CHRONIC PASSIVE HEAT THERAPY AS A NOVEL MEANS OF IMPROVING VASCULAR FUNCTION IN SEDENTARY HUMANS

by

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A DISSERTATION

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DISSERTATION ABSTRACT

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Cardiovascular disease is the leading cause of death in the developed world. The majority of cardiovascular diseases are characterized by disorders of the arteries, predominantly caused by endothelial dysfunction and arterial stiffening. Passive heat stress results in elevations in core temperature (inducing heat shock protein expression) and changes in cardiovascular hemodynamics, such as increased cardiac output and shear stress, that are similar to exercise. Thus, repeated passive heat stress ("heat therapy") may provide an alternative means of improving cardiovascular health, particularly for patients with limited exercise tolerance and/or capabilities. Therefore, the goal of this dissertation was to perform integrative studies to determine the effects of heat therapy on vascular function and the associated cellular pathways in young, sedentary humans.

Twenty subjects were assigned to participate in 8 weeks (4-5x/week) of heat therapy (N=10; immersion in a 40.5°C bath sufficient to maintain rectal temperature ≥38.5°C for 60 min/session) or thermoneutral water immersion (N=10; sham).

As discussed in Chapter V, we found that heat therapy improved numerous well-established biomarkers of conduit vessel/macrovascular function, including flow-mediated dilation (a measure of endothelial function), arterial stiffness, intima media

thickness, and blood pressure. Heat therapy also improved microvascular function, as discussed in Chapter VI, measured as improved cutaneous thermal hyperemia and nitric oxide-dependent dilation (the difference between microdialysis sites receiving Lactated Ringer's [control] and nitric oxide synthase inhibition). No changes were observed in any variables in sham subjects. In Chapter VII, we showed that both direct cellular heating and serum collected from human subjects following heat therapy improved nitric oxide bioavailability and angiogenesis in cultured endothelial cells, providing potential mechanisms by which heat therapy improves vascular function *in vivo*.

Therefore, the studies described herein provide comprehensive evidence that passive heat therapy improves vascular health and insight into the mechanisms involved. Our data presented in Chapters IV-VII, combined with pilot data we conducted in spinal cord injured individuals (Chapter VIII), strongly indicate that passive heat therapy could be used as a simple and effective tool to improve cardiovascular health in a variety of patient populations.

This dissertation includes published and unpublished co-authored material.

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2. Brunt VE, Minson CT. Book Chapter. Thermoregulatory considerations for the performance of exercise in SCI. In: *The Physiology of Exercise in Spinal Cord Injury.* 2016; *In Review.*

3. Brunt VE, Howard MJ, Francisco MA, Ely BR, Minson CT. Passive heat therapy improves endothelial function, arterial stiffness, and blood pressure in sedentary humans. J Physiol. 2016; doi:10.1113/JP272453. *In Press.*

4. Fujii N, Meade RD, Minson CT, Brunt VE, Boulay P, Sigal RJ, Kenny GP. Cutaneous blood flow during intradermal NO administration in young and older adults: roles of calcium-activated potassium channels an cyclooxygenase? Am J Physiol Regul Integr Comp Physiol. 2016; doi:10.1152/ajpregu.00041.2016. *In Press.*

5. Zarling JA, Brunt VE, Vallerga AK, Li W, Tao A, Zarling DA, Minson CT. Nitroxide pharmaceutical development for age-related degeneration and disease. Frontiers Genet. 2015; 6: 325.

6. Brunt VE, Fujii N, Minson CT. Endothelial-derived hyperpolarization contributes to acetylcholine-induced vasodilation in human skin in a dose-dependent manner. J Appl Physiol. 2015; 119 (9): 1015-22.

7. Ely BR, Brunt VE, Minson CT. Invited commentary. Can targeting glutamate receptors with long-term heat acclimation improve outcomes following hypoxic injury? Temperature. 2015; 2(1), 51-52.

8. Choi PJ*, Brunt VE *, Fujii N, Minson CT. New approach to measure cutaneous microvascular function: an improved test of NO-mediated vasodilation by thermal hyperemia. J Appl Physiol. 2014; 117 (3), 277-283. *Co-first authors

9. Fujii N, Brunt VE, Minson CT. Tempol improves thermal hyperemia through increasing nitric oxide bioavailability in young smokers. Am J Physiol Heart Circ Physiol. 2014; 306: H1505-11.

10. Brunt VE, Miner JA, Kaplan PF, Halliwill JR, Strycker LA, Minson CT. Short-term administration of progesterone and estradiol independently alter carotid-vasomotor, but not carotid-cardiac, baroreflex function in young women. Am J Physiol Heart Circ Physiol. 2013; 305: H1041-H1049.

11. 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. 2013; 115: 1290-6.

12. 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. 2013; 304: H667-73.

13. Brunt VE, Minson CT. KCa channels and EETs: major contributors in cutaneous thermal hyperaemia in humans. J Physiol. 2012; 590: 3523-3534.

14. Miner JA, Martini ER, Smith MM, Brunt VE, Kaplan PF, Halliwill JR, Minson CT. Short-term oral progesterone administration antagonizes the effect of transdermal estradiol on endothelium-dependent vasodilation in young healthy women. Am J Physiol Heart Circ Physiol. 2011; 301(4), H1716–H1722.

15. Brunt VE and Minson CT. Invited commentary. Cutaneous thermal hyperemia: more than skin deep. J Appl Physiol. 2011; 111(1), 5-7.

16. Brunt VE, Miner JA, Meendering JR, Kaplan PF, Minson CT. 17β-estradiol and progesterone independently augment cutaneous thermal hyperemia but not reactive hyperemia. Microcirculation. 2011; 18(5), 347–355.

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CHAPTER I

INTRODUCTION

BACKGROUND

Cardiovascular disease is the number one cause of death in the developed world, contributing to 34% of deaths in the United States in 2011 [1]. As the average life span of Americans is increasing, the prevalence of cardiovascular disease is increasing proportionally. Furthermore, the prevalence of risk factors for cardiovascular disease, such as obesity and hypertension, are increasing at an even greater rate [1]. In 2011, only 18% of Americans had >5 traditional risk factors that fell in the ideal range (out of 11) [1]. There is clearly desperate need for novel interventions to reduce risk of cardiovascular disease and incidence of cardiovascular-related mortality.

Exercise training has long been considered to be the ideal lifestyle intervention for reducing risk of cardiovascular-related and other diseases. However, there are many populations who are either unable to exercise to a great enough extent to induce cardiovascular adaptation or who are unwilling to do so. For example, patients who have sustained a spinal cord injury (SCI) are only able to perform some limited modalities of exercise training, many of which do not confer the same benefits as whole-body dynamic exercise [2,3]. Many other disease states are characterized by impaired exercise tolerance, e.g., patients with heart failure, peripheral artery disease, and obesity. Alternative therapies are needed to reduce cardiovascular risk in these patients.

Heat therapy, in the form of chronic use of hot baths and saunas, is an intervention which may confer similar cardiovascular benefits to exercise training. Both heat exposure and exercise result in an increase in body core temperature, which induces the expression of heat shock proteins (HSPs). In turn, HSPs upregulate and stabilize a variety of proteins important to the cardiovascular system. These include proteins which improve nitric oxide (NO) signaling [4], reduce oxidative stress [5] and reduce vascular inflammation [6], all of which greatly influence cardiovascular health. Additionally, both heat exposure and exercise increase heart rate and cardiac output, thereby increasing

blood flow and shear stress on the arteries [7]. Intermittent increases in shear stress are known to promote beneficial vascular remodeling. Indeed, it has been proposed that many of the beneficial effects of exercise training are mediated through increases in core temperature [8,9] and subsequent increases in blood flow and shear stress [10,11]. Therefore, heat therapy may offer a novel therapeutic tool for improving cardiovascular health.

While heat therapy has been used for centuries in several cultures, with common reports of improved well-being and quality of life [12], relatively few studies have investigated the physiological health benefits in humans. Recently, a 30-year prospective study revealed lifetime sauna use resulted in significantly reduced incidence of cardiovascular-related mortality, sudden cardiac death, and all-cause mortality [13], providing strong evidence that heat therapy could be used feasibly and effectively to treat cardiovascular risk. Additionally, studies in animals have demonstrated extensive cardioprotective benefits of >4 weeks of continuous heat exposure [14,15].

STATEMENT OF THE PROBLEM

Very few studies have investigated the physiological effects of heat therapy in humans. There is evidence that heat therapy can be used to treat clinical symptoms of cardiovascular diseases, but these studies were short-term (2-3 weeks) and offer little mechanistic insight. Only two studies have investigated changes in vascular function following greater than 3 weeks of heat therapy [16,17], and experimental limitations of these studies warrant further investigation. No studies have previously investigated the molecular mechanisms behinds improvements associated with heat therapy.

Therefore, the studies described in this dissertation are the first to comprehensively investigate the physiological benefits of long-term heat therapy in humans. As such, we chose to study a non-patient population in order to gather a thorough understanding of the mechanisms by which heat therapy improves vascular health. Since exercise training has profound CV effects on sedentary individuals, we expected to observe similar improvements with heat therapy. As the majority of CV diseases are characterized by vascular dysfunction [1], including impaired endothelial-dependent dilation and arterial stiffening, we chose to focus on vascular function. A

period of 8 weeks was chosen as studies in animals have demonstrated >4wks of passive heat exposure are required to induce complete cellular adaptation [14].

The overall purpose of this dissertation was to investigate the effects of 8 weeks of passive heat therapy on biomarkers of vascular function and on the associated molecular mechanisms in young, sedentary humans. Importantly, we were interested in both conduit vessel (macro-)vascular function and microvascular function, as these two sections of the vasculature are affected differently by cardiovascular disease progression and can respond differently to interventions.

In order to address this purpose, we performed one large study, in which twenty subjects were assigned to participate in 8 weeks of either heat therapy or thermoneutral water immersion, which served as a sham. The physiological responses experienced by subjects during heat therapy and thermoneutral water immersion sessions, and the adaptations they experienced across the 8 weeks, are characterized in Chapter IV. Subjects reported to the laboratory before, every 2 weeks during, and after the 8-week intervention for experimental testing. Subjects participated in two types of experimental sessions. In the first type of session, we investigated the effects of heat therapy on clinically relevant biomarkers of macrovascular function, the results of which are discussed in Chapter V. In the second type of session, we investigated the effects of heat therapy on cutaneous microvascular function, discussed in Chapter VI. During these studies, we performed cutaneous microdialysis, a technique which allows for the delivery of pharmacological agents to a localized area of skin. Using this technique, we were able to investigate some of the mechanisms behind potential improvements in microvascular function. In order to further elucidate the molecular mechanisms behind potential improvements in vascular function, we utilized a cell culture model. We isolated the effects of physiological elevations in temperature from the effects of circulating factors upregulated by heat therapy by incubating cultured endothelial cells at 39°C (to match the body temperature reached by subject undergoing heat therapy) or with serum collected from the human subjects before and after heat therapy. The results of these studies are discussed in Chapter VII. By performing these studies, we hoped to gain a comprehensive understanding of how heat therapy affects the vasculature.

Lastly, in order to test the translatability of our findings to a patient population, we conducted a pilot study in a small group of subjects who had sustained complete spinal cord injury (SCI), discussed in Chapter VII. SCI is a clinical population with a very high rate of cardiovascular-related deaths (~40%), who demonstrate impairments in many biomarkers of cardiovascular health, and who are unable to exercise to an extent great enough to gain the full cardioprotective effects of exercise. By studying these subjects, we were able to confirm that heat therapy is a viable treatment option for at-risk patient populations.

HYPOTHESES

The studies described in this dissertation were designed to test the following hypotheses, summarized in Figure 1.1 (next page):

1. In Chapter IV, we hypothesized that, relative to thermoneutral water immersion, 8 weeks of heat therapy would result in similar adaptations as are observed with exercise heat acclimation. Specifically, we hypothesized that resting core temperature and resting heart rate would be reduced, sweat rate during heat stress would be increased, and basal HSP expression in primary peripheral blood mononuclear cells would be increased. As a sub-aim, we also hypothesized that plasma volume would be expanded (measured in a sub-set of subjects).
2. In Chapter V, we hypothesized that, relative to thermoneutral water immersion, 8 weeks of heat therapy would improve clinically relevant biomarkers of macrovascular function. Specifically, we hypothesized that brachial artery flow-mediated dilation, carotid and superficial arterial compliance, aortic pulse wave velocity, carotid and superficial femoral intima media thickness, and blood pressure would all be improved.
3. In Chapter VI, we hypothesized that relative to thermoneutral water immersion, 8 weeks of heat therapy would improve cutaneous microvascular function. Specifically, we hypothesized that plateau cutaneous vascular conductance during local skin heating and NO-dependent dilation (difference in plateau between microdialysis sites receiving Lactated Ringer's [Control] and a NO synthase inhibitor) would be improved and that Tempol-mediated dilation (difference in plateau between

microdialysis sites receiving Lactated Ringer's and Tempol, a superoxide dismutase mimetic; an indicator of oxidative stress) would be reduced.

4. In Chapter VII, we hypothesized that simulating heat therapy in cultured endothelial cells would result in improved NO signaling, reduced oxidative stress, and increased angiogenesis. Specifically, we hypothesized that cultured endothelial cells exposed to physiological levels of direct heat (relative to cells incubated at 37°C) or to sera from heat-acclimated human subjects (relative to exposure to sera collected prior to heat therapy) would result in increased expression of endothelial NO synthase and superoxide dismutase, reduced superoxide production, and increased endothelial tubule formation (indicative of angiogenesis).

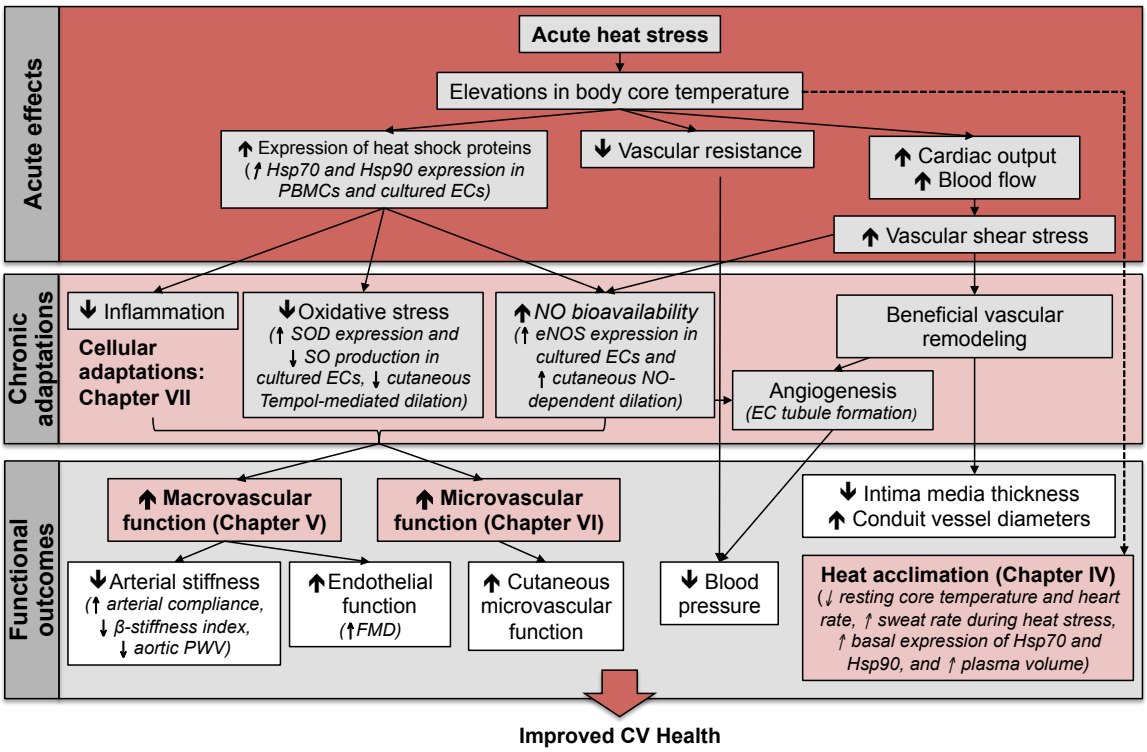

Figure 1.1 Schematic of our hypotheses for how heat therapy will improve cardiovascular health. Text in parentheses denotes outcome variables measured as part of this dissertation. Abbreviations: HSP, heat shock protein; PBMCs, peripheral blood mononuclear cells; ECs, endothelial cells; SOD, superoxide dismutase; SO, superoxide; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; PWV, pulse wave velocity; FMD, flow-mediated dilation.

SIGNIFICANCE

Although hot tubs and saunas have been used for their health benefits for centuries, very few studies have explored the physiological effects of heat therapy, and only two investigations have studied effects beyond 3 weeks, and none have investigated the mechanisms behind improvements. The studies described in this dissertation provide novel comprehensive insight into the physiological benefits of heat therapy.

The long-term goal of this line of research is to develop a model for improving cardiovascular health, which could be used as an alternative or adjunctive therapy for patient populations who are unable to exercise to an extent great enough to gain cardioprotective effects. While we studied SCI patients as add-on experiment to this dissertation, there are many other patient populations who have limited exercise capabilities (e.g., amputees, advanced diabetics, morbidly obese, heart failure patients, etc.). Heat therapy has the potential to improve cardiovascular health and mortality in all of these patient populations. Therefore, our research seeks to shift the face of clinical practice regarding the treatment of these patient groups, hopefully thereby improving quality of life, reducing morbidity and mortality, and reducing the societal healthcare burden of treating cardiovascular diseases in these populations.

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PUBLISHED MATERIAL

Material contained in Chapter V has been published in the *Journal of Physiology*. Material contained in Chapter VI has been submitted for publication in the *Journal of Applied Physiology* and is currently under review (submitted May 5, 2016).

CHAPTER II

REVIEW OF THE LITERATURE

INTRODUCTION

While heat therapy has been used for centuries in several cultures, with common reports of improved well-being and quality of life [12], relatively few studies have investigated the physiological health benefits in humans. As introduced in the previous chapter, we believe that the mechanisms underlying potential improvements in vascular function with heat therapy are two fold. First, increases in body core temperature induce expression of heat shock proteins (HSPs), which in turn stabilize a variety of proteins important to the cardiovascular system. Secondly, hyperthermia increases cardiac output and therefore increases shear stress on the vasculature, which is known to promote beneficial vascular remodeling [18]. The cellular effects of both HSPs and of increases in shear stress have been well characterized. Therefore, in this chapter, we will start by characterizing the acute effects of heat stress. We will then present the evidence for how increased expression of HSPs improves vascular health, focusing on both data characterizing the direct intracellular effects of HSPs and the essential role of HSPs in whole-organism adaptation and protection against novel stressors, as demonstrated in animal studies. Next, we will present evidence for how increases in shear stress improve vascular function, demonstrating why shear stress is essential for arterial adaptation. Lastly, we will describe the studies that have been performed thus far in humans. While the human studies are limited in number, the evidence supporting heat therapy as a viable treatment option for cardiovascular risk is compelling.

PHYSIOLOGICAL EFFECTS OF ACUTE PASSIVE HEATING

Although the long-term effects of heat therapy have been under-studied, the acute effects of passive heat stress have been well characterized. There are a few different modalities available for eliciting whole-body passive heat stress. In laboratory settings, the most commonly used method for passive heating has been water-perfused suits.

Subjects wear a nylon suit that has tubes sewn into the lining, which covers their entire body with the exception of their hands, feet, and face. Water can then be circulated through the tubing at any temperature, thereby increasing skin temperature.

Saunas and hot tubs are the modalities for heating most-widely used in community settings. There are two predominant types of saunas: traditional Finnish saunas, and far infrared saunas popularized by the Japanese (also referred to as 'Waon therapy'). Finnish saunas are typically maintained with air temperatures of 80-100°C. Increases in humidity can be achieved by pouring water over hot rocks. The Finns typically enter the sauna for 1-5 min at a time in order to increase body core temperature and initiate sweating. They will then exit and rest outside the sauna for 15-30 min. They may repeat these cycles 1-5 more times, depending on how accustomed they are to sauna use. In far infrared saunas, body temperature is increased by infrared waves that penetrate the body, and thus the air temperature is kept cooler at around 50-60°C. Individuals typically remain in these saunas for 15-30 min at a time.

Although widely used and available for community-use in many cultures, less has been documented regarding hot baths and Jacuzzis. Hot water immersion is advantageous because the conduction of heat in water is approximately 24x that in air, meaning that water temperature can be considerably lower than air temperature needs to be to achieve similar increases in body core temperature. Water temperature in hot tubs is typically kept around 38-41°C. A key difference of hot water immersion compared to other types of passive heating is hydrostatic pressure, which is known to have some effects on cardiovascular hemodynamics [19,20].

Core temperature

The goal of all modes of passive heating is to increase body core temperature. The rate at which increases occur will be dependent on the transfer of heat from the environment to the body and the effectiveness of the body's heat loss mechanisms. In both air and water, heat is transferred from the environment to the body through conduction, or the transfer of heat energy between objects. Via convection in the blood, this heat is transferred from the skin to the body core. Infrared saunas transfer heat in a

different way, through radiation. Infrared waves are able to penetrate the skin, effectively heating the body from the inside out.

Under resting thermoneutral conditions, heat is lost from the body to the environment primarily through radiation. During hyperthermia, increases in skin blood flow routes warm blood from the core to the surface of the skin and sweating allows for this heat to be lost by evaporation. In the majority of environmental conditions found on Earth, humans are able to dissipate heat so effectively (in air) that it is difficult to achieve increases in body core temperature at rest. As such, traditional saunas have to be as hot as 80-100°C in order to increase core temperature at a reasonable rate. Increases in hot water are much easier to achieve. Not only is heat transferred at a faster rate, but the body's cooling mechanisms do not work under water - a phase change from liquid to air is required for sweating to remove heat and the core-to-skin temperature gradient must favor heat loss (whereas it favors heat gain when skin temperature, i.e., hot water temperature, is greater than core temperature).

In typical Finnish sauna sessions, rectal temperatures increase by 0.2-1.0°C, depending on air temperature and length of stay (70-100°C for 15-30 min) [21]. This is a fairly modest increase in core temperature relative to theoretical thresholds that are considered to be necessary in order to elicit long-term adaptation, and yet a multitude of long-term benefits have been reported. Based on animal and some human work, the general consensus in the field is that body core temperature needs to reach 38.0-38.5°C during sessions in order to confer adaptation [22]. With far infrared sauna, core temperature has been reported to increase by 1.0-1.2°C in 15 min sessions [23,24]. The rate of rise in core temperature with hot water immersion depends on the water temperature and how deeply immersed individuals are in the water. Increases of 1.0-1.2°C in just 10 min have been reported in 41°C water [24].

Sweating and skin blood flow

The thermoregulatory centers in the hypothalamus respond to elevations in skin and body core temperature by first withdrawing cutaneous adrenergic sympathetic vasoconstriction and then by proportionally increasing cutaneous cholinergic sympathetic output. The cutaneous cholinergic sympathetic nerves co-release acetylcholine and

unknown neurotransmitter(s) [25], stimulating the sweat glands to increase sweat output and the cutaneous microvessels to vasodilate. Acetylcholine is the primary stimulus for inducing sweating, but inhibition of acetylcholine has little effect on skin blood flow during hyperthermia [25]. Instead, the unknown neurotransmitter(s) are thought to be the primary signal for active vasodilation. These unknown neurotransmitter(s) are thought to be vasointestinal peptide (VIP) [26], pituitary adenylate cyclase activating peptide (PACAP) [27], and/or substance P [28]. Additionally, ~30-45% of the vasodilation can be blocked with NOS inhibition [29-31]. During passive heating, neuronal NOS appears to be the primary isoform responsible for NO production [32,33], suggesting NO may be one of the unknown 'neutrotransmitters' released from the sympathetic nerves.

Active skin vasodilation and sweating are initiated once body core temperature reaches a temperature threshold, usually ~0.4°C above resting core temperature [7]. Skin temperature modulates this, in that, the higher the skin temperature, the lower this threshold [34]. With passive heat stress, skin blood flow can increase as much as 4.5-7.0L above resting [35].

Cardiovascular hemodynamics

Cardiac output must increase to support the increased skin blood flow and sweating. The magnitude of increase will be dependent on the rise in core temperature, but increases to as high as 13 L/min have been reported with passive heat stress [35]. With traditional Finnish sauna, there have been varying reports from only a small increase in cardiac output (0.47L/min) [36] up to a 75% increase [37,38]. This variability is likely attributable to the varying air temperatures and duration of sauna exposure used by community members. Far infrared sauna and hot water immersion elicit similar increases in cardiac output, with increases of 30-50% typically reported with both [24].

The effects on other cardiovascular hemodynamics can differ between different modalities of heat stress. In all modalities, cutaneous vasodilation shifts blood volume towards the skin for thermoregulation. Blood is redistributed away from the splanchnic and renal circulations in order to support the increased skin blood flow [39,40]; however, venous return to the heart and thus cardiac filling pressure are still reduced relative to normothermia. The reduced venous return limits increases in stroke volume despite the

effects of the sympathetic nervous system on contractility. Changes in stroke volume will depend on the balance between reductions in filling pressure, sympathetically-mediated increases in contractility, and the effects of temperature of myocardial function (contractility can be impaired during severe hyperthermia, i.e., core temperature >40°C [41,42]). As such, studies have shown that stroke volume either does not change during heat stress or only increases minimally [43-46]. With hot water immersion, the compressive hydrostatic force exerted on the body by water should aid venous return and allow for greater increases in stroke volume; however, the one study which has directly compared hemodynamic effects between sauna and hot water immersion observed no difference in stroke volume between the two heating modalities [24].

Heart rate can increase fairly high, to levels similar to moderate intensity exercise. Since increases in stroke volume are minimal, the high heart rates are necessary to support increases in cardiac output. Heat may also have a direct chronotropic effect on the sinoatrial node [47,48], although this will have much less a contribution to the increased heart rate compared to neural input. In general in humans, heart rate increases by approximate 30 beats per minute for every degree increase in core temperature [7].

As a result of the cutaneous vasodilation, peripheral vascular resistance generally decreases, although this is offset by increases in splanchnic and renal vascular resistance [40]. Systolic blood pressure typically increases due to increases in cardiac output and heart rate [40]; however, reductions in systolic blood pressure may be observed with sauna [24,49]. Diastolic blood pressure can decrease considerably, particularly with sauna [24,49]. Hydrostatic pressure with hot water immersion may help limit decreases in diastolic blood pressure. Importantly, intermittent reductions in peripheral vascular resistance and diastolic blood pressure with passive heat stress (along with other mechanisms, such as improved endothelium-dependent dilation and angiogenesis) may help facilitate reductions in resting blood pressure with chronic use of heat therapy.

Intracardiac pressures

As discussed, the result of redistribution of blood to the skin for thermoregulation is a reduction in central venous pressure [35,40,50]. Left ventricular filling pressure, as measured by pulmonary capillary wedge pressure, has been shown to decrease in parallel

[50,51]. Pulmonary artery pressure may either increase [52,53] or decrease [45,54,55], depending on the balance between increases in right heart cardiac output and reductions in pulmonary vascular resistance. In contrast, intracardiac pressures increase during hot water immersion as the hydrostatic compressive force of the water aids venous return to the heart [24,56]. Due to this difference, it has been proposed that sauna bathing may be safer for heart failure patients than hot water immersion, as strain on the heart is reduced [24]. However, the patterns observed with hot water immersion mimic the hemodynamic effects of exercise more closely and so may be more effective at inducing cardiovascular adaptations.

Potential negative effects of passive heat stress

In Finland, the vast majority of residents participate in sauna bathing at least once per week. Sauna bathing is a cultural activity that families and communities participate in together, and thus individuals begin participating as children and tend to continue to do so their entire lives. Because of the widespread prevalence of sauna bathing, and because of the contention in Western medicine that passive heat stress is potentially dangerous, the Finns have performed a multitude of studies demonstrating no adverse effects of sauna bathing across many populations (see Kukkonen-Harjula & Kauppinen, 2006 [57] for a review). These include patients with stable cardiovascular diseases [58,59], such as hypertension and heart failure, children [60,61], and pregnant women [62,63]. Additionally, long-term sauna has no effect on fertility, which has been regarded as a concern in the Western world. Although some studies have reported reductions in sperm count following acute sauna bathing [64,65], Finnish men on average have higher sperm counts than men in other countries [66] and time to pregnancy (a indicator of fertility) is shorter in Finland compared to other countries [67]. Especially for individuals who are already heat adapted, as most Finns are, sauna is very safe.

The primary contention with passive heat stress is that it may increase risk of cardiac arrhythmias, and thus in Western medicine, it has been contraindicated for patients with cardiovascular diseases. However, in a study monitoring 98 acute myocardial infarction patients and age-matched control subjects, only 8% experienced arrhythmias during and after sauna bathing; whereas, 18% of subjects experienced

arrhythmias during sub-maximal exercise [68]. Furthermore, of all sudden deaths that occurred in Finland in 1970, only 1.7% occurred within 24 h after sauna bathing (this includes those that may be related to sauna and those that definitely are not, e.g., motor vehicle accidents) [68]. Of the non-accidental deaths, the majority were caused by myocardial infarction related to alcohol consumption, which is known to increase risk of cardiac events even without hyperthermia [69].

There are some cardiovascular contraindications for heat therapy. Primarily, these include unstable conditions, for which exercise would also be contraindicated. Those such conditions which have been studied include severe aortic stenosis, unstable angina pectoris, recent myocardial infarction, recent stroke or transient ischemic attack, and elderly individuals prone to orthostatic hypotension [58,70].

Similarities to exercise

In general, the cardiovascular effects of passive heat stress are similar to low-to-moderate intensity aerobic exercise. Both increase body core temperature to similar extents. As described in more depth in the next section, elevated body core temperature increases the expression of HSPs which upregulate a variety of other proteins beneficial to the cardiovascular system, allowing for long-term cellular adaptation and protection against stressors. The increase in core temperature causes a subsequent increase in cardiac output, again similar to exercise. Although with passive heat exposure, the increased blood flow is routed to the skin for thermoregulation rather than to skeletal muscle, both exercise and passive heating result in similar increases in shear stress on the conduit arteries, which is essential for arterial adaptation. The mechanisms for how repeated increases in shear result in arterial adaption will be reviewed in a later section.

HEAT SHOCK PROTEINS & THEIR ROLE IN VASCULAR FUNCTION

Heat shock proteins (HSPs) are a class of proteins synthesized by all organisms in response to heat or other stressors. They were first discovered in 1962 in drosophila by Ritossa [71]. Although HSPs were named for the observation that their expression is increased rapidly and intensely at the onset of heat shock, they are also expressed in

response to acute hypoxia [72], ultraviolet radiation [73], cytokines [74,75], cold stress [76], and exercise [8,77,78].

It is believed that HSPs have two major roles. First, HSPs are thought to protect cells from the damaging effects of heat and other stressors. In support of this notion is the observation that HSP expression is increased rapidly and intensely at the onset of heat shock [79]. Upon exposure to milder doses of stressors, HSPs also confer tolerance against subsequent otherwise lethal stress. For example, thermotolerance refers to the ability of cells which have previously been exposed to non-lethal heat shock to survive a subsequent otherwise lethal shock. In addition to conferring thermotolerance, HSPs have been shown to also confer tolerance against a variety of other stressors, including ischemia [80,81], ultraviolet radiation [82], and toxicity from cytokines such as tumor necrosis factor (TNF)-α [83]. It is believed that much of this conferred tolerance stems from HSPs role in processing stress-denatured proteins [84] and preventing disruption of structural proteins [85], as described in more depth below.

Secondly, HSPs are also expressed under non-stressed conditions and play important roles in cell function, namely in translocating proteins to other locations within the cell, chaperoning proteins across cell membranes, and stabilizing various proteins and receptors. Many of these processes involve unfolding and refolding of proteins. HSPs can be found, usually associated with other proteins, in the nucleus, cytosol, mitochondria, endoplasmic reticulum, and in close proximity to the plasma membrane [86].

HSPs are named for their molecular weight. The different families of HSPs have been found to play different roles within the cells, although all aimed at stabilizing, chaperoning, and refolding of proteins.

HSP90 family. The HSP90 family consists of proteins which are constitutively expressed and abundant at normal temperatures, but which can be further induced by heat. Their primary role is to associate with other proteins. Through association, HSP90 proteins aid in translocation and stabilization of other proteins. In some such proteins (e.g., plasma membrane proteins and tyrosine kinases), Hsp90 associates immediately after the protein has been synthesized and, as soon as Hsp90 dissociates, the protein is phosphorylated and becomes active [87,88]. These observations led to the proposed role that Hsp90 aids with

transport of proteins from the nucleus to other locations in the cells, keeping these proteins stable and inactive until they are in their appropriate place. In other types of proteins (for example, nitric oxide synthase as described below), association with Hsp90 allows for phosphorylation. Hsp90's third role is with stabilization of steroid hormone receptors. Hsp90 association with estrogen, progesterone, and glucocorticoid receptors (and likely others, although not yet specifically shown) ensures activation of the receptors only when the actual hormone is present [89,90].

Although HSP90 proteins are constitutive expressed, they are also inducible. Hsp90 expression is increased in cells and intact animals following stressors, including following acute bouts of exercise [8]. Hsp90 has been shown to translocate into the cellular nucleus with heat shock [91], suggesting these proteins play a role in either gene transcription or chaperoning newly formed proteins out of the nucleus [79].

HSP70 family. The HSP70 family consists of highly inducible proteins which are essential for cell growth and repair. For example, *Escherichia coli* cells cannot grow at temperatures of 42°C if they have a genetic deletion of one of the HSP70-related proteins, dnaK [92]. Within the HSP70 family, four have been identified in mammals: Hsp70, Hsp72, p72, and Grp78 [79]. Hsp70 and Hsp72 are the primary heat inducible proteins [93], although Hsp70 is also involved in regulating the cell cycle [94]. P72 (Hsp73) is highly abundant in growing cells. Grp78 (glucose-related protein) is generally found in the endoplasmic reticulum [95], where it binds to a variety of transmembrane proteins, typically those that are unfolded or misfolded [79]. Grp78 protein content is increased with starvation, anoxia, viral infection, but typically not heat shock [96].

HSP70 proteins are involved in a variety of cell processes, including DNA replication, post-translational transport of proteins across membranes in the endoplasmic reticulum and mitochondria [97], and binding proteins within the endoplasmic reticulum. Most of these processes involve hydrolysis of ATP [98] and HSP70 proteins are known to be able to bind ATP with high affinity [99,100]. Translocation of proteins also requires the protein to be unfolded to cross the membrane and then refolded on the other side [101].

These roles all work towards protecting the cell against damage. As such, it is thought that thermotolerance is most likely attributable to the processing of stress denatured proteins by HSP70s [84]. HSP70 proteins are highly inducible with heat shock and other stressors. With heat shock, Hsp70 concentrates mainly in the nucleus and in close proximity to cell membranes [102]. In the nucleus, Hsp70 and p72 associate with the nucleoli, which experiences severe structural changes with hyperthermia, aiding nucleolar recovery after heat shock [103]. In intact animals, Hsp70 protein content has been shown to be increased in rodent tissues following acute hyperthermia [104-108], ischemia [105,107], peripheral injection of pyrogens to simulate fever [109,110], local injections of neurotoxins [111], and exercise [112,113]. In humans, Hsp70 has been shown to be inducible by exercise [77,78] and exercise in the heat [114,115]. Interestingly, Hsp70 is also inducible in cultured endothelial cells by shear stress [116,117].

Small HSPs. All other known HSPs smaller than ~30kDa have been grouped into the 'small HSP' family. As such, they are a much more diverse group. They have been studied to a much lesser extent than the other families of HSPs and so much less is currently understood about their roles in the cell. They are expressed at different stages of cell development under normal temperatures and are inducible in response to heat shock and other stresses [118,119]. Hsp27 has been shown to prevent actin microfilament disruption under stress conditions [85], and so a role of small HSPs has been proposed in thermotolerance. Heat-induced cytokine production also independently signal increased Hsp27 expression, which is in turn involved in the signal transduction of these cytokines, including interleukin (IL)-1 and TNF-α, perhaps dampening their harmful effects on the cell [74].

Besides heat, increases in fluid shear stress have also been shown to increase Hsp27 phosphorylation [116]. In isolated arteries, increased Hsp20 protein content is correlated with improved flow-mediated vasodilation [120] and nitroglycerine-induced vasodilation is associated with Hsp20 phosphorylation [121]. This link offers another mechanism for how repeated elevations in shear stress can improve vascular function (reviewed in depth below).

Magnitude and time course of HSP expression. In general, the greater the duration of heat exposure and the higher the ambient temperature, the greater the magnitude of HSP expression. For example, Harris et al. [122] observed no change in Hsp70 protein following 1h of heat shock at 42°C in bovine aortic endothelial cells, but observed an 8-fold increase in Hsp70 with 45°C heat shock of the same duration. Similarly, in rats, the longer the duration of heat stress (measured following 40, 60, and 90min) and the higher core temperature rose (37, 39, 42, and 45°C), the greater the induction of Hsp70 and Hsp27 mRNA [104]. Cultured cells often require higher ambient temperatures to observe increases in HSPs compared to intact organisms, or require extended periods of recovery time following heat exposure before peak expression of HSPs [79,123]. For example, Harris et al. [122] observed that 15min heat stress at 42°C in rats was sufficient to obtain a similar fold increase in Hsp70 in aortic tissue as what was obtained in cultured aortic cells following 1h at 45°C.

Typically, HSP mRNA is increased immediately following heat exposure. Both Hsp70 and Hsp27 mRNA have been shown to peak within 1h following heat stress in rodents, returning to baseline levels by ~6h [104,107]. This has been shown across various tissue types, including brain, lung, liver, kidney, and skin tissue [104,107]. However, protein abundance generally does not peak for hours after heat stress.

The time course of HSP induction may differ across species and tissue types. In skeletal muscle, Oishi et al. [124] showed that the timing for when Hsp72 protein abundance peaked following 1h heat stress (muscle temp reached 42°C) depended on the type of muscle fibers. In the soleus muscle of the rat, a predominantly slow-twitch muscle, Hsp72 protein content was elevated immediately following heat stress, with peak content at 4h; whereas, in the plantaris muscle, which is predominantly fast-twitch, Hsp72 protein content was not elevated until 24-48h following heat stress. Similar findings were observed in deep (more slow-twitch) and superficial (more fast-twitch) regions of the gastrocnemius [125]. In exercising humans, Hsp70 content in the vastus lateralis muscle does not increase until ~48h after exercise and is maintained elevated above baseline levels for up to 6-7 days [126,127]; however, the exercise stimulus may produce a different pattern of expression compared to passive heat stress. The one study that measured muscle HSP content in heat stressed humans observed no change in Hsp70

content [128], but they studied physically active men who may have had a blunted HSP response to acute stress. Interestingly, HSP content in heat stressed human peripheral blood mononuclear cells increases much more quickly, peaking following 1h after heat stress and returning back to baseline levels after ~5h [129].

Roles of HSPs in vascular function

The primary signaling pathways that are altered with the progression of cardiovascular diseases and which have the greatest impact on vascular function are the nitric oxide pathway, oxidative stress, and vascular inflammation. HSPs can interact with all three, stabilizing proteins which result in a phenotype that has more bioavailable NO, is anti-oxidative, and is anti-inflammatory. The interactions of HSPs with these pathways are reviewed below and summarized in Figure 2.1 (page 20).

