human dermal microvascular endothelial cells. *Clin Exp Rheumatol* 22: S24-7, 2004.

153. **Minson CT**, **Berry LT**, **Joyner MJ**. Nitric oxide and neurally mediated regulation of skin blood flow during local heating. *J Appl Physiol* 91: 1619–26, 2001.

154. **Minson CT**, **Wladkowski SL**, **Cardell AF**, **Pawelczyk JA**, **Kenney WL**. Age alters the cardiovascular response to direct passive heating. *J Appl Physiol* 84: 1323–32, 1998.

155. **Miyamoto T**, **Petrus MJ**, **Dubin AE**, **Patapoutian A**. TRPV3 regulates nitric oxide synthase-independent nitric oxide synthesis in the skin. *Nat Commun* 2: 369, 2011.

156. **Moorhouse VHK**. Effect of increased temperature of the carotid blood. *Am J Physiol Content* 28: 223–234, 1911.

157. **Nadel ER**, **Fortney SM**, **Wenger CB**. Effect of hydration state of circulatory and thermal regulations. *J Appl Physiol* 49: 715–21, 1980.

158. **Nagashima K**, **Nakai S**, **Tanaka M**, **Kanosue K**. Neuronal circuitries involved in thermoregulation. *Auton Neurosci* 85: 18–25, 2000.

159. **NICHOLSON C**, **RICE ME**. The Migration of Substances in the Neuronal Microenvironment. *Ann N Y Acad Sci* 481: 55–68, 1986.

160. **Nielsen BYB**, **Hales JRS**, **Strange S**, **Juel N**. Thermoregulatory adaptations. *J Physiol* 460: 467–485, 1993.

161. **Nielsen JB**, **Benfeldt E**, **Holmgaard R**. Penetration through the Skin Barrier. *Curr Probl Dermatol* 49: 103–11, 2016.

162. **Nixon PGF**. Human circulation regulation during physical stress. L. B. Rowell. *Stress Med* 4: 124–125, 1988.

163. **Niyonambaza SD**, **Kumar P**, **Xing P**, **Mathault J**, **Koninck P De**, **Boisselier E**, **Boukadoum M**, **Miled A**. A Review of neurotransmitters sensing methods for neuro-engineering research. *Appl Sci* 9: 1–31, 2019.

164. **Ogedegbe G**, **Pickering T**. Principles and techniques of blood pressure measurement. *Cardiol Clin* 28: 571–86, 2010.

165. **Pappireddi N**, **Martin L**, **Wühr M**. A Review on Quantitative Multiplexed Proteomics. *Chembiochem* 20: 1210–1224, 2019.

166. **Peier AM**, **Reeve AJ**, **Andersson DA**, **Moqrich A**, **Earley TJ**, **Hergarden AC**, **Story GM**, **Colley S**, **Hogenesch JB**, **McIntyre P**, **Bevan S**, **Patapoutian A**. A heat-sensitive TRP channel expressed in keratinocytes. *Science (80-)* 296: 2046–2049, 2002.

167. **Pérgola PE**, **Johnson JM**, **Kellogg DL**, **Kosiba WA**. Control of skin blood flow by whole body and local skin cooling in exercising humans. *Am J Physiol* 270: H208-15, 1996.

168. **Pérgola PE**, **Kellogg DL**, **Johnson JM**, **Kosiba WA**. Reflex control of active cutaneous vasodilation by skin temperature in humans. *Am J Physiol* 266: H1979-84, 1994.

169. **Peters JL**, **Michael AC**. Modeling Voltammetry and Microdialysis of Striatal Extracellular Dopamine: The Impact of Dopamine Uptake on Extraction and Recovery Ratios. *J Neurochem* 70: 594–603, 2002.

170. **Pierzga JM**, **Frymoyer A**, **Kenney WL**. Delayed distribution of active vasodilation and altered vascular conductance in aged skin. *J Appl Physiol* 94: 1045–1053, 2003.

171. **Prior JC**, **McKay DW**, **Vigna YM**, **Barr SI**. Medroxyprogesterone increases basal temperature: a placebo-controlled crossover trial in postmenopausal women. *Fertil Steril* 63: 1222–6, 1995.

172. **Ramathan NL**. A New Weighting System for Mean Surface Temperature of the Human Body. *J Appl Physiol* 19: 531–33, 1964.

173. **Ribera J**, **Marsal J**, **Casanovas A**, **Hukkanen M**, **Tarabal O**, **Esquerda JE**. Nitric oxide synthase in rat neuromuscular junctions and in nerve terminals of Torpedo electric organ: Its role as regulator of acetylcholine release. *J Neurosci Res* 51: 90–102, 1998.

174. **Roberts MF**, **Wenger CB**, **Stolwijk JAJ**, **Nadel ER**. Skin blood flow and sweating changes following exercise training and heat acclimation. *J Appl Physiol Respir Environ Exerc Physiol* 43: 133–137, 1977.

175. **Roddie IC**, **Shepherd JT**, **Whelan RF**. The vasomotor nerve supply to the skin and muscle of the human forearm. *Clin Sci* 16: 67–74, 1957.

176. **Roddie IC**, **Shepherd JT**, **Whelan RF**. The contribution of constrictor and dilator nerves to the skin vasodilatation during body heating. *J Physiol* 136: 489–97, 1957.

177. **Roosterman D**, **Goerge T**, **Schneider SW**, **Bunnett NW**, **Steinhoff M**. Neuronal control of skin function: The skin as a neuroimmunoendocrine organ. *Physiol Rev* 86: 1309–1379, 2006.

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

179. **Rowell LB**. Reflex control of the cutaneous vasculature. *J. Invest. Dermatol.* 69: 154–166, 1977.

180. **Rowell LB**. *Human circulation: Regulation during physical stress*. New York: Oxford Press, 1986.

181. **Rowell LB**. Cardiovascular Adjustments to Thermal Stress. Hoboken, NJ, USA: John Wiley & Sons, Inc., 2011.

182. **Sagawa S**, **Shiraki K**, **Yousef MK**, **Miki K**. Sweating and cardiovascular responses of aged men to heat exposure. *J Gerontol* 43: M1-8, 1988.

183. **Savage M V**, **Brengelmann GL**, **Buchan AM**, **Freund PR**. Cystic fibrosis, vasoactive intestinal polypeptide, and active cutaneous vasodilation. *J Appl Physiol* 69: 2149–54, 1990.

184. **Sawka MN**, **Coyle EF**. Influence of body water and blood volume on thermoregulation and exercise performance in the heat. *Exerc Sport Sci Rev* 27: 167–218, 1999.

185. **Sawka MN**, **Francesconi RP**, **Young AJ**, **Pandolf KB**. Influence of hydration level and body fluids on exercise performance in the heat. *JAMA* 252: 1165–9, 1984.

186. **Schittek B**, **Hipfel R**, **Sauer B**, **Bauer J**, **Kalbacher H**, **Stevanovic S**, **Schirle M**, **Schroeder K**, **Blin N**, **Meier F**, **Rassner G**, **Garbe C**. Dermcidin: A novel human antibiotic peptide secreted by sweat glands. *Nat Immunol* 2: 1133–1137, 2001.

187. **Schulze E**, **Witt M**, **Fink T**, **Hofer A**, **Funk RH**. Immunohistochemical detection of human skin nerve fibers. *Acta Histochem* 99: 301–9, 1997.

188. **Shastry S**, **Dietz NM**, **Halliwill JR**, **Reed AS**, **Joyner MJ**. Effects of nitric oxide synthase inhibition on cutaneous vasodilation during body heating in humans. *J Appl Physiol* 85: 830–4, 1998.

189. **Shastry S**, **Minson CT**, **Wilson SA**, **Dietz NM**, **Joyner MJ**. Effects of atropine and L-NAME on cutaneous blood flow during body heating in humans. *J Appl Physiol* 88: 467–72, 2000.

190. **Shibasaki M**, **Aoki K**, **Morimoto K**, **Johnson JM**, **Takamata A**. Plasma hyperosmolality elevates the internal temperature threshold for active thermoregulatory vasodilation during heat stress in humans. *AJP Regul Integr Comp Physiol* 297: R1706–R1712, 2009.

191. **Sjögren F**, **Anderson CD**. Sterile trauma to normal human dermis invariably induces IL1β, IL6 and IL8 in an innate response to "danger." *Acta Derm Venereol* 89: 459–465, 2009.

192. **Smith CJ**, **Santhanam L**, **Bruning RS**, **Stanhewicz A**, **Berkowitz DE**, **Holowatz LA**. Upregulation of inducible nitric oxide synthase contributes to attenuated cutaneous vasodilation in essential hypertensive humans. *Hypertension* 58: 935–942, 2011.