Nitric oxide signaling. NO is produced by the enzyme nitric oxide synthase (NOS), of which there are three primary isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). When activated under coupled conditions, NOS catalyzes the conversion of L-arginine into L-citrulline and NO. Hsp90 is essential for the activation and stabilization of both eNOS [4] and nNOS [130]. When Hsp90 is inhibited with geldanamycin, eNOS activity in response to bradykinin, VEGF, histamine, and fluid shear stress is reduced by 50-90% [131,132]. eNOS binds to the middle region (residues 259-615) of Hsp90 [131].

eNOS activation is dependent on the calcium state of the cell. While inactive, eNOS is bound to the caveolar cell membrane by caveolin-1. When bound with calcium, calmodulin can dissociate eNOS from caveolin-1, a process which is mediated by eNOS association with Hsp90 [133,134]. Once free in the cytoplasm, eNOS can then be activated, which can be achieved by various kinases, including mitogen-activated protein kinases (MAPK) and Akt (protein kinase B). Hsp90 is also important for activation. For example, Akt phosphorylation in endothelial cells is dependent on Hsp90 [135,136]. When activated, eNOS catalyzes the conversion of L-arginine to NO and L-citrulline. In order to enable proper flow of electrons during this conversion, several bound cofactors must be present, one of which is Hsp90. Another important and commonly studied one is

tetrahydrobiopterin. In the absence of one of these cofactors (termed 'uncoupling'), eNOS instead produces the damaging free radical superoxide ($O_2^{\cdot -}$) [4].

Through these extensive roles of Hsp90, eNOS activity and therefore NO production can be enhanced by increased Hsp90 protein independent of changes in total eNOS protein. For example, Harris et al. [137] observed increased eNOS activity in mice following 10 weeks of exercise training with no change in total eNOS protein. However, Hsp90 was increased following exercise training, as well as Hsp90 association with eNOS.

Oxidative Stress. Reactive oxygen species, such as superoxide and hydrogen peroxide, are produced as byproducts of oxidative metabolism in the mitochondria and by other intracellular enzymes, such as NADPH oxidases. Under normal, healthy conditions, the production of ROS is balanced by their reduction and removal from the cell by anti-oxidative enzymes. However, when this balance is disrupted, either by excessive production of ROS or impaired anti-oxidative mechanisms, ROS build up and can have damaging effects on the cells, known as 'oxidative stress'. Oxidative stress occurs with normal aging and is thought to be involved in the pathogenesis of many other diseases.

In the vasculature, NADPH oxidases are a primary source of ROS, which transfer electrons from NADPH to O_2, forming superoxide [138]. Endothelial cells primarily express the NOX4 isoform of NADPH oxidase, which is found on intracellular organelles. NOX2, which spans cell membranes, is a major contributor to systemic ROS [138]. White blood cells, particularly peripheral blood mononuclear cells [139], are also a major source of ROS, especially in disease states. Many disease states are characterized by high levels of circulating angiotensin-II, which activates NADPH oxidases. While electron leakage at complexes I and III within the electron transport chain can substantially contribute to ROS production in other tissue types, this mechanism is not thought to play as large a role in the vasculature, where >96% of superoxide produced through the electron transport chain is rapidly reduced to water [140].

While transient increases in ROS can be beneficial (for example, ROS are transiently produced following acute exercise, which may help mediate adaptations such as exercise-induced angiogenesis [141]), chronically elevated levels of ROS are

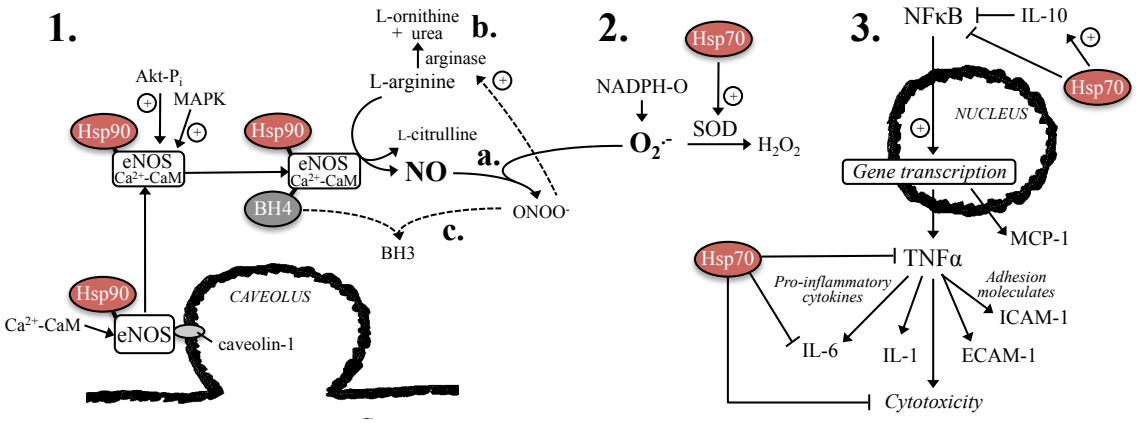

Figure 2.1. Interactions of heat shock proteins (HSPs) in endothelial cells with three primary pathways associated with vascular function: the nitric oxide (NO) pathway, oxidative stress, and inflammation. **1.** Hsp90 is essential for activation of endothelial NO synthase (eNOS) by calcium-calmodulin (Ca^{2+}-CaM) and Akt (also known as protein kinase B). **2.** Hsp70 upregulates superoxide dismutase (SOD), which reduces superoxide ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2), such that the damaging effects of $O_2^{\cdot-}$ are attenuated. These damaging effects include, **a.** scavenging of NO, **b.** upregulation of arginase, which then decreases available L-arginine for synthesis of NO, and **c.** scavenging of tetrahydrobiopterin (BH4), and thus uncoupling of eNOS. **3.** Heat stress, most likely via Hsp70, suppresses nuclear factor kappa B (NFκB), a master regulator of pro-inflammatory gene transcription, and its downstream effects mediated by pro-inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL-6). Hsp70 also upregulates the anti-inflammatory cytokine IL-10, which can suppress NFκB activation. Other abbreviations: NADPH-O, NADPH oxidase; MAPK, mitogen-activated protein kinase; ONOO-, peroxynitrite; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule-1; ECAM-1, endothelial cell adhesion molecule-1.

associated with impairments in vascular function and disease progression [140]. Superoxide is especially damaging to endothelial cells. Superoxide can combine with NO to produce peroxynitrite, reducing the amount of bioavailable NO and therefore impairing the functional effects of NO, such as reducing NO-dependent vasodilation, impairing angiogenesis, and promoting the progression of atherosclerosis [142]. As mentioned, superoxide also impairs NO production by uncoupling eNOS. This is primarily achieved by the actions of peroxynitrite on tetrahydrobiopterin, one of the essential cofactors for eNOS [143]. Additionally, peroxynitrite upregulates arginase activity [144], which reduces the availability of L-arginine, the substrate used by eNOS for NO production. Therefore, the actions of superoxide on the NO pathway form a positive feedback loop promoting greater and greater impairments in vascular function.

ROS are reduced by anti-oxidative enzymes. For example, superoxide dismutase reduces superoxide into hydrogen peroxide, which is further reduced to water by catalase [145]. Glutathione peroxidase reduces glutathione, a byproduct of lipid peroxidation and another source of oxidative stress, to glutathione disulfide and water. The primary mechanism by which HSPs ameliorate oxidative stress is by upregulating expression of anti-oxidative enzymes. Hsp70 upregulates superoxide dismutase expression [146,147] and Hsp25/27 helps facilitate the actions of glutathione peroxidase [5]. As described, removal of superoxide by upregulation of superoxide dismutase can have profound effects on vascular function.

Inflammation. Vascular dysfunction and cardiovascular disease are strongly associated with chronic systemic and vascular inflammation [148]. Patients with cardiovascular diseases have elevated levels of circulating pro-inflammatory cytokines [149,150] and the pathogenesis of many diseases states is considered to be mediated by chronic low-grade inflammation [148].

A mechanism that is thought to play a primary role in inflammation-mediated vascular dysfunction is activation of nuclear factor κ-B (NFκB) [151,152]. NFκB is a transcription factor which, when activated, regulates the production of various pro-inflammatory molecules, such as TNFα, IL-6, monocyte chemoattractant protein 1 (MCP-1), and adhesion molecules [151-154]. Some of these cytokines can in turn activate NFκB, contributing to another positive feedback loop [151].

Several studies have shown that prior heat stress suppresses NFκB activation in response to various pro-inflammatory stimuli, including angiotensin-II, as shown in heart tissue in rats [155], induced pancreatitis in rat pancreatic cells [156], and TNFα in cultured bovine aortic endothelial cells [157]. Heat stress also suppresses TNFα release in response to pro-inflammatory stimuli in cultured macrophages [158,159] and in intact animals [156,160,161]. In the study by Snyder et al, the authors observed a reciprocal relationship between Hsp70 and TNFα [159]. Additionally, cells expressing elevated levels of Hsp70 are more resistant to the cytotoxic effects of TNFα [83,162]. Although not addressed in these studies, it is thought that heat stress suppresses TNFα production by suppressing NFκB.

Heat stress has also been shown to downregulate other pro-inflammatory molecules, many of which are downstream to NFκB and TNFα. For example, endotoxin-induced release of IL-1 is suppressed in heat-treated cells [158,159]. IL-1- or TNFα-mediated production of IL-6 can be suppressed by both prior heat stress and treatment with Hsp70, but not treatment with Hsp60 [6]. Heat stress also suppresses TNFα-mediated expression of intracellular (ICAM-1) [163] and endothelial cell adhesion molecules (ECAM) [157], and pancreatitis-mediated production of ICAM-1 [156]. Adhesion molecule expression is known to be partially dependent on NFκB activation [164].

Heat stress may also increase anti-inflammatory defenses. Treatment of arthritic mice with Hsp70 increased IL-10 levels and suppressed inflammatory responses in various immune cells that are key for promoting arthritis [165]. Interestingly, Hsp70 had no effect on suppressing arthritis in IL-10 knockout mice. Given that IL-10 is thought to be able to suppress NFκB activation, this offers a potential mechanism by which Hsp70 elicited such profound effects on systemic inflammation.

WHOLE-ORGANISM HEAT ACCLIMATION

The concept of heat acclimation is different from thermotolerance. While thermotolerance refers to the ability of cells to withstand otherwise lethal heat shock, heat adaptation refers to improved overall function of the organism following repeated exposures to mild heat stress. Furthermore, thermotolerance is only present while HSP expression remains elevated after initial milder stress, whereas, the benefits of heat adaptation can be maintained for prolonged periods of time. Heat acclimation is an evolutionarily conserved adaption and can be observed in a variety of species, from invertebrate worms [166] and insects [167] to fish [168], rodents [14], and humans [169].

The whole-organism heat acclimated phenotype results in decreased heat production, decreased core temperature and heart rate for any given ambient temperature or exercise intensity, decreased core temperature threshold for initiating heat loss mechanisms, and an increased capacity for heat loss, although some variations in the adaptive responses can be observed across species. For example, in humans, there is no evidence that heat production is decreased, but this is offset by a robust capacity for heat

loss, achieved via augmented skin blood flow and sweating responses. Other systemic effects of complete heat acclimation, as determined in rats chronically exposed to heat, include increased cardiac work efficiency through improved excitation-contraction coupling [170,171] and plasma volume expansion, resulting in increased stroke volume and a subsequent reduction in heart rate [172].

Another important hallmark of whole-organism heat acclimation is enhancement of cytoprotective networks. These include upregulation of HSP-mediated pathways [173,174], upregulation of anti-oxidative and anti-apoptotic proteins, and earlier activation of these pathways during stressful conditions [173,175]. In animals who live in warm environments, HSP expression is elevated under non-stressed conditions (i.e., thermoneutral core temperature) [176,177]. This protects the animals against sudden increases in core temperature associated with bouts of physical activity in a hot environment. In fact, core temperature has to rise considerably higher in these animals before they exhibit a stress response. Upregulation of these pathways also protects organisms against novel stressors, termed "heat acclimation-mediated cross tolerance." In addition to heat stress, heat acclimated animals have conferred protection against stressors such as ischemia-reperfusion [178], traumatic brain injury [179], hyperoxia [180], hypohydration imposed on heat stress [181], and ionized irradiation [14]. While enhanced heat loss mechanisms associated with heat acclimation (e.g., increased sweat rates, increased skin blood flow, decreased core temperature) can be achieved within a relatively short period of time (e.g., in humans, these adaptations are achieved within 10-14 days of 60-90 min per day exercise in the heat [22]), cross-tolerance involves reprogramming of a variety of genes and requires much longer periods of heat exposure.

Dr. Michal Horowitz and colleagues have performed an elegant body of work investigating the genomic changes that occur with heat acclimation. To do so, they have primarily utilized a rat model in which they have investigated the protective effects of 4 weeks of continuous heat exposure on ischemic insult in heart tissue. In the heart, they have shown that heat acclimation upregulates a variety of genes associated with: 1) anti-apoptosis [182], 2) maintenance of DNA and chromatin integrity [183,184], 3) chaperones (e.g., HSPs) [175], and 4) reactive oxygen species scavengers [185]. These molecular pathways are common to the majority of stress responses, which are generally

characterized by high levels of oxidative stress and apoptosis. As such, Horowitz and colleagues have shown that heat acclimation protects heart tissue from apoptosis associated with ischemia reperfusion injury.

A number of metabolic changes in heart tissue facilitate this protection. For example, the genomic changes associated with heat acclimation shift the cells towards a greater reliance on anaerobic metabolism. Intracellular glycogen reserves are increased [186], upregulation of GLUT-1 and GLUT-4 facilitates greater intake of glucose into the cell [186], 6-phosphfructo-2-kinase-2 expression is increased [186], and glyceraldehyde-3-phosphate dehydrogenase activation is increased [186]. During ischemia, these changes favor anaerobic glycolysis. Additionally, the cell becomes more metabolically efficient and so rate of glycogen depletion is decreased [186]. The drop in pH that normally occurs rapidly with ischemia and CO_2 build-up and which would inhibit glycolysis is prevented. Many of these metabolic changes are believed to be mediated through hypoxia inducible factor (HIF)-1α, which is essential for complete heat acclimation [166] and which is stabilized by Hsp72.

SHEAR STRESS

Shear stress is the mechanical frictional force exerted by the blood on the arterial walls, determined by the velocity and viscosity of the blood. Importantly, shear stress and the adaptive effects of changes in shear stress are greatest in the conduit vessels. While some effects of shear stress can be observed in the microvasculature, this section will focus most on the conduit vessels.

With perfect forward laminar flow, shear stress will be completely anterograde. However, due to the pulsatile nature of blood flow *in vivo* (in conduit vessels), there are times in the cardiac cycle where blood flow will be halted or may move in a retrograde fashion back towards the heart. The shear patterns that occur as a result of alternating anterograde and retrograde flow is referred to as oscillatory shear. Stimuli that increase blood flow, such as exercise, typically increase anterograde shear. Stimuli or disease progression which result in increased peripheral vascular resistance will increase retrograde or oscillatory shear, as there will be greater resistance to the forward movement of blood. Many studies have investigated the effects of different shear patterns

(i.e., more or less retrograde or anterograde shear) on endothelial function and adaptation. In general, high retrograde or oscillatory shear (also referred to as "disturbed flow") is associated with endothelial dysfunction and a pro-atherogenic profile, and high anterograde shear improves endothelial function and is anti-atherogenic. The opposing effects of retrograde and anterograde shear stress on the vasculature are discussed below and summarized in Figure 2.2 (page 27).

Detrimental effects of high retrograde/oscillatory shear

There is now a wealth of information that disturbed flow under resting conditions can promote unhealthy processes. Studies in cultured endothelial cells have indicated that high oscillatory shear upregulates a host of pro-atherogenic genes, including those that are pro-inflammatory, pro-apoptosis, and pro-coagulant [117,187,188]. This results in increased expression of pro-atherogenic factors, such as adhesion molecules [117,187,189], endothelin-1 [190], reactive oxygen species-producing enzymes like NADPH oxidase [191,192], and superoxide [192]. Expression of eNOS is also reduced [190,191], and leukocyte adhesion to endothelial cells [193] and endothelial cell turnover [194] are both increased. Expression of eNOS is also reduced in isolated arteries exposed to high oscillatory shear, resulting in impaired endothelium-dependent dilation [195].

In vivo rodent models of high oscillatory shear, induced either by carotid artery partial ligation [196] or casting of the carotid artery to cause partial stenosis [197], produces rapid and profound endothelial dysfunction [196] and vascular remodeling (intimal thickening, reduced number of smooth muscle cells, and greater lipid content) [197], upregulation of pro-atherogenic genes [196] and pro-inflammatory cytokine production [197], and downregulation of anti-atherogenic genes [196]. These rodents also quickly develop atherosclerotic plaques in regions of high retrograde shear; whereas, no plaques form in regions of normal or high anterograde shear [196,197].

In humans, disturbed shear patterns and increases in retrograde shear may occur as a result of increased vascular tone in the downstream microvasculature in various patients populations, such as those with advanced age [198,199], obesity [200], and hypertension [201]. The extent of disturbance in flow has been shown to be related to reduced NO bioavailability [202] and alpha-adrenergic mediated vasoconstriction [203].

Conduit vessel endothelial dysfunction, likely secondary to greater oscillatory shear, is also present in these patient populations [204-206] and there is a well-established link between endothelial dysfunction and the progression of atherosclerosis [207,208]. In support of a causal link between disturbed flow and endothelial dysfunction, acutely disturbing flow in young, healthy individuals by inflating a distal blood pressure cuff to increase retrograde shear (without affecting anterograde shear) in the brachial artery reduces flow-mediated dilation [209] and causes release of endothelial microparticles (EMPs) CD62E (E-selectin) and CD31 [210], biomarkers of pro-inflammatory endothelial activation and apoptosis, respectively [211]. EMPs, in turn, can release C-reactive protein [212], carry regulatory microRNAs [213], reduce eNOS [214], and promote thrombosis, inflammation, and reactive oxygen species production [215]. Low mean shear rates are also associated with higher levels of circulating EMPs in patient populations [216].

Beneficial effects of increases in anterograde shear

On the contrary, repeated increases in anterograde shear, such as occurs during exercise or heat stress, have been shown to be anti-atherogenic and to improve endothelial function. In cultured endothelial cells, physiologically-relevant increases in shear stress (e.g., at levels that would be achieved in conduit vessels during exercise in humans) increase expression of eNOS [217-219], the essential eNOS cofactor tetrahydrobiopterin [220], and the anti-oxidative enzyme superoxide dismutase [217], and therefore increases NO production. Shear stress can also increase phosphorylation of eNOS through a few mechanisms, including through activating the receptor for vascular endothelial growth factor (VEGFR-2) [221] and activating phosphoinositide (PI)3-kinase, which in turn activates protein kinase A and then eNOS [222]. Additionally, shear stress (and VEGF and histamine) can increase association of Hsp90 with eNOS [132].

Increases in shear stress also decrease endothelin-1 expression [223,224] and are anti-inflammatory, reducing expression of adhesion molecules [225,226] and protecting cells against TNFα-induced insult [225]. Importantly, these results have been confirmed in isolated arteries, in which physiological increases in shear stress have been shown to

increase eNOS and superoxide dismutase expression [227,228], resulting in improved endothelial-dependent dilation [228].

In humans, Dr. Daniel Green and colleagues have performed a series of studies indicating that increases in shear stress are essential for arterial adaptation to various stimuli. These authors have utilized a model in which they assess vascular function before and after an intervention in both arms. One arm is allowed to adapt normally; whereas, a blood pressure cuff is placed on the other arm and inflated to resting systolic pressure throughout the intervention sessions, such that blood flow, and therefore shear stress, is prevented from increasing above resting levels. Using this model, they have shown that arterial adaptation in both conduit vessels and the microvasculature, as measured by increases in brachial artery flow-mediated dilation, cutaneous microvascular endothelial function, or brachial artery diameter (i.e., arteriogenesis), is prevented in the cuffed arm following 8 weeks of local arm heating [229], whole-body heating [16,17], lower limb exercise training [230], and handgrip exercise training [18]. Reductions in brachial artery wall thickness with handgrip exercise training, a marker of structural remodeling, is also prevented in the cuffed arm [231]. These data argue for an obligatory role of shear stress in arterial adaptation, even in response to stimuli which should induce HSP expression, i.e., stimuli such as whole-body heating and exercise which increase body core temperature.

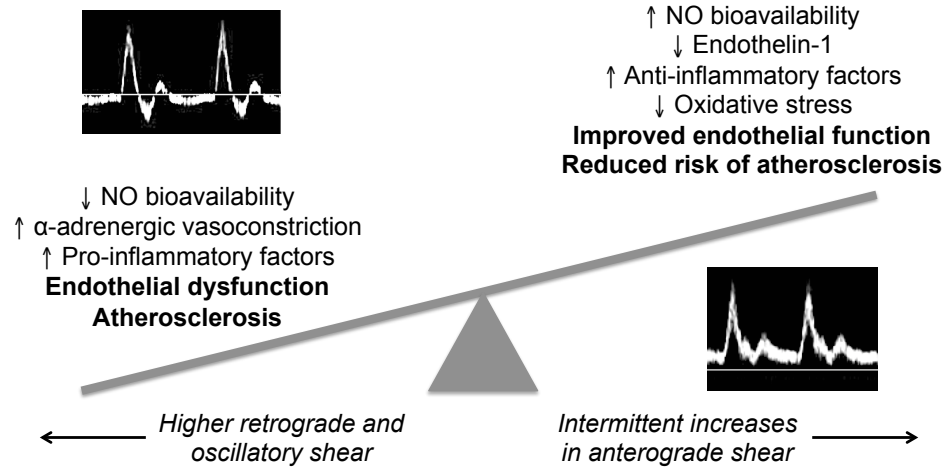

Figure 2.2. Summary of opposing effects of retrograde and anterograde shear stress on the vasculature.

Based on these data and data from other laboratories, the general consensus in the field is that shear stress is the primary stimulus for inducing arterial adaptation to exercise training [232-234] Indeed, exercise training is well-known to improve endothelial function [235] and to be anti-atherogenic [113,236]. Additionally, exercise training can induce arteriogenesis (i.e., increases in diameter of the conduit vessels) [237,238], which is also thought to be shear-mediated. In contrast, it is important to note that angiogenesis, the growth of blood vessels in the microvasculature, is much less dependent on shear stress.

Typically, eNOS protein is increased [234,235]. However, some studies have failed to observe increases in eNOS and endothelium-dependent dilation following exercise training [239-241]. The differential effects of exercise of conduit arteries throughout the body may help explain these differing results. For example, anterograde and total shear are increased and retrograde shear is decreased in the abdominal aorta during exercise [242], but depending on the mode and intensity of the exercise, oscillatory shear may be increased in peripheral conduit arteries [243,244]. It is also important to recognize that increases in flow can also increase arterial diameters, and so shear stress may not have increased in these studies if diameter increased proportionally with velocity.

Increases in local temperature also seem to be important. Interestingly, in the same studies from Dr. Green's lab, adaptations to repeated whole-body heating [16] were prevented when local skin temperature was clamped to 30°C using a water bath, even though shear stress was allowed to increase. As such, it appears that the interactive effects of elevations in shear stress and temperature are necessary for adaptation. This evidence suggests that heat therapy may offer an even more powerful mode for inducing arterial adaptation.

HEAT THERAPY STUDIES IN HUMANS

Although the acute effects of passive heat stress have been well characterized, very few studies have investigated the long-term benefits of heat therapy, with only 6 studies investigating physiological effects beyond 4 weeks. As shown in animals, greater than 4 weeks of heat exposure are necessary in order to induce the full protective

phenotype [14]. While the available data is highly convincing of the benefits of heat therapy, further investigations are needed.

Short-term studies: 2-4 weeks

The majority of shorter-term heat therapy studies have been conducted by a group in Japan utilizing Waon therapy, which consists of 15 min in a 60°C far infrared sauna followed by 30 min recovery in blankets to keep body core temperature elevated.

Most studies from this research group have been conducted on heart disease patients. Waon therapy significantly reduces the severity of these diseases, greatly improving myocardial perfusion in chronically-occluded coronary artery-related ischemia patients [245] and improving cardiac function in left-sided heart failure. Improvements in the latter group include improved left-ventricular ejection fraction [23,246,247], improved cardiothoracic ratio [247], reduced incidence of arrhythmias [247], reduced atrial natriuretic peptide [247], and improved autonomic function, as measured by reduced low frequency (sympathetic) and increased high frequency (parasympathetic) components of heart rate variability [248]. In congestive heart failure, Waon therapy reduces mitral regurgitation and cardiothoracic ratio, and increases left ventricular ejection fraction [246].

Waon therapy also improves biomarkers of vascular function. Flow-mediated dilation is improved in heart failure patients [23] and patients with chronically-occluded coronary artery-related ischemia [245]. Systemic vascular resistance is reduced acutely [24] following hot water immersion or sauna, and remains reduced chronically, measured 24h following 2 weeks of heat therapy treatment [249]. Reductions in vascular resistance, along with improvements in angiogenesis that have been observed in rats undergoing 5 weeks of Waon therapy [250], may underscore chronic improvements in blood pressure [251].

Perhaps one of the most compelling arguments that passive heat therapy could be used as an alternative or adjunctive therapy to exercise is the observation that just 3 weeks of Waon therapy improves VO_2 peak [23] and exercise tolerance, measured using both 6-minute walk distance [23] and time-to-fatigue on a modified Bruce test [245]. In the study by Sobajima et al. [245], improvements were observed in every heart failure

patient studied, and 16 out of 20 patients improved in the study by Ohori et al. [23]. In both of these studies, improvements in exercise tolerance were correlated with improvements in FMD. Previous studies in animals have demonstrated that NOS inhibition reduces exercise tolerance, suggesting that improvements in vascular function are a primary mediator for heat therapy-induced improvements in exercise tolerance.

Longer-term studies: 8-12 weeks

One study has investigated the effects of longer-term Waon therapy. Following 10 weeks of therapy in peripheral artery disease patients, Tei et al. [252] reported improved pain scores, walking distance, ankle-to-brachial pressure index, and resting leg skin perfusion (laser-Doppler). Waon therapy was also successful at inducing the formation of new collateral vessels in the affected legs of these patients, as shown by angiography [252]. In one patient, Waon therapy greatly improved the rate of healing of a large skin ulcer, preventing amputation of his leg [253].

With Finnish dry sauna, 12 weeks of biweekly sauna use has been shown to increase left-ventricular ejection fraction by 7-8% in heart failure patients [254] and to reduce blood pressure in heart failure patients [254] and hypertensives [254,255].

In young, healthy subjects, two studies from the same research group have been published investigating the effects of repeated hot water immersion on vascular function [16,17]. In these studies, subjects were immersed to the waist in 40°C hot water for 30min per session, 3 times per week for 8 weeks. Following the 8 weeks, these authors observed an improvement in cutaneous microvascular function, measured using slow local skin heating to 42°C [16]. They also observed an improvement in brachial artery FMD after 4 weeks of leg heating; however, FMD returned to baseline values by 8 weeks [17]. However, the method of heating resulted in a fairly mild heat stress. Rectal temperature reached about 38.1°C during the first session, but by 8 weeks, only reached ~37.5°C, which was only 0.4°C above resting core temperature. While these results indicate a beneficial effect of hot water immersion therapy on vascular function, due to limitations, these studies warrant further investigation.

Lifelong sauna use

While extensive studies have been performed in Finland characterizing the acute effects of sauna bathing, very few have prospectively (or cross-sectionally) studied individuals using sauna regularly. In acute studies, the main focus has been on disproving myths that sauna bathing is dangerous. There have been a few long-term studies, but none have systematically assessed cardiovascular health. For example, a ten-year follow-up study of patients who had suffered a myocardial infarction reported that although 60% of patients experienced symptoms of angina pectoris throughout their daily lives, only one patient experienced these symptoms associated with sauna bathing [58]. In another study, the authors followed patients with essential hypertension and coronary artery disease who used sauna regularly and reported a positive effect on blood pressure at 1 and 3 years of follow-up [251]. Several studies have also reported anecdotal evidence that regular sauna bathing improves symptoms associated with asthma [256], joint pain [38], rheumatoid arthritis [257], and chronic obstructive pulmonary disease [258], and the incidence of common colds and upper respiratory tract illnesses [259].

The most convincing study on the health benefits of sauna bathing was published recently by Laukkanen et al. [13]. These authors studied 2,315 Finnish men, aged 42-60 years, who used sauna regularly. Subjects participated in baseline testing and completed a questionnaire on their sauna bathing habits. The authors then followed these subjects for 30 years, or until time of death. Both the frequency and duration per session of sauna bathing significantly predicted incidence of sudden cardiac death, fatal cardiovascular disease, fatal coronary artery disease, and all-cause mortality. The risk of cardiovascular-related mortality was 48-50% lower in subjects who used sauna 4-7 times per week compared to those who used it only once per week.

CONCLUSIONS

It has been well established that exercise training improves vascular function and reduces risk of cardiovascular-related morbidity and mortality. Both increases in core temperature and the subsequent increases in shear stress are considered to be the primary mechanisms by which exercise induces arterial adaptation. The similarities in acute effects on the cardiovascular system between heat therapy and exercise argue strongly in

favor of heat therapy also inducing profound improvements in cardiovascular health. This argument is supported by the extensive data in animals that has shown long-term heat acclimation to be protective for the cardiovascular system, and the existing data in humans demonstrating improvements in vascular and cardiac function, as well as reduced incidence of all-cause mortality, with sauna therapy.

CHAPTER III

OVERVIEW OF METHODOLOGY

OVERVIEW

The overarching goal of this dissertation was to assess the effects of long-term heat therapy on vascular function and the underlying molecular mechanisms in young, healthy, sedentary humans. To investigate this goal, one large study was conducted in which 20 young, healthy, sedentary subjects were assigned to participate in one of the following 4-5x per week for 8 weeks (36 sessions total):

Group 1: Heat Therapy (hot water immersion; N=10)
Group 2: Sham (thermoneutral water immersion; N=10)

Before, during, and after the 8 weeks, we performed the following experimental measures. A timeline of the human study is provided in Figure 3.1 (next page).

1) We measured various well-established clinically relevant biomarkers of macrovascular health, including flow-mediated dilation, arterial stiffness, intima media thickness, and blood pressure. The results of these studies are detailed in Chapter V.
2) We investigated the effects of heat therapy on microvascular function and the mechanisms behind improvements by performing a cutaneous microdialysis study. The skin serves as an ideal site to easily and effectively pharmacodissect the molecular pathways involved. The results of these studies are detailed in Chapter VI.
3) We collected venous blood in order to measure circulating factors which are affected by heat therapy and for use in cell culture experiments in which we sought to elucidate the cellular mechanisms behind improvements in vascular health. The results of these studies are detailed in Chapter VII.
4) In a subset of subjects, we measured blood volume, hemoglobin, and hematocrit in order to characterize whether passive heat therapy results in similar classical

adaptations to exercise heat acclimation. These results, along with other data that characterizes the overall physiological response to passive heat therapy (e.g., changes in core temperature, sweat rates, and heart rate) are detailed in Chapter IV.

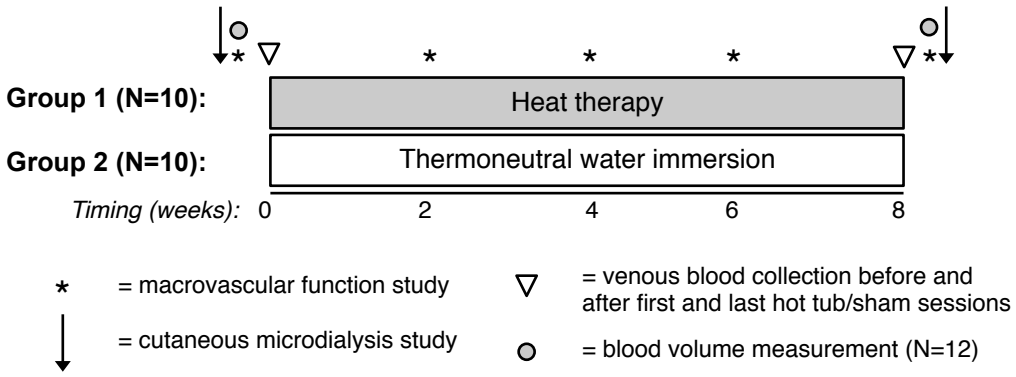

Figure 3.1. A timeline of the human studies. Macrovascular and cutaneous studies were performed 36-48h after the previous hot tub session. Blood volume measurements were performed in a subset of subjects on the same day as macrovascular function studies.

HUMAN SUBJECTS

All protocols in human subjects were approved by the Institutional Review Board at the University of Oregon (IRB #09272013.025, see Appendix pages 153-216 for informed consent documents). All subjects gave oral and written consent prior to participation as set forth by the *Declaration of Helsinki*. The study was registered as a clinical trial on clinicaltrials.gov. Identifier: NCT02518399; https://www.clinicaltrials.gov/ct2/show/NCT02518399?term=heat+therapy&rank=1.

Figure 3.2 (next page) summarizes progression of subjects through the study. Seventy-six potential human subjects were assessed for qualification in the study. Subject inclusion criteria included age 18-40 years, healthy body mass index (BMI between 18-25kg/m^2), sedentary lifestyle (<2h aerobic exercise per week), not taking any prescription medications with the exception of hormonal contraceptives, no cigarette smoking, and no history of cardiovascular, metabolic, or other chronic diseases. Of these seventy-six subjects, thirty-eight met inclusion criteria. Eleven declined to participate. Thus, twenty-seven (17 women, 10 men) young (age 21.5 ± 0.5 yrs old), healthy (BMI 22.4 ± 0.3 kg/m^2), sedentary individuals enrolled in the study. These subjects underwent an

additional health screening to further confirm they met all inclusion criteria and to ensure they were healthy enough to undergo heat therapy. Following the health screening, they were assigned to participate in either heat therapy or thermoneutral water immersion. Because of the relatively small cohort size, subjects were not randomly assigned to interventions, but were instead assigned by investigators to match for age, sex, BMI, and time of year across the two groups (Table 3.1 - next page). Of the 27 subjects who were initially enrolled, 24 subjects completed part or all of the 0-week testing, 23 initiated heat therapy or thermoneutral water immersion (sham), and 20 completed the full 8-week protocol. These numbers resulted in N=10 in each group. Data from subjects who dropped out of the study partway has been excluded, unless otherwise specified.

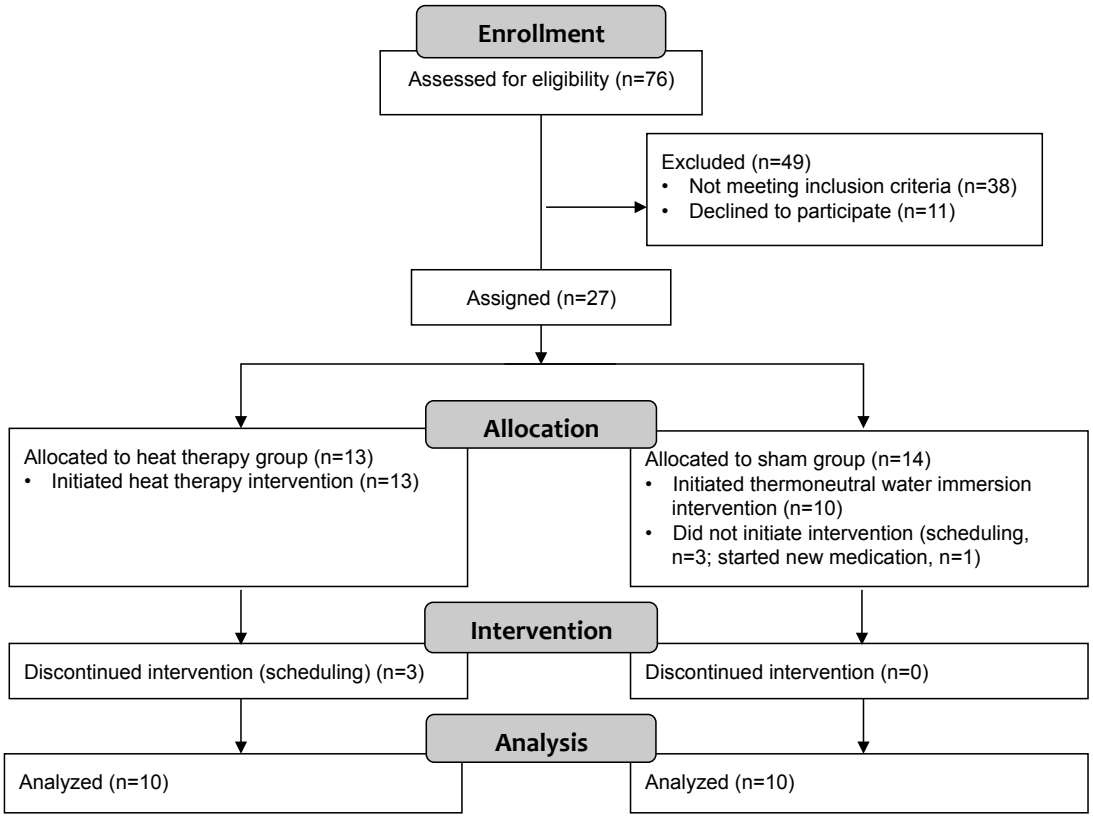

Figure 3.2. Progression through the phases of the study.

Prior to all study days (at 0, 2, 4, 6, and 8 weeks), subjects abstained from food for 4h, caffeine and alcohol for 12h, over-the-counter medications, vitamins, and supplements for 24h, and exercise for 24h. No subjects were taking prescription

medications at the start of the study; however, one subject had to take antibiotics for a 7-day period during the 8 weeks, which was discontinued 8 days prior to the next study day. Anther subject took amitriptyline for 3 days for migraine-like symptoms, which was discontinued 2 days prior to the next study day. All female subjects provided a negative pregnancy test within 24h of each study day. Women taking hormonal contraceptives (N=10) were always studied during the active (hormone) phase in order to control hormone status. Women who were naturally menstruating (N=2) were studied during the same phase of the menstrual cycle for 0, 4, and 8 weeks and at 2 and 6 weeks. Although women have been studied under low hormone conditions (i.e., in the early follicular phase of the menstrual cycle or during the placebo week if taking contraceptives) in the majority of recent investigations, it was not possible to do so in a study with such frequent experimental sessions. Studying women in the active phase of contraceptives provided more consistent hormonal conditions across all experimental days. Additionally, the magnitude of changes in vascular function we hoped to observe, which would indicate clinically relevant improvements in cardiovascular health, are of greater magnitude than the changes observed across the menstrual cycle or with the active vs. placebo phases of hormonal contraceptives [260,261].

Table 3.1. Demographics of subjects who completed all 8 weeks of heat therapy or thermoneutral water immersion (sham). Data are mean ± S.E.

	Heat Therapy (N=10)	Sham (N=10)
# of women	6	6
Age (yrs)	22.0 ± 1.1	21.7 ± 1.3
Height (cm)	173 ± 4	174 ± 3
Weight (kg)	67 ± 3	67 ± 4
BMI (kg/m^2)	22.4 ± 0.6	21.9 ± 0.8
# of subjects studied in Summer months	4	4

HEAT THERAPY

Subjects in both groups reported to the Evonuk Environmental Physiology Core in Esslinger Hall on the University of Oregon campus 4-5 times per week for 8 weeks,

for a total of 36 sessions. Upon arrival, subjects were instrumented with a sterile rectal thermistor probe (YSI Series 400; Yellow Spring Instruments, Yellow Springs, OH) inserted ~10cm past the anal sphincter to monitor core temperature and a chest strap (Polar Electro Inc., Lake Success, NY) to monitor heart rate. Dry nude body weight was measured before and immediately after sessions in order to calculate mean whole-body sweat rate, after correcting for water intake.

For heat therapy, subjects were immersed up to the shoulder in a 40.5°C hot tub until rectal temperature (T_{re}) reached 38.5°C, which took ~20-30 min (Figure 3.3). Subjects then sat up on a bench such that the water reached waist-level in order to maintain T_{re} between 38.5-39.0°C for another 60 min (up to 90 min total in the tub). Following hot water immersion, subjects were monitored for another 10 min, or until T_{re} had fallen below 38.5°C. Subjects in the sham group were immersed up to the shoulder in a 36°C tub for 30 min and then to waist-level for another 60 min in order to mimic the same hydrostatic pressures as were experienced by subjects in the heat therapy group, but without the heat exposure. This water temperature was selected to match reference values for mean body temperature (calculated at $0.2*T_{sk} + 0.8*T_c$, where T_{sk} = mean skin temperature and T_c = body core temperature, using reference values of T_{sk} = 32.0°C and T_c = 37.0°C) in order to minimize core-to-skin temperature gradients [262], and was successful at avoiding any changes in rectal temperature >0.2°C across the 90 min in all subjects.

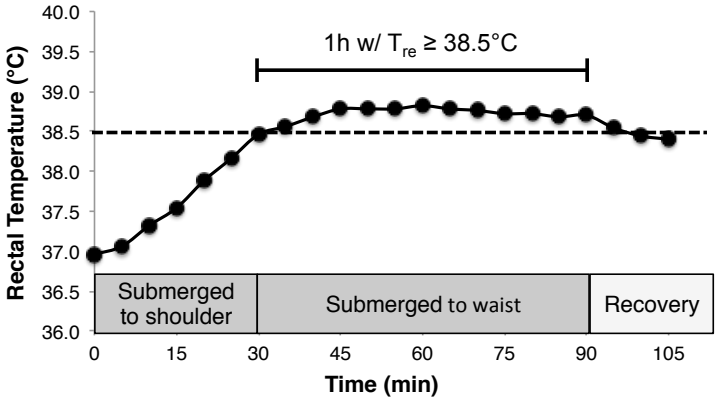

Figure 3.3. Representative rectal temperature response to the heat therapy protocol from one subject.

The goal of heat therapy was to raise body core temperature to 38.5°C and maintain it at or above this threshold for 60 min. This threshold was selected as HSP expression is dependent upon time spent above a core temperature threshold, which in humans has most commonly been reported to be in the range of 38.0-38.5°C [22]. This protocol (>38.5°C for 60 min) has also been shown to be the ideal core temperature and length of time for attaining complete heat acclimation when using controlled passive hyperthermia [263]. A frequency of 4-5 sessions per week was selected to match exercise training regimens. There were no constraints on when sessions could be held so long as they were completed within the week (as in, all five sessions could be consecutive or could be spread out across the 7-day week). In general, subjects completed five sessions in odd weeks and four sessions in even weeks when a macrovascular function study was also held. However, schedules were customized to fit subjects' needs. A time period of 8 weeks was selected because periods of heat exposure greater than 4 weeks are required to induce many of the cellular adaptations [182], and the majority of exercise training studies are conducted over at least 8 weeks. Water immersion was selected as the preferred method of heating since it is difficult to achieve increases in core temperature solely in air. Water immersion is capable of increasing core temperature at a rate similar to moderate-intensity exercise [264] and it allows for core temperature to be continually elevated to a given threshold, even once individuals are adapted to the heat.

Prior to all sessions, subjects provided a first-morning urine sample to confirm euhydration via urine specific gravity <1.02 (Analog Refractometer; Atago, Tokyo, Japan). If USG was >1.02, subjects drank 5ml/kg of water prior to entering the hot tub. While in the tub, subjects drank water *ad libitum*. If subjects did not drink enough water to compensate for sweat loss such that body weight loss was >1%, they drank additional fluids to make up the difference prior to leaving the laboratory.

HEMODYNAMIC MONITORING

For vascular function studies, heart rate and rhythm were monitored using 3-lead electrocardiogram (CardioCap; Datex Ohmeda, Louisville, CO). Baseline blood pressure was measured in triplicate following at least 20 min of supine rest and periodically throughout the study via brachial oscillation. Beat-by-beat blood pressure was measured

using photopletysmography (Nexfin; BMEye, Amsterdam, the Netherlands), with a cuff placed around the middle finger of the left hand. Beat-by-beat blood pressure measurements were calibrated back to the brachial blood pressure. For cutaneous microvascular studies, brachial blood pressure was measured periodically throughout the study, timed with key points into the experimental measures.

MACROVASCULAR FUNCTION

As the majority of cardiovascular diseases are associated with disorders of the arteries, we chose to focus this project on vascular function. The two primary components of vascular function are endothelium-dependent dilation and arterial stiffness. Endothelium-dependent dilation refers to the ability of the endothelial cells to synthesize and release vasodilators in response to a given stimulus; whereas, arterial stiffness refers to the distention of the arteries that occurs due to changes in pressure. Arterial stiffness can be impacted by endothelial-dependent dilation, but is also affected by elastic fiber composition, collagen content, vascular smooth muscle hypertrophy, and monocyte adherence. The structural properties and function of the conduit vessels can be non-invasively assessed using a combination of Doppler ultrasonography and applanation tonometry. The two techniques are described in depth below, whereas the specific measurements we made using these techniques are described in subsequent sections.

Ultrasonography

An ultrasound transducer probe emits ultrasonic waves at a given frequency that penetrate through tissue. The lower the frequency, the deeper the waves can penetrate. For imaging the conduit arteries, which lie in the range of 1-6cm below the surface of the skin, transducer probes with a maximum pulsed frequency in the range of 10.0-12.0-MHz are most commonly used. As the ultrasound waves travel through a medium, they displace particles at their given frequency. When these waves meet a solid interface (e.g., soft tissue, muscle, blood cells), they are reflected back at an intensity equal to the acoustic impedence of the material. Modern ultrasounds have two modes. The first is B-mode, or brightness mode, which is used to obtain an image of the vessel. Images are constructed by determining the amplitude of reflected energy at each depth through the

tissue. The beam of ultrasonic waves can be focused to a given depth (i.e., the depth where the vessel lies) to improve resolution within that focal zone. Secondly, the Doppler mode can be used to obtain blood velocity. In Doppler mode, ultrasound waves are transmitted at an angle of insonation. Velocity of the blood cells is determined based on the Doppler shift that occurs when waves are back-scattered. While an insonation angle of 0° provides maximal ability to detect a Doppler shift, this is not possible without being inside the body. Thus, an insonation angle of 60° is most commonly used [265].

For the studies described in this dissertation, a high-resolution Doppler ultrasound (Terason t3000cv, Teratech, Burlington, MA) equipped with 10.0-MHz linear array ultrasound transducer probe was used to image the common carotid, superficial femoral, and brachial arteries 1-2 cm distal to the carotid bulb, 2-3 cm distal to the femoral bifurcation, and 3-10 cm proximal to the antecubital fossa, respectively. Probe placement (distances and angles) and subject position (including limb-trunk angles) were recorded to ensure consistency between experimental sessions. B-mode images were optimized using ultrasound contrast controls which were consistent across experimental days for each individual subject [266].

Doppler blood velocity tracings were obtained using an insonation angle of 60° from a region of interest centered between the arterial walls in order to capture peak blood velocity, which occurs in the center of the vessel due to laminar flow. The gates of the region of interest were set to a width just less than the diameter of the vessel in order to prevent capturing the vessel walls, which can create artifacts and thus inaccuracies in the velocity tracing. Pulse repetition Doppler frequency was set to 6.0-10.0 MHz, depending on the depth of the vessel of interest (higher frequency pulsed Doppler was used for more shallow arteries and vice versa).

Customized wall-tracking analysis software. Ultrasound data were collected at 20 frames/sec using a video recording software (Camtasia Studio, TechSmith, Okemos, MI). Videos were later transferred to a computer operating a custom-designed edge-detection and wall tracking analysis software (DICOM, Perth, Australia). The software tracks vessel walls and peak blood velocity within a region of interest set by the investigator (Figure 3.4 – next page), providing frame-by-frame average diameter and peak blood

velocity values. Data can then be used to calculate blood flow and shear rate. For calculations, mean blood velocity is estimated as half peak blood velocity. Peak blood velocity is typically used as it can be captured more accurately than mean blood velocity. In order to accurately measure mean blood velocity, velocity across the entire width of the vessel must be captured without capturing the vessel walls or regions outside the walls. This is technically challenging, particularly in arteries that are constantly pulsing across the cardiac cycle. However, this estimation that mean blood velocity is equivalent to half peak blood velocity assumes that flow is perfectly laminar, which may or may not be true across all vessels and conditions (e.g., close to arterial bifurcations or when cardiac output is increased).

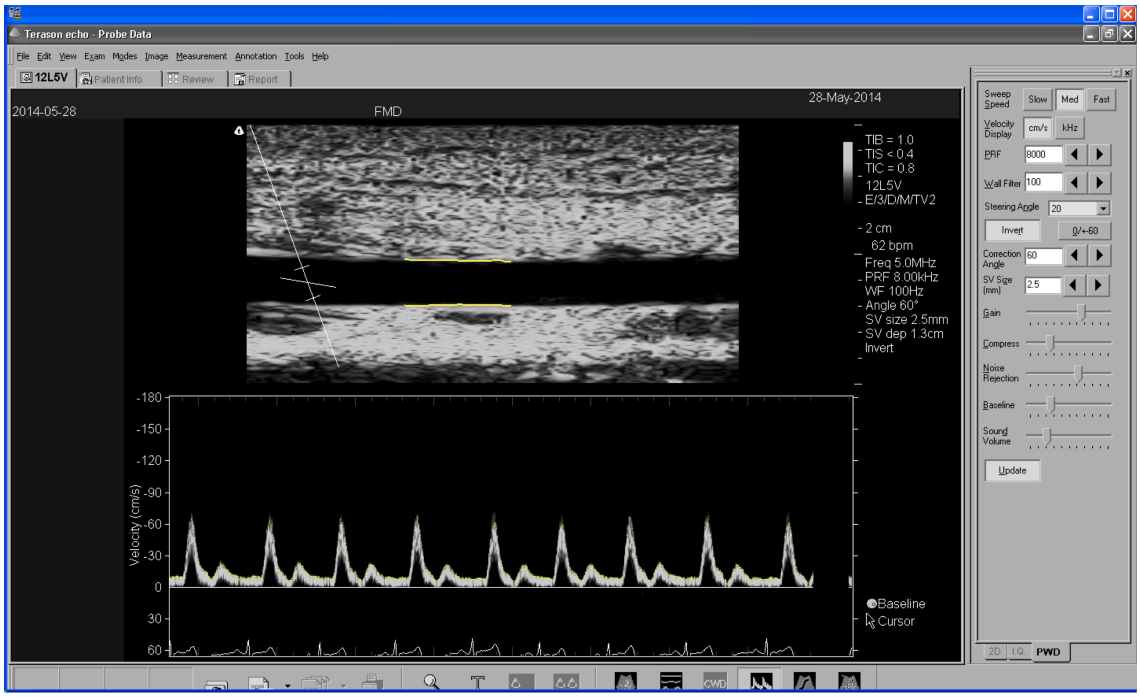

Figure 3.4. Ultrasound 2D image of the brachial artery (top panel) and Doppler blood velocity (bottom panel) from one representative subject approximately 1 min following release of a 5-min arterial occlusion. Wall tracking software was used to quantify changes in vessel diameter (yellow lines) and peak blood velocity (yellow dots).

Applanation tonometry

Applanation tonometry was used to obtain arterial pressure tracings of the common carotid, common femoral, posterior tibial/dorsal pedal, brachial, and radial arteries for measurements of arterial stiffness.

Applanation tonometry was developed in conjunction with Millar Instruments as a simple, non-invasive technique for accurately obtaining intra-arterial pressure [267]. The probes consist of Millar strain gauge pressure transducer mounting on the tip of a pencil-like probe. To measure intra-arterial pressure, the artery is flattened (applanated) over the point of maximal pulsation between the probe and an underlying rigid structure, ideally bone. Flattening of the artery allows for even force distribution across the pressure-sensing surface of the probe - a curved wall would distort the pressure tracing due to circumferential forces. When performed properly (as described below), tonometry pressure tracings are equivalent to those obtained with intra-arterial pressure transducers [267].

In studies which verified accuracy between applanation tonometry and intra-arterial pressure, the authors identified criteria that must be followed in order to obtain accurate pressure tracings (summarized in Figure 3.5 – next page). First, movement of the probe must be minimized. The site of measurement on the subject must be immobilized and the investigator holding the probe must prevent movement of their hand. Second, hold-down force should be optimized to achieve adequate applanation of the artery, but should not be excessive as this could impact arterial pressure. Kelly et al. [267] reported that excessive hold-down pressure most commonly results in a gradual increase in diastolic pressure, a sharp negative deflection immediately preceding the next systolic upstroke, or sometimes inversion of the systolic peak. Others have confirmed that when the artery is applanated with appropriate hold-down pressure, there is no effect on arterial pressure and in the carotid artery, there is no effect on the baroreceptors [268]. Third, the probe should ideally be held perpendicular to the artery. Inappropriate angling of the probe, particularly in the same direction as blood flow (i.e., pointing distally) can underestimate systolic pressure.

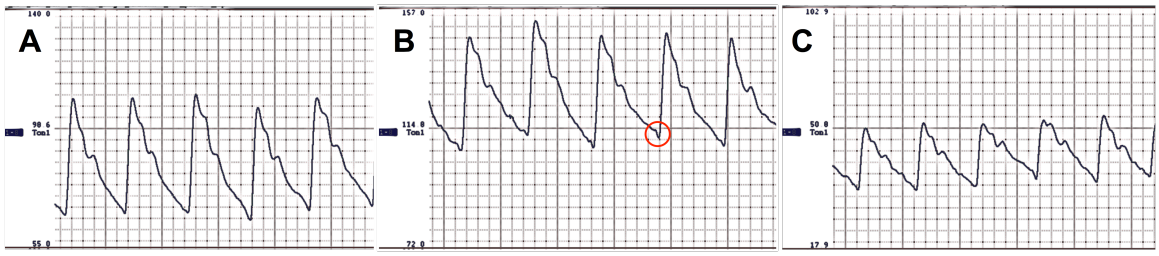

Figure 3.5. Applanation tonometry tracings from the brachial artery, applanated over the medial condyle of the humerus. **A)** Tracings with optimized hold-down force with the probe held perpendicular to the artery. **B)** Tracings with excessive hold-down force demonstrating a sharp negative deflection preceding the systolic upstroke. **C)** Tracings demonstrating distortion of the pressure tracing when the probe is angled distally.

To account for differences in hold-down force, pressure waves are commonly calibrated to brachial diastolic and mean arterial pressures, obtained via brachial auscultation or oscillation [268,269]. Mean arterial and diastolic pressures are used based on the assumption that mean pressure does not change along the large conduit arteries and the observation that diastolic pressure is the same across the carotid and femoral arteries [268]. In contrast, systolic pressure is affected by the elastic properties of the arteries and so can be different between different conduit arteries.

Flow-mediated dilation

The most commonly used test of conduit vessel endothelial function is brachial artery flow-mediated dilation (FMD). FMD has been shown to be highly correlated with CV morbidity and mortality [270-272] and to parallel endothelial function in the coronary arteries [273].

FMD is quantified as the percent change in brachial artery diameter following a distal arterial occlusion, achieved by inflating a blood pressure cuff placed just distal to the elbow to 250mmHg for 5 min. Upon release of the arterial occlusion, blood flow rapidly increases due to the downstream metabolic vasodilation, increasing shear stress on the brachial artery. Shear stress, which is the mechanical fluid force of the blood exerted on the blood vessel walls, activates mechanically-gated calcium channels on the endothelium, stimulating synthesis and release of NO [274,275] and endothelial-derived hyperpolarizing factors (EDHFs) [276], resulting in vasodilation. Peak brachial artery

dilation typically occurs approximately 40 to 90 sec post-cuff release [277,278] (Figure 3.6).

FMD measurements were made in accordance with previously published guidelines [279]. Subjects lay supine with their right arm extended to 80-90° at heart level. An inflatable occlusion cuff was placed 0-2 cm distal to the antecubital fossa and inflated to 250mmHg for 5 min (E20 Rapid Cuff Inflater, D. E. Hokanson, Bellevue, WA). Brachial artery diameter and blood velocity were measured via Doppler ultrasonography for 1 min of baseline prior to cuff inflation and for 3 min following release of the cuff. Immediately following the test, ultrasound scans were analyzed using the wall-tracking software and FMD was calculated as the percent change in diameter from baseline to peak dilation post-occlusion. Using this software, 2.5% changes in FMD can be detected with an N of 8 and power of 80% [280]. In the event that the first FMD was considered non-physiological (i.e., the tissue moved during occlusion and did not return to the same position post-release), the test was repeated at least 20 min after the first FMD. Subjects remained supine during this rest period.

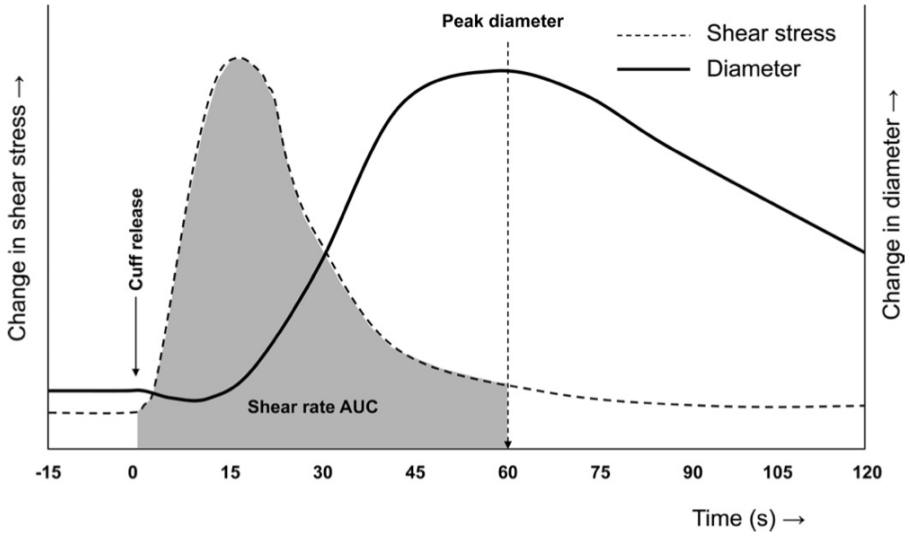

Figure 3.6. Taken from Thijssen et al. [278] (no permission needed from this publisher for reuse in a dissertation). Schematic presentation of diameter and shear rate responses following release of the 5-min arterial occlusion. The grey area represents the area under the curve (AUC) shear rate response that is believed to be the main stimulus for eliciting the peak dilation.

Shear rate was calculated frame-by-frame from diameter and velocity measurements. The shear stimulus responsible for eliciting dilation was calculated as area under the curve above baseline shear rate from the time of release to peak dilation (SR_{AUC}). FMD was then normalized for shear stimulus by dividing FMD by SR_{AUC}, as described and validated previously [281,282].

(1) Shear rate = 4 x mean blood velocity / diameter

(2) Shear corrected FMD (%/SR_{AUC}) = FMD (%) / SR_{AUC} x 10^3

Endothelium-independent dilation. When measuring endothelial-dependent dilation, it is important to also measure endothelium-independent dilation (EIVD). Doing so allows for effects on the endothelium versus the underlying smooth muscle to be teased apart. For example, if FMD increases across an intervention, but EIVD remains unchanged, we can conclude that the intervention specifically improved endothelial-dependent dilation. Conduit vessel EIVD is most commonly assessed using sublingual nitroglycerine, which produces a maximal dilation of the conduit arteries. In solution (i.e., in the blood), nitroglycerine is rapidly converted to NO, which can then diffuse directly to the vascular smooth muscle, bypassing the endothelium. For the measurement of EIVD, brachial artery diameter was measured for 1 min of baseline and for another 10 min following administration of 0.4 mg of sublingual nitroglycerine (Nitrolingual; Sciele Pharma, Atlanta, GA). EIVD was calculated as both the peak diameter and the percent change in diameter from baseline to peak dilation post-nitroglycerine administration.

Reactive hyperemia

Reactive hyperemia refers to the large transient increase in blood flow that occurs following a period of ischemia secondary to profound vasodilation of the microvasculature distal to the site of arterial occlusion. Vasodilation occurs due to a combination of metabolic vasodilators, including NO, prostaglandins, adenosine, and ADP [283-285], and myogenic responses [284]. Reactive hyperemia has most commonly been studied in the forearm, where blood flow to the limb can be occluded safely and easily for a period time, as done with the FMD protocol described above. Forearm post-

occlusive reactive hyperemia (PORH) has been established as a predictor of risk of CV disease and mortality [286-288].

A peak rise in blood flow is observed within a few seconds of release of the occlusion, which is thought to be representative of maximal vasodilator capacity of the microvasculature, and thus changes in peak PORH are thought to reflect structural remodeling of the resistance vessels [289,290]. The theory that peak PORH represents maximal vasodilator capacity was based on observations that handgrip exercise performed during the arterial occlusion, which augments the metabolic stimulus for vasodilation, elicits no further increase in peak reactive hyperemia [291,292]. Blood flow then remains elevated for an extended period of time following release of the cuff, which is dependent on the ability of the resistance vessels to continue to vasodilate in response to the metabolic stimulus. Following a 5-min forearm occlusion, blood flow typically remains elevated for 2-3 min following release of the occlusion (Figure 3.7 – next page).

It is important to note that PORH is not considered to be the gold-standard method for assessing microvascular function, but it is one that is used commonly due to being non-invasive and relatively easy to measure. Intra-arterial infusion of vasoactive agents allow for much better isolation of endothelial-dependent (e.g., acetylcholine) and -independent (e.g., sodium nitroprusside) vasodilation. However, this technique is invasive and requires a skilled physician to place the intra-arterial catheter. Additionally, structural changes in the microvasculature can be quantified more accurately using other methods, such as taking skin or muscle biopsies to quantify vascularization. Again, these techniques are invasive and so we chose not to perform them in the present investigation. We additionally wanted to use techniques that are used clinically or have clinical relevance in order to be comparable to other studies that have determined cardiovascular risk.

In this dissertation, PORH was measured following 5-min arterial occlusion at the same time as FMD by averaging blood flow measurements from ultrasound tracings and arterial pressure data (from Nexfin beat-by-beat measurements) over each cardiac cycle (R to R wave). Forearm vascular conductance (FVC) was calculated as blood flow / mean arterial pressure and zero-hold interpolated to 5Hz. As blood flow is dependent on changes in mean arterial pressure, conductance is more representative of the degree of

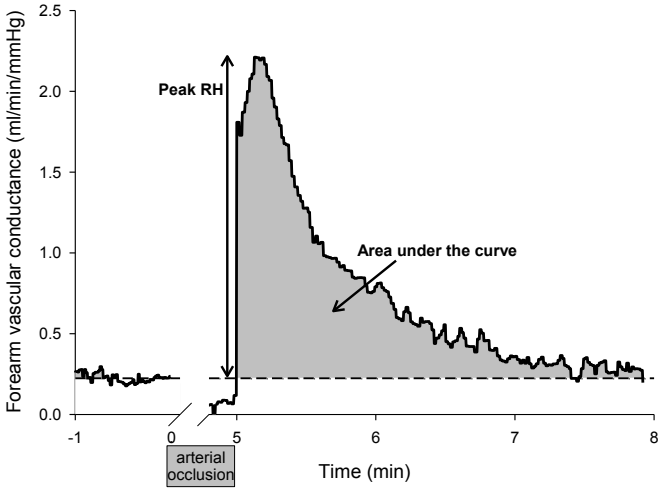

Figure 3.7. Representative post-occlusive forearm reactive hyperemia response from one heat therapy subject.

vasodilation/vasoconstriction in the downstream resistance vessels. Peak PORH was identified as the cardiac cycle with the highest FVC value. Area under the curve (AUC) PORH was calculated as the integral of FVC values minus baseline FVC until FVC had returned to baseline values (range ~90-150 sec following release of the occlusion). Peak PORH and AUC PORH provide different information regarding microvascular function (i.e., structural versus functional changes, respectively) and so were treated as separate variables.

Measures of arterial stiffness

Stiffening of the conduit arteries occurs with healthy aging, beginning as young as adolescence [293,294], and with disease progression due to a combination of elastic fiber degeneration, increased collagen content, impaired endothelial function, and structural changes to the arteries, including vascular smooth muscle cell hypertrophy [295]. As such, arterial stiffness is also an independent risk factor for cardiovascular disease [296-298]. Pathological arterial stiffening, as in stiffening to a greater extent than what would occur with healthy aging, is also often accompanied by atherosclerosis, left ventricular hypertrophy, and increased incidence of vascular events [299].

A variety of measures have been developed to measure arterial stiffness in humans. As all measures look at slightly different properties of the arterial walls, it is

important to measure more than just one [300]. Furthermore, as the various arteries themselves have different properties (e.g. elastic vs. muscular) and are subjected to different shear profiles, it is also important to make measurements at more than just one artery, in order to obtain a more complete view of arterial stiffness changes [300]. For this dissertation, we chose to assess three different measures, all of which have been validated for their abilities to predict CV morbidity and mortality [296-298,301,302]: pulse wave velocity, dynamic arterial compliance, and β-stiffness index. Compliance and β -stiffness were measured at the common carotid artery (a more elastic artery) and the superficial femoral artery (a more muscular artery). Pulse wave velocity was measured across the aorta (elastic) and across the leg and arm (peripheral muscular arteries).

Dynamic arterial compliance. Arterial compliance is defined as the change in arterial volume that occurs for a given change in pressure, and is a direct measure of the elastic properties of the arteries. It has been previously shown to decrease progressively with age [303] and is predictive of cardiovascular risk.

As changes in the cross-sectional area of a cylinder (i.e., a blood vessel) are equivalent to changes in volume, cross-sectional compliance is most commonly used for calculating arterial compliance. Additionally, changes in cross-sectional area are proportional to changes in circumference of the artery. As elasticity is dependent on the square of initial arterial diameter, compliance is usually presented relative to diastolic or mean diameter.

In this dissertation, dynamic cross-sectional arterial compliance relative to mean diameter was measured at the common carotid and superficial femoral arteries and calculated as,

$$(3)\ \text{Cross-sectional compliance} = [(\Delta D/D) / \Delta P\ ] * \pi(D/2)^2$$

Where, ΔD = change in diameter across one cardiac cycle from diastolic trough to systolic peak measured using ultrasonography, D = diameter averaged across each cardiac cycle, ΔP = pulse pressure (systolic = diastolic blood pressure), measured beat-by-beat using finger photoplethysmography (Nexfin device).

Changes in arterial diameter and pressure were related to one another on a beat-by-beat basis (i.e., dynamically) as averaged over at least 20 cardiac cycles.

β-stiffness index. While arterial compliance is a direct measure of arterial elasticity, there is a non-linear relationship between pressure and diameter and so compliance is heavily dependent on changes in arterial pressure [304]. With interventions that may alter blood pressure (e.g., heat therapy), it is impossible to distinguish whether changes in compliance are due to changes in arterial elasticity or pressure. β-stiffness index is also a direct measure of arterial stiffness, but is much less dependent of blood pressure. As such, it has become much more commonly used clinically [305]. It has also been validated for its ability to predict future cardiovascular morbidity and mortality [306,307]. As it is been more-widely measured than compliance, reference values have been determined. In young, healthy subjects, β-stiffness index is typically ~5±2 and it increases linearly with healthy aging up to ~8 by age 70 [308]. β-stiffness index is calculated as,

(4) $\beta\text{-stiffness index} = \ln(SBP/DBP) * D/\Delta D$

Where, SBP = systolic blood pressure, DBP = diastolic blood pressure, ΔD = change in diameter across one cardiac cycle from diastolic trough to systolic peak measured using ultrasonography, and D = diameter averaged across each cardiac cycle.

Pulse wave velocity. Pulse wave velocity (PWV) is an indirect measure of arterial stiffness, but one which has been shown to have greater predictive value than other available methods [296,302]. While compliance and β-stiffness index offer more direct measures, they provide characteristics of the arteries only at a specific point where the measurement is taken [304]. As the properties of different arterial segments can vary, indices of stiffness across entire arteries may provide a more complete view of arterial health.

PWV is measured as the time delay between the arrival of the pressure wave at two arteries of interest (i.e., the systolic upstroke on tonometry tracings) divided by the distance traveled (Figure 3.8 – page 51). Mathematically, PWV is equivalent to arterial

impedance, as impedance = PWV x density of the blood, which is uniform within any given individual [309]. Arterial impedance is effectively the resistance the artery exhibits against pulsatile pressure and blood flow. It is directly proportional to Young's modulus of stiffness, the theoretical pressure required for maximal elongation.

Most commonly, pulse wave velocity is measured at the carotid and femoral arteries, i.e., across the aorta. Aortic pulse wave velocity is considered to be the gold-standard method for measuring arterial stiffness due to the relative ease of making measurements compared to other methods and due to its value in predicting cardiovascular morbidity and mortality [310-312].

There are two approaches for determining distance travelled. One uses the linear distance between the two probes, and the other is the subtracted distance, the difference between the sternal notch of the femoral artery minus the distance between the sternal notch and the carotid artery. A study which compared measured distances between the carotid and femoral arteries to actual arterial distances determined with magnetic resonance imaging found that 80% of the time, the linear distance was an accurate estimate of the real distance traveled, whereas the subtracted distance underestimated the actual distance [313]. Therefore, linear distance was used in this dissertation. Normal values for aortic pulse wave velocity in young, healthy individuals ranges from 6.0-10.0 m/s [314], and values >13.0 m/s are associated with significantly increased cardiovascular disease risk [302].

In addition to being measured across the aorta, pulse wave velocity can be measured across any segment of conduit vessels where tonometry pressure tracings can be obtained. Some disease states are characterized by greater dysfunction in peripheral arteries compared to central arteries (e.g., peripheral artery disease), and so there has been interest in measuring stiffness in peripheral arteries, even though stiffness in the more muscular arteries tends to not be as predictive as that in the central arteries across the general population [315]. Leg and arm pulse wave velocity are most common, measured from the femoral to dorsal pedal or posterior tibial arteries and from the brachial to radial arteries, respectively. Arterial stiffness increases from the central aorta down through the peripheral arteries, and so normal values in healthy young individuals

range from 8.4 to 1.2 m/s for arm pulse wave velocity and 9.2 to 1.05 m/s for leg pulse wave velocity [314].

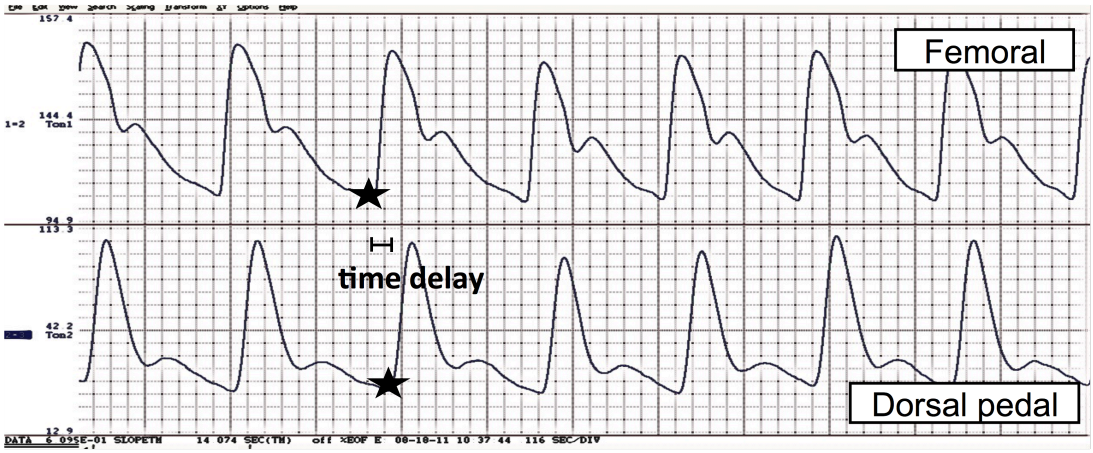

Figure 3.8. Concurrent applanation tonometry tracings taken from the common femoral and dorsal pedal arteries. Pulse wave velocity is calculated as the time delay between the systolic upstrokes at each artery divided by the linear distance between the probes. Femoral-ankle pulse wave velocity measured from this tracing was 9.3 m/sec, which falls within what is considered to be a healthy range [314].

In this dissertation, we measured leg and arm pulse wave velocity in addition to aortic primarily to be able to compare to spinal cord injured (SCI) subjects. We believed that the paralyzed limbs may respond differently to heat therapy due to loss of sympathetic innervation and due to much greater baseline impairment. Chapter VIII describes pilot work performed in SCI subjects. Leg and arm pulse wave velocity data from able-bodied young healthy subjects is included in that chapter for comparison. We hypothesized that we would observe no difference in leg or arm pulse wave velocity in able-bodied subjects; whereas, we would observe a robust improvement in leg pulse wave velocity in SCI subjects. Consistent with this hypothesis is the observation that 16 weeks of exercise training, which significantly reduced aortic pulse wave velocity, had no effect on leg pulse wave velocity [316].

Conduit vessel structure

In addition, we quantified structural changes in the arteries, namely vessel diameter, resting blood flow, and intima media thickness (IMT; also referred to as "wall

thickness"). IMT is another independent risk factor for cardiovascular disease as it is highly associated with the progression of atherosclerosis [297,301]. Thickening of the intimal lining of the arteries (the innermost layer consisting of endothelial cells, connective and elastic tissue, and sometimes other adhered cells) thickens with primary aging due to accumulation of collagen and adherence of white blood cells, primarily monocytes. Under pathogenic/pro-atherosclerotic conditions, monocytes that have differentiated into macrophages can accumulate cholesterol and become foam cells, resulting in atherosclerotic plaques. As these plaques grow, IMT becomes greater and greater. Although our subjects were young and healthy, some wall thickening can occur even in young subjects as a result of a sedentary lifestyle [310].

To measure IMT, the common carotid artery was imaged 1-2cm distal to the carotid bulb in three planes: anterior, lateral, and posterior. The superficial femoral artery was imaged 2-3 cm distal to the femoral bifurcation in two planes: anterior and lateral. Clearly demarcated intimal-medial boundaries were obtained while focusing on the far wall. Ultrasound contrast settings remained constant across all experimental sessions [317]. Images were frozen in diastole, enlarged, and calipers were used to make three repeat measurements of the wall thickness from the lumen-intima interface to the media-adventitia interface (Figure 3.9 – next page). All measurements (3 from each angle) were averaged.

When studying interventions which may change diameter and/or studying groups which may have different diameters (e.g., able-bodied versus spinal cord injured individuals), wall-to-lumen (W/L) ratio can be calculated to normalize for differences in diameter across groups and/or interventions [318]. For these measurements, average diameter was analyzed using the wall-tracking software (DICOM) over a period of 60 to 90 sec immediately following wall thickness measurements.

(5) W/L = wall thickness (mm) / average diameter (mm)

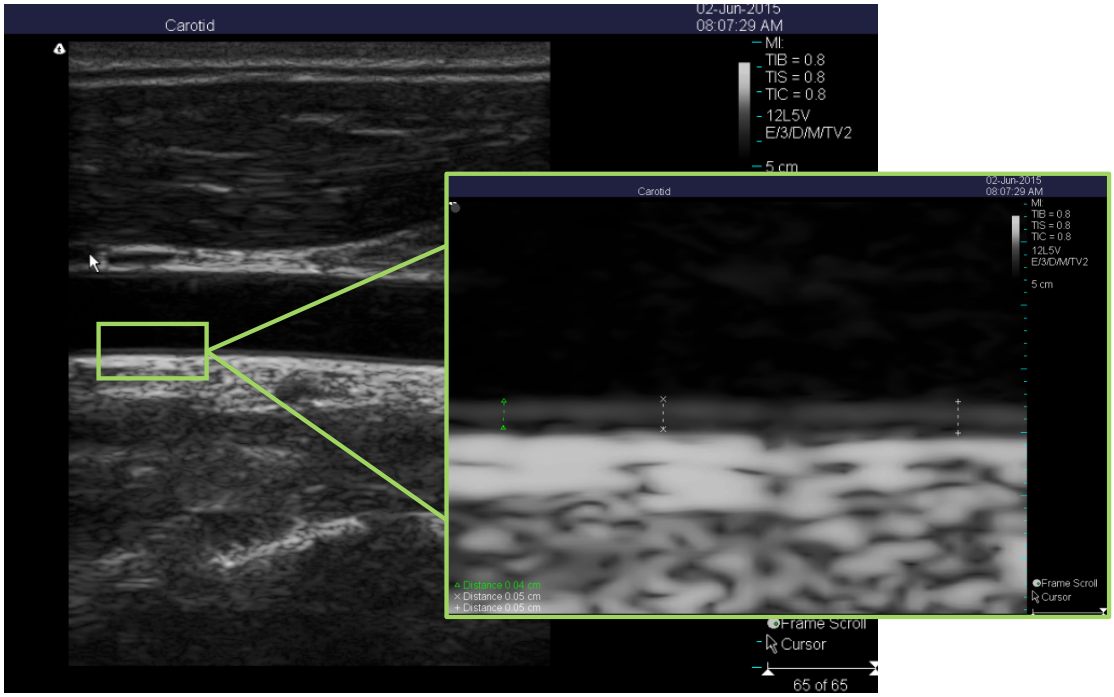

Figure 3.9. B-mode ultrasound image focusing on the distal wall of the common carotid artery, imaged from the posterior angle. A region of interest was magnified and calipers within the Terason software were used to measure from the lumen-intima interface to the media-adventitia interface in triplicate.

CUTANEOUS MICROVASCULAR FUNCTION

There is now a wealth of evidence to suggest that cardiovascular disease begins in the microvasculature. In a variety of cardiovascular-related disease states, impairments in the microvasculature can be detected prior to that in the large conduit vessels. As such, any comprehensive assessment of vascular health must include measurement of microvascular function. Techniques now exist to measure blood flow to almost all regions of the body, and, in some, vasoactive stimuli can be delivered (e.g., pharmacological agents, ischemia, hypoxia) in order to assess the reactivity of the microvasculature, or microvascular function.

In humans, the cutaneous circulation offers an ideal place to study microvascular function. Due to its accessibility, blood flow can be easily quantified using techniques such as laser-Doppler flowmetry and the underlying molecular mechanisms can be studied easily and relatively non-invasively using cutaneous microdialysis.

Microdialysis

The microdialysis technique allows for vasoactive substances to be delivered locally to a small area of skin with minimal systemic effects, thereby elucidating the molecular mechanisms behind vasodilator responses *in vivo*. Substances released from the tissue can also be collected from the distal end of the microdialysis fibers in the dialysate, although this was not performed as part of this dissertation. Microdialysis fibers consist of a hollow tube with a semi-permeable membrane in the center (Figure 3.10). Vasoactive substances dissolved in a vehicle (most commonly Lactated Ringer's solution) can be infused through the fiber and will be delivered to an area of tissue approximately 1cm in diameter around the semi-permeable membrane.