193. **Smith GD**, **Gunthorpe MJ**, **Kelsell RE**, **Hayes PD**, **Reilly P**, **Facer P**, **Wright JE**, **Jerman JC**, **Walhin J-PJ-P**, **Ooi L**, **Egerton J**, **Charles KJ**, **Smart D**, **Randall AD**, **Anand P**, **Davis JB**. TRPV3 is a temperature-sensitive vanilloid receptor-like protein. *Nature* 418: 186–190, 2002.

194. **Sokolnicki LA**, **Strom NA**, **Roberts SK**, **Kingsley-Berg SA**, **Basu A**, **Charkoudian N**. Skin blood flow and nitric oxide during body heating in type 2 diabetes mellitus. *J Appl Physiol* 106: 566–70, 2009.

195. **Sonkusare S**. A novel TRPV4-eNOS signaling pathway in pulmonary endothelium.

196. **Stanhewicz AE**, **Alexander LM**, **Kenney WL**. Oral sapropterin acutely augments reflex vasodilation in aged human skin through nitric oxide-dependent mechanisms. *J Appl Physiol* 115: 972–8, 2013.

197. **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: 791–797, 2012.

198. **Stanhewicz AE**, **Greaney JL**, **Larry Kenney W**, **Alexander LM**. Sex- and limb-specific differences in the nitric oxide-dependent cutaneous vasodilation in response to local heating. *AJP Regul Integr Comp Physiol* 307: R914–R919, 2014.

199. **Stapleton JM**, **Yardley JE**, **Boulay P**, **Sigal RJ**, **Kenny GP**. Whole-body heat loss during exercise in the heat is not impaired in type 1 diabetes. *Med Sci Sports Exerc* 45: 1656–1664, 2013.

200. **Stephenson LA**, **Kolka MA**. Menstrual cycle phase and time of day alter reference signal controlling arm blood flow and sweating. *Am J Physiol* 249: R186-91, 1985.

201. **Stephenson LA**, **Kolka MA**. Thermoregulation in women. *Exerc Sport Sci Rev* 21: 231–62, 1993.

202. **Stephenson LA**, **Kolka MA**. Esophageal temperature threshold for sweating decreases before ovulation in premenopausal women. *J Appl Physiol* 86: 22–8, 1999.

203. **Stephenson LA**, **Wenger CB**, **O'Donovan BH**, **Nadel ER**. Circadian rhythm in sweating and cutaneous blood flow. *Am J Physiol* 246: R321-4, 1984.

204. **Takamata A**, **Nagashima K**, **Nose H**, **Morimoto T**. Role of plasma osmolality in the delayed onset of thermal cutaneous vasodilation during exercise in humans. *Am J Physiol* 275: R286-90, 1998.

205. **Tankersley CG**, **Nicholas WC**, **Deaver DR**, **Mikita D**, **Kenney WL**. Estrogen replacement in middle-aged women: thermoregulatory responses to exercise in the heat. *J Appl Physiol* 73: 1238–45, 1992.

206. **Taylor WF**, **Johnson JM**, **Kosiba WA**, **Kwan CM**. Graded cutaneous vascular responses to dynamic leg exercise. *J Appl Physiol* 64: 1803–9, 1988.

207. **Thomas CM**, **Pierzga JM**, **Kenney WL**. Aerobic training and cutaneous vasodilation in young and older men. *J Appl Physiol* 86: 1676–86, 1999.

208. **Thompson AC**, **Justice JB**, **McDonald JK**. Quantitative microdialysis of neuropeptide Y. *J Neurosci Methods* 60: 189–198, 1995.

209. **Ulker P**, **Özen N**, **Abdullayeva G**, **Köksoy S**, **Yaraş N**, **Basrali F**. Extracellular ATP activates eNOS and increases intracellular NO generation in Red Blood Cells. *Clin Hemorheol Microcirc* 68: 89–101, 2018.

210. **Vaalasti A**, **Tainio H**, **Rechardt L**. Vasoactive intestinal polypeptide (VIP)-like immunoreactivity in the nerves of human axillary sweat glands. *J Invest Dermatol* 85: 246–8, 1985.

211. **Vandewauw I**, **Owsianik G**, **Voets T**. Systematic and quantitative mRNA expression analysis of TRP channel genes at the single trigeminal and dorsal root ganglion level in mouse. *BMC Neurosci* 14: 21, 2013.