Placement of microdialysis fibers is performed using aseptic technique. In this dissertation, fibers were placed in the dermal layer of skin on the ventral side of the non-dominant forearm, although other locations can be used (the calf is also common). Before placement, a tourniquet should be used to identify superficial veins to be avoided in order to ensure fibers are placed within the microvasculature. Fibers are typically inserted at least 5cm apart by first introducing a 25-g needle ~1mm below the surface of the skin, with entry and exit points ~2.5cm apart (Figure 3.11 – next page). As pharmacological agents infused through the fibers typically reach an area of skin ~1 cm in diameter around the fiber, this placement ensures little to no interaction between drugs infused at different sites. Once the needle is placed, fibers are then threaded through the lumen of the needle

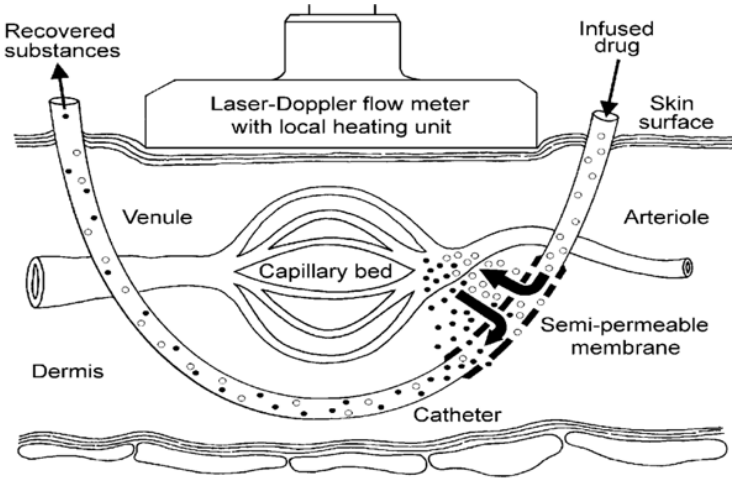

Figure 3.10. Depiction of cutaneous microdialysis. Artwork by Bob Lorenz.

and the needle is removed leaving just the fiber in place under the skin, with the semi-permeable membrane centered between entry and exit points. Fibers are typically infused with Lactated Ringer's solution at a rate of 2.0 μl/min (CMA 102 Syringe Pump; CMA Microdialysis AB, Solna, Sweden) until the start of study drugs. Since the needle trauma causes inflammation and a transient substantial increase in skin blood flow, a period of at least 60-90 min should be allowed prior to the start of experimental protocols.

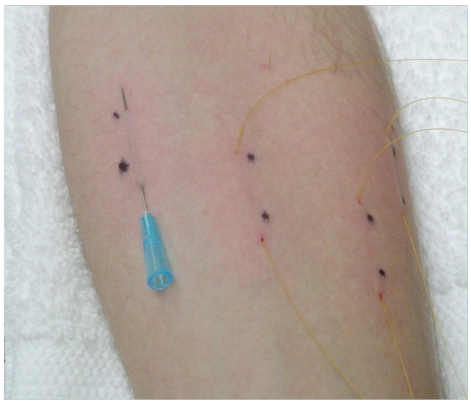

Figure 3.11. Placement of four intradermal microdialysis fibers in the ventral forearm using a 25-gauge needle. Photo by Brett Wong.

Cutaneous thermal hyperemia

Using cutaneous microdialysis, the molecular mechanisms underlying vasodilation and vasoconstriction to a variety of stimuli have been extensively explored. Perhaps most widely assessed has been the vasodilator response to locally applied heat, known as "thermal hyperemia," in which the skin is typically heated to 42°C at a rate of 0.1°C/sec. Local heating produces a biphasic vasodilator response consisting of an initial peak that occurs within ~3-5min into heating and a prolonged secondary plateau phases which stabilizes approximately 30-40 min into heating (Figure 3.13 – page 57). A brief nadir occurs between the two phases.

Cutaneous thermal hyperemia has been shown to be impaired under a variety of disease states, including coronary artery disease [319], kidney disease [320], primary aging [321], and hypertension [322], as summarized in Figure 3.12 (next page). As these impairments mirror declines in microvascular function in other microvascular beds, the cutaneous microcirculation has often been proposed to be representative of globalized

microvascular health [323-325]. As a result, many studies have aimed at discovering the underlying mechanisms that contribute to the response. So doing helps to establish impairments in specific molecular pathways that are present in disease states, which then helps to identify potential targets for pharmacological and/or lifestyle treatments.

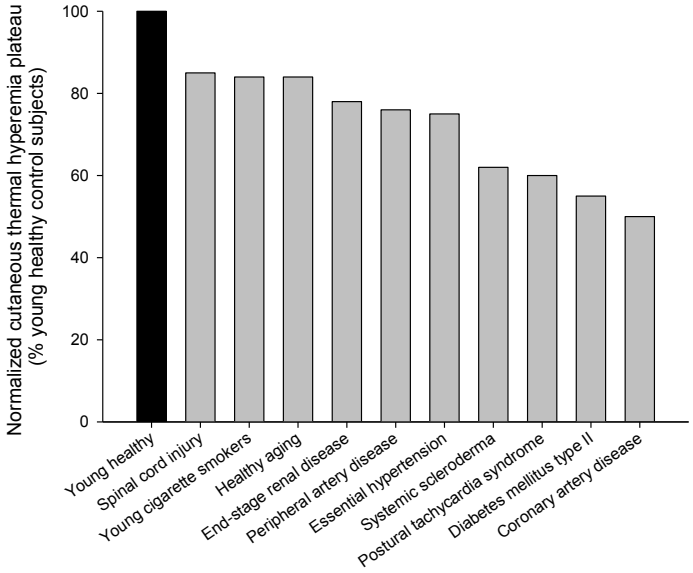

Figure 3.12. Normalized plateau cutaneous vascular conductance across a variety of disease conditions. In general, the disease states which are characterized by the greatest impairments in cardiovascular function also exhibit the greatest impairments in cutaneous microvascular function. Data adapted from Van Duijnhoven et al. [2], Fujii et al. [326], Minson et al. [321], Kruger et al. [320], Hodges et al. [327], Smith et al. [322], Boignard et al. [328], Stewart et al. [329], Colberg et al. [330], and Agarwal et al. [319].

The initial peak is predominantly mediated by a sensory nerve axon reflex [331], most likely initiated via transient receptor potential vanilloid type-1 (TRPV-1) channels located on the sensory nerves [332]. The sensory nerves are thought to release calcitonin gene-related peptide and substance P, although this has not been confirmed experimentally [333].

The plateau phase is approximately 60% dependent on nitric oxide (NO) [331,334]. Several other factors, including adenosine receptors [335], TRPV-1 receptors [332], and reactive oxygen species [336], have been shown to play a role, primarily through altering bioavailable NO. The cyclooxygenase pathway, a predominant vasodilatory pathway in response to other stimuli, does not appear to be involved [337].

For a long time, the remaining ~40% of dilation was unexplained. However, in 2012 [338], we showed that almost the entirety of the remaining vasodilation could be abolished by blocking the actions of endothelial-derived hyperpolarizing factors (EDHFs), a class of vasodilators that cause smooth muscle hyperpolarization and therefore relaxation primarily by stimulating calcium-activated potassium (KCa) channels (Figure 3.13). In this same study, we went on to elucidate which types of EDHFs may be involved. Of the various EDHFs, epoxyeicosatrienoic acids (EETs) have been most studied and have been shown to elicit vasodilation in humans in other vascular beds [276,339,340]. We showed that approximately half of the EDHF component was attributable to EETs [338].

Despite how well the molecular underpinnings of this response have been teased out, some limitations do exist. For example, in young, healthy subjects, the plateau commonly reaches ~90–95% of maximal cutaneous vascular conductance (CVC), making it difficult to evaluate the effects of potentially beneficial interventions due to a

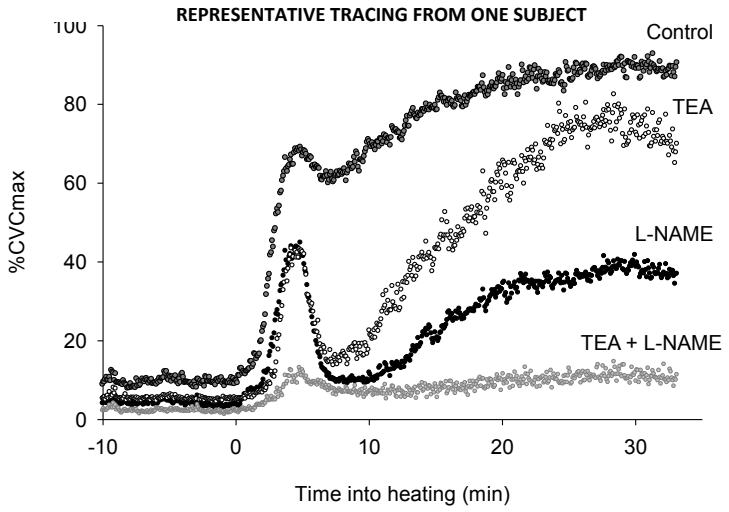

Figure 3.13. Taken from Brunt & Minson, 2012 [338] with permission. A representative tracing from one subject during local heating of the skin at four microdialysis sites receiving: 1) Lactated Ringer's (control), 2) Tetraethylammonium (TEA), a calcium-activated potassium (KCa) channel blocker, 3) NG-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor, and 4) combined TEA + L-NAME. Data are presented as a percentage of maximal cutaneous vascular conductance (%CVCmax), as determined by infusion of 56mM sodium nitroprusside and max heating to 44°C. Combined blockade of KCa channels and nitric oxide synthase with TEA + L-NAME abolished the majority of thermal hyperemia and plateau CVC at this site was not different from baseline CVC.

ceiling effect. Furthermore, as the plateau is substantially dependent on both NO and EDHF, it is not possible to attribute impairments and/or improvements to either pathway without the use of pharmacological techniques, such as microdialysis. We therefore developed a new protocol for local heating in which the skin was rapidly heated to just 39°C [341]. Using this protocol, we were successfully able to isolate the NO component of the response, creating a test of microvascular function that is more clinically relevant as vascular dysfunction in the majority of disease states is characterized by impaired NO. Additionally, plateau CVC reaches ~40-50% of max, allowing for the assessment of interventions, such as heat therapy, to improve the response (Figure 3.14). For these reasons, and those described above in this section, we selected local heating to 39°C as the best approach for assessing the effects of heat therapy on microvascular function.

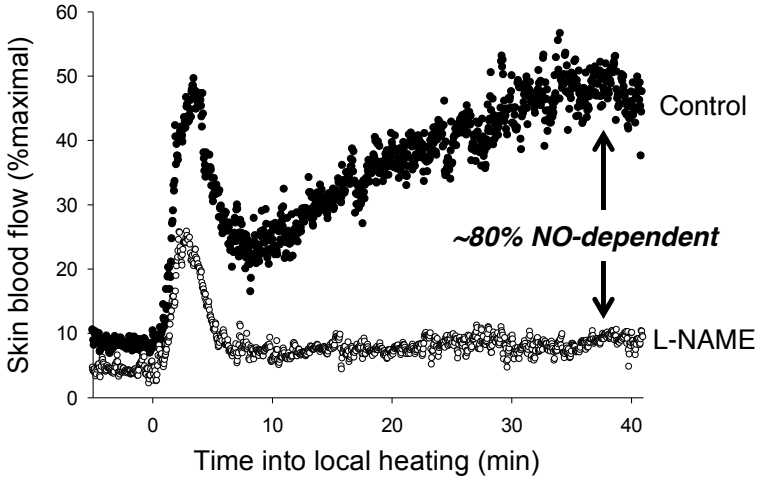

Figure 3.14. Adapted from Choi et al. 2014 [341]. Representative response to local heating to 39°C at a rate of 0.1°C/sec at microdialysis sites receiving 1) Lactated Ringer's (Control), and 2) NG-nitro-L-arginine methyl ester (L-NAME), a non-specific nitric oxide synthase inhibitor. This local heating protocol results in vasodilation which is ~80% dependent on nitric oxide and which reaches a plateau skin blood flow of ~40-50% of maximal.

Pharmacological Interventions

In this dissertation, local heating to 39°C was assessed at three microdialysis sites randomly assigned to be infused with: 1) Lactated Ringer's solution (sham site), 2) 10mM Nω-nitro-L-arginine (L-NNA), or 3) 10μM Tempol. L-NNA is an analog of L-arginine, which non-specifically inhibits NO synthase. Inhibiting NOS, and therefore NO

production, allows us to investigate the contribution of NO to the overall vasodilator response and whether NO-dependent dilation is improved following heat therapy. Tempol is a superoxide dismutase mimetic. In the presence of Tempol, the free radical superoxide is converted to oxygen of hydrogen peroxide. Superoxide, which normally combines with NO to form peroxynitrite, is a primary contributor to oxidative stress and reduced NO bioavailability. Therefore, infusion of Tempol allows us to determine how oxidative stress limits the vasodilator response and how heat therapy may reduce oxidative stress. Drug concentrations were selected as the minimum doses for eliciting maximal inhibition, based on previous studies [336,342].

All drugs obtained from manufactures at the highest chemical purity available. Solutions and dilutions to the appropriate concentration were made the day of the study by the primary investigator with sterile Lactated Ringer's solution in a fashion that yields sterile perfusates. Furthermore, perfusates were filtered through a 0.2μm filter prior to administration to the human subjects. An investigational new drug approval was obtained through the U.S. Food & Drug Administration in order to use these drugs in these studies (IND# 124,303; PI: Minson CT).

Laser-Doppler flowmetry

As previously mentioned, skin blood flow can be easily quantified using laser-Doppler flowmetry. These probes sit flush with the skin and can be seated in the center of local heaters (Figure 3.15 – next page). Laser-Doppler probes emit a single beam of infrared light which penetrates the skin and reflects off the red blood cells. Returning light is received by collecting fibers on the probe. The intensity of light received gives a measure of concentration of red blood cells, and the velocity of the red blood cells is determined using a Doppler shift analysis. Red blood cell flux is then calculated as the product of concentration and velocity.

Flux is an arbitrary measure (versus absolute blood flow) and is dependent on probe placement, as the density of blood vessels will vary substantially across the skin. Therefore, it is important to normalize all flux measurements. Most commonly, flux is normalized to maximal flux, obtained by locally heating the skin to >43°C and/or microdialysis infusion of high doses of sodium nitroprusside, an endothelium-

independent nitric oxide donor. In this dissertation, we used a combination of local heating to 43.5°C and infusion of 56mM sodium nitroprusside. Flux may also be presented as a percent change from baseline, although this approach is questionable when performing microdialysis studies as many pharmacological agents alter baseline. Since flux is dependent on changes in blood pressure, data are often converted to cutaneous vascular conductance (CVC = flux / mean arterial pressure) and then presented as a percentage of maximal CVC. CVC more closely reflects the vasodilator/constrictor state of the blood vessels.

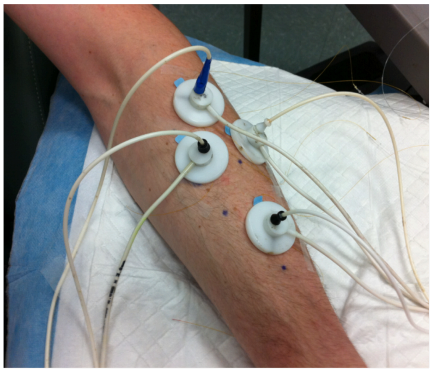

Figure 3.15. A subject instrumented with four laser-Doppler probes (MoorLab; Moor Instruments, Axminster, UK) seated in the center of local heaters (Skin Heater/ Temperature Monitor SH02; Moor Instruments) which cover ~0.78cm^2 of tissue. Photo by Vienna Brunt.

VENOUS BLOOD COLLECTION & ANALYSIS

During the first and last water immersion sessions, blood was collected from an antecubital vein prior to entering the hot tub and 1h after exiting the hot tub. Collection at 1h after exiting the hot tub was chosen as pilot studies indicated this time frame was ideal for detecting peak changes in factors in the serum and peak increases in HSP content in peripheral blood mononuclear cells (PBMCs). Pilot data is presented in Chapter IV (page 73).

Venous blood was collected into three types of vacutainers: 1) EDTA (anticoagulant)-containing tubes for the measurement of hemoglobin and hematocrit, 2) serum-separating tubes (for cell culture experiments and other analyses described in Chapter VII), and 3) Ficoll Hypaque-containing cell preparation tubes with sodium citrate (CPT Vacutainer; BD, Franklin Lakes, NJ) for the separation of mononuclear cells from

whole blood. Whole blood samples in EDTA tubes were immediately analyzed in triplicate for hemoglobin (Hb 201 Hemoglobin Analyzer; HemoCue, Brea, CA) and hematocrit (microcapillary centrifugation). Serum-separating and CPT tubes were centrifuged 30 min post-collection, and serum or plasma containing PBMCs were separated, aliquoted, and stored at –80°C until later analysis.

Blood volume

Plasma volume expansion is a hallmark of exercise heat acclimation [343]. It has been shown to also occur in animals following chronic heat exposure [172], but has not yet been measured in humans undergoing passive heat acclimation.

Red cell and plasma volume were measured in a subset of subjects (N=4 in the heat therapy group and N=8 in the sham group) prior to the first hot tub session and 36-48h after the last hot tub session using the optimized carbon monoxide rebreathing method developed by Schmidt & Prommer [344]. Due to the technical challenges of performing this technique, we were only able to begin using it part way through data collection (equipment had to be shipped from Germany and a technician had to come out to train us on the technique). Unfortunately, due to timing of when subjects in each of the groups began the 8-week intervention, this resulted in measurements being obtained in uneven numbers of subjects in each group.

Subjects reported to the laboratory having abstained from food for at least 4h, caffeine and alcohol for at least 12h, and exercise and any medications, vitamins, or supplements for 24 h. Subjects provided a first-morning urine sample to confirm euhydration via urine specific gravity <1.02 (refractometer). If USG was >1.02, subjects drank 5ml/kg of water at least 30 min before blood volume measurements were obtained.

Subjects rested seated for >20 min while an intravenous catheter was placed in an antecubital vein. A baseline venous blood sample was collected into an EDTA-containing vacutainer (BD, Franklin Lakes, NJ) and baseline end-tidal carbon monoxide (CO) was measured using a CO monitor (Dräger Pac 3500; Dräger, Lübeck, Germany).
Red cell volume was then measured using the CO-rebreathing method developed by Schmidt & Prommer [344]. Briefly, at the end of a maximal exhalation, subjects fully inhaled a mixture of CO and oxygen (O_2) through the spirometer device shown in Figure

3.16. A syringe (D) was pre-filled with 100% CO in a volume equivalent to 1ml/kg of the subject's body weight. A 3L anesthetic bag (I) was pre-filled with 100% O_2 to a volume of approximately 2.5L (not necessary for it to be exact). After full inhalation, subjects held their breath for 10 seconds and then rebreathed through the device (with the syringe now closed off) for another minute and fifty seconds. At the end of the rebreathing period, subjects maximally exhaled and the air was captured in the anesthetic bag by closing the main two-way valve (C). End-tidal CO was again measured 2 min after subjects completed the rebreathing period (4 min after receiving the bolus of CO), and another venous blood sample was collected 5 min after subjects completed the rebreathing period (7 min after receiving the bolus of CO). HbCO in venous blood is equilibrated with arterial blood by this time [344]. CO concentration of the exhaled air in the bag was measured, and the volume of air in the bag was measured using a 3L calibration syringe (Hans Rudolph, Inc., Shawnee, KS). Baseline and post-CO blood samples were analyzed in triplicate for CO bound to hemoglobin (HbCO), O_2 bound to hemoglobin (O_2Hb), and total hemoglobin (Hb) (OSM3 Blood Gas Analyzer; Radiometer, Copenhagen, Denmark). Hematocrit was measured in triplicate by microcapillary tube centrifugation.

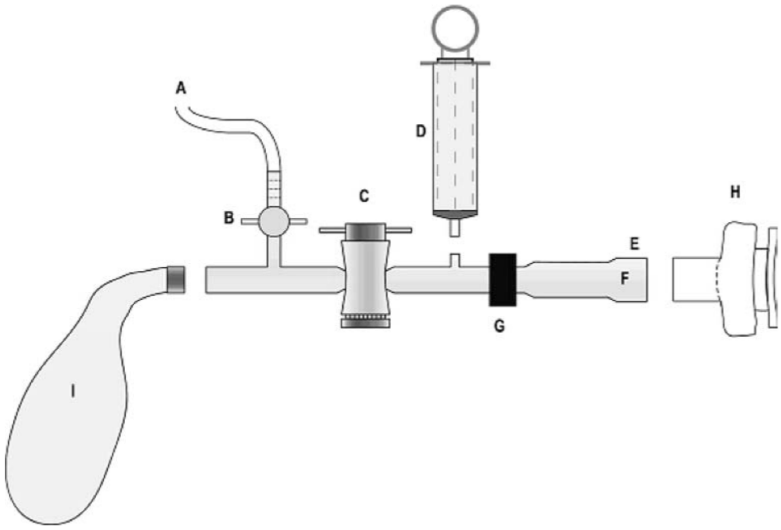

Figure 3.16. Schematic of the carbon monoxide (CO)-rebreathing spirometer device, taken from Schmidt & Prommer [344] with permission. A: tube for filling bag with oxygen; B: oxygen port valve (closed during test); C: valve for oxygen reservoir (open during test); D: CO syringe; E: adapter to connect mouthpiece; F: anti-microbial filter and soda-lime to absorb carbon dioxide; G: connector to glass spirometer; H: mouthpiece; I: anesthetic bag filled with 100% oxygen.

Total hemoglobin mass (t-Hb-mass) was calculated using the below equations. Red cell volume was calculated from t-Hb-mass using the ratio of Hb concentration to hematocrit. Total blood volume and plasma volume were calculated from red cell mass using hematocrit. Two correction factors were applied to hematocrit values. A factor of 0.96 was applied to account for plasma trapped between red cells as a result of centrifugation [345]. A factor of 0.91 was applied to account for differences in hematocrit in blood sampled from the venous periphery compared to whole-body hematocrit [346].

(6) $\text{t-Hb-mass} = K \times MCO \times 100 \times (\Delta HbCO\% \times 1.39)^{-1}$

Where, $\Delta HbCO\%$ = the difference in venous blood HbCO between baseline and post-CO administration

1.39 = Hüfner's number (ml CO x g Hb^{-1})

K = current barometric pressure x 760^{-1} x $[1 + (0.003661 \times \text{current temperature})]^{-1}$

MCO = CO_{adm} - (CO_{sys} (at time of disconnection) + $CO_{exhaled}$ (at 5 min))

CO_{adm} = volume of CO administered using syringe (equal to 1ml/kg body weight)

CO_{sys} = concentration of CO in the bag x (bag volume + estimated lung residual volume)

$CO_{exhaled}$ = end-tidal CO concentration (change from baseline to post-CO administration) x estimated alveolar ventilation x time since delivery of bolus (i.e. 5 min)

Estimated residual volume (L) = (0.0275 x age) + (0.0189 x height) - 2.6139

Estimated alveolar ventilation (L) = (0.075 x height) - 7.75

CELL CULTURE & *IN VITRO* TECHNIQUES

In addition to demonstrating functional vascular adaptations in human subjects are conferred with long-term heat therapy, a major goal of this dissertation was to identify some of the molecular mechanisms that potentiate functional improvements. While direct (acute) effects of HSPs have been extensively demonstrated, and intracellular genomic

changes in animal models of heat acclimation have been well-studied, the changes in these pathways that link back to changes in vascular function have not yet been elucidated, and these changes have not been investigated in humans.

Furthermore, the majority of models in cells and animals have utilized what would be considered 'extreme' stressors in humans. For example, changes in protein abundance associated with heat shock to >42°C has been well-studied, but very few studies have investigated effects to more mild levels of heat stress that would match temperatures commonly reached by humans.

Therefore, we utilized cell culture of human endothelial cells paired with collection of human sera and primary cells to elucidate the changes in the molecular pathways associated with our specific model of heat acclimation. In order to keep to techniques that could be performed feasibly for this dissertation, endothelial cells for culture were purchased and treated (as described below) to simulate physiological heat acclimation. A better approach would have been to collect primary endothelial cells from our human subject undergoing heat therapy; however, the techniques available to do this in humans result in very small yields of endothelial cells such that is it challenging to investigate changes in protein content and it is impossible to investigate stimulated responses (e.g., changes in protein activity). For all experiments, we focused on the molecular pathways that are associated with vascular function (i.e., the NO pathway, oxidative stress, and inflammation).

We performed three types of experiments:

1) We investigated changes in protein content in human umbilical vein endothelial cells (HUVECs) following 24h of incubation at 39°C. This temperature was matched to the rectal temperatures obtained by human subjects participating in heat therapy. An incubation period of 24h was selected to represent a chronic time frame in terms of the lifespan of the cells. In preliminary studies, we demonstrated that the magnitude of HSP induction was similar following 24h at 39°C (compared to shorter durations) to HSPs measured in primary mononuclear cells collected from human subjects, a cell line which can be isolated from venous blood, which is exposed to the same

temperature conditions as endothelial cells, and which has been shown to exhibit an HSP stress response to exercise heat stress [114].

2) We investigated changes in protein content in HUVECs exposed to human sera collected from human subjects pre- and 1h post- the first session of heat therapy (acute response) and pre- and 1h post- the last session of heat therapy at 8wks (chronic response). Serum at 8wks was collected 24-48h after the penultimate heat therapy session. As demonstrated in animal models, heat acclimation results in widespread changes in gene activation and protein expression in a variety of tissues. Many of these changes include upregulation (or downregulation) of proteins that can traverse the cell membranes and enter the circulation (e.g., cytokines), thereby influencing endothelial cells[1].
3) We investigated the effects of heat acclimation on serum angiogenic balance, utilizing an endothelial tubule formation assay in which HUVECs were exposed to sera from human subjects collected at the four time points described above. Performing this assay allowed us to demonstrate the downstream effects of heat therapy on cellular processes, rather than just demonstrating changes in protein content.

Specific techniques were performed as follows:

Cell culture experiments

Primary human umbilical vein endothelial cells (HUVECs) were purchased from ATCC. Cells were cultured in 75 cm^2 culture flasks under standard conditions (37°C, 5% CO_2, 20% O_2) in vascular cell basal medium that was supplemented with an endothelial growth kit containing growth factors, nutrients, and fetal bovine serum ("complete media"). Cells were grown until ~80% confluence and then passaged. For passaging, cells were washed in warm phosphate buffered saline (PBS), lifted from the flask with an enzymatic dissociation buffer (TrypLE Express, Gibco, Gaithersburg, MD), and

[1] Besides direct heat and circulating factors, endothelial cells *in vivo* in our human subjects were also exposed to intermittent increases in shear stress. The effects of fluid shear stress on cultured endothelial cells have been well studied by others (as reviewed in Chapter 2) and so we chose not to repeat those experiments for this dissertation.

resuspended in complete media before being split between 2-3 new flasks. Cells were used for experiments after being passaged 3-4 times.

For direct heat and serum exposure experiments, cells were plated in 6-well cell culture plates and incubated under standard conditions until ~40% confluence. Cells were then serum starved for 4h by replacing complete media with 1ml per well raw media (no endothelial growth kit). This step synchronizes the cell proliferation phase across all plates. After serum starve, media in plates used for direct heating experiments was replaced with 1ml complete media and plates were incubated at 39°C or 37°C (control) for 24h. For plates used for serum exposure experiments, 100μl human sera were added to each well and plates were incubated under standard conditions for 24h. Sera collected from human subjects in just the heat therapy group at each of the 4 time points were used (0wks preHT, 0wks postHT, 8wks preHT, 8wks postHT). Each sample was introduced to 3 wells of cells, which were combined at collection.

For each condition (temperature or serum sample), samples from all 3 wells were combined. Cells were washed with warm PBS, lifted with TrypLE, and collected into sterile cryotubes. Cells were pelleted via centrifugation and washed again with PBS. Cell pellets were combined with radioimmunoprecipitation assay buffer (RIPA) plus protease inhibitor and lysed via sonication.

Peripheral blood mononuclear cell isolation

Venous blood was collected into Ficoll-Hypaque containing vacutainers (BD Vacutainer CPT Cell Preparation Tube; Becton Dickinson, Franklin Lakes, NJ) immediately prior to and 1h following the first and last hot tub sessions (4 time points). PBMCs were isolated in vacutainers by centrifugation (3,000rpm for 15 min at room temperature). PBMCs suspended in plasma were separated and stored frozen at –80°C until analysis. Prior to analysis, thawed PBMCs were separated from plasma by centrifugation and washed with PBS four times. Cell pellets were then combined with RIPA buffer plus protease inhibitor and lysed via sonication. Due to insufficient cell lysing in some samples, soluble protein was separated from any insoluble particles and transferred to clean cryotubes before protein analysis.

Western blotting

The concentration of total protein in HUVEC and PBMC lysates was quantified using a Bradford dye-binding protein assay (Bio-Rad Protein Assay; Bio-Rad, Hercules, CA). Samples were diluted with DI water to achieve a concentration of 20-50ug total protein in 20µl, and 4µl 2mM DTT + 2µl 4X LDS sample buffer (NuPAGE®, Invitrogen, Carlsbad, CA) was added to each sample. After being heated at 80°C for 20 min to facilitate unfolding of proteins, samples were loaded into each well of 4-20% SDS polyacrylamide separating gels (Life Technologies, Grand Island, NY) and separated by electrophoresis at 200V in MOPS or MES SDS running buffer (NuPage®). Proteins were then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) and Ponceau-stained to access transfer across each gel. The images of the Ponceau-stained membranes were digitized using a flatbed scanner.

Nitrocellulose membranes were incubated for 1h in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) and then incubated overnight at 4°C in blocking buffer containing primary antibodies (detailed below). Membranes were then washed and incubated with the appropriate secondary antibodies (LI-COR Biosciences) for 1h at room temperature. The fluorescent bands were digitized using a LI-COR Odyssey infrared imaging system (LI-COR Biosciences). Digitized images were quantified using LI-COR Image Studio^{TM} software. Primary antibodies for one protein at a time were used and antibodies were stripped using NewBlot^{TM} Nitro Stripping Buffer (LI-COR Biosciences) in between probing for other proteins of interest. For direct heating and serum exposure experiments, primary antibodies used were: 1) anti-endothelial NO synthase (eNOS) (1:1,000; Cell Signaling Technology, Danvers, MA) 2) anti-superoxide dismutase-2 (MnSOD/SOD2) (1:1,000; Sigma-Aldrich, St. Louis, MO) 3) anti-Hsp90 [S88] (1:200; Abcam, Cambridge, MA) and 4) anti-Hsp70 [BRM-22] (1:5,000; Abcam), and 5) anti-vinculin (loading control; 1:1,000; Cell Signaling Technology). For PBMCs, primary antibodies used were: 1) anti-Hsp70 [BRM-22] (1:5,000; Abcam) 2) anti-Hsp90 [S88] (1:200; Abcam), or 3) anti- β actin (loading control; 1:1,000; Cell Signaling Technology).

Superoxide production assay

In order to determine whether direct heat and exposure to serum from heat acclimated subjects reduces superoxide production, we used a cellular superoxide detection assay in live HUVECs (Cellular Superoxide Detection Assay Kit; Abcam). Since superoxide is generally only present in cells transiently (it is rapidly either reduced to hydrogen peroxide and then water or combined with NO to form peroxynitrite), assays must detect real-time superoxide production. The superoxide detection stain we used is cell permeable and reacts rapidly and specifically with superoxide, generating an orange fluorescent product.

In separate experiments from the ones described above, HUVECs were plated in 96-well culture plates at a concentration of 2 x 10^4 cells per well and exposed to the direct heat or serum exposure conditions as described in triplicate. After 24h, media was replaced with 20µl of 1µM superoxide detection stain diluted in either complete (for direct heating cells) or raw (for serum exposure cells) media. Cells were incubated for 1h under the same conditions as before (37 or 39°C, or 37°C with 10% serum). Additional wells were left untreated (in complete media) and unstained (blank) or were treated with 20µl of 500µM pyocyanin (reactive oxygen species inducer), which served as a positive control. Cell fluorescence was determined with a phase-contrasted fluorescent microscope with Cy3 filter at 10X optical zoom (Axio Observer.D1; Zeiss, Oberkochen, Germany). Total cell fluorescence per frame was determined using ImageJ analysis software (National Institutes of Health; Bethesda, MD). Total fluorescence of blank wells was subtracted from treated wells to obtain values of corrected total cell fluorescence and values from triplicate were averaged.

Angiogenesis assay

An endothelial tubule formation assay was used as a marker of serum angiogenic balance. This assay was first developed by Kubota et al. in 1988 [347] and has been used widely since to quantify angiogenesis [348-350]. Briefly, endothelial cells are plated onto Matrigel, a gel comprised of extracellular matrix isolated from Engelbreth-Holm-Swarm mouse sarcoma cells and pro-angiogenic factors, which promotes the cells to proliferate, migrate, and form tubules and lumen-like structures. After a period of incubation

(commonly 6-12h), the cells resemble a cross-section of a capillary bed (Figure 3.17). Angiogenesis can be quantified by imaging the cells under a microscope. Number of tubules, number of lumen, and total tubule length are common methods of quantification.

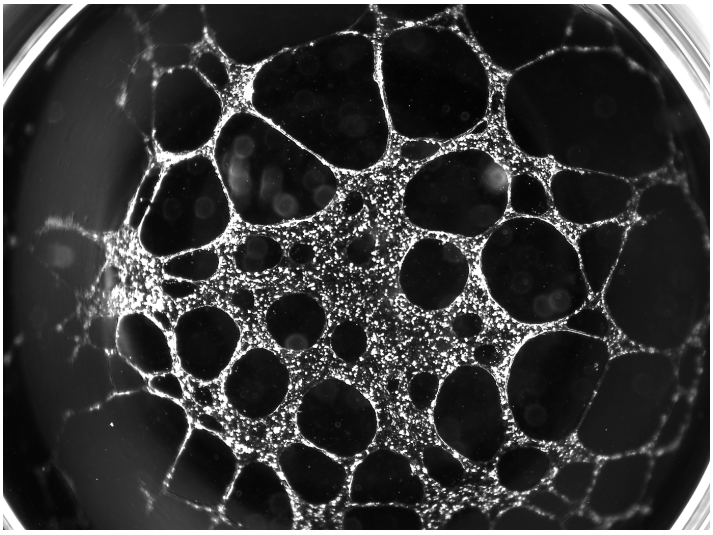

Figure 3.17. A representative phase-contrasted image at 2.5X magnification of HUVECs plated onto Matrigel following 10h of incubation with serum from a young, healthy human subject under standard conditions.

The following were pipetted to each well of a 96-well cell culture plate, in this order:

1) 40µl of phenol-red free growth factor reduced Matrigel (BD Biosciences, San Jose, CA)
2) 100µl of HUVECs (American Type Culture Collection [ATCC], Manassas, VA), suspended in serum-free vascular cell growth media at a concentration of $1x10^6$ cells/ml
3) 10µl of serum from human subjects (triplicate wells per sample)
4) For a subset of experiments: 10µl of 12mM L-NNA (final concentration of 1mM; SigmaAldrich, St, Louis, MO)

Matrigel was incubated for at least 30min at 37°C prior to plating cells in order to allow time for the Matrigel to solidify and set. HUVECs used had undergone 2-3 passages at the time experiments were conducted. To determine cell concentrations, a small sample of cells were stained with trypan blue to determine the concentration of live

cells with a Countess™ automated cell counter (Invitrogen; ThermoFisher Scientific, Inc., Grand Island, NY), and additional raw media was added to achieve a concentration of $1x10^6$ cells/ml. Sera were collected from human subjects at 4 time points: immediately prior to their first heat therapy session (0wks pre), 1h following their first heat therapy session (0wks post), immediately prior to their last heat therapy session which was also at least 36h following their penultimate session (8wks pre), and 1h following their last heat therapy session (8wks post). Each serum sample was added to 3 wells on 2 separate experiments.

In a subset of experiments, L-NNA was added to inhibit NO synthase and to determine whether the pro-angiogenic effects of heat therapy are mediated via NOS upregulation. A concentration of 1mM was selected based on pilot studies in order to reduce NOS activity without completely abolishing it (10mM is typically considered to produce complete inhibition), as NO is essential for angiogenesis [351].

Cells were then incubated under standard conditions (37°C, 5% CO_2, 20% O_2) for 10h. This time frame was determined in pilot studies to coincide with peak tubule formation (tubules began to dissociate shortly thereafter). Wells were then imaged with a phase-contrasted microscope at 2.5X magnification (Axio Observer.D1; Zeiss, Oberkochen, Germany). Images were cropped to 600x750 pixels to avoid edges of the wells where Matrigel became concave. Total tubule length was determined independently by two blinded investigators using ImageJ analysis software (National Institutes of Health, Bethesda, MD). Results were averaged first, for each well across investigators, second, across the 3 wells for each serum sample, and third, across the two experiments that used each serum sample.

CHAPTER IV

HALLMARK ADAPTATONS TO HEAT ACCLIMATION CAN BE OBTAINED THROUGH EIGHT WEEKS OF PASSIVE HEAT THERAPY IN SEDENTARY HUMANS

INTRODUCTION

This dissertation utilized passive heat exposure as a therapy for inducing cardiovascular adaptation over time. Although the physiological responses to acute passive heat exposure are well understood, few studies have investigated the effects of repeated bouts of passive heat exposure, and only two have done so for a time period >3 weeks in humans [16,17].

Exercise heat acclimation, which typically consists of 100 min of low-to-moderate intensity exercise in a hot environment (35-50°C) for 7-14 consecutive days, has been extensively studied and shown to induce a number of "classical" adaptations. These include reductions in resting core temperature, improved thermoregulation (e.g., higher sweat rate and skin blood flow for a given core temperature), and increased plasma volume [22,343,352]. Additionally, basal expression of heat shock proteins (HSPs) have been demonstrated to increase, particularly Hsp70 which is highly inducible [169]. HSPs in turn upregulate a variety of proteins important for improved cardiovascular health [4-6]. This increased expression of HSPs is likely a key mechanism behind the chronic vascular adaptations observed with heat therapy.

Prior to presenting data regarding the vascular adaptations observed in this dissertation (Chapters V-VII), we sought to characterize whether 8 weeks of passive heat therapy also results in a heat-acclimated phenotype, as does exercise heat acclimation. Specifically, we hypothesized that, compared to a sham group, heat therapy would result in reduced resting core temperature, increased sweat rate during heat exposure, plasma volume expansion, and elevated basal expression of HSPs.

METHODS

Human Subjects

As described in Chapter III, twenty young, healthy subjects were assigned to participate in and completed either 8 weeks of heat therapy or thermoneutral water immersion (N=10 in each group; see Chapter III, pages 36-38 for descriptions of interventions). All subjects provided oral and written informed consent prior to participation in the study, as set forth by the *Declaration of Helsinki*. All experimental procedures were approved by the Institutional Review Board at the University of Oregon.

Venous blood collection

During the first and last water immersion sessions, blood was collected from an antecubital vein prior to entering and 1h after exiting the hot tub. Collection at 1h after exiting the hot tub was chosen based on pilot data (Figure 4.1 – next page) in which we identified 1h post-hot water immersion as an ideal time point to observe increased HSP content in peripheral blood mononuclear cells (PBMCs) and improved anti-oxidative capacity in the serum. A previous study which conducted a more extensive time course analysis also showed that HSP protein content in PBMCs peaks at 1h following heat stress [129]. Additionally, Zuhl et al. [353] found Hsp70 protein in PBMCs to peak 2h after acute exercise in the heat, but observed no change from baseline immediately following or 4h after exercise.

At the four time points, venous blood was collected into three types of vacutainers: 1) EDTA (anticoagulant)-containing tubes for the measurement of hemoglobin and hematocrit, 2) serum-separating tubes (for cell culture experiments and other analyses described in Chapter VII), and 3) Ficoll Hypaque-containing cell preparation tubes with sodium citrate (CPT Vacutainer; BD, Franklin Lakes, NJ) for the separation of mononuclear cells from whole blood. Whole blood samples in EDTA tubes were immediately analyzed in triplicate for hemoglobin (Hb 201 Hemoglobin Analyzer; HemoCue, Brea, CA) and hematocrit (microcapillary centrifugation). Serum-separating and CPT tubes were centrifuged 30 min post-collection, and serum or plasma containing peripheral blood mononuclear cells (PBMCs) were separated, aliquoted, and stored at –80°C until later analysis.

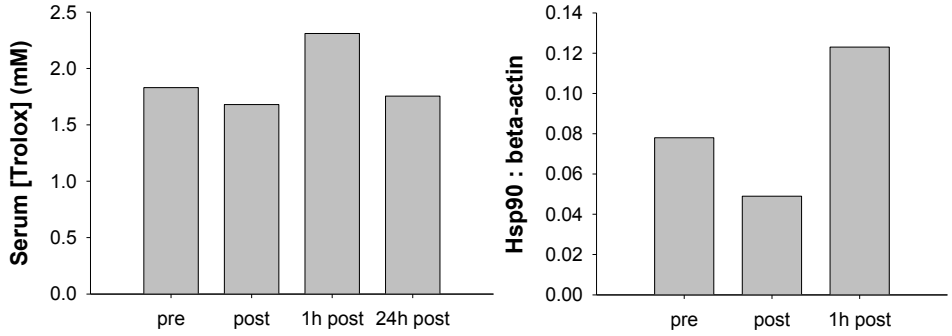

Figure 4.1. Pilot data from a subject who participated in one acute 90-min bout of hot water immersion. Venous blood was drawn before the session (pre), and immediately (post), 1h, and 24h after exiting the hot tub. **A)** Serum anti-oxidative capacity, measured as Trolox concentration using a commercially-available ELISA kit, peaked 1h after the hot water immersion session and had returned to baseline values by 24h. **B)** Heat shock protein (Hsp)90 content in primary peripheral blood mononuclear cells decreased immediately post-hot water immersion, but increased above baseline levels by 1h post hot water immersion. Data were obtained using Western blot and normalized to a β-actin loading control.

Blood volume

Prior to the first hot tub session and 48-72h after the last hot tub session, a subset of subjects (N=4 in the heat therapy group and N=8 in the sham group) reported to the laboratory having abstained from food for at least 4h, caffeine and alcohol for at least 12h, and exercise and any medications, vitamins, or supplements for 24h. Subjects provided a first-morning urine sample to confirm euhydration via urine specific gravity <1.02 (Analog Refractometer; Atago, Tokyo, Japan). If USG was >1.02, subjects drank 5ml/kg of water at least 30 min before blood volume measurements were obtained.

Subjects rested seated for >20 min while an intravenous catheter was placed in an antecubital vein. A baseline venous blood sample was collected into an EDTA-containing vacutainer (BD, Franklin Lakes, NJ) and baseline end-tidal carbon monoxide (CO) was measured using a CO monitor (Dräger Pac 3500; Dräger, Lübeck, Germany). Hemoglobin (Hb 201 Hemoglobin Analyzer; HemoCue), hematocrit by microcapillary centrifugation, and CO bound to hemoglobin (HbCO; OSM3 Blood Gas Analyzer; Radiometer, Copenhagen, Denmark) were immediately analyzed from the blood sample. Red cell mass was then measured using the CO-rebreathing method developed by

Schmidt & Prommer [344], and described in detail in Chapter III (pages 61-63). Total blood volume and plasma volume were calculated from red cell mass using hematocrit.

Heat Shock Proteins

PBMCs from subjects in the heat therapy group were separated from plasma and washed with phosphate-buffered saline (PBS) four times. Cell pellets were then combined with RIPA buffer plus protease inhibitor and lysed via sonication. Soluble protein (25-50ug – consistent within subjects) was separated by electrophoresis on 4-20% SDS polyacrylamide separating gels (Life Technologies, Grand Island, NY) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were Ponceau-stained to assess transfer. Membranes were incubated for 1h in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) and then incubated overnight at 4°C in blocking buffer containing either 1) anti-Hsp70 [BRM-22] (1:5,000; Abcam), 2) anti-Hsp90 [S88] (1:200; Abcam), or 3) anti-β actin (loading control; 1:1,000; Cell Signaling Technology). Membranes were washed and incubated with the appropriate secondary antibodies (LI-COR) for 1h at room temperature. The fluorescent bands were digitized using a Odyssey infrared imaging system (LI-COR). Digitized images were quantified using LI-COR Image Studio^{TM} software. Antibodies were stripped using NewBlot^{TM} Nitro Stripping Buffer (LI-COR) in between probing for primary antibodies.

Due to increased variability in human PBMCs, we confirmed all results by running samples on duplicate blots. Data have been averaged.

Statistics

Rectal temperature and heart rate during the first water immersion session were compared using two-way mixed design ANOVA with a between factor of group (heat therapy vs. sham) and a within factor of time into water immersion. Resting rectal temperature, resting heart rate, peak rectal temperature and sweat rate during water immersion sessions were compared using two-way mixed design ANOVA with a between factor of group and a within factor of weeks into the intervention. Time to reach a T_{re} of 38.5°C and HR at a T_{re} of 38.5°C were compared using one-way repeated measures ANOVA across weeks into heat therapy (0, 4, and 8 weeks), as these variables

were only measured for the heat therapy group. For all analyses, when significant main effects or interactions were detected, pairwise comparisons within group were made using Bonferroni's post-hoc text. Comparisons across groups were made using Student's unpaired t-test.

Linear regression analysis was performed to compare HR and rectal temperature data that were pooled from one session at 0, 4, and 8 weeks from subjects in the heat therapy group.

Due to the smaller sample sizes, fasted/resting hemoglobin and hematocrit and blood volume, plasma volume, and red cell volume were compared within groups across weeks into heat therapy (0 vs. 8wks) with Student's paired t-test. We were not powered to make across group comparisons for these variables.

We were also underpowered for comparing changes in HSP protein content in PBMCs using ANOVA (power on two-way repeated measures ANOVA for Hsp90 in the heat therapy group was 0.21). Instead, differences were compared using Student's paired t-test for 0wks pre vs. 0wks post, 0wks pre vs. 8wks pre, and 8wks pre vs. 8wks post.

All data are presented at mean ± S.E., unless otherwise specified. Significance was set at α=0.05.

RESULTS & DISCUSSION

Core Temperature

During the first hot water immersion session (0wks), T_{re} reached 38.5°C in 24 ± 2 min with subjects immersed up to the shoulder (Table 4.1 – next page). After this threshold was obtained, subjects sat up such that the water reached approximately waist-level and such that T_{re} was maintained between 38.5-39.0°C. At the end of the water immersion period, subjects exited the hot tub and T_{re} and HR were measured for another 10 min, or until T_{re} had dropped to <38.5°C, whichever was longer. For subjects in the sham group, T_{re} was maintained within ±0.2°C of resting throughout the protocol, regardless of body position. Average T_{re} across the first session of heat therapy or thermoneutral water immersion is presented in Figure 4.2 (next page).

By 4 weeks into heat therapy, subjects experienced a significant reduction in resting T_{re} (p=0.004), which stayed low through 8 weeks (Table 4.1 – next page);

whereas no significant difference in resting T_{re} was observed in sham subjects (p=0.15). Reduced resting core temperature is a hallmark sign of heat adaptation, indicating passive heat therapy is capable of eliciting similar thermoregulatory adaptations as classical exercise heat acclimation.

Table 4.1. Rectal temperature (T_{re}) measurements during water immersion sessions

	Heat Therapy (N=10)			Sham (N=10)		
	0wks	4wks	8wks	0wks	4wks	8wks
Resting T_{re} (°C)	37.3±0.1	36.9±0.1*†	36.9±0.1*	37.3±0.1	37.4±0.1	37.2±0.1
Time to reach 38.5°C (min)	24±2	26 ± 2	27±1	-	-	-
Peak T_{re} (°C)	39.0±0.03	38.8±0.05*	38.8±0.04*	37.5±0.1	37.4±0.1	37.4±0.1

Data are mean ± S.E. * P<0.05 vs. 0wks (within group), † P<0.05 vs. sham at same time point.

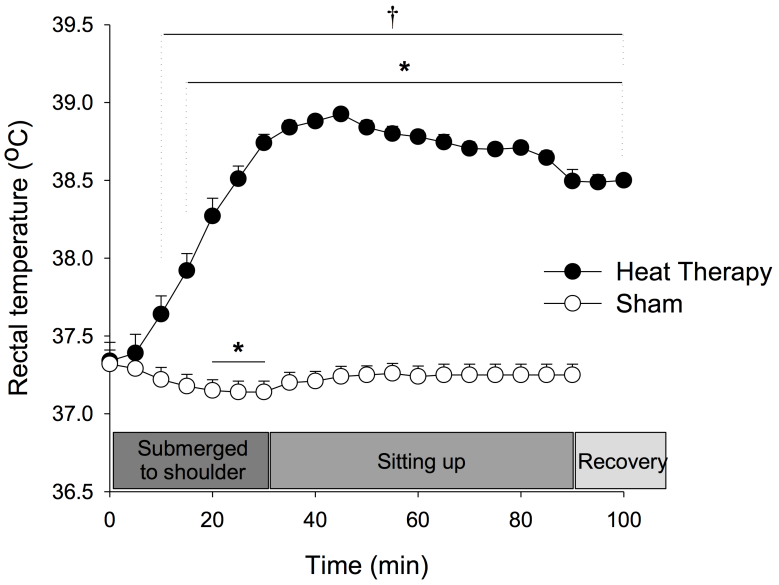

Figure 4.2. Average rectal temperature during the first water immersion session. Subjects were fully submerged for either 30 min (sham group) or until T_{re} reached 38.5°C (24±0.1 min; heat therapy group), and then sat up so that the water reached approximately waist-level for the remainder of the session (90 min for the sham group or until T_{re} had been at or above 38.5°C for 60 min for the heat therapy group). Data are mean ± S.E. *P<0.05 vs. resting (time 0) within group. † P<0.05 vs. sham group at same time point.

Peak T_{re} obtained during heating was slightly higher during the first session compared to the sessions analyzed at 4 ($p=0.008$) and 8 weeks ($p=0.003$), but this was only a difference of 0.2°C (Table 4.1). This is likely attributable to improved heat loss mechanisms once the subjects became acclimated. Once they sat up out of the water (once T_{re} reached 38.5°C), they were more effective at losing heat through sweating and increased skin blood flow such that further increases in core temperature were limited. However, the time required to reach our target threshold of 38.5°C did not significantly differ across the 8 weeks ($p=0.43$) as subjects' improved heat loss mechanisms were not effective while the majority of skin surface area was submerged. A major challenge with heat acclimation performed in a hot environment (i.e., in air instead of water) is that it becomes difficult to elevate core temperature to the same levels reached during a naive session as heat loss mechanisms become more robust as individuals acclimate. The conductive heat transfer in water is 24x that in air. Furthermore, evaporative cooling (which occurs with sweating) cannot take place in a 100% humid environment, leaving only a relatively small area of skin (e.g., the shoulders and head) from which heat could be lost via sweating. While some heat can be dissipated via respiratory water loss, it is minimal and this is not a mechanism that can be altered with heat acclimation. Therefore, despite presumably improved skin blood flow and sweating responses, the rate at which heat could be dissipated to the environment during the initial water immersion period changed minimally in our subjects across the 8 weeks. Thus, the target core temperature threshold could be attained within a similar amount of time across the 8 weeks. This further supports the appropriateness of using water immersion as the modality for inducing heat stress for long-term use.

Heart rate

The initial full immersion during heat therapy resulted in a continual increase in HR as core temperature increased. Once subjects sat up, HR dropped partially and stayed relatively stable throughout the remainder of the heating period, although there was variability in HR during this time due to subject movement (subjects could freely adjust their sitting position in the tub for comfort so long as T_{re} was maintain within the desired range). In the sham group, HR was not different from initial HR (prior to getting in the

tub) at any time point. Average HR across the first water immersion session is presented in Figure 4.3.

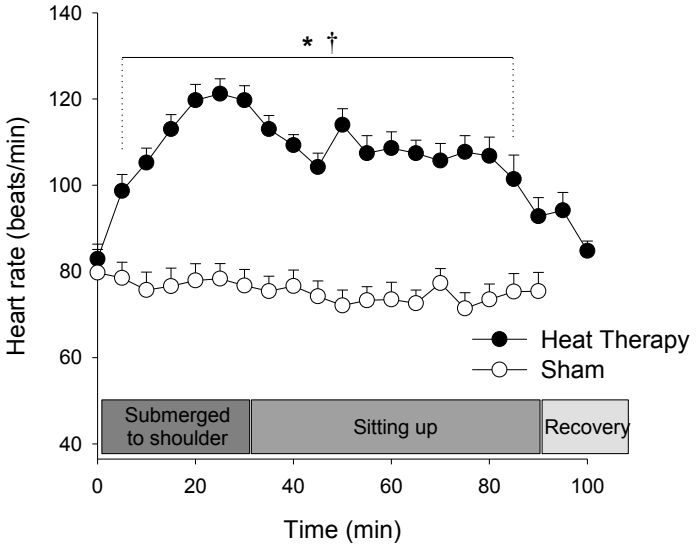

Figure 4.3. Average heart rate during the first water immersion session. Subjects were fully submerged for either 30 min (sham group) or until rectal temperature (T_{re}) reached 38.5°C (24±0.1 min; heat therapy group), and then sat up so that the water reached approximately waist-level for the remainder of the session. Data are mean ± S.E. *P<0.05 vs. resting (time 0) within group. † P<0.05 vs. sham group at same time point.

One of our primary long-term research goals regarding heat therapy is for it to be translatable to clinical practice. As such, we were interested in investigating whether HR could be used as an accurate indicator for when core temperature reaches our chosen threshold for inducing adaptation. Rectal temperature measurements are not feasible for use outside the laboratory and non-invasive methods of measuring temperature (e.g., oral or tympanic) are generally not reflective of true body core temperature. Since cardiac output during heat stress is determined by thermoregulatory drive (i.e., core temperature since skin temperature is held constant at 40.5°C while submerged in water), we thought HR may provide a non-invasive and easy-to-measure surrogate for core temperature.

We used several approaches for comparing HR and rectal temperature within the heat therapy group. For these analyses, we used HR and T_{re} data from one session at 0, 4, and 8 weeks. First, we investigated whether HR predicts T_{re} across all data pooled from these sessions. Overall (i.e., throughout the entire heating period and during all three sessions), there was only a weak, albeit significant, relationship between HR and T_{re} ($p<0.001$, $r^2=0.071$). However, when we included data from just the initial 20-30 min of

full immersion (when subjects maintained a more consistent body positions), we observed a modest relationship between HR and T_{re} ($p<0.001$, $r^2=0.40$; Figure 4.4).

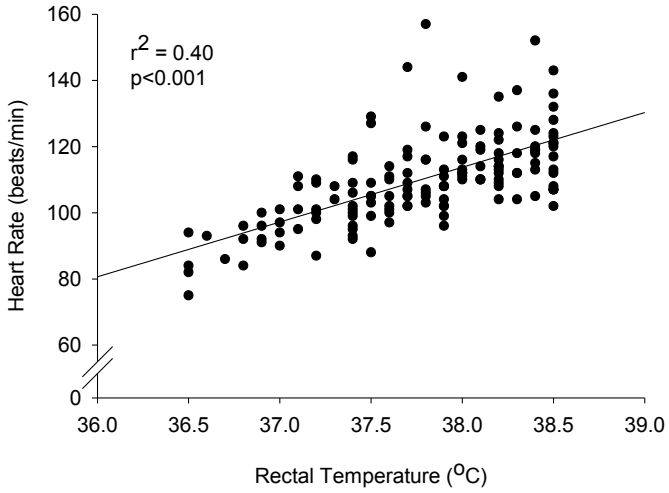

Figure 4.4. Relationship between heart rate and rectal temperature during the initial full immersion. Data are pooled from one session at 0, 4, and 8 weeks into heat therapy from all subjects in the heat therapy group (N=151 observations across 10 subjects).

We also investigated whether HR could be used as a predictor of when core temperature reached our target threshold. In our subjects, HR when T_{re} reached 38.5°C (while still fully submerged) was not significantly different across the 8 weeks (0wks: 120 ± 3 beats/min, 4wks: 118 ± 3 beats/min, 8wks: 116 ± 2 beats/min; p=0.25). However, within individual subjects, HR at this time point was not very consistent (average range in HR at T_{re}=38.5°C across the 8 weeks: 9 ± 2 beats/min).

A likely reason behind why we only observed a modest relationship between HR and T_{re} in our two analyses (both across the full immersion period and just at the point when T_{re} reached 38.5°C) is because HR and core temperature adaptations to the heat did not occur in tandem. We observed a reduction in resting T_{re} over the 8 weeks (Table 4.1 – page 76); whereas, we observed no change in initial HR (immediately prior to each hot tub session; p=0.16) or true resting HR (measured on a separate day after at least 30 min of supine rest; p=0.95) (Table 4.2 – next page). Furthermore, we assume the rate at which adaptations occur varies across individuals. Therefore, based on our data, we believe it is possible that HR could be used as a rough estimate for when T_{re} reaches 38.5°C, but further studies are needed to conclude whether the same vascular adaptations could be

conferred by heat therapy if HR were used as a marker of when subjects should sit up out of the water instead of a T_{re} of 38.5°C. Future studies should also investigate whether non-invasive temperature measurements (e.g., oral or tympanic temperature) could be used to determine this threshold, allowing heat therapy to be translated to non-laboratory settings.

Table 4.2. Initial heart rate (HR) taken immediately prior to water immersion sessions and HR measured on the vascular function day after at least 20 min supine rest.

	Heat Therapy (N=10)			Sham (N=10)		
	0wks	4wks	8wks	0wks	4wks	8wks
Initial HR (bpm)	83±1	86±3	79±3	80±5	92±4	81±3
Supine resting HR (bpm)	59±3	57±4	59±3	62±3	64±3	61±2

Data are mean ± S.E. * P<0.05 vs. 0wks (within group), † P<0.05 vs. sham at same time point.

Sweat rate

Mean whole-body sweat loss was calculated during each session by measuring pre- and post- nude body weight, which was corrected for fluid intake (Table 4.3 – next page). During heat therapy sessions (up to 90 min immersion + 10 min recovery), subjects lost 0.84 ± 0.09 L to sweat during the first session (range 0.49 - 1.30 L). This corresponded to a 1.26 ± 0.12 % body weight loss (range 0.86-1.97%). Following 8 weeks of heat therapy, these values more than doubled to 2.08 ± 0.20 L ($p<0.001$; range 1.11-3.37 L) and 3.09 ± 0.25% body weight loss ($p<0.001$; range 1.96-4.75%). Subjects in the sham group experienced minimal sweat loss, which was not significantly different from 0L and did not change across the 8 weeks. In order to be comparable to previous studies, we also calculated mean whole-body sweat rate per hour, presented in Figure 4.5 (next page).

Increased sweat rate is a classic and important adaptation to heat acclimation. Although we only measured mean whole-body sweat rate in the current study, others have shown that heat acclimation lowers the body core temperature threshold for initiation of sweating and increases the sensitivity and output of the sweat glands, such that sweat rate is increased for any given core temperature [354].

Table 4.3. Sweat loss during water immersion sessions at 0, 4, and 8 weeks into heat therapy or sham, presented as absolute sweat loss in the 90 min period and as a percentage of body weight (BW).

	Heat Therapy (N=10)			Sham (N=10)		
	0wks	4wks	8wks	0wks	4wks	8wks
Sweat loss (L)	0.84±0.09	1.69±0.16*	2.08±0.20*‡	0.05±0.02	0.06±0.04	0.03±0.02
Sweat Loss (%BW)	1.3±0.1	2.5±0.2*	3.1±0.3*‡	0.1±0.03	0.1±0.03	0.0±0.03

Data are mean ± S.E. * P<0.05 vs. 0wks (within group), ‡ P<0.05 vs. 4wks (within group). Sweat loss (both absolute and as a % BW) were significantly different between the groups at every time point.

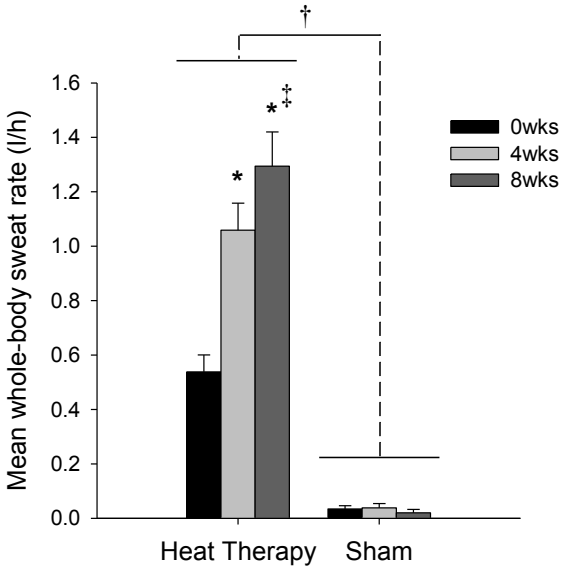

Figure 4.5. Mean whole-body sweat rate during the first (0wks) and last (8wks) water immersion sessions, and one mid-way session (4wks). *P<0.001 vs. 0wks (within group), ‡ P<0.05 vs. 4wks (within group), † P<0.001 vs. sham group at the same time point.

Tolerance

In general, subjects tolerated the heating protocol well. The most common side effects reported included light-headedness and slight headache (Table 4.4 – next page). These symptoms occurred most frequently during the first two weeks (26 out of the 43 total reported cases occurred in the first 9 sessions). This suggests subjects adapt very rapidly to the heat stress.

Table 4.4. Symptoms of heat exposure reported by subjects throughout the 8 weeks of heat therapy. Ten subjects participated in 36 sessions each for a total of 360 sessions.

Symptom	Subjects reporting	Times reported	Median session reported during	
Light-headedness/dizziness	5	15	8	Subsided upon sitting up in 8 of 15 cases
Slight headache	4	11	9	
Tingling sensation in hands and/or feet	2	7	5	
Nausea	3	4	13	Due to recent food intake in 3 of 4 cases
Uncomfortable flushing of the face	1	2	1.5	
Moderate and persistent headache	1	1	5	

Hemoglobin/hematocrit

Venous blood was collected for hemoglobin and hematocrit measurements under resting fasted conditions for N=7 subjects in the heat therapy group and N=10 subjects in the sham group. Unfortunately, we did not perform these measurements on the first three subjects we studied (which were all in the heat therapy group). Subjects also abstained from caffeine and alcohol for at least 12h, all medications, vitamins, and supplements for at least 24h and heavy exercise for at least 4h. All subjects were assessed for euhydration via first-morning USG and drank 5ml/kg of water prior to when the blood sample was collected if USG was >1.02. These fasted blood draws occurred immediately prior to the first and last water immersion session (24-48h after the penultimate session) in N=3 subjects in the heat therapy group and N=2 subjects in the sham group. In the remaining subjects (N=4 heat therapy and N=8 sham subjects), fasted blood draws were taken on a separate day prior to the blood volume measurement.

In addition, venous blood was collected for hemoglobin and hematocrit measurements before and after the first and last hot tub sessions in order to assess the acute effects of heat stress/thermoneutral water immersion. The subjects who performed the blood volume measurement were not fasted for these sessions, but maintained time of day and approximate food and caffeine intake consistent between the two sessions.

Subjects tended to tolerate the first session of hot water immersion better when they were not fasted.

There was no effect of heat therapy or sham on either fasted hemoglobin (p=0.35) or hematocrit (p=0.62) (Table 4.5). There was also no significant acute effect of heat therapy on hemoglobin or hematocrit at either 0 or 8 weeks. However, when considering individual subjects, we observed fairly large changes pre to post heat exposure on the very first heat therapy session (absolute change in hemoglobin from pre to post heat exposure averaged across heat therapy subjects: 1.5 ± 0.5 mg/dl); whereas, both hemoglobin and hematocrit remained relatively consistent pre to post heat exposure on the last heat therapy session (absolute change in hemoglobin: 0.6 ± 0.1 mg/dl, p=0.12 vs. change at 0wks), as shown by the individual data presented in Figure 4.6 (next page). As acute changes in hemoglobin and hematocrit are reflective of plasma volume shifts, these data suggest subjects became better able to gauge the volume and rate at which they needed to drink water in order to maintain hydration as they became heat adapted.

Table 4.5. Resting fasted hemoglobin and hematocrit values. Venous blood for measurements was collected at the same time of day for each subject, following 4h fast, and after subjects abstained from caffeine and alcohol for 12h, medications, vitamins, supplements, and heavy exercise for 24h. All subjects were euhydrated, measured by first-morning urine specific gravity.

	Heat Therapy (N=7)		Sham (N=10)	
	0 wks	8 wks	0 wks	8 wks
Hemoglobin (mg/dl)	14.8 ± 0.9	14.0 ± 0.6	14.1 ± 0.7	14.2 ± 0.6
Hematocrit (%)	41.7 ± 1.4	41.4 ± 0.7	40.2 ± 1.7	40.7 ± 1.7

Data are mean ± S.E.

Plasma volume

In subjects undergoing heat therapy (N=4), blood volume increased significantly (p=0.05); whereas both plasma volume (p=0.12) and red cell volume (p=0.09) tended to increase (Table 4.6 – next page). Importantly, we observed increases in blood and plasma volume in all four heat therapy subjects. Red cell volume increased in three subjects, with one subject showing no change. No consistent changes were observed in the sham group.

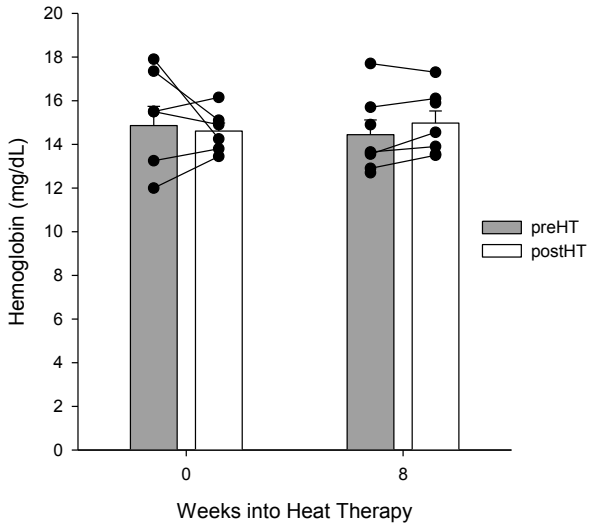

Figure 4.6 Hemoglobin concentration measured immediately prior to (preHT) and following (postHT) the first (0 weeks) and last (8 weeks) heat therapy session. Bars are mean ± S.E. (N=7). Circles are data from individual subjects.

Table 4.6. Changes in blood, plasma, and red cell volume following 8 weeks of heat therapy or thermoneutral water immersion (sham). *P<0.05 from 0wks (within group). First-morning urine specific gravity (USG) was measured on the days when blood volume measurements were made. Subjects with USG >1.020 drank 5ml/kg water ≥30min before the first blood draw.

	Heat Therapy (N=4)		Sham (N=8)	
	0 wks	8 wks	0 wks	8 wks
Blood volume (L)	5.03 ± 0.30	5.45 ± 0.35 *	4.90 ± 0.23	4.64 ± 0.34
Plasma volume (L)	3.15 ± 0.12	3.49 ± 0.20	3.22 ± 0.17	3.01 ± 0.21
Red cell volume (L)	1.88 ± 0.19	1.96 ± 0.16	1.68 ± 0.11	1.63 ± 0.16
USG	1.019 ±0.002	1.017 ± 0.002	1.020 ± 0.002	1.021 ± 0.001

Plasma volume expansion is another hallmark adaptation of heat acclimation. It allows for increases in blood flow to the skin for a given body core temperature to support increased sweating and heat loss. To our knowledge, no studies have investigated changes in plasma volume with heat acclimation in humans beyond 3 weeks. In exercise training studies, plasma volume increases fairly rapidly, but has been shown to return back to baseline levels after ~3-4 weeks [343]. This likely occurs because the stimulus for maintaining plasma volume expansion is reduced progressively as individuals adapt to exercise training, i.e., core temperature does not increase by as great an extent in later sessions. From our preliminary results, it seems plasma volume expansion with passive

heat acclimation is maintained for longer durations. This may have occurred in our study because we continued to raise core temperature to the same threshold throughout the 8 weeks. However, further studies are needed to fully characterize this adaptation.

Interestingly, red cell volume also tended to increase with heat therapy. This explains why we observed no change in hematocrit or hemoglobin. Red cell volume expansion is controlled by erythropoietin, which can be stimulated by long-term exercise training, ischemia, and hypoxia, among other stimuli [355]. Previous studies using short-term exercise heat acclimation have failed to observe any change in red cell volume [356,357]; however, longer-term exposure is likely required to induce this phenotype [14]. Hypoxia increases red cell production via activation of hypoxia inducible factor-1 (HIF-1)-α [355]. Interestingly, HIF-1-α is stabilized by Hsp70, providing a mechanism by which heat confers cross tolerance against hypoxia [15]. Although not tested experimentally in this study, it is possible increases in Hsp70 with heat therapy also activated HIF-1-α and therefore stimulated erythropoiesis.

Heat Shock Proteins

HSP content was analyzed in primary PBMCs, isolated from venous blood using the Ficoll Hypaque method before and 1h following the first and last session of heat therapy. By collecting PBMCs at these times, we hoped to capture both the acute HSP responses to individual bouts of hot water immersion and the chronic effects of heat therapy. We chose to measure HSPs in PBMCs because they can be isolated easily and relatively non-invasively from human subjects, they are exposed to the same temperatures and circulating factors *in vivo* as endothelial cells, and they exhibit a stress response in humans during exercise hyperthermia [114] and exertional heat injury [115]. Hsp70 and Hsp90 have also been shown to be elevated basally in human primary PBMCs following 10 days of exercise heat acclimation [169].

Unfortunately, we observed considerable variability in responses, even when data were normalized as fold changes from baseline within individuals. For example, the range in acute responses in Hsp70 following the last hot water immersion session was a 0.5-fold reduction up to a 6.4-fold increase. Despite this variability, we did observe a significant elevation in basal Hsp70 following heat therapy ($p=0.05$). Additionally, we

observed trends towards increases in Hsp70 with acute hot water immersion at 0wks (p=0.08) and 8wks (p=0.09). We observed a trend towards an increase in Hsp90 following acute water immersion at 0wks (p=0.08), but Hsp90 only remained chronically elevated in half of the subjects at 8wks (p=0.25 vs. 0wks pre) (Figure 4.7).

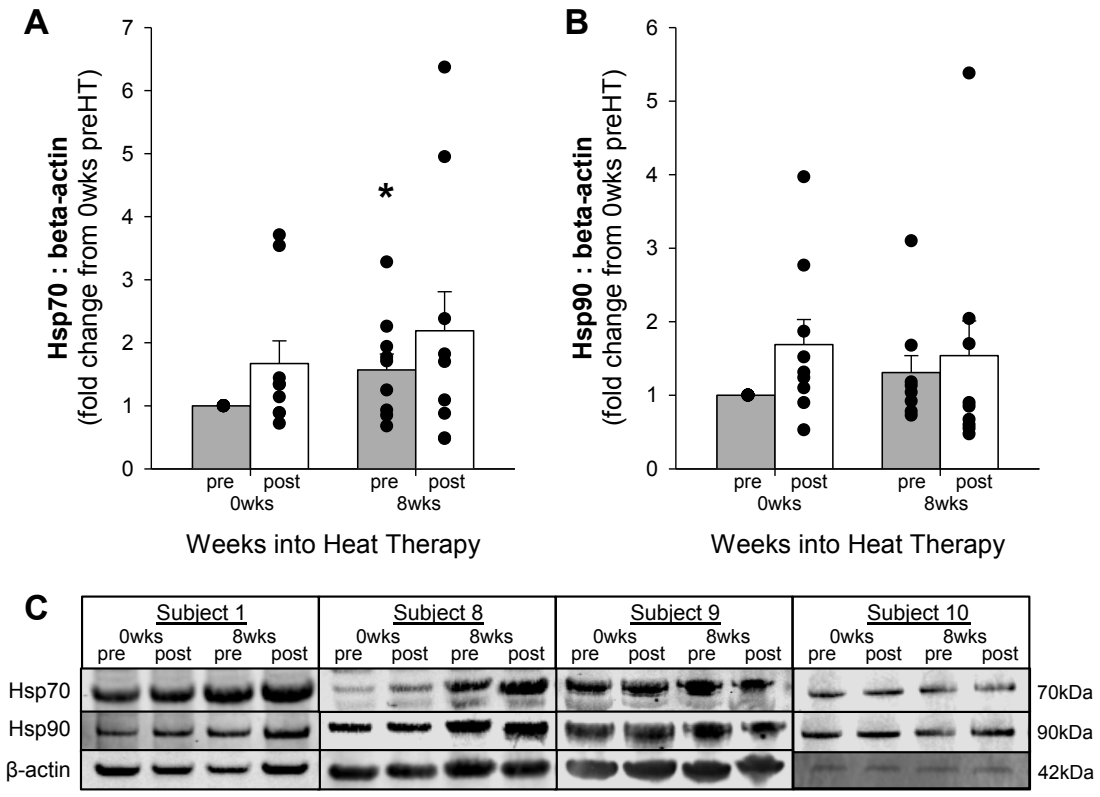

Figure 4.7. A) Heat shock protein (Hsp)-70 and **B)** Hsp90 protein content in peripheral blood mononuclear cells (PBMCs) collected from subjects before (pre) and 1h after (post) the first (0wks) and last (8wks) heat therapy sessions. Data are mean±S.E. (bars) and from individual subjects (black symbols), presented as fold changes from the 0wks pre condition within subjects. *P<0.05 vs. 0wks pre. **C)** Western blots performed with PBMC lysates from four representative subjects. Although we observed trends towards increases in HSPs across time into heat therapy, individual responses varied, as shown by individual data on graphs and Western blots.

It is possible variability in acute responses was due to the timing of blood collection. We selected to draw blood 1h post hot water immersion based on our (albeit limited) pilot data and previous reports of when HSP protein peaks in heat stressed PBMCs [129]. However, there may be individual variability in peak time for HSP abundance. For example, we observed a reduction in Hsp90 immediately post hot water immersion in our pilot subject (Figure 4.1, page 73). One hour may not have been long

enough for this transient reduction to recover in some subjects. Additionally, more time is often required for protein synthesis to occur. Had we measured HSP mRNA, we may have observed more robust responses. However, we chose to measure protein, as it is more indicative of functional effects.

We are not sure of the reason behind variability in the chronic responses, besides physiological inter-individual variability. All Western blots were run in duplicate and in general, responses were consistent across the two blots (e.g., reductions in HSP responses observed in some subjects were consistently observed across both blots), suggesting technical limitations were not the source of the variability. There were no trends in chronic HSP responses for sex or time since the second to last water immersion session (all within a 24-48h range).

Regardless of this variability, we did observe a significant increase in Hsp70 following 8 weeks of heat therapy. This is consistent with findings in animals chronically exposed to heat [173] and humans following exercise heat acclimation [169,358]. Furthermore, the magnitude of basal change in Hsp70 that we observed was similar to changes reported following 10 days of exercise heat acclimation (60% increase vs. 67% increase observed by Amorim et al. [358] and a 21% increase observed by McClung et al. [169]). Of course, our study differed from exercise heat acclimation studies in a number of ways (e.g., passive heat stress, longer duration, increase in core temperature per session); however, the comparison of our data to theirs further supports the notion that passive heat acclimation results in the same hallmark adaptations as classic exercise heat acclimation.

Lastly, we observed increases in basal Hsp90 in only half of the heat therapy subjects. As Hsp90 is constitutively expressed (versus Hsp70 is highly inducible), increases in Hsp90 will be of smaller magnitude than increases in Hsp70, making it difficult to detect significant changes. For example, increases in Hsp90 abundance were lower than increases in Hsp70 in rats continuously exposed to passive heat for 5 days in all tissues harvested (e.g., brain: Hsp90 1.2-fold increase vs. Hsp70 3.5-fold increase; heart: Hsp90 1.9-fold increase vs. Hsp70 3.1-fold increase) [359]. Because of this difference, few studies have investigated changes in Hsp90 protein in humans following exercise or heat stress/acclimation [169,360]. The one study that has measured changes in

Hsp90 protein following 10 days of exercise heat acclimation demonstrated a similar increase (21%) to the average increase observed in our subjects (30%).

CONCLUSIONS

Overall, eight weeks of passive heat acclimation results in reduced resting core temperature, increased sweat rate for a heat stress stimulus, and elevated basal HSP content, similarly to classical exercise heat acclimation [22], and similarly to what has been shown with long-term passive heat acclimation in animals [14]. Additionally, our preliminary data indicates that plasma volume is expanded. Differently from classical exercise heat acclimation, HR for a given heat stress stimulus was unchanged. We also observed a trend towards an increase in red cell volume with heat therapy, which has not previously been observed with short-term exercise heat acclimation [356,357], supporting the claim that longer term heat acclimation is required to induce the full phenotype [14].

Importantly, no changes were observed in the sham group. During thermoneutral water immersion sessions, there were no changes in rectal temperature >0.2°C from resting, no significant changes in HR, and no sweat loss. As a result, we observed no long-term adaptations in this group. This allows us to conclude that all adaptations observed in the heat therapy group were caused by the heat stress itself and not by hydrostatic or placebo effects.

The data included in this chapter demonstrates that the hyperthermic protocol and duration of time that we chose for performing passive heat therapy in humans is capable of inducing the hallmark adaptations associated with chronic heat exposure in animals and exercise heat acclimation in humans. As such, we expect heat therapy to have similar effects on vascular function as those that have been shown with exercise training, which itself confers long-term heat acclimation. Furthermore, we have shown that Hsp70 is chronically elevated in PBMCs following 8 weeks of heat therapy. Assuming HSP responses in PBMCs are similar to those in endothelial cells *in vivo*, this provides evidence that the pathways associated with improved vascular function which can be improved by HSPs (i.e., NO signaling, anti-oxidative stress, and anti-inflammation, as discussed in Chapter II) will also be improved *in vivo*, leading to the improvements in vascular function that will be presented in the following chapters.

CHAPTER V

PASSIVE HEAT THERAPY IMPROVES ENDOTHELIAL FUNCTION, ARTERIAL STIFFNESS, AND BLOOD PRESSURE IN SEDENTARY HUMANS

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CO-AUTHOR CONTRIBUTIONS

While I was the primary contributor to all aspects of the work described in this chapter, Matthew J. Howard, Michael A. Francisco, Brett R. Ely, and Christopher T. Minson all made substantial contributions to the work. M.J.H., M.A.F., and B.R.E. contributed to the data collection for this project. M.J.H. assisted greatly with data analysis. C.T.M. contributed to conception and design of experiments and interpretation of data. While I drafted the manuscript in its entirety, C.T.M. revised it critically for intellectual content.

INTRODUCTION

The majority of cardiovascular (CV) diseases are characterized by vascular dysfunction [1], including impaired endothelial-dependent dilation and arterial stiffening. To assess the progression of CV diseases, several biomarkers of conduit vessel function have been developed and verified to be independently predictive CV morbidity and mortality across a range of disease conditions.

Heat exposure increases core temperature, heart rate and contractility, redistribution of blood flow, and changes in conduit vessel endothelial shear stress [7], all

of which are believed to potentiate long-term improvements in vascular function. Additionally, heat exposure induces the expression of heat shock proteins (HSPs), which in turn stabilize a variety of other proteins important to the CV system. These include proteins which improve nitric oxide (NO) signaling [4], reduce oxidative stress [5], and reduce vascular inflammation [6], all of which greatly influence vascular function.