212. **Venturini G**, **Colasanti M**, **Fioravanti E**, **Bianchini A**, **Ascenzi P**. Direct effect of temperature on the catalytic activity of nitric oxide synthases types I, II, and III. *Nitric Oxide - Biol Chem* 3: 375–382, 1999.

213. **Wallengren J**, **Ekman R**, **Sundler F**. Occurrence and distribution of neuropeptides in the human skin. An immunocytochemical and immunochemical study on normal skin and blister fluid from inflamed skin. *Acta Derm Venereol* 67: 185–192, 1987.

214. **Wenger CB**, **Roberts MF**, **Stolwijk JA**, **Nadel ER**. Nocturnal lowering of thresholds for sweating and vasodilation. *J Appl Physiol* 41: 15–9, 1976.

215. **Westerink BHC**, **Hofsteede HM**, **Damsma G**, **de Vries JB**. The significance of extracellular calcium for the release of dopamine, acetylcholine and amino acids in conscious rats, evaluated by brain microdialysis. *Naunyn Schmiedebergs Arch Pharmacol* 337: 373–378, 1988.

216. **Wick DE**, **Roberts SK**, **Basu A**, **Sandroni P**, **Fealey RD**, **Sletten D**, **Charkoudian N**. Delayed threshold for active cutaneous vasodilation in patients with Type 2 diabetes mellitus. *J Appl Physiol* 100: 637–641, 2006.

217. **Wilkins BW**, **Chung LH**, **Tublitz NJ**, **Wong BJ**, **Minson CT**. Mechanisms of vasoactive intestinal peptide-mediated vasodilation in human skin. *J Appl Physiol* 97: 1291–1298, 2004.

218. **Wilkins BW**, **Holowatz LA**, **Wong BJ**, **Minson CT**. Nitric oxide is not permissive for cutaneous active vasodilatation in humans. *J Physiol* 548: 963–9, 2003.

219. **Wilkins BW**, **Martin EA**, **Roberts SK**, **Joyner MJ**. Preserved reflex cutaneous vasodilation in cystic fibrosis does not include an enhanced nitric oxide-dependent mechanism. *J Appl Physiol* 55905: 2301–2306, 2007.

220. **Wilkins BW**, **Wong BJ**, **Tublitz NJ**, **McCord GR**, **Minson CT**. Vasoactive intestinal peptide fragment VIP10-28 and active vasodilation in human skin. *J Appl Physiol* 99: 2294–2301, 2005.

221. **Wilson TE**, **Tollund C**, **Yoshiga CC**, **Dawson EA**, **Nissen P**, **Secher NH**, **Crandall CG**. Effects of heat and cold stress on central vascular pressure relationships during orthostasis in humans. *J Physiol* 585: 279–285, 2007.

222. **Wong BJ**. Sensory nerves and nitric oxide contribute to reflex cutaneous vasodilation in humans. *Am J Physiol - Regul Integr Comp Physiol* 304: R651-656, 2013.

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

224. **Wong BJ**, **Minson CT**. Neurokinin-1 receptor desensitization attenuates cutaneous active vasodilatation in humans. *J Physiol* 577: 1043–51, 2006.

225. **Wong BJ**, **Tublitz NJ**, **Minson CT**. Neurokinin-1 receptor desensitization to consecutive microdialysis infusions of substance P in human skin. *J Physiol* 568: 1047–56, 2005.

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

227. **Wong BJ**, **Wilkins BW**, **Minson CT**. H1 but not H2 histamine receptor activation contributes to the rise in skin blood flow during whole body heating in humans. *J Physiol* 560: 941–8, 2004.

228. **Van Wylen DGL**, **Park TS**, **Rubio R**, **Berne RM**. Increases in cerebral interstitial fluid adenosine concentration during hypoxia, local potassium infusion, and ischemia. *J Cereb Blood Flow Metab* 6: 522–528, 1986.

229. **Zelis R**, **Mason DT**, **Braunwald E**. Partition of blood flow to the cutaneous and muscular beds of the forearm at rest and during leg exercise in normal subjects and in patients with heart failure. *Circ Res* 24: 799–806, 1969.

230. **Zhang HQ**, **Fast W**, **Marletta MA**, **Martasek P**, **Silverman RB**. Potent and Selective Inhibition of Neuronal Nitric Oxide Synthase by N^{ω} -Propyl- l -arginine. *J Med Chem* 40: 3869–3870, 1997.