Therefore, we investigated the effects of 8 weeks of heat therapy on well-established biomarkers of macrovascular health, including endothelial-dependent dilation, arterial stiffness, intima media thickness, and blood pressure in young, sedentary (otherwise healthy) individuals. We hypothesized that, relative to thermoneutral water immersion (sham), heat therapy would improve endothelial-dependent dilation, arterial stiffness, blood pressure, and intima media thickness (also referred to as 'wall thickness'). This is the first clinical trial to comprehensively investigate the physiological benefits of long-term heat therapy in humans.

METHODS

Human subjects

As described in Chapter III, twenty young (18-30 yrs of age), sedentary (<2h of aerobic exercise per week), healthy subjects completed 8 weeks of heat therapy (N=10) or thermoneutral water immersion (N=10) (see Figure 3.2, page 35, for a flow chart of progression through the phases of the study; see Chapter III, pages 36-38 for descriptions of heat therapy and thermoneutral water immersion protocols). Subjects were well matched across groups for age, height, weight, BMI, and the distribution of males vs. females (see Table 3.1, page 36). Data from subjects who dropped out of the study partway have been excluded.

All subjects provided oral and written informed consent prior to participation in the study, as set forth by the Declaration of Helsinki. All experimental procedures were approved by the Institutional Review Board at the University of Oregon.

Outcome measures

Subjects in both groups reported to a temperature-controlled laboratory before, every 2 weeks during, and immediately following the 8-week intervention for

experimental testing. All experimental sessions took place 36-48h after the previous heat therapy session to ensure the chronic, rather than acute, effects of heat therapy were being investigated. Prior to all experimental days, subjects refrained from all over-the-counter medications, including vitamins and supplements, for 24h, alcohol and caffeine for 12h, food for 4h, and heavy exercise for 24h. Female subjects taking hormonal contraceptives were always studied during the active phase (N=10). Naturally menstruating females (N=2) were studied in the same menstrual phase during Weeks 0, 4, and 8 and during Weeks 2 and 6.

Upon arrival at the laboratory, subjects voided their bladder and subjects' height and weight were measured. Subjects were instrumented with 3-lead electrocardiogram (CardioCap; Datex Ohmeda, Louisville, CO), a blood pressure cuff on the left brachium, and a finger cuff for measurement of beat-by-beat blood pressure via photoplethysmography (Nexfin; BMEye, Amsterdam, the Netherlands). Brachial blood pressure was measured in triplicate following at least 20 min of supine rest. Biomarkers of vascular function were then measured using a high-resolution Doppler ultrasound (Terason t3000cv; Teratech, Burlington, MA) equipped with 10.0-MHz linear array ultrasound transducer probe (Doppler insonation angle of 60°) and using applanation tonometry (PCU-2000; Millar, Inc., Houston, TX) to obtain arterial pressure tracings [361], as follows (in this order, and as described in more detail in Chapter III, pages 39-53):

Wall thickness (i.e., intima media thickness) was measured in diastole in the common carotid artery 1-2cm distal to the carotid bulb and in the superficial femoral artery 2-3cm distal to the femoral bifurcation. Concurrent ultrasound images and tonometry pressure tracings were obtained in the contralateral common carotid and superficial femoral arteries in order to calculate dynamic cross-sectional arterial compliance and β-stiffness index. Concurrent tonometry pressure tracings were obtained from the common carotid and common femoral arteries to calculate aortic pulse wave velocity [362]. Brachial artery flow-mediated dilation (FMD) and post-occlusive reactive hyperemia (PORH) were measured following 5-min arterial occlusion according to published guidelines [278,279]. Lastly, endothelium-independent dilation was measured as the percent change in brachial artery diameter following administration of 0.4 mg of

sublingual nitroglycerine (Nitrolingual; Sciele Pharma, Atlanta, GA), which elicits maximal dilation of the conduit arteries.

FMD was calculated as the percent change in brachial diameter from baseline to peak dilation post-occlusion. The shear stimulus responsible for eliciting dilation was calculated as area under the curve above baseline shear rate from the time of release to peak dilation (SR_{AUC}). FMD was then normalized for shear stimulus by dividing FMD by SR_{AUC}, as described and validated previously [281,282]. PORH was characterized as the peak forearm vascular conductance (FVC = flow / mean arterial pressure) following release of the occlusion and as the area under the curve (AUC) PORH, calculated as the integral of FVC values above baseline FVC (average FVC across the 1-min baseline) until return to baseline. Due to higher variability in 0-week PORH values across subjects, data are presented as fold changes across the 8 weeks.

Statistics

A priori, sample size analyses were conducted for each outcome variable using differences in mean values that would be considered clinically relevant and standard deviations from young, healthy subjects who have previously been studied in our lab. We determined a sample size of N=10 per group would ensure a power of 0.80 with α=0.05.

Outcome variables were compared across groups and time into heat therapy or thermoneutral water immersion using two-way 2x5 mixed design analysis of variance (ANOVA). When significant main effects or interactions were detected, significant differences between paired variables across time were determined using Bonferroni's post-hoc test. Statistical significance was set to $\alpha = 0.05$. P-values given denote interaction between group x time, unless otherwise noted. Data are presented as mean±S.E.

RESULTS

Endothelial-dependent dilation

We observed a robust improvement in FMD with heat therapy (p=0.003), which was significantly elevated by 2 weeks (p=0.015 vs. 0wks within heat therapy group). FMD then decreased back down to baseline at 4 weeks (p=0.64 vs. 0wks), but continued

to increase afterwards through 6 and 8 weeks (Figure 5.1 – next page). No changes were observed in the sham group at any time point. FMD corrected for the shear rate stimulus continually increased across the 8 weeks of heat therapy (p=0.003), with no dip at the 4-week time point (Table 5.1 – next page), and was significantly different from the sham group at all time points into the intervention (Weeks 2-8) We observed a significant difference in SR_{AUC} between 4 and 6 weeks into heat therapy (p=0.04, 4 vs. 6wks within group; group x time: p=0.02). No changes were observed in baseline brachial artery diameter (p=0.43). There was no effect of heat therapy or thermoneutral water immersion on endothelium-independent dilation across the 8 weeks when data (p=0.24) (Table 5.1 – next page).

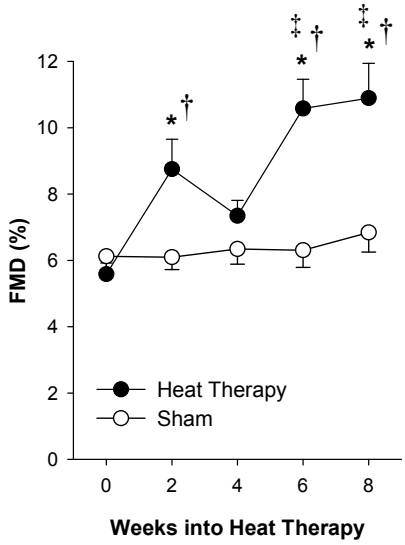

Figure 5.1. Changes in brachial artery flow-mediated dilation over 8 weeks of heat therapy (closed symbols) or thermoneutral water immersion (sham; open symbols). Data are mean ± S.E. Symbols denote results of post-hoc analyses when significant main effects were observed. *p<0.05 from 0wks (within group). ‡p<0.05 from 4wks (within group). †p<0.05 vs. sham group at the same time point.

Post-occlusive reactive hyperemia

Heat therapy significantly increased AUC PORH by 6 weeks (p=0.004 vs. 0wks; group x time: p=0.047) (Table 5.1 – next page), while no changes were observed in the sham group. We observed no significant changes in Peak PORH (p=0.39).

Table 5.1. Shear rate stimulus, flow-mediated dilation corrected for the shear stimulus, endothelium-dependent dilation, post-occlusive reactive hyperemia, conduit vessel diameters, and blood pressure

	Weeks into Heat Therapy (N=10)					Weeks into Sham (N=10)				
	0	2	4	6	8	0	2	4	6	8
SR_{AUC} ($10^3 \cdot s^{-1}$)	17±2	17±2	14±2	20±3‡	17±3	20±2	21±2	19±1	17±1	24±2
Corr. FMD (%/SR_{AUC})	4.0±0.8	6.1±1.0†	6.8±1.4*†	6.9±1.3*†	7.5±1.1*†	3.1±0.3	3.1±0.3	3.4±0.3	3.8±0.3	3.1±0.4
GTN (%)	18±1	18±2	18±2	18±2	16±2	16±2	16±2	20±1	19±2	16±2
PORH (fold change)										
Peak	1.0	1.2±0.1	1.2±0.1	1.4±0.2	1.3±0.1	1.0	1.1±0.1	1.2±0.1	1.2±0.1	1.1±0.1
AUC	1.0	1.5±0.2	1.3±0.2	2.2±0.5*	2.0±0.3*	1.0	1.8±0.5	1.5±0.3	1.3±0.2	1.5±0.3
Diameter (mm)										
Carotid	6.1±0.1	6.2±0.1	6.2±0.2	6.2±0.2	6.2±0.2	6.0±0.1	6.0±0.1	6.0±0.1	6.0±0.1	6.0±0.1
Femoral	6.1±0.2	6.2±0.2	6.2±0.2	6.1±0.1	6.1±0.2	6.3±0.2	6.3±0.2	6.2±0.2	6.3±0.2	6.2±0.2
Brachial	3.5±0.1	3.6±0.2	3.5±0.2	3.5±0.2	3.7±0.1	3.6±0.2	3.7±0.2	3.7±0.1	3.7±0.2	3.6±0.1
Blood pressure (mmHg)										
Systolic	112±2	109±3	107±2	108±3	108±2	110±3	105±3	109±3	106±3	107±3
Diastolic	69±1	66±1	65±1*	67±2	65±2*	67±1	67±2	68±1	68±2	68±2
Mean	83±1	80±2*	79±1*	79±2*	79±2*	81±1	80±2	81±1	80±2	81±2
Heart Rate (beats/min)	59±3	59±4	57±4	55±3	59±3	62±3	65±3	64±3	63±2	61±2

Data are mean ± S.E. Symbols denote results of post-hoc analyses when significant main effects were observed. *$p<0.05$ vs. 0wks (within group), †$p<0.05$ vs. Sham group at the same time point, ‡$p<0.05$ between 4 vs. 6wks (within group). SR_{AUC}, area under the curve above baseline of the shear rate stimulus from release of the arterial occlusion to peak dilation; Corr. FMD, flow-mediated dilation corrected for the shear stimulus; GTN, glycerol tri-nitrate, endothelial-independent dilation; PORH, post-occlusive reactive hyperemia.

Arterial stiffness

There was no effect of heat therapy or themoneutral water immersion on carotid artery dynamic arterial compliance (p=0.10) or β-stiffness index (p=0.41). However, in the superficial femoral artery, dynamic arterial compliance became significantly elevated from baseline by 4 weeks into heat therapy (p=0.02 vs. 0wks within heat therapy group), while β-stiffness index became significantly reduced from baseline by 8 weeks (p=0.03 vs. 0wks). Heat therapy also reduced carotid-femoral pulse wave velocity (p=0.03), but this effect only became significantly reduced from baseline at 8 weeks (p=0.046 vs. 0wks). No changes were observed in the sham group. Data are summarized in Figure 5.2.

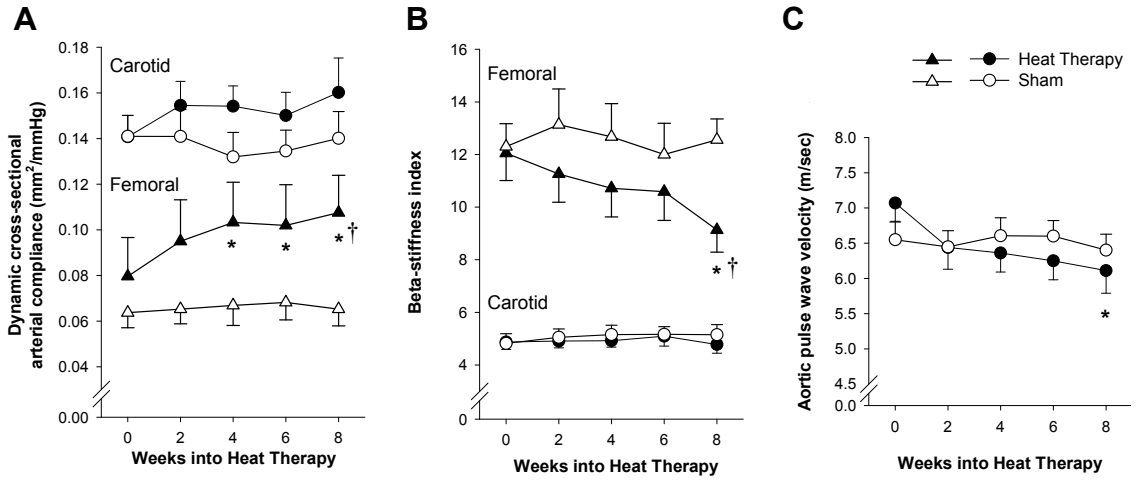

Figure 5.2. Changes in **A)** dynamic cross-sectional arterial compliance, **B)** β-stiffness index, and **C)** aortic pulse wave velocity over 8 weeks of heat therapy (closed symbols) or thermoneutral water immersion (sham; open symbols). Data are mean ± S.E. Symbols denote results of post-hoc analyses when significant main effects were observed. *p<0.05 from 0wks (within group). †p<0.05 vs. sham group at the same time point.

Vessel wall thickness & diameter

Heat therapy reduced carotid artery wall thickness significantly by 8 weeks (p=0.003 vs. 0wks within heat therapy group), but had no effect on superficial femoral artery wall thickness (p=0.26) (Figure 5.3 – next page). Heat therapy had no effect on resting superficial femoral artery diameter (p=0.73), although we observed a trend towards an increase in carotid artery diameter (p=0.09) (Table 5.1 – previous page).

Blood pressure & heart rate

We observed significant decreases in resting diastolic and mean arterial blood pressures across the 8 weeks (Table 5.1); whereas no changes were observed in systolic blood pressure (p=0.77) or resting heart rate (p=0.95).

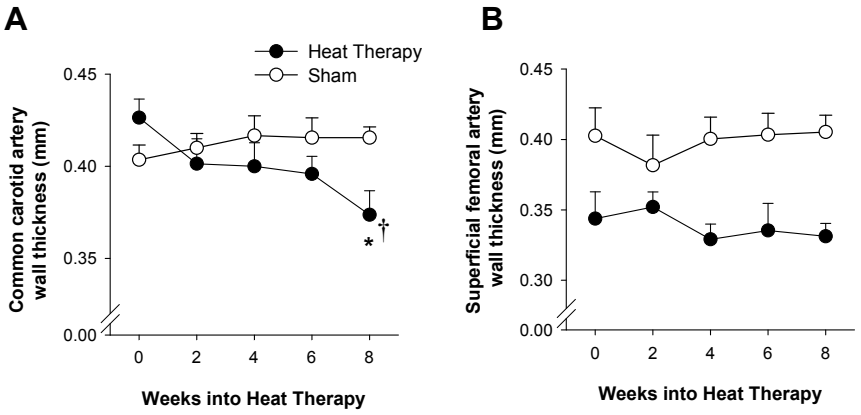

Figure 5.3. Changes in arterial wall thickness in the **A)** common carotid and **B)** superficial femoral arteries over 8 weeks of heat therapy (closed symbols) or thermoneutral water immersion (sham; open symbols). Data are mean ± S.E. Symbols denote results of post-hoc analyses when significant main effects were observed. *p<0.05 from 0wks (within group). †p<0.05 vs. sham group at the same time point.

DISCUSSION

In the present study, we sought to determine the effects of 8 weeks of passive heat therapy in young, sedentary individuals. Our major findings were that passive heat therapy results in robust and clinically relevant improvements in endothelium-dependent dilation, arterial stiffness, wall thickness, and blood pressure, which continually improved across the 8 weeks. Furthermore, we observed no changes in any variables in response to 8 weeks of thermoneutral water immersion. This is the first study to comprehensibly investigate the physiological effects of long-term whole-body passive heat therapy in human subjects. Our results suggest that these improvements in vascular function likely underlie the reduced incidence of CV disease and all-cause mortality associated with lifelong heat therapy.

Endothelium-dependent dilation

FMD is a commonly used and clinically relevant test of endothelial function which is primarily dependent on NO [274] and highly correlated with CV morbidity and mortality [270-272]. Furthermore, endothelial function in the brachial artery has been shown to parallel that in the coronary arteries [273], adding to its prognostic value. In the present study, we observed a robust improvement in FMD by the first time-point into heat therapy (2 weeks), consistent with previous studies investigating the effects of 2 weeks of infrared sauna therapy in patients with elevated CV risk [249,363]. By the end of the 8 weeks, FMD had increased by 5.31%, which represents a substantial improvement in vascular health, as improvements of just 2% have been correlated with a 15% reduction in CV risk [364]. These findings are in contrast to those observed following 8 weeks of 3x per week leg heating, in which FMD was significantly improved by 4wks, but had returned back to baseline values by 8 weeks [17]. However, core temperature only reached ~38.0°C per session in that study, perhaps demonstrating the importance of reaching a higher threshold core temperature. Our findings also contrast with exercise training studies, in which early improvements in FMD typically resolve and return back towards baseline values by 8 weeks [365], suggesting heat therapy may offer a more robust stimulus for endothelial adaptation. However, exercise training is also known to increase conduit vessel diameter [237,238]. As such, it has been proposed that FMD presented as a percent change in diameter returns back towards baseline values due to this structural remodeling rather than because improvements in endothelial function have been reversed [366]. Interestingly, we did not observe a change in conduit vessel diameter with heat therapy, possibly due to hydrostatic effects or because increases in cardiac output and blood flow during hot water immersion were not as great as may be seen during dynamic exercise. The differential effects of heat therapy on exercise training on conduit vessel diameter may explain the difference in the pattern of FMD adaptations to the two interventions.

Heat therapy results in repeated episodic elevations in core temperature and perfusion, both of which may upregulate NO-dependent dilation and therefore improve FMD. HSPs, which are activated by acute bouts of heat stress and chronically increased following heat acclimation [169], directly improve NO signaling. For example,

association of Hsp90 with eNOS is essential for eNOS activation and NO production [4]. Thus, increases in Hsp90 result in enhanced NOS activity independent of total eNOS protein [137]. Repeated episodes of increased shear rate, as occurs due to elevated blood flow during heat stress, has also been shown to increase FMD [18]. Furthermore, shear stress is known to upregulate transcription of HSPs, independently of elevations in temperature [117]. Importantly, limiting increases in shear with an arm cuff during 8wks of repeated leg heating prevented brachial artery FMD from improving over time [17], demonstrating elevations in shear, either alone or in combination with elevations in core temperature, is essential to arterial adaptation.

We also evaluated the reactive hyperemia response, which is a common test of microvascular function and an independent predictor of future CV events [286,288]. Furthermore, the shear stimulus, and therefore FMD, are dependent on PORH and baseline diameter. Although we did not observe any significant change in baseline brachial artery diameter, heat therapy did improve AUC PORH at the 6- and 8-week time points. However, as we observed improvements in FMD as early as 2 weeks into heat therapy despite no changes in baseline diameter or PORH, we can conclude these improvements represent augmented vasodilator function of the brachial artery. The prolonged downstream vasodilation that occurs following release of arterial occlusion (measured as AUC PORH) is thought to be the combined result of myogenic responses and release of metabolic vasodilators, including adenosine, ADP, prostanoids, and NO [283-285]. Although we did not investigate the mechanisms in the present study, it is likely increases in AUC PORH with heat therapy were caused by HSP- and shear stress-induced improvements in NO-dependent dilation, as described above.

Although we observed a trend towards improved Peak PORH with heat therapy, this trend was not statistically significant when compared to the sham group. Peak PORH has been suggested to be representative of maximal vasodilator capacity [291,292], and so our findings suggest that there were no structural effects of heat therapy in the microvasculature, i.e., heat therapy did not induce angiogenesis. It is possible this is the case, despite pro-angiogenic effects of heat therapy [250,367], HSPs [368], and NO [351] that have been demonstrated in tissue culture and in animals. It is more likely that 8 weeks was not long enough to detect angiogenesis *in vivo* using Peak PORH, which has

never been validated for this purpose. In fact, Peak PORH has only been validated to represent maximal vasodilator capacity using venous occlusion plethysmography to measure blood flow [291,292], which may underestimate Peak PORH. Therefore, other approaches are required to conclusively determine the effects of heat therapy on microvascular structure *in vivo*.

Lastly, we did not observe any changes in endothelium-independent dilation to nitroglycerine administration across the 8 weeks of heat therapy. Thus, improvements in FMD can be attributed to changes in the brachial artery endothelium rather than changes in the response of the underlying vascular smooth muscle to a NO stimulus.

Arterial stiffness

Arterial stiffness is an independent risk factor for CV disease [296,298,301], and increased arterial stiffness is often accompanied by atherosclerosis, left ventricular hypertrophy, and increased incidence of vascular events [369]. We assessed three different measures, all validated for their abilities to predict CV morbidity and mortality [298,301,307]. With heat therapy, superficial femoral arterial compliance increased, femoral β-stiffness index decreased, and aortic pulse wave velocity decreased, all indicating reduced arterial stiffness. Furthermore, all of these improvements were of a great enough magnitude to be considered clinically relevant, which is compelling given we studied a young, healthy population, albeit a sedentary one. Consistent with our results, previous studies have demonstrated a reduction in arterial stiffness with acute passive heat stress [370] and following 8 weeks of Bikram (hot) yoga [371].

Arterial stiffness can be impacted by a variety of factors, including elastic fiber degeneration, increased collagen content, structural changes to the arteries (e.g. vascular smooth muscle cell hypertrophy and hyperplasia), and impaired endothelial function [372], several of which were likely affected by heat therapy. HSPs are known to inhibit smooth muscle cell hyperplasia and hypertrophy [373,374], to reduce oxidative stress [5] and vascular inflammation [6]. Improved NO bioavailability and endothelial function (by the before-mentioned mechanisms) also reduce arterial stiffness [375]. Sympathetic nervous activity has been shown to increase stiffness [376]. Resting sympathetic activity is lower in the summer compared to winter despite no difference in body or room

temperature at the time of testing [377], although it is unknown whether heat therapy would have similar effects.

Lastly, blood pressure is well known to affect the visco-elastic properties of the arterial wall. As blood pressure is reduced, compliance increases. In the present study, we observed a reduction in diastolic and mean arterial blood pressure. For this reason, we chose to calculate β-stiffness index, which is less dependent on changes in blood pressure. In the carotid artery, we observed no change in β-stiffness index with heat therapy, suggesting that improvements in compliance were likely the result of reductions in blood pressure. However, both compliance and β-stiffness index were significantly improved in the superficial femoral artery. The superficial femoral artery is a more muscular artery than the carotid and, as such, may be more affected by nervous activity, hormones, or locally produced vasoactive substances, such as nitric oxide [378]. It is possible we were able to better observe the effects of heat therapy on the arteries in the superficial femoral compared to the more elastic carotid and aorta. Our results also demonstrate the importance of assessing arterial stiffness at more than one artery and using more than one method as each has limitations [314].

Blood Pressure

We observed significant reductions in diastolic and mean arterial blood pressure with heat therapy. Although the magnitude of these reductions were only ~4mmHg, increases of 10mmHg in diastolic pressure (or increases on 20mmHg in systolic) are associated with a two-fold increases in CV-related mortality, even in individuals as young as age 40 [379]. We expect the effects of heat therapy on blood pressure would be even greater in a (pre-)hypertensive population. Although exercise is considered a primary treatment for hypertension, exercise training generally has little or no effect on blood pressure in young individuals. Given that heat therapy is capable of lowering blood pressure even in young, normotensive individuals, heat therapy (or exercise combined with heat therapy) could prove more powerful than exercise alone as a treatment for hypertension.

Thermoneutral water immersion

Acute thermoneutral water immersion has previously been shown to induce changes in CV hemodynamics, such as increased cardiac output and mean arterial blood pressure [19], changes in conduit vessel diameter [20], and increased arterial compliance [380]. As such, we thought a true sham group which underwent chronic thermoneutral water immersion was necessary in order to isolate the effects of repeated rises in core temperature in the experimental group.

We selected a water temperature of 36°C to match mean body temperature and therefore minimize core-to-skin temperature gradients. We were successful in preventing changes in core temperature in sham subjects, with no greater than +/-0.2°C changes in T_{re} from resting in any session. However, as mean skin temperature is typically closer to 33°C, subjects may have experienced some cutaneous vasodilation during thermoneutral water immersion sessions, resulting in minor changes in blood flow and shear rate. Additionally, by having subjects in a seated position in the tub, we minimized hydrostatic effects on blood flow and cardiac output redistribution.

Regardless of any acute changes, we observed no long-term adaptation in sham subjects.

SUMMARY & BRIDGE

The data presented in this chapter demonstrates that passive heat therapy is capable of inducing robust improvements in macrovascular health, even in sedentary, young (otherwise healthy) individuals. Furthermore, the magnitude of improvements in vascular function and blood pressure observed in the present study was similar to what is typically observed in young, healthy, sedentary subjects with exercise training [365,381,382], and in some cases, even greater (e.g. FMD). As such, improved endothelial function and reduced arterial stiffness and blood pressure likely underlies the cardioprotective effects of lifelong heat therapy, similarly to exercise training.

In this chapter, we investigated the effects of passive heat therapy on well-established biomarkers of macrovascular health. While these were all clinically relevant measures that have been validated in multiple large-cohort studies for their ability to predict CV morbidity and mortality, other evidence suggests CV disease progression

begins in the microvasculature [383]. Thus, in order to provide a comprehensive assessment of global vascular health, we also investigated the effects of passive heat therapy on microvascular function, presented in the next chapter.

CHAPTER VI

PASSIVE HEAT THERAPY IMPROVES CUTANEOUS MICROVASCULAR FUNCTION IN SEDENTARY HUMANS VIA IMPROVED NITRIC OXIDE-DEPENDENT DILATION

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Brunt VE, Eymann TM, Francsico MA, Howard MJ, and Minson CT. Passive heat therapy improves cutaneous microvascular function in sedentary humans via improved nitric oxide-dependent dilation. J Appl Physiol. 2016.

CO-AUTHOR CONTRIBUTIONS

While I was the primary contributor to the work described in this chapter, Taylor M. Eymann, Michael A. Francisco, Matthew J. Howard, and Christopher T. Minson all made substantial contributions to the work. T.M.E., M.J.H. and M.A.F. contributed to the data collection for this project. C.T.M. contributed to conception and design of experiments and interpretation of data. While I drafted the manuscript in its entirety, C.T.M. revised it critically for intellectual content.

INTRODUCTION

Dysfunction in the microvasculature often precedes dysfunction in the conduit vessels [383]. Therefore, developing novel strategies to specifically target this important vascular bed is of critical importance. Few studies to date have investigated the effects of heat therapy on microvascular function in humans and none have investigated the molecular mechanisms that underscore potential adaptions.

In humans, the cutaneous circulation offers an ideal site to study microvascular function. The accessibility of the skin allows the molecular pathways to be studied easily and relatively non-invasively using cutaneous microdialysis. A commonly-used test of cutaneous microvascular function is thermal hyperemia in which the skin is locally heated to 39-42°C, producing a biphasic vasodilator response [331,336,341,384]. Importantly, thermal hyperemia has been shown to be impaired under a variety of disease conditions [2,321,328,385] and to mirror microvascular dysfunction in other microvascular beds. For example, thermal hyperemia is substantially impaired in diseases such as end-stage renal disease [320], essential hypertension [322], type II diabetes mellitus [330], and coronary artery disease [319], all diseases which are characterized by impaired microvascular function independent of changes in the cutaneous circulation. Furthermore, the secondary plateau phase is primarily dependent on nitric oxide (NO) [331,334,341]. Given that the majority of disease states are characterized by impaired NO-dependent dilation and that the skin offers an accessible means of investigating the underlying mechanisms of microvascular function, thermal hyperemia is an ideal test for assessing the potential therapeutic effects of heat therapy on the microvasculature.

Only one study has so far investigated the effects of chronic passive heating on cutaneous thermal hyperemia. Carter et al. [16] showed that 8 weeks of lower limb heating sufficient to raise body core temperature by 0.5-1.0°C increased the cutaneous forearm response to a local thermal stimulus. However, a few limitations to this study warrant further investigation. For example, maximal vasodilation at the skin sites were not obtained, a commonly performed practice to account for limitations in the laser-Doppler flowmetry technique [386]. In this setting, recording maximal skin blood flow is necessary to determine whether any observed changes were due a structural or functional improvement. Additionally, to test microvascular function, a slow local heating protocol to 42°C was utilized, which has been shown in microdialysis studies to be only ~30% NO-dependent [341] and to be confounded by axon reflex involvement [387]. Furthermore, the underlying mechanisms were not investigated, so it is unknown whether the improvements in skin blood flow were due to improved NO bioavailability.

NO bioavailability and cutaneous thermal hyperemia can also be limited by oxidative stress [336]. The effects of oxidative stress can by investigated via

microdialysis using Tempol, a superoxide dismutase mimetic. While Tempol has been shown to have no effect in young, recreationally active subjects when locally heating to 42°C [336], this protocol elicits vasodilation to ~85-95% of maximal, and so a ceiling effect may have been observed. The effects of Tempol have not yet been investigated using local heating to 39°C, which reaches a plateau of ~40-60% of maximal [341], allowing the effects of pharmacological agents and/or interventions which may increase vasodilation to be observed. Additionally, Tempol has not been investigated in sedentary subjects, nor following long-term heat therapy. It has been reported that short-term heat acclimation (≤14 days) can increase total peroxide concentration and oxidative stress index [388]. However, heat shock proteins, which are elevated following heat acclimation [169], can increase expression of antioxidative enzymes, including superoxide dismutase [147].

Therefore, we investigated the effects of eight weeks of passive heat therapy on cutaneous microvascular function, consisting of 4-5 sessions per week in which rectal temperature was maintained at ≥38.5°C for 60 min per session throughout the 8 weeks. The arm used for microdialysis studies was kept out of the water the entire time so that adaptations would be induced by systemic increases in body core temperature rather than increases in local skin temperature. To assess cutaneous microvascular function, we utilized rapid local heating to 39°C, a thermal hyperemia protocol which is ~80% dependent on NO [341]. Specifically, we hypothesized that, compared to a sham group (thermoneutral water immersion), eight weeks of heat therapy would improve the overall plateau response of 39°C cutaneous thermal hyperemia by improving NO-dependent dilation (investigated by delivering a NO synthase (NOS) inhibitor via microdialysis). Secondly, we hypothesized that Tempol would augment thermal hyperemia in sedentary subjects prior to heat therapy, and that Tempol-mediated dilation (the difference between Control and Tempol microdialysis sites, indicative of oxidative stress) would decrease with eight weeks of heat therapy.

METHODS

Human subjects

Of the twenty-five young (18-30 yrs of age), sedentary (<2h of aerobic exercise per week), healthy subjects who were initially enrolled in the study, twenty-two completed the initial (0wk) cutaneous microdialysis study, and twenty completed the full 8-wk intervention. However, data from eighteen who completed the intervention are included in this chapter (N=9 in each group). Microdialysis data was not collected on one of these subjects (heat therapy group) and data was excluded from one subject in the sham group due to technical difficulties. All experimental procedures were approved by the Institutional Review Board at the University of Oregon. All subjects provided oral and written informed consent prior to participation in the study, as set forth by the *Declaration of Helsinki*.

As before, subjects were assigned to participate in either 8 weeks of heat therapy or thermoneutral water immersion (sham) (see Chapter III, pages 36-38 for descriptions of intervention protocols), and were well matched by age, sex, height, weight, BMI, and time of year across groups (see Table 3.1, page 36).

Experimental Measures

Prior to and following the eight weeks of heat therapy or thermoneutral water immersion, subjects reported to the lab having refrained from all over-the-counter medications, including vitamins and supplements for 24h, alcohol and caffeine for at least 12h, food for at least 4h, and exercise for at least 24h. Female subjects provided a negative pregnancy test prior to studies, measured using urine hCG. Studies were held at the same time of day within subjects and the post study was held at least 48h after the last water immersion session in order to capture the chronic effects of heat therapy. Female subjects taking hormonal contraceptives (N=10) were studied during the active phase. We did not control for menstrual phase in naturally menstruating females (N=2), but the phase they were in was consistent between the pre and post studies. Studies were held on separate days from macrovascular function testing (described in Chapter V), separated by at least 24h.

Upon arrival at the laboratory, subject height and weight was obtained. Subjects then rested semi-supine and three microdialysis fibers (MD2000, 30-kDa cutoff membrane; Bioanalytical Systems, Inc., West Lafeyette, IN, or CMA 31 Linear Probe, 55-kDa cutoff membrane; CMA Microdialysis AB, Kista, Sweden) were placed at least 5 cm apart in the ventral skin of the non-dominant forearm. Fibers were introduced with a 25-gauge needle, which was inserted ~1 mm below the surface of the skin with entry and exit sites ~2.5 cm apart. Fibers were then threaded through the lumen of the needle and the needle was removed, leaving the fiber in place under the skin. Fibers were secured with tape and infused with Lactated Ringer's solution at a rate of 2.0μl/min (CMA 102 Microdialysis Pump; CMA Microdialysis AB) until the start of study drugs. Due to manufacturing issues, we had to switch to a different microdialysis fiber manufacturer mid-way through the study. However, the same fibers were used pre and post within each subject. All drugs delivered have molecular weights well below the membrane cutoffs, the concentrations of drugs delivered were great enough to cause maximal inhibition, and the infusion rate was low enough to allow for equilibration with the tissue. Therefore, this switch should not have impacted our results.

A period of at least 60 min was allowed for the trauma associated with needle insertion to subside. During this time, subjects were instrumented with local heaters (SH02 Skin Heater/Temperature Monitor; Moor Instruments, Axminster, UK) over each microdialysis site. Laser-Doppler flowmeter probes (MoorLab; Moor Instruments) were seated in the center of each local heater flush with the skin in order to measure red blood cell flux, an index of skin blood flow. A fourth local heater with laser-Doppler probe was placed at a fourth site that did not have a microdilaysis fiber, which served as a control site. A blood pressure cuff was placed on the brachium of the opposite arm (Datex Ohmeda CardioCap; GE Medical Systems, Tampa, FL).

After flux had returned to baseline values, local heaters were set to 33°C and a 5-min baseline was recorded. After this, microdialysis fibers were randomly assigned to receive: 1) Lactated Ringer's (sham site), 2) 10mM Nω-nitro-L-arginine (L-NNA; Sigma-Aldrich, St. Louis, MO), a non-specific NO synthase inhibitor, or 3) 10μM 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol; EMD Millipore Chemicals, Billerica, MA), a superoxide dismutase mimetic to reduce oxidative stress. All drugs were

dissolved in Lactated Ringer's solution. Concentrations of drug were selected based on previous studies as the minimum concentrations capable of producing maximal inhibition [336,389]. Drugs were delivered for 60 min after which a second 'post-drug' 5-min baseline was recorded, immediately prior to the start of local heating.

The local heaters were increased to a temperature of 39°C at a rate of 0.1°C/s and maintained at this temperature until skin blood flux reached a stable plateau for at least 5 min, which took approximately 30-45 min. Once a stable plateau in skin blood flux was reached, the local heaters were furthered increased to 43.5°C at a rate of 0.1°C/s and all fibers were infused with 56mM sodium nitroprusside (US Pharmacopeia, Rockville, MD) in order to obtain maximal flux.

Pilot Studies to Ensure Efficacy of Tempol

In order to ensure non-effects were real and not due to technical or drug limitations, we studied an additional two pilot subjects with chronic complete spinal cord injury (SCI) (sustained ASIA A complete injuries at T11 and T12, 7 and 8 years ago, respectively). SCI is known to result in elevated levels of oxidative stress [390], making them an ideal population in which to characterize the efficacy of Tempol. These two subjects underwent identical experimental testing to the able-bodied subjects, and were of similar age and BMI. Additionally, we have previously studied the effects of Tempol in young smokers in our laboratory using the same techniques and concentration of Tempol [326]. In these subjects, 10μM Tempol fully reversed impairments in thermal hyperemia.

Data Analysis

Data were digitized and recorded to a computer using data acquisition software (Windaq; Dataq Instruments, Akron, OH). The local heating response was characterized by initial peak, nadir, and plateau. The initial peak was determined as the highest 30s period of flux values occurring within the first 5 min of local heating. Nadir was determined as the lowest 30s period of flux values occurring within 5 min following the initial peak. Plateau was averaged over the last 5 min of stable flux values prior to moving on to obtaining maximal flux. All flux values were then converted to cutaneous vascular conductance (CVC = flux/mean arterial pressure) and normalized to a

percentage of maximal CVC (%CVCmax). There was no difference between CVC at the Lactated Ringer's site and the site which had no fiber (paired t-test across all 0wk data: p=0.49) and so data were averaged ("Control"). NO-dependent dilation was calculated as the difference in normalized plateau CVC between the Control and L-NNA sites. Tempol-mediated dilation was calculated as the difference in normalized plateau CVC between the Tempol and Control sites.

Statistics

Subject demographic data (age, height, weight, BMI) was compared across groups using Student's unpaired *t*-test. Mean arterial blood pressure was compared across groups and across time (0 vs. 8 weeks) using two-way mixed design analysis of variance with a between factor of group and a within factor of week.

Effects of the drugs using data pooled across all subjects who completed 0wk testing. Changes in normalized CVC were compared using two-way repeated measures (RM) analysis of variance (ANOVA) with factors of drug site (Control, L-NNA, and Tempol) and phase into local heating (baseline, peak, nadir, and plateau). Comparisons across paired variables were made with Bonferroni's post-hoc test. Maximal CVC was compared across drug sites using one-way RM ANOVA. Pre- to post-drug baseline was compared at each site using Student's paired two-tailed *t*-test.

Effects of the interventions. Changes in normalized CVC at the Control site (e.g. the normal response) were compared using three-way mixed design ANOVA with a between factor of group and within/paired factors of week (0 vs. 8) and phase into local heating (baseline, peak, nadir, and plateau). Changes in NO-dependent dilation (difference in plateau between Control and L-NNA sites) and Tempol-mediated dilation (difference in plateau between Control and Tempol sites) were compared using two-way mixed design ANOVA with a between factor of group and a within/paired factor of week. Maximal CVC was compared using three-way mixed design ANOVA with between factors of group and drug site and a within factor of week. For all analyses, when significant main effects or interactions were detected, comparisons across paired variables (within group) were made with Bonferroni's post-hoc test and comparisons

across groups were made using Student's unpaired two-tailed *t*-test. For all analyses, the level of significance was set at α=0.05. Data are mean ± S.E.

RESULTS

Subject demographic data is presented in Table 6.1. Subjects were well matched across the two groups for sex, age, height, weight, and BMI ($p>0.64$ between groups for all demongraphic variables).

Table 6.1. Demographics of subjects who participated in cutaneous microdialysis studies

	All subjects (N=22)	Heat therapy group (N=9)	Sham group (N=9)
Females (N)	12	5	5
Age (years)	22 ± 1	22 ± 1	22 ± 1
Height (cm)	172 ± 2	174 ± 3	172 ± 3
Weight (kg)	66 ± 2	68 ± 3	66 ± 4
BMI ($kg \cdot m^{-2}$)	22.4 ± 0.4	22.6 ± 0.6	22.2 ± 0.6
Mean arterial pressure (mmHg)[2]			
0 weeks	84 ± 1	83 ± 2	83 ± 2
8 weeks	-	81 ± 2	85 ± 2

Local heating of the skin elicited a biphasic vasodilator response, consisting of an initial peak and plateau with a brief nadir in between the two phases (Figure 6.1 – page 111). When data were pooled across all subjects who completed the initial 0wk testing (N=22), average plateau in the Control site was 43±3%CVCmax, which is similar to what we have reported previously [341]. There was a significant interaction between drug site and phase into local heating ($p<0.001$). L-NNA significantly attenuated initial peak, nadir, and plateau compared to both the Control and Tempol sites ($p<0.001$ for all) (Figure 6.1 – next page), such that NO accounted for 78±4% of the overall plateau increase in CVC above baseline. There was no effect of Tempol on any phase of the local heating response compared to the Control site ($p=0.53$).

Heat therapy significantly improved the local heating response (group x week x phase of local heating: $p=0.04$). Within the heat therapy group, initial peak ($p<0.001$),

[2] Blood pressure measurements included in Table 6.1 differ from those reported in Chapter V Table 5.1 as they were taken on different days, and in different positions. The semi-recumbent position may explain why we observed no significant reduction in these blood pressure measurements across heat therapy.

nadir (p=0.03), and plateau (p<0.001) were all significantly elevated after 8 weeks of heat therapy, whereas no changes were observed in the sham group (week x phase within group: p=0.22) (Figure 6.2A – next page, and Table 6.2).

Table 6.2. Initial peak and nadir cutaneous vascular conductance (CVC) and Tempol-mediated dilation

	All Subjects (N=22)	Heat Therapy (N=9)		Sham (N=9)	
		0wks	8wks	0wks	8wks
Initial peak (%CVCmax)	42 ± 3	44 ± 4	59 ± 4*	39 ± 4	45 ± 5
Nadir (%CVCmax)	20 ± 2	21 ± 4	26 ± 4*	17 ± 3	19 ± 3
Tempol-mediated dilation	4.0±3.3	0.2±6.1	-4.3±7.6	5.6±5.2	0.0±2.4

Data are mean ± S.E. *p<0.05 vs. 0wks within group. L-NNA, Nω-nitro-L-arginine; Tempol-mediated dilation, difference in plateau CVC between Control and Tempol microdialysis sites.

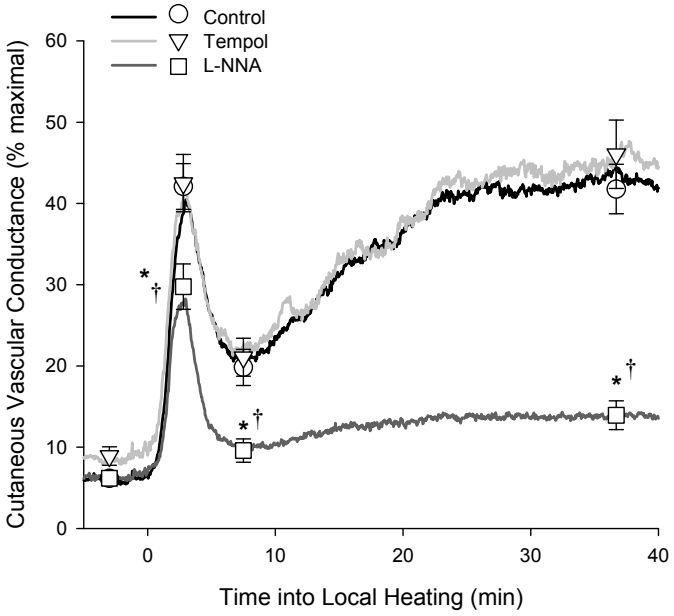

Figure 6.1. Average normalized cutaneous vascular conductance across time into local heating in all subjects who completed 0wk testing (N=22) at three microdialysis sites receiving 1) Control (Lactated Ringer's), 2) Tempol, and 3) Nω-nitro-L-arginine (L-NNA). Data over time were averaged across subjects at 4Hz (solid lines). Baseline, initial peak, nadir, and plateau were analyzed for each subject and presented as mean ± S.E. (symbols). Note, for initial peak, the symbols do not line up exactly with the solid lines since they were analyzed at the subject's true peak CVC, rather than at a certain time point into local heating. *p<0.05 vs. Control. †p<0.05 vs. Tempol.

Heat therapy significantly improved NO-dependent dilation, the difference between the Control and L-NNA sites (group x week: p=0.049; 0 vs. 8wks within heat therapy group: p<0.01), whereas no change in NO-dependent dilation was observed in the sham group (p=0.93) (Figure 6.2B – next page). No changes were observed in Tempol-mediated dilation, the difference between the Tempol and Control sites, across heat therapy or thermoneutral water immersion (p=0.58) (Table 6.2 – previous page). Sample size analysis using the difference in Tempol-mediated dilation between 0 and 8wks of heat therapy and the standard deviation within the heat therapy group showed that we would have needed 260 subjects to show a significant difference with a power of 0.80 and alpha level of 0.05. In the two SCI subjects (data averaged across the two), plateau reached 50.7%CVCmax in the Ringer's site. Tempol increased plateau in both subjects to an average of 61.0%CVCmax.

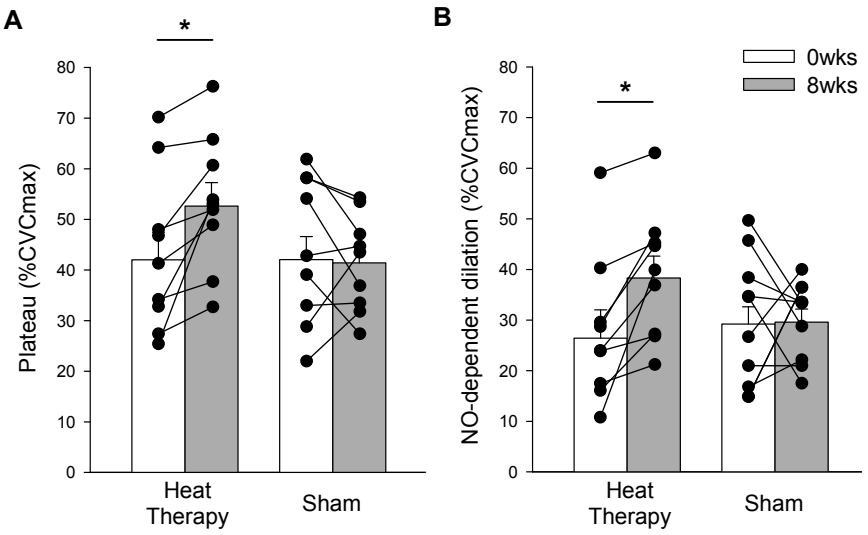

Figure 6.2. A) Local heating plateau and **B)** and nitric oxide (NO)-dependent dilation [difference in plateau between microdialysis sites receiving Lactated Ringer's (Control) and Nω-nitro-L-arginine (L-NNA)] across 8 weeks of heat therapy or thermoneutral water immersion (sham). Heat therapy increased both plateau and NO-dependent dilation in every subject, as indicated by the individual data. Data are mean ± S.E. *p<0.05 0 vs. 8wks within group.

Maximal CVC and baseline

There were no significant effects of the drugs on maximal CVC within the pooled 0wk data (p=0.20) (Table 6.3 – next page). Across the intervention, there was a

significant interaction between group, week, and drug site (p=0.02), but the only significant difference in paired variables that was found was the increase in maximal CVC at the Tempol site in the heat therapy group (p=0.01). L-NNA significantly decreased baseline CVC (p=0.02) and Tempol significantly increased baseline CVC (p=0.03) (Table 6.4).

Table 6.3. Maximal cutaneous vascular conductance (CVC)

	All Subjects (N=22)	Heat Therapy (N=9)		Sham (N=9)	
		0wks	8wks	0wks	8wks
Maximal CVC (mV/mmHg*100)					
Control	253 ± 15	302 ± 26	285 ± 33	214 ± 14	271 ± 12
L-NNA	198 ± 14	185 ± 22	218 ± 41	197 ± 15	235 ± 31
Tempol	241 ± 32	199 ± 26	339 ± 46*	214 ± 18	180 ± 16

Data are mean ± S.E. *p<0.05 vs. 0wks within group. L-NNA, Nω-nitro-L-arginine

Table 6.4. Baseline drug effects

	Pre-drug baseline	Post-drug baseline	P-value
Control	5.5 ± 0.4	6.1 ± 0.5	0.13
L-NNA	7.8 ± 0.9	6.2 ± 0.9*	0.02
Tempol	7.4 ± 0.8	8.5 ± 1.1*	0.03

Baseline data pooled across all subjects completed 0wk testing (N=22). Data are mean ± S.E. cutaneous vascular conductance (%max). *p<0.05 from pre-drug baseline

DISCUSSION

This is the first study to investigate the molecular mechanisms by which passive heat therapy improves microvascular function. Our major finding is that passive heat therapy improves cutaneous thermal hyperemia via improved NO bioavailability. We observed no change in NO-dependent dilation in our sham subjects and no change in the sites receiving Tempol in either group, demonstrating that the observed findings in the experimental group were due to the heat therapy intervention through improved NO-dependent dilation.

Local heating of the skin produces a biphasic vasodilator response, consisting of an initial peak occurring within the first 5 min into heating and a secondary plateau, which is achieved after approximately 20-40 min into heating. The initial peak is a sensory nerve axon reflex which elicits dilation via both NO and endothelial-derived hyperpolarizing factors (EDHFs) [331,338,387], most likely initiated via transient receptor potential vanilloid type-1 (TRPV-1) channels [332] located on the sensory nerves, which are thought to release calcitonin gene-related peptide and/or substance P [333]. The plateau phase is heavily dependent on NO [331,334] and also EDHFs to varying extent depending on the temperature used [338,341].

Heat Therapy

Heat therapy increased all phases of the local heating response, indicating improved microvascular function. Furthermore, these effects were NO-dependent as no changes in the NOS inhibited site were observed. No changes were observed in the sham group, suggesting that improvements in microvascular function in the heat therapy group were the result of the heat exposure itself and not due to possible hemodynamic changes secondary to changes in hydrostatic pressure or visitation to the lab environment.

We believe the mechanisms behind this adaptation are two-fold. Heat stress induces elevations in core temperature and subsequent increases in blood flow, particularly to the skin. Elevations in core temperature upregulate expression of heat shock proteins. In particular, Hsp90 is an essential cofactor for endothelial NOS (eNOS) [4], meaning that changes in Hsp90 can alter eNOS activity and NO production independent of changes in total eNOS protein [137]. In the skin, inhibition of Hsp90 with geldanamycin attenuates thermal hyperemia [391], indicating Hsp90 is a primary player in the response. Secondly, increases in blood flow and shear stress are essential for cutaneous microvascular adaptation. For example, chronic localized arm heating in a water bath improves cutaneous microvascular function, but these improvements are prevented when a blood pressure cuff is used on the upper arm to prevent increases in blood flow above baseline during heating [229]. Shear stress can stimulate NO production via phosphorylation of eNOS through a few mechanisms, including through activating the receptor for vascular endothelial growth factor (VEGFR-2) [221] and activating phosphoinositide 3 (PI3)-

kinase, which in turn activates protein kinase A and then eNOS [222]. Therefore, not only does heat therapy likely result in increased association of eNOS with Hsp90, but the pathways necessary for eNOS activation are also likely upregulated.

Importantly, we kept the arm used for microdialysis experiments out of the hot tub to better isolate the systemic effects of heat therapy rather than effects of chronic elevations in local skin temperature. By doing so, we believe our results may better reflect changes that occurred in other microvascular beds as the thermal hyperemia response has been shown to reflect globalized microvascular function [323-325]. In support of this, we also showed that forearm reactive hyperemia is improved following 8 weeks of heat therapy (Brunt et al. 2016). Although we did not investigate this in the present study, the skin under the water (e.g. lower torso and legs) presumably received an even greater stimulus for adaptation as it would have been subject to both the systemic stimuli (e.g. elevated core temperature, exposure to circulating factors upregulated in the blood, and increased shear stress) and to elevations in local temperature. Shear stress may have been even greater in the legs as vasodilation would likely have been greater given the combined core temperature elevation and local skin temperature elevation. As such, heating the legs may be valuable for improving microvascular health in patient populations such as spinal cord injured and peripheral artery disease patients who suffer from a variety of complications secondary to poor circulation in their legs. However, it is important to recognize that we observed improved function in a microvascular bed which experienced large magnitude vasodilation during hot water immersion sessions. Microvascular beds that do not vasodilate during heat stress (e.g., skeletal muscle or kidneys) may not have experienced as great of improvements in microvascular function as did the skin (although, we did observe improved area under the curve forearm post-occlusive reactive hyperemia, which is reflective of microvascular function predominantly in the skeletal muscle of the forearm, described in Chapter V).

Effects of Tempol

Superoxide, a predominant reactive oxygen species, binds to NO to produce peroxynitrite, reducing NO bioavailability. Additionally, superoxide uncouples tetrahydrobiopterin, an essential cofactor for eNOS, causing eNOS to produce superoxide

instead of NO, further depleting NO bioavailability [142]. Tempol, a superoxide dismutase mimetic, reduces superoxide to hydrogen peroxide, thus improving NO bioavailability [392]. Oxidative stress, the accumulation of reactive oxygen species such as superoxide, occurs with aging and with the progression of vascular disease. In the skin, impairments in vasodilator responses with primary aging and hypertension can be reversed by administration of the antioxidant ascorbate [393,394], and by restoring tetrahydrobiopterin levels [395] or inhibiting arginase [393,396], which breaks down L-arginine, the substrate necessary for conversion of NO by eNOS, and is upregulated by oxidative stress. However, the effects of Tempol, which targets oxidative stress more specifically than antioxidants such as ascorbate via its direct effects on superoxide, has been studied relatively little in the skin, despite being used extensively in other preparations.

When locally heating the skin to 42°C, Tempol has been shown to have no effect in young, recreationally active subjects [336], but to augment the plateau in young smokers [326], patients with chronic kidney disease [397], and young healthy subjects following angiotensin II infusion (a model of vascular disease) [336], restoring plateau back to the level of young, healthy individuals. However, in young, healthy subjects, plateau CVC when heating to 42°C reaches ~85-95% of CVCmax and so non-effects of Tempol may have been due to a ceiling effect. Heating to 39°C better isolates the NO-component compared to the more commonly used protocol heating to 42°C (~80% NO vs. ~50-60% NO with 42°C local heating), and it reaches a plateau of ~40-60%CVCmax allowing for the investigation of interventions which may improve NO-dependent dilation, such as heat therapy [341]. In the present study, our data show that Tempol also has no effect in young, sedentary but otherwise healthy subjects when using this local heating protocol, either across all subjects pooled at 0wks or following heat therapy or thermoneutral water immersion. As such, this establishes these methods for use in future studies in patient populations who have elevated oxidative stress in which heat therapy (or other interventions) may be able to alleviate oxidative stress-associated impairments in endothelial function.

In order to ensure non-effects of Tempol were truly because oxidative stress was minimal in our young, sedentary subjects and not because the drug didn't work in our

hands, we studied two SCI subjects. SCI is associated with elevated oxidative stress [390], which is likely exacerbated due to increases in circulating angiotensin-II [398], which increases superoxide production via NADPH-oxidase [399]. This population was selected over other patient groups known to have high oxidative stress because they are young (matched to the mean age of our other subjects) and free of co-morbidities which might alter the effects of Tempol. For example, we have previously shown this concentration of Tempol was highly effective at reversing oxidative stress-associated impairments in young smokers [326]; however, oxidative stress is considerably higher in smokers than in any other disease state as multiple compounds in the cigarettes themselves induce oxidative stress [400]. By studying SCI subjects, we wanted to ensure the effects on vasodilation of more physiological levels of oxidative stress could also be detected using this concentration of Tempol via microdialysis.

Although our data suggest Tempol is having the effect we believe it is, some non-specific effects of Tempol have been observed in animal studies. For example, Tempol has been shown to open ATP-sensitive potassium channels [401] and calcium-sensitive potassium channels [402], the latter of which is highly involved in the cutaneous thermal hyperemia response when heating to 42°C [338]. In our previous study in young smokers, we demonstrated no effect of Tempol when co-infused with L-NNA, indicating no NO-independent effects [326].

Maximal CVC

Although we observed some trends in maximal CVC across drug sites and interventions, only the increase in maximal CVC in the Tempol site with heat therapy was statistically significant. Previous studies from our lab and others [326,397] have observed no effects of Tempol on maximal CVC, and since this effect was only observed in one site, we do not believe it was due to heat therapy. More likely, these differences are due to limitations in the laser Doppler flowmetry technique rather than physiological structural changes in the cutaneous microvasculature. For example, microvessel density can vary greatly from site to site, highlighting the importance of normalizing laser Doppler flux values [386].

CONCLUSIONS, PERSPECTIVES & BRIDGE

In the present study, we have demonstrated that passive heat therapy improves cutaneous microvascular function via improved NO-dependent dilation. Many disease states are characterized by impaired endothelial function, particularly in the microvasculature, and the usual cause is impaired NO bioavailability secondary to chronic oxidative stress and inflammation. Although exercise is a potent means of improving vascular function, many patient populations are either unable or unwilling to exercise to an extent necessary for inducing protective adaptation. Our results demonstrate that passive heat therapy may offer a powerful alternative for improving microvascular function and NO bioavailability that could be implemented by a wide range of patient populations.

While we have demonstrated a role for NO in the adaptations to heat therapy in this chapter, the specific cellular mechanisms that result in improved NO bioavailability are still unknown. In the next chapter, we sought to discover the intracellular pathways associated improved NO bioavailability and vascular function in cultured endothelial cells.

CHAPTER VII

BOTH DIRECT HEAT AND EXPOSURE TO SERUM FROM YOUNG, SEDENTARY HUMANS WHO HAVE UNDERGONE PASSIVE HEAT THERAPY IMPROVES NITRIC OXIDE SIGNALING, OXIDATIVE STRESS, AND ANGIOGENESIS IN CULTURED ENDOTHELIAL CELLS

CO-AUTHOR CONTRIBUTIONS

While I was the primary contributor to the work described in this chapter, Karen M Wiedenfeld-Needham, Lindan N. Comrada, Taylor M. Eymann, Michael A. Francisco, and Christopher T. Minson all made substantial contributions. The cell culture experiments and analyses would not have been possible without Ms. Wiedenfeld-Needham, who provided both her technical expertise and dedicated assistance. L.N.C. performed the majority of venous blood draws. L.N.C. and T.M.E. assisted with cell culture experiments and Western blot analyses. M.A.F. optimized image capture and analysis for angiogenesis experiments, and both M.A.F. and L.N.C. put in countless hours assisting me with performing those analyses. C.T.M. contributed to conception and design of experiments and interpretation of data. While I drafted the manuscript in its entirety, C.T.M. revised it critically for intellectual content.

INTRODUCTION

As detailed in Chapter V and VI, eight weeks of passive heat therapy improves biomarkers of conduit vessel and microvascular function in young, sedentary individuals. In particular, we observed robust improvements in brachial artery flow-mediated dilation

(FMD) (Chapter V), which is heavily dependent on nitric oxide (NO) [274], and cutaneous microvascular NO-dependent dilation (Chapter VI). As reviewed in Chapter II, we believe the mechanisms behind these improvements are two-fold. First, heat exposure increases cardiac output, blood flow, and therefore shear stress on the vasculature. Shear stress is well-known to upregualte eNOS activity and therefore NO bioavailability through several mechanisms. Secondly, elevations in body core temperature induce expression of heat shock proteins (HSPs), which in turn stabilize a variety of proteins important to the cardiovascular system. These include eNOS [4] and anti-oxidative enzymes such as superoxide dismutase (SOD) [147]. Together, these changes may explain the improvements in NO-dependent dilation we observed in humans. Therefore, the overall purpose of the experiments described in this chapter were to elucidate the molecular mechanisms by which heat therapy improves NO bioavailability and vascular function.

The ideal way to investigate which molecular pathways are altered following heat therapy would be to collect endothelial cell biopsies from subjects before and after heat therapy. Although it is possible to collect endothelial cells from human subjects using a J-wire technique [403], this technique yields a very small sample of cells, making it difficult to quantify changes in protein. We therefore utilized a cell culture model.

Endothelial cells *in vivo* in subjects undergoing heat therapy are exposed to three different stimuli: 1) the *direct* intracellular effects of elevations in temperature, 2) the *indirect* effects of circulating factors upregulated elsewhere in the body, and 3) the effects of intermittent increases in shear stress. The effects of shear stress on cultured endothelial cells have been studied extensively, as reviewed in Chapter II, and so for these experiments, we focused on the effects of direct heat and of circulating factors.

Therefore, in order to investigate the molecular mechanisms by which heat therapy functionally improves NO-dependent dilation, we utilized a cell culture model to isolate the direct effects of heat from the effects of upregulated circulating factors. We hypothesized that incubating cultured endothelial cells either 1) at 39°C (to match core temperatures reached by human subjects during heat therapy sessions) or 2) with sera collected from human subjects before and after 8 weeks of heat therapy, would increase HSP, eNOS and SOD protein abundance, and would reduce production of superoxide.

To support this purpose, we collected primary peripheral blood mononuclear cells (PBMCs) from human subjects before and 1h after the first and last water immersion sessions in order to compare the HSP response in cultured cells exposed to direct heat to intracellular HSP levels achieved *in vivo* in cells exposed to the same conditions as endothelial cells. We hypothesized that heating cultured endothelial cells at 39°C for 24h would induce a similar increase in Hsp70 and Hsp90 as observed in primary PBMCs following 8 weeks of heat therapy.

As a second purpose, we utilized an endothelial tubule formation bioassay to investigate whether heat therapy improves serum angiogenic potential. In our human subjects, we observed a reduction in blood pressure, which is often associated with angiogenesis. As NO is essential for angiogenesis, endothelial tubule formation is also a functional test of improved NO bioavailability. Therefore, by performing this assay, we can relate changes in protein abundance observed in experiments performed for our first hypothesis to functional improvements in NO bioavailability. We hypothesized that incubating cultured endothelial cells with sera collected from human subjects before and after 8 weeks of heat therapy would increase endothelial tubule formation, and that this increase would be prevented by the addition of 1mM Nω-nitro-L-arginine (L-NNA; a non-specific NOS inhibitor) to the cell culture media. We additionally hypothesized that incubating cultured endothelial cells with sera from human subjects before and after 8 weeks of thermoneutral water immersion (sham) would have no effect on endothelial tubule formation.

Lastly, we compared changes in some of the variables obtained through cell culture to *in vivo* biomarkers of vascular function using regression analysis. We hypothesized that improvements in FMD and cutaneous NO-dependent dilation would be correlated with improvements in eNOS and SOD protein in serum-exposed cultured endothelial cells, and that improvements in blood pressure (particularly diastolic and mean arterial pressure) would be correlated with improvements in serum angiogenic potential (as measured by endothelial tubule formation).

METHODS

Human Subjects

All subjects provided oral and written informed consent prior to participation in the study, as set forth by the *Declaration of Helsinki*. All experimental procedures were approved by the Institutional Review Board at the University of Oregon. Twenty young (18-30 yrs of age), healthy (no history or cardiovascular-related diseases), sedentary (<2h aerobic exercise per week) subjects, who were non-smokers and were not taking any prescription medications except for contraceptives, completed 8 weeks of either heat therapy (N=10) or thermoneutral water immersion (sham; N=10), as described in Chapter III (pages 36-38).

On the first (0wks) and last (8wks) water immersion sessions, venous blood was drawn into serum-separating vacutainers (BD, Franklin Lakes, NJ) and Ficoll Hypaque-containing cell preparation tubes with sodium citrate (CPT Vacutainer; BD, Franklin Lakes, NJ) immediately before (pre hot tub [preHT]) and 1h after water immersion (postHT). This time frame was selected because based on pilot data (reported in Chapter IV, page 71) and previous reports [129]. The session at 8wks was held 36-48h after the penultimate water immersion session in order to capture the chronic rather than acute effects of heat therapy. Subjects reported to the laboratory fasted from food for at least 2h and having abstained from exercise for 24h. Sessions were held at the same time of day and subjects were asked to maintain food prior to 2h before and caffeine intake similarly between the two sessions. After collection, venous blood in serum-separating tubes was kept at room temperature for 30min and then separated by centrifugation. Serum was stored at –80°C until later use in cell culture experiments. PBMCs were isolated from whole blood in CPT vacutainers by centrifugation (3,000rpm for 15 min at room temperature).

Direct heat & serum exposure experiments

Following 4h serum starve, human umbilical vein endothelial cells (HUVECs; ATCC, Manassas, VA) were incubated for 24h under the following conditions: 1) 37°C in complete media, 2) 39°C in complete media, or 3) 37°C in non-supplemented media with 10% human sera collected at each of the 4 time points (0wks preHT, 0wks postHT,

8wks preHT, 8wks postHT) from all 10 subjects in the heat therapy group. Following the 24h, cells were lifted with TrypLE, combined with radioimmunoprecipitation assay buffer (RIPA) plus protease inhibitor, and lysed via sonication. Cells used for experiments had undergone 2-3 passages.

Western blotting

Protein (20-50ug) from HUVEC and primary PBMC lysates was separated by electrophoresis on 4-20% SDS polyacrylamide separating gels (Life Technologies, Grand Island, NY) and then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were Ponceau-stained to access transfer across each gel. Membranes were incubated for 1h in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) and then incubated overnight at 4°C in blocking buffer containing primary antibodies. Membranes were then washed and incubated with the appropriate secondary antibodies (LI-COR Biosciences) for 1h at room temperature. The fluorescent bands were digitized using a LI-COR Odyssey infrared imaging system (LI-COR Biosciences). Digitized images were quantified using LI-COR Image Studio^{TM} software. Antibodies were stripped using NewBlot^{TM} Nitro Stripping Buffer (LI-COR Biosciences) in between probing for primary antibodies.

Primary antibodies for HUVECs were: 1) anti-endothelial NO synthase (eNOS) (1:1,000; Cell Signaling Technology, Danvers, MA) 2) anti-superoxide dismutase-2 (MnSOD/SOD2) (1:1,000; Sigma-Aldrich, St. Louis, MO) 3) anti-Hsp90 [S88] (1:200; Abcam, Cambridge, MA) and 4) anti-Hsp70 [BRM-22] (1:5,000; Abcam), and 5) anti-vinculin (loading control; 1:1,000; Cell Signaling Technology).

Primary antibodies for PBMCs were: 1) anti-Hsp70 [BRM-22] (1:5,000; Abcam) 2) anti-Hsp90 [S88] (1:200; Abcam), or 3) anti-β-actin (loading control; 1:1,000; Cell Signaling Technology).

Superoxide production

HUVECs were plated in 96-well culture plates at 2 x 10^4 cells per well and exposed to the direct heat or serum exposure conditions as described in triplicate. After 24h, media was replaced with 20μl of 1μM superoxide detection stain (Cellular

Superoxide Detection Assay Kit; Abcam), which permeates the cell membrane and reacts with specifically with superoxide. Cells were incubated for 1h under the same conditions as before (37 or 39°C, or 37°C with 10% serum). Additional wells were left untreated (in complete media) and unstained (blank) or were treated with 20µl of 500µM pyocyanin (reactive oxygen species inducer), which served as a positive control. Cell fluorescence was determined with a phase-contrasted fluorescent microscope with Cy3 filter at 10X optical zoom (Axio Observer.D1; Zeiss, Oberkochen, Germany). Total cell fluorescence per frame minus blank fluorescence was determined using ImageJ analysis software (National Institutes of Health; Bethesda, MD).

Endothelial tubule formation assay

As a functional test of improved NO bioavailability, serum angiogenic potential was quantified as previously described [348]. HUVECs were plated in 96-well cell culture plates onto solidified phenol-red free growth factor reduced Matrigel (BD Bioscience, San Jose, CA) at a concentration of 1 x 10^5 cells/ml in serum-free media. Cells were treated with 10% serum from human subjects collected at each of the four time points and from each of the two groups (in triplicate per sample), and incubated for 10h. Wells were imaged with a phase-contrasted microscope at 2.5X optical zoom and total length of tubule formation per frame was assessed with ImageJ analysis software by two blinded investigators. Results were averaged across investigators and across two separate experiments for verification.

In a subset of experiments, cells were additionally treated with 1mM Nω-nitro-L-arginine (L-NNA; Sigma Aldrich, St. Louis, MO) to inhibit NOS and to determine whether the pro-angiogenic effects of heat therapy are mediated via NOS upregulation. A concentration of 1mM was selected based on pilot studies in order to reduce NOS activity without completing abolishing angiogenesis.

Enzyme-linked immunosorbent assays

To attempt to identify factors in the serum affected by heat therapy that could be responsible for inducing changes in endothelial cells, we measured serum concentrations of free vascular endothelial growth factor (VEGF) and soluble VEGF Receptor (sFlt)-1

using commercially-available enzyme linked immunosorbent assay kits (R&D Systems, Quantikine, Minneapolis, MD) according to manufacturer's instructions. The ratio of sFlt-1 to VEGF was calculated as a marker of bioavailable VEGF.

Statistical analyses

Changes in protein abundance and superoxide production in direct heating experiments (37°C vs. 39°C) were compared using Student's unpaired t-test. Changes in protein abundance and superoxide production in serum exposure experiments, serum [VEGF], [sFlt-1], and sFlt-1/VEGF, and endothelial tubule formation with heat therapy sera, sham group sera, and heat therapy sera + L-NNA, were compared using two-way repeated measures ANOVA with factors of week (0 vs. 8wks) and time (pre vs. post hot tub [HT]). When significant main effects were observed, pairwise comparisons were made using Tukey's post hoc test. Comparisons in endothelial tubule formation across groups were made using Student's paired (heat therapy with vs. without L-NNA) and unpaired (heat therapy vs. sham) t-tests. We were underpowered for comparing changes in HSP protein in PBMCs using ANOVA (power on two-way RM ANOVA for Hsp90 was 0.21) and so differences were instead compared using Student's paired t-test for 0wks pre vs. 0wks post, 0wks pre vs. 8wks pre, and 8wks pre vs. 8wks post.

Linear regression analysis was used to compare changes in molecular measures to changes in *in vivo* outcomes across the 8 weeks of intervention. Specifically, we compared changes in serum angiogenic potential (pooled across all 20 subjects) to changes in mean arterial, systolic, and diastolic pressure, and we compared changes in eNOS and SOD protein in serum exposed cells (heat therapy group only) to changes in FMD, cutaneous NO-dependent dilation.

RESULTS

Effects of direct heating on protein abundance & superoxide production

Exposing endothelial cells to 39°C for 24h significantly increased Hsp70 ($p<0.001$), Hsp90 ($p=0.02$), and MnSOD ($p=0.02$) protein, but had no effect on eNOS protein ($p=0.65$), all relative to endothelial cells incubated at 37°C (Figure 7.1 – next

page). Additionally, direct heating significantly reduced superoxide production (p=0.03) (Figure 7.3A – page 128).

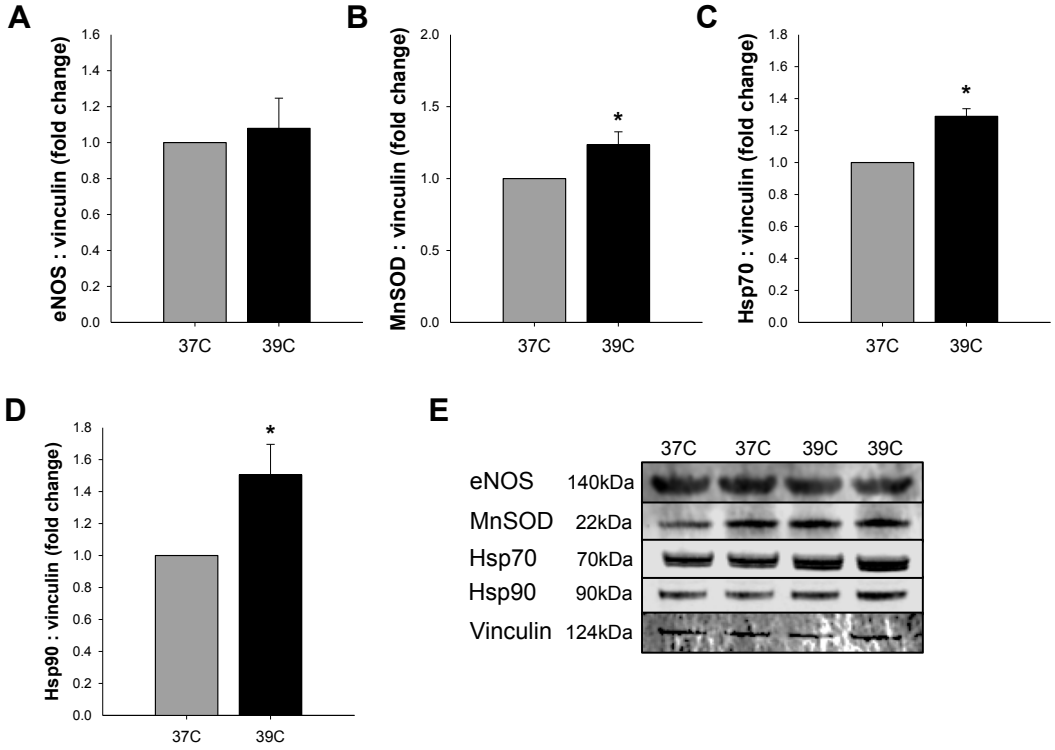

Figure 7.1. Heating endothelial cells to 39°C for 24h had no effect on endothelial nitric oxide synthase protein expression (eNOS) **(A)**, but increased protein expression of manganese superoxide dismutase (MnSOD) **(B)**, heat shock protein (Hsp)-70 **(C)** and Hsp90 **(D)**. All data was normalized to a vinculin loading control and are presented as mean±S.E. fold changes from cells exposed to 37°C. N=9 across 4 separate experiments. *P<0.05 vs. 37°C (control). **E)** Western blots performed with HUVEC lysates incubated at 37°C or 39°C for 24h.

Effects of serum exposure on protein abundance & superoxide production

Exposing endothelial cells to sera collected following the first hot water immersion session (0wks postHT) had no effect on eNOS (p=0.37) or MnSOD (p=0.58) protein, relative to cells exposed to sera collected prior to the same session (0wks preHT). However, sera collected following 8 weeks of heat therapy (chronic condition; 8wks preHT) induced significant increases in both eNOS (p=0.04) and MnSOD (p=0.02) protein in endothelial cells. These increases were maintained when endothelial cells were exposed to sera collected for the last hot water immersion session (8wks postHT vs. 8wks

preHT, eNOS: p= 0.57, MnSOD: p=0.99). These improvements occurred independent of intracellular changes in HSPs, as there was no effect of serum exposure on either Hsp70 (p=0.50) or Hsp90 (p=0.45). Results are summarized in Figure 7.2.

Conversely, both sera collected following the first hot water immersion (p=0.001) and chronically following the 8 weeks of heat therapy (p=0.004) reduced superoxide production (Figure 7.3B – next page).

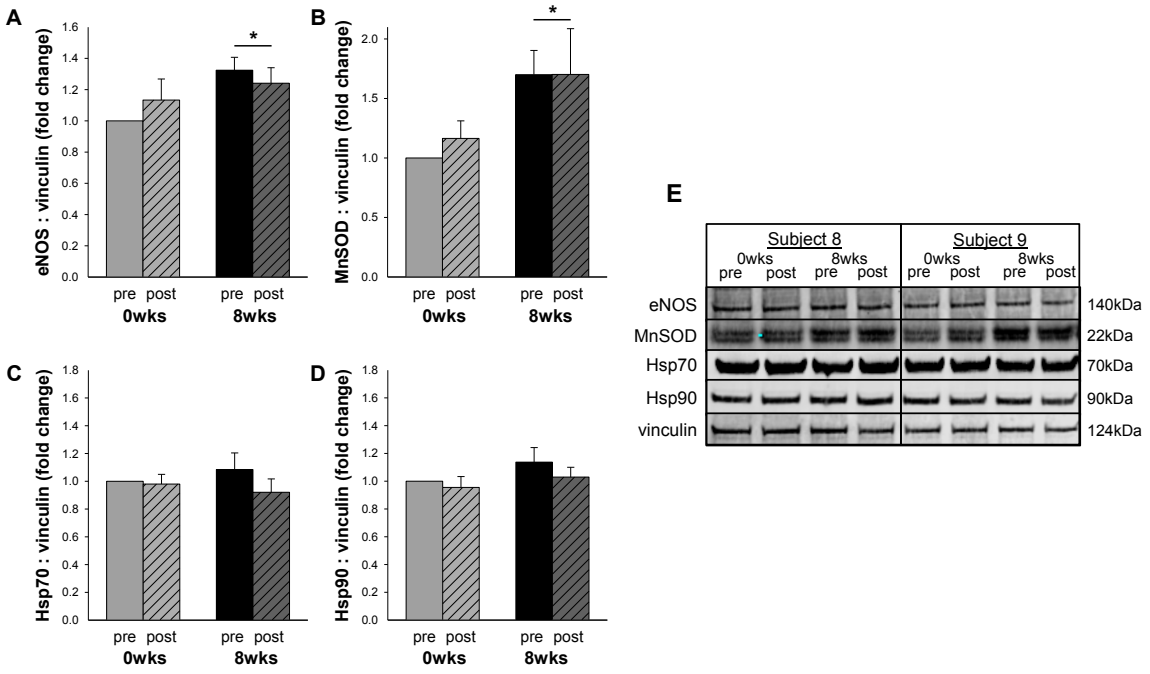

Figure 7.2. A) Endothelial nitric oxide synthase (eNOS), **B)** manganese superoxide dismutase (MnSOD), **C)** heat shock protein (Hsp) 70, and **D)** Hsp90 protein abundance in endothelial cells exposed to sera from human subjects collected before (pre) and 1h after (post) the first (0wks) and last (8wks) heat therapy sessions. Data were normalized to a vinculin loading control and are presented as mean±S.E. fold changes from cells exposed to sera from the same human subjects prior to heat therapy (0wks pre). *P<0.05 vs. 0wks pre. **E)** Western blots performed with HUVEC lysates exposed to sera from two representative heat therapy subjects.

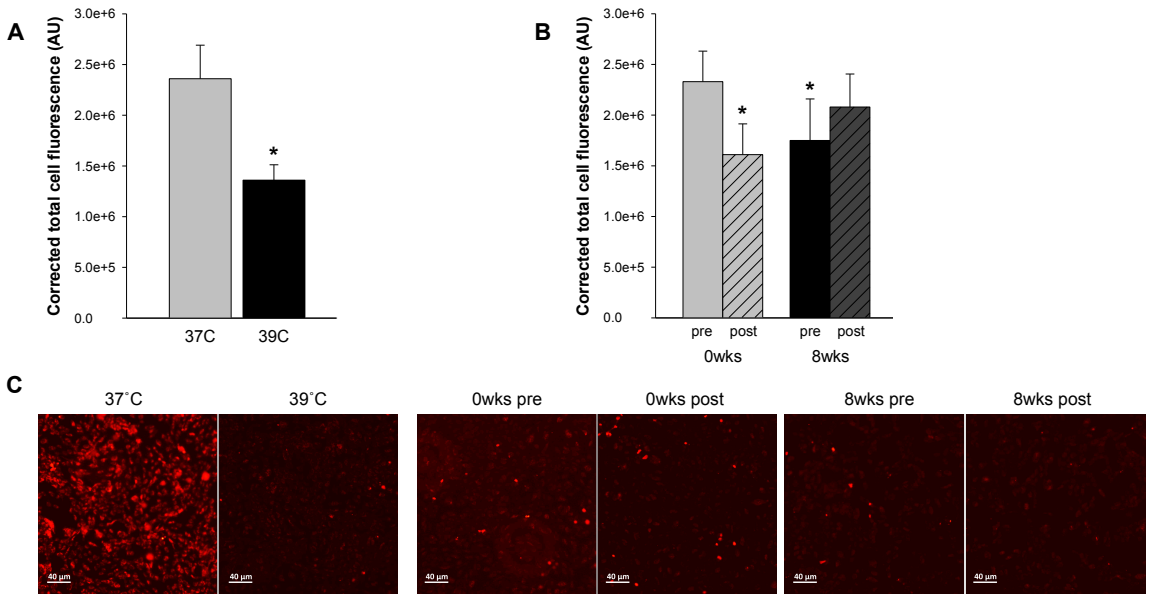

Figure 7.3. Superoxide production in cultured endothelial cells incubated for 24h at **A)** 37°C or 39°C, or **B)** with 10% sera collected from human subjects pre (solid bars) and 1h post (dashed bars) the first (0wks) and last (8wks) heat therapy sessions. Data are mean±S.E., *p<0.05 from 37°C or 0wks pre conditions. **C)** Representative Cy3 fluorescent images taken at 10X optical zoom of cultured endothelial cells stained for superoxide and exposed to 37°C, 39°C, or sera from one representative subject who underwent heat therapy.

Heat shock proteins in primary mononuclear cells

As confirmation that changes we observed with direct heating experiments were comparable to changes that may occur *in vivo*, we measured HSP protein abundance in PBMCs collected from human subjects. Although not statistically significant, Hsp70 and Hsp90 were increased in PBMCs 1h following the first heat therapy session (Hsp70: p=0.12, Hsp90: p=0.09). Following 8 weeks of heat therapy, Hsp70 remained significantly elevated (p=0.05); whereas, Hsp90 tended to return back towards baseline (p=0.24). Importantly, fold changes in both Hsp70 and Hsp90 in PBMCs were similar to changes observed in cultured endothelial cells incubated at 39°C for 24h (Figure 7.4 – next page).

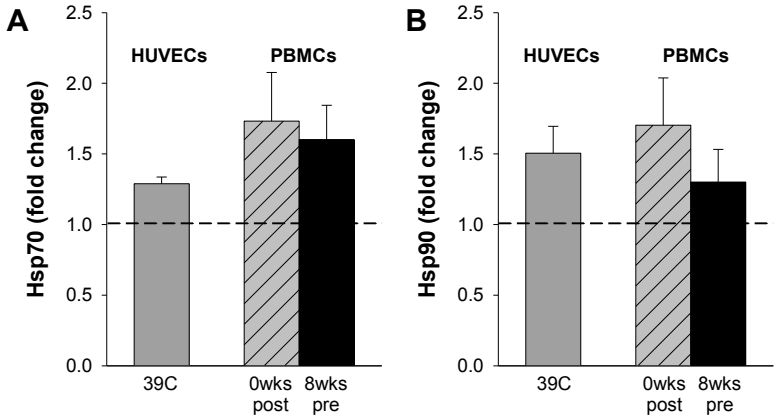

Figure 7.4. Heat shock protein (Hsp)-70 **(A)** and Hsp90 **(B)** protein abundance was similar between human umbilical vein endothelial cells (HUVECs) incubated at 39°C for 24h and peripheral blood mononuclear cells collected from human subjects after the first hot water immersion session (0wks post) and chronically following 8 weeks of heat therapy (8wks pre). Data are mean±S.E. and presented as a fold change from control conditions (37°C or exposure to sera from subjects prior to heat therapy, 0wks pre), as indicated by the dashed lines.

Endothelial Tubule Formation

The effects of heat therapy and thermoneutral water immersion (sham) are summarized in Figure 7.5 (next page). Endothelial tubule formation was significantly improved in cells exposed to sera collected from subjects following one bout of hot water immersion (0wks postHT; p=0.04) and following 8 weeks of heat therapy (8wks preHT; p=0.03), relative to cells exposed to sera from the same subjects prior to heat therapy (0wks preHT). Although endothelial tubule formation in cells exposed to 8wks postHT sera trended to come back down towards baseline, this was not significantly reduced from 8wks preHT (p=0.52). The addition of 1mM L-NNA to the culture media had no effect on baseline endothelial tubule formation (exposure to 0wks preHT sera from subjects in the heat therapy group; with vs. without L-NNA: p=0.41). However, L-NNA prevented the improvement in tubule formation associated with heat therapy (p=0.50). There were also no effects of sham on endothelial tubule formation (0wks preHT vs. 0wks postHT: p=0.39; vs. 8wks preHT: p=0.98; vs. 8wks postHT: p=0.15), however, we did observe a significant difference in tubule formation in cells exposed to sera from sham subjects between the 0wks postHT and 8wks postHT time points (p=0.004).

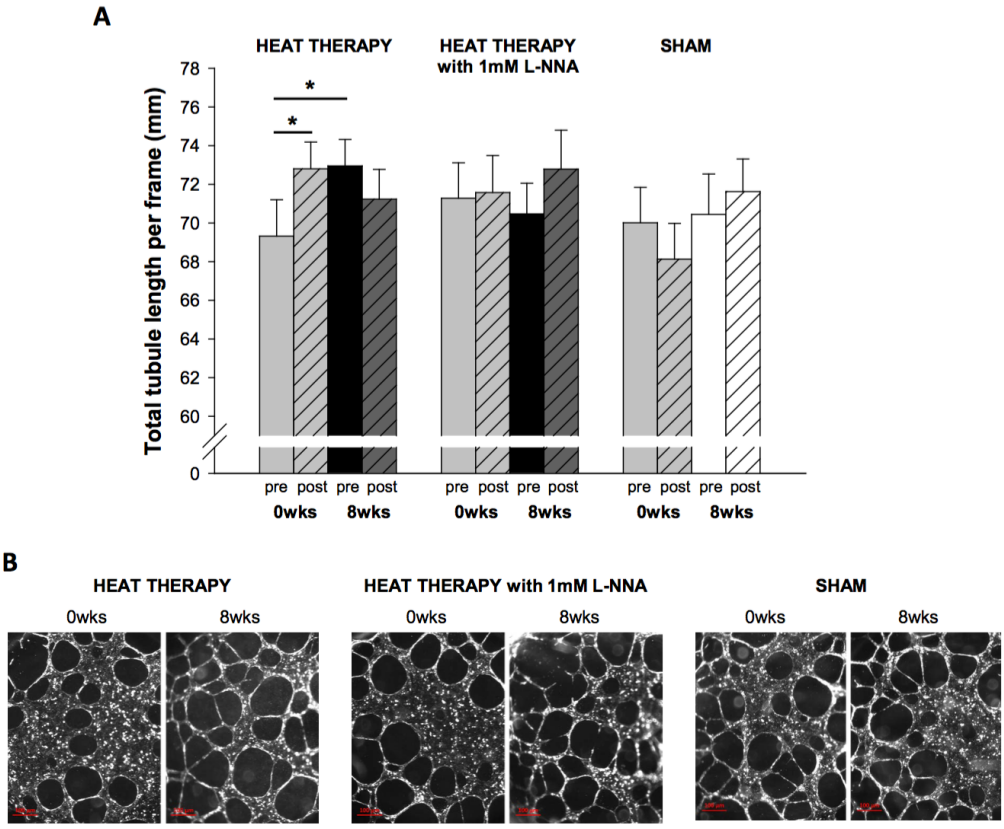

Figure 7.5. A) Changes in endothelial cell tubule formation following treatment with sera from heat therapy (with and without 1mM Nω-nitro-L-arginine [L-NNA]) and sham subjects collected pre (solid bars) and 1h post (dashed bars) the first (0wks) and last (8wks) water immersion sessions. Endothelial tubule formation was significantly improved following treatment with sera from subjects in the heat therapy group following acute and chronic hot water immersion. No other significant changes were observed. Data are mean±S.E., *$p<0.05$. **B)** Representative phase contrasted images at 2.5X magnification of endothelial cells exposed to sera collected from the same subject in the heat therapy group before and after 8wks of heat therapy, with and without L-NNA, and from a subject in the sham group (all pre-water immersion).

Serum VEGF and sFlt-1 concentrations

Heat therapy had no effect on serum concentration of VEGF ($p=0.31$) or sFlt-1 ($p=0.92$), or on the ratio of sFlt-1/VEGF ($p=0.68$) (Figure 7.6 – next page).

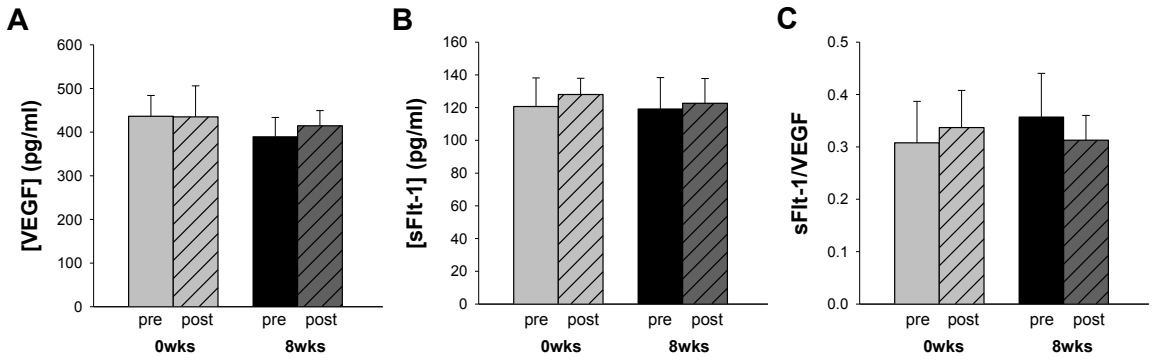

Figure 7.6. Serum concentrations of **A)** vascular endothelial growth factor (VEGF) and **B)** soluble VEGF receptor (sFlt)-1, and **C)** the ratio of sFlt-1/VEGF, indicative of bioavailable VEGF, across time points into heat therapy. Serum was collected before (pre; solid bars) and 1h after (post; dashed bars) the first (0wks) and last (8wks) heat therapy session. Data are mean±S.E. No significant differences were observed.

Correlations between cellular and in vivo vascular function data

Changes in serum angiogenic potential were significantly correlated with changes in mean arterial pressure (r^2=0.28, p=0.02) (Figure 7.7), but not with changes in diastolic (r^2=0.08, p=0.23) or systolic blood pressure (r^2=0.05, p=0.34). We found no significant correlations between changes in eNOS or SOD protein with *in vivo* outcomes, presumably because the sample size was lower for these measures (performed in only heat therapy subjects).

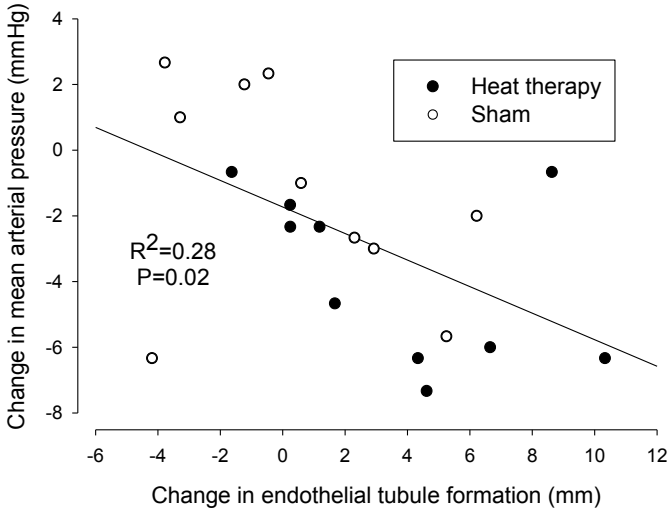

Figure 7.7. Changes in serum angiogenic balance across the 8 weeks of heat therapy or thermoneutral water immersion (sham), as measured by endothelial tubule formation in cells exposed to sera from human subjects, significantly predicted changes in mean arterial pressure.

DISCUSSION

In the present study, we showed that 1) exposing endothelial cells to physiological levels of heat (39°C) increases SOD, Hsp70, and Hsp90 protein abundance, and reduces superoxde production, 2) exposing endothelial cells to serum collected from human subjects who have undergone 8 weeks of passive heat therapy increases eNOS and SOD protein abundance and reduces SO production, independent of changes in HSPs, and 3) serum from heat-acclimated human subjects increases endothelial tubule formation, a functional assay of NO bioavailability. Presumably *in vivo*, these effects are combined, offering multiple mechanisms by which passive heat therapy improves NO bioavailability and presumably overall vascular function. Additionally, we observed that circulating factors upregulated following acute hot water immersion were able to induce some changes in endothelial cells (e.g., reduced superoxide production and increased endothelial tubule formation), but not all, suggesting that repeated bouts of hyperthermia are required to acquire all cellular adaptations of interest.

Direct effects of heat

To our knowledge, this is the first study to investigate changes in endothelial cells in response to lower levels of heat (i.e., similar to temperatures that endothelial cells *in vivo* may commonly be exposed to). Many studies have investigated the effects of exposure to higher levels of heat (41-45°C). While these studies induce greater changes in protein abundance, they are less translatable to what occurs *in vivo* in humans.

In the present study, physiological levels of heat were sufficient to induce increases in HSP abundance in cultured endothelial cells at magnitudes similar to what we observed in blood mononuclear cells in human subjects (approximately 1.5-fold). PBMCs were chosen as an *in vivo* marker for measuring HSPs because they are exposed to the same conditions *in vivo* as endothelial cells, they can be collected from human subjects easily and relatively non-invasively, they exhibit a stress response to exercise hyperthermia [114,115], and HSPs are upregulated under non-stressed conditions in human PBMCs following 10 days of exercise heat acclimation [169].

Heating endothelial cells at 39°C for 24h increased MnSOD protein and reduced superoxide production. MnSOD has been previously shown to be increased in association

with Hsp70 in gerbil hippocampal neurons [146] and to be suppressed in Hsp70-knockout mice [147], as such, it is likely that MnSOD increased with direct heating in the present study as a direct result of increased Hsp70. HSPs are known to stabilize other anti-oxidative enzymes, such as glutathione peroxidase [5]. However, we chose to focus on SOD for the current study as it is the most relevant for regulating NO bioavailability.

Interestingly, we observed no effect of direct heat on eNOS protein, although we observed approximately a 1.5-fold increase in Hsp90. Hsp90 is an essential cofactor for eNOS activation, which controls the balance of NO and superoxide production [4]. Therefore, increased Hsp90 abundance can increase NO bioavailability, independent of changes in total eNOS protein. The predominant mechanism by which Hsp90 is thought to regulate eNOS activity is through enhancing Akt phosphorylation [136] and calcium-calmodulin binding to eNOS [134]. In rats, Harris et al. [137] demonstrated that 10 weeks of exercise training had no effect on eNOS protein, but increased Hsp90 content and association of Hsp90 with eNOS, therefore increasing eNOS activity and NO production. Based on our results, the same may occur with physiological levels of direct heating.

We did not investigate the effects of direct heat on tubule formation in the present study as it has previously been well established that Hsp90 regulates angiogenesis. Rattan et al. [404] showed that 24h exposure to 41°C improves tubule formation in HUVECs using the same assay as we used in the present study. Furthermore, inhibition of both NOS with 1mM L-NAME and Hsp90 with allyamino-17-demethoxygeldanamycin (17-AAG) inhibits VEGF-stimulated endothelial tubule formation in HUVECs to similar extents [368]. In rats, inhibition of Hsp90 with 17-dimethylaminoethylamino-17demethoxygeldanamycin (17-DMAG) prevents increases in eNOS protein content and phosphorylation and hindlimb angiogenesis induced by repeated sauna therapy [367].

Effects of serum exposure

Exposing endothelial cells to serum from heat-acclimated subjects increased eNOS and MnSOD protein, reduced superoxide production, and increased functional NO bioavailability (assessed by endothelial tubule formation), independent of changes in HSPs. Importantly, NOS inhibition with L-NNA prevented heat-therapy induced improvements in endothelial tubule formation, suggesting that these improvements were

due to the increases in eNOS and SOD protein that we observed (i.e., improved capacity to produce NO and anti-oxidative mechanisms to prevent NO from being scavenged by superoxide).

This begs the question as to what did heat therapy upregulate in the serum that could have such profound effects on NO bioavailability? Interestingly, the well-established pro-angiogenic factor VEGF, which induces angiogenesis by increasing eNOS protein and activation [405], was unchanged in serum following heat therapy. This finding is consistent with studies investigating the effects of repeated sauna therapy in rats [250] and humans [406]. In particular, Akasaki et al. [250] observed no change in serum VEGF in rats following 3 weeks of sauna therapy, despite NOS-dependent improvements in hindlimb angiogenesis.

There are other known pro-angiogenic factors that can induce NOS-dependent angiogenesis independent of VEGF; however, whether they may be upregulated following heat stress is unclear. Both transforming growth factor (TGF)-β [407] and basic fibroblast growth factor (bFGF) [408] can induce angiogenesis independently of VEGF by upregulating eNOS protein content and activity. Genes involved in the TGF-β pathway have been shown to be upregulated following acute heat stress [409]; however, overexpression of Hsp70/72 has also been shown to inhibit TGF-β signaling [410,411]. Heat stress has been shown to increase bFGF mRNA in cancer cells via protein kinase C [412].

Angiopoietin-1 (Ang-1) is a ligand for Tie-2 receptors expressed on the surface of endothelial cells. Although, Ang-1-induced angiogenesis generally occurs downstream to VEGF, Ang-1 enhances eNOS activity via the PI 3-kinase [413], Akt and MAP-kinase pathways [414], and so heat therapy-induced increases in Ang-I could promote angiogenesis independent of changes in VEGF. Recently, exogenous Ang-1 administration has been shown to induce angiogenesis and muscle repair in injured skeletal muscle in mice [415]. Heat stress in cultured cells has been shown to increase Ang-1 mRNA [416], but it is presently unknown whether whole-body heat acclimation would do the same.

Repeated sauna therapy has been shown to increase the number of circulating CD34+ cells in heart failure [23] and peripheral artery disease patients [406]. CD34+ cells are

endothelial progenitor cells which are thought to promote angiogenesis through the secretion of angiogenic growth factors and/or their incorporation into developing blood vessels [417]. However, the exact mechanisms for how these processes occur are presently unknown. Although CD34+ cells would not be present in the human sera with which we treated endothelial cells, it is possible mobilized CD34+ cells released signaling molecules that could have been present in the sera.

However, since investigating what specifically was upregulated in the sera was beyond the scope of this project, all we can conclude definitively from our data is that treating endothelial cells with sera from heat acclimated subjects increases NO bioavailability in a VEGF-independent manner.

Considerations

As discussed, we observed no changes in circulating VEGF, and so improvements in NO bioavailability in serum exposed endothelial cells were VEGF-independent. However, we cannot rule out VEGF as a mediator of improvements in vascular function *in vivo*. Acute hyperthermia [418] and repeated sauna therapy [419,420] have been shown to increase VEGF expression in myocardial tissue, although these changes have not been observed in skeletal muscle [250].

We chose to study HUVECs in the present study, which are venous cells and so are habituated to lower levels of oxygen and shear stress than arterial endothelial cells. However, many of the other commercially available cell lines are harvested from patients who all have some level of disease progression. In order to be more similar to endothelial cells in our young, healthy subjects, we wanted to avoid cell lines harvested from patient populations.

Relevant to the cardiovascular system and vascular function, HSPs are known to suppress oxidative stress and inflammation. In the present study, we chose to focus on the effects of heat therapy on NO signaling because both healthy subjects and healthy cells have minimal levels of oxidative stress and inflammation, and because we observed the greatest improvements in our human subjects in measures of vascular function which were the most dependent on NO. In pilot studies, we observed no effects of either direct heat or serum exposure on expression of markers oxidative stress (e.g., nitrotyrosine) or

inflammation (e.g., nuclear factor kappa-B) (data not shown). However, given the known effects of HSPs, heat therapy may be capable of reducing oxidative stress and inflammation in patient populations who have elevated levels of these markers to begin with.

We only performed experiments measuring changes in protein abundance using sera from human subjects in the heat therapy group. This prevents us from being able to isolate the effects of whole-body heat exposure from hydrostatic and placebo effects by comparing changes with serum from heat therapy vs. sham subjects. However, we observed no effects of serum from sham subjects on endothelial tubule formation, suggesting that we also would not have observed any changes in protein abundance with serum from these subjects.

Perspectives: Potential combined effects in vivo

During heat therapy sessions, endothelial cells *in vivo* are exposed to direct heat, increases in shear stress, and factors upregulated elsewhere in the body. These circulating factors, which may be upregulated due to HSPs, increases in shear, or by unknown mechanisms, are then released into the blood and may have acute and prolonged effects on endothelial cells. In our direct heating experiments, we were able to determine the intracellular effects of heat exposure. In our serum exposure experiments, we investigated the effects of circulating factors in the blood released both acutely following hot water immersion and chronically following 8 weeks of heat therapy. The mechanisms that underscore improvements in vascular function *in vivo* will be a combination of these effects, along with the effects of repeated elevations in shear stress, which is also known to upregulate eNOS expression. Combining data from our two sets of experiments, we believe that, *in vivo*, heat therapy results in upregulation of eNOS and SOD protein. Increased Hsp90 further increases eNOS activity. This provides three separates mechanisms by which increases in NO bioavailability are achieved, which likely explains why we were able to induce such robust improvements in NO-dependent dilation *in vivo*.

CHAPTER VIII

PILOT STUDY IN SPINAL CORD INJURED INDIVIDUALS

INTRODUCTION

In the previous chapters, I have presented data that strongly argues heat therapy can be used to drive robust arterial adaptation. The motivation behind conducting studies in this line of research is to establish heat therapy as an effective alternative means for improving cardiovascular health, particularly for use by patients with limited exercise capabilities. Patients who have sustained spinal cord injury (SCI) are one such population. Just in the United States, an estimated 276,000 individuals are living with a SCI, with an estimated 12,500 new cases per year [421]. SCI results in an estimated lifetime healthcare cost of up to $4.7 million per individual [421]. Despite advancements in medical treatment following SCI, these patients experience significantly higher rates of mortality than the general population [422], with cardiovascular (CV) diseases accounting for >40% of deaths beyond the first year post-injury [423]. The incidence and severity of ischemic cardiovascular events is exceptionally high, leading to 2-3x higher risk of stroke [424] and 2x higher risk of heart attack [425,426] compared to their able-bodied peers. Although SCI is characterized by an amplification of risk of CV disease, this increased risk cannot be explained by traditional risk factors [427]. Instead, the increased CV risk may relate to the direct effects of SCI on arterial health. SCI is associated with rapid vascular remodeling including decreased conduit-artery diameters [428,429] and increased wall thickness [430], increased arterial stiffness [431,432], and increased peripheral vascular resistance [433]. Oxidative stress is also greater [390,434] and circulating levels of vasoconstrictors, such as angiotensin-II [398] and endothelin-I [435], are increased. These changes result in impairments in vasodilator ability, including impaired lower limb reactive hyperemia [436] and cutaneous endothelial-dependent dilation [2,437].

Although some modalities of exercise are available to SCI patients (e.g. arm exercise and functional electrical stimulation [FES]), they do not confer the same benefits

as dynamic whole- body exercise. For example, FES, in which denervated muscles are electrically stimulated to produce rhythmic contractions, is able to partially reverse the conduit vessel remodeling [290], but has been unsuccessful at improving endothelium-dependent dilation in conduit and resistance vessels [2,3]. A potential explanation is that FES is not accompanied by the normal changes in cardiovascular hemodynamics that are observed during voluntary exercise (e.g. increases in HR, cardiac output, and stroke volume, and decreases in peripheral vascular resistance [438]), which are key stimuli for arteries to adapt [18]. Similarly, upper body exercise in SCI only results in minimal increases in cardiac output since complete SCIs lack the ability to redistribute blood from the inactive lower body [439], severely limiting exercise intensities that can be reached. Thus, there is high demand for new therapies to better manage CV risk in these patients.

Therefore, we performed pilot data in two subjects who had sustained complete SCI, with one completing 8 weeks of heat therapy and the other serving as a time control. By doing so, we hoped to gain insight into whether heat therapy could successfully be translated into a clinical population. We hypothesized that we would observe improvements in all primary outcome variables assessed in young, sedentary, able-bodied subjects. Specifically, we hypothesized that we would observe increased brachial artery flow-mediated dilation, reduced arterial stiffness, reduced intima media thickness, reduced blood pressure, increased cutaneous microvascular function and nitric oxide-dependent dilation, and increased serum angiogenic potential.

METHODS

Two young, otherwise healthy (non-smokers and no overt cardiovascular-related diseases), paraplegic subjects participated in the study. Subject demographics are provided in Table 8.1 – next page. A physician familiar with treating SCI patients screened subjects prior to participation in order to ensure they would tolerate heat therapy. Subjects provided oral and written informed consent prior to participation in the study, as set forth by the Declaration of Helsinki. All experimental procedures were approved by the Institutional Review Board at the University of Oregon.

Subject 1 participated in 8 weeks of heat therapy, exactly as performed by able-bodied subjects, as described in Chapter III (pages 36-38). Subject 2 served as a time

Table 8.1. Characteristics of spinal cord injured (SCI) pilot subjects

	SCI subject 1	SCI subject 2
Sex	Male	Female
Age	23	25
Body mass (kg)	66	59
BMI (kg/m^2)	20.8	25.4
SCI level	T11 (ASIA A/complete)	T12 (ASIA A)
Years since injury	7	8

ASIA, American Spinal Injury Association classification system

control and reported to the laboratory for all experimental days every 2 weeks but did not participate in thermoneutral water immersion sessions. Since we observed no differences in any of our outcome variables over the 8 weeks of sham in able-bodied subjects, we believed it would suffice to perform just a time control in SCI subjects. This helped to compensate for the higher difficulty of recruiting and retaining SCI subjects.

Both subjects reported to the laboratory every 2 weeks for macrovascular function studies, which were performed exactly as described in Chapter V, and pre- and post-the 8-week intervention for cutaneous microdialysis studies, as described in Chapter VI. Additionally, venous blood was drawn from both subjects at the 0 and 8-week time points. After collection, blood was serum separated and stored at -80°C until later use in serum angiogenic potential experiments, as described in Chapter VII.

RESULTS

Macrovascular function

We observed clinically relevant improvements in biomarkers of macrovascular function in our pilot SCI subject who underwent 8 weeks of heat therapy (Figure 8.1 – next page), including improved brachial artery flow-mediated dilation (FMD), improved superficial femoral arterial compliance, reduced intima media thickness of both the carotid and superficial femoral arteries (presented as wall-to-lumen ratio in Figure 8.1, which normalizes for differences in diameter across SCI and able-bodied subjects [318]), and reduced mean arterial pressure. No changes were observed in the SCI time control subject in any of these variables beyond fluctuations that would be expected with hormonal variations across the menstrual cycle.

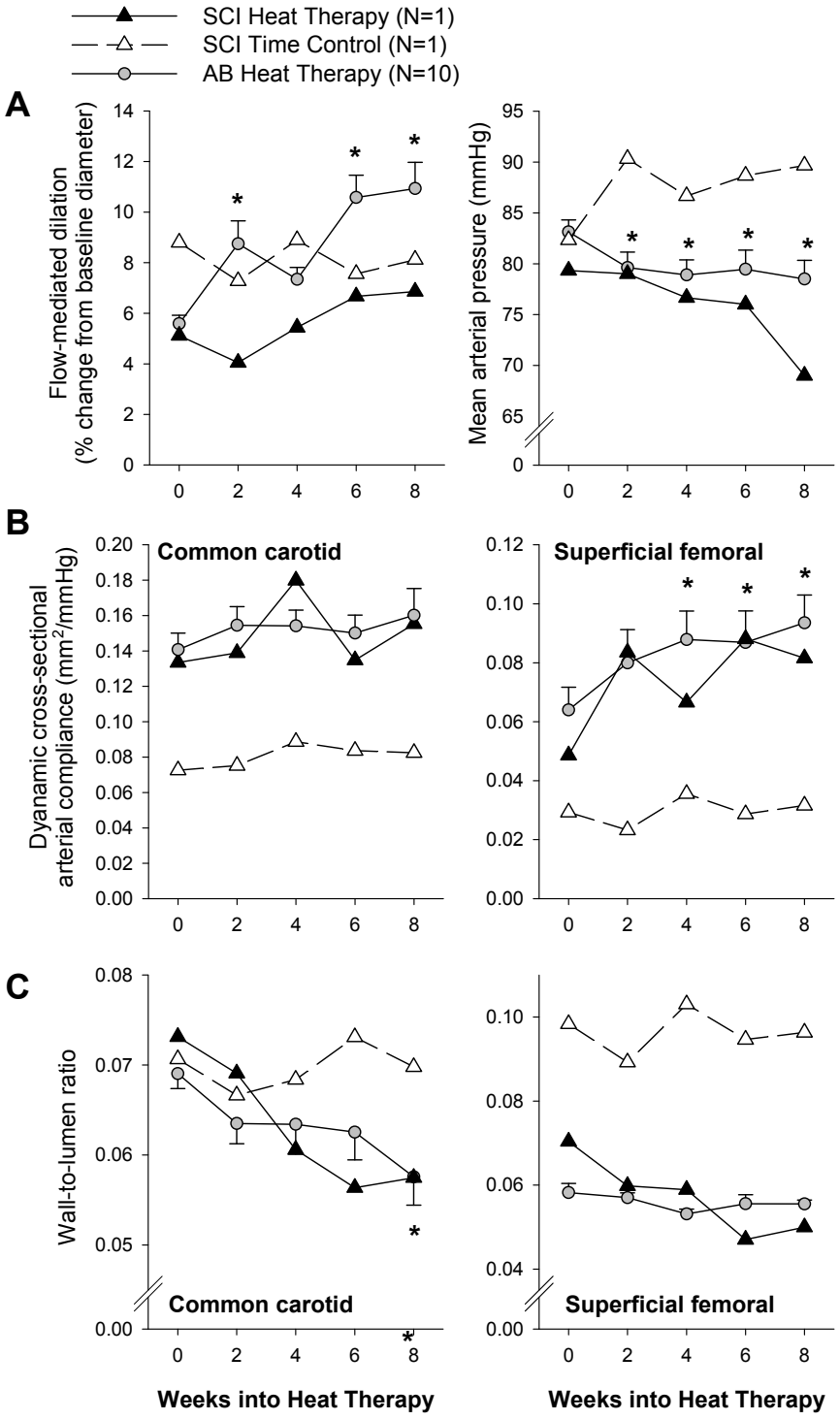

Figure 8.1. **A)** Brachial artery flow-mediated dilation, mean arterial blood pressure, **B)** carotid and superficial femoral dynamic arterial compliance, and **C)** carotid and femoral wall-to-lumen ratio in pilot spinal cord injury (SCI) subjects who underwent 8 weeks of heat therapy or time control. Data (mean±S.E.) from able-bodied (AB) subjects are included for comparison (also reported in Chapter V). *P<0.05 vs. 0wks within AB group.

Additionally, we observed a reduction in leg pulse wave velocity in the SCI heat therapy subject, measured from the femoral to dorsal pedal arteries, whereas we observed no changes in leg PWV in able-bodied subjects who underwent heat therapy (Figure 8.2) (this measure was not reported in Chapter V since it was included purely for comparison to SCI subjects).

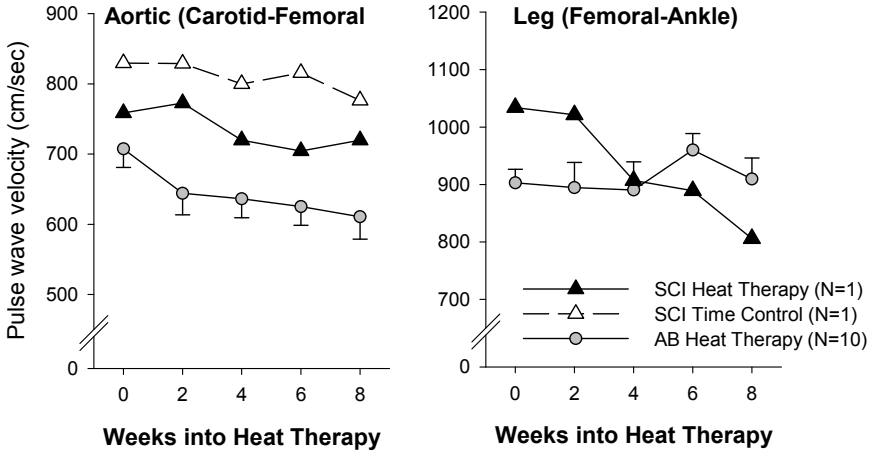

Figure 8.2. Aortic and leg pulse wave velocity in spinal cord injury (SCI) subjects who underwent 8 weeks of heat therapy or time control. Data (mean±S.E.) from able-bodied (AB) subjects are included for comparison. There was no effect of heat therapy on leg pulse wave velocity in able-bodied subjects, but we observed a robust improvement in the SCI pilot subject. *P<0.05 vs. 0wks within AB group.

Microvascular function

Interestingly, we observed no change in overall plateau cutaneous vascular conductance (CVC) during local skin heating in the subject who underwent heat therapy. However, we observed a substantial reduction in plateau CVC at the microdialysis site receiving L-NNA (NO synthase inhibition) and thus a large improvement in NO-dependent dilation (the difference between Control and L-NNA microdialysis sites) (Figure 8.3 – next page). We observed no changes in plateau in the SCI time control subject (change in plateau CVC across 8 weeks of time control at the Ringer's/Control site: +2.3%CVCmax, and at the L-NNA site: –1.4%CVCmax).

In both SCI pilot subjects, Tempol increased plateau CVC at the 0-week time point (Tempol-mediated dilation, Subject 1: 9.0%CVCmax, Subject 2: 11.7%CVCmax), indicating baseline oxidative stress. In the SCI subject who underwent heat therapy, Tempol-mediated dilation was effectively abolished (Figure 8.3C). However, we

observed a similar reduction in Tempol-mediated dilation in the SCI time control subject, and so we cannot conclude an exclusive effect of heat therapy on this variable.

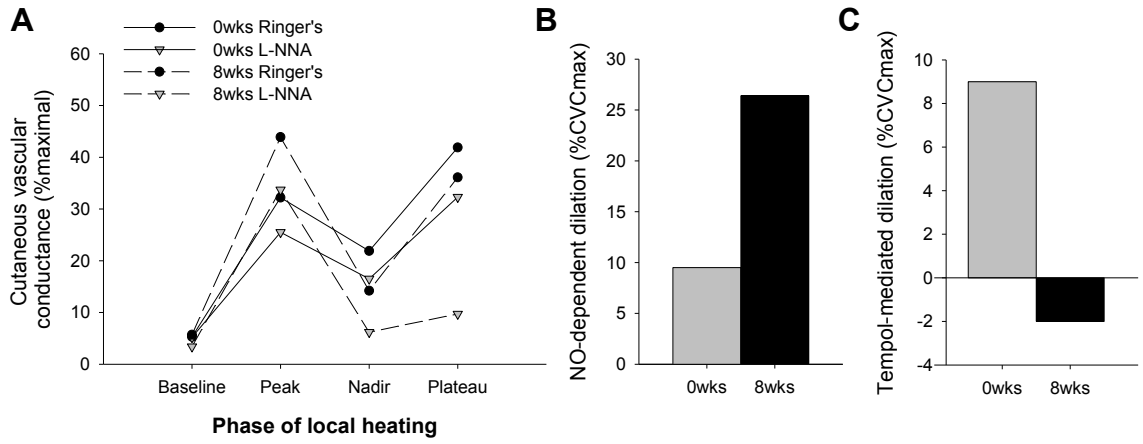

Figure 8.3. A) Cutaneous vascular conductance (CVC) at the four phases into local skin heating in our pilot spinal cord injury (SCI) subject who underwent heat therapy in microdialysis sites receiving Lactated Ringer's (control) and Nω-nitro-L-arginine (L-NNA). **B)** Nitric oxide (NO)-dependent dilation, the difference between microdialysis sites receiving Lactated Ringer's and L-NNA, and **C)** Tempol-mediated dilation, the difference between microdialysis sites receiving Lactated Ringer's and Tempol, in our SCI pilot subject who underwent heat therapy.

Serum angiogenic potential

Endothelial tubule formation was impaired in cells exposed to baseline 0-week sera from SCI subjects compared to cells exposed to 0-week sera from able-bodied subjects (average total tubule length per frame: 61.0 vs. 69.3±1.9 mm). Heat therapy resulted in a robust improvement in serum angiogenic potential, whereas no change was observed in the SCI time control subject (Figure 8.4 – next page).

DISCUSSION

In a young, otherwise healthy SCI pilot subject, we observed clinically relevant improvements in conduit vessel and microvascular endothelial function, reductions in arterial stiffness, intima media thickness, and blood pressure, and improvements in serum angiogenic potential, while minimal changes were observed in the SCI time control subject. The magnitude of many of the improvements we observed was on par with

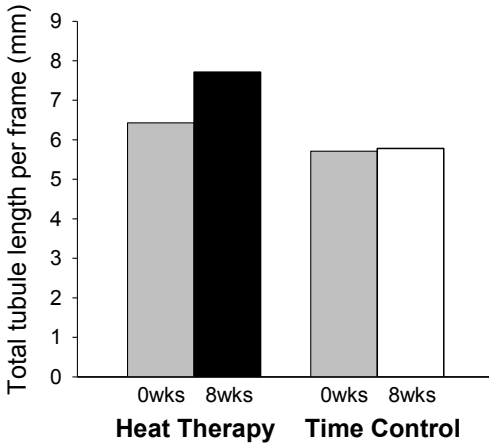

Figure 8.4. Total tubule formation in cultured endothelial cells exposed to sera from spinal cord injury subjects collected before and after heat therapy or time control interventions.

improvements observed in AB subjects; however, some were of considerably greater magnitude and warrant discussion.

First, the reduction in mean arterial blood pressure with heat therapy was approximately double in the SCI subject compared to the AB subjects. Hypertension is a major problem with chronic SCI, particularly for paraplegics. Despite a loss of sympathetic control below the level of the injury and a period of profound hypotension that accompanies acute SCI, prevalence of hypertension among individuals with chronic SCI [440] is on par with prevalence in the general population [1]. SCI results in rapid and extensive remodeling of the vasculature to smaller and stiffer arteries [428]. Additionally, circulating levels of angiotensin-II [398] and endothelin-1 [435] are upregulated soon after the initial injury, which is thought to occur to combat the loss of sympathetic vasoconstrictor tone. These adverse changes in the vasculature predispose SCIs to developing hypertension. Our data indicate that heat therapy may useful in combatting hypertension in the SCI population.

Secondly, heat therapy improved some biomarkers of vascular function in the lower limb to a greater extent in our SCI subject than we observed in AB subjects, including superficial femoral wall-to-lumen ratio and leg pulse wave velocity. Vascular impairments are often more extensive in the lower limbs compared to the rest of the body following SCI due to complete lower body inactivity (although systemic impairments are

also present). For example, remodeling of vessel diameters occurs to a greater extent in the lower body compared to upper body [428] and impairments in cutaneous microvascular function are worse in the lower body compared to the upper body [437]. As SCIs are prone to complications secondary to poor circulation in their legs, such as pressure ulcers [441,442] and deep vein thrombosis [443,444], interventions that can target improvement in the lower legs is particularly helpful for this population. Unfortunately, exercise training modalities that are available to SCIs have very little effect on the lower body, likely because these forms of exercise do not sufficiently increase shear rate in the legs of SCIs [2,445].

In contrast to AB subjects, both of the SCI subjects were physically active, engaging in aerobic and resistance exercise 2-5x per week. Despite them participating in regular exercise and being young and otherwise healthy, we observed baseline impairments in these subjects in many of the biomarkers of vascular function we measured compared to our young, able-bodied, sedentary subjects (e.g., arterial compliance, intima media thickness, pulse wave velocity, and serum angiogenic potential). This finding stresses the important of developing novel therapies to improve cardiovascular health in this population. Also importantly, both subjects maintained their exercise programs throughout the 8-week interventions. Heat therapy induced additional changes in vascular function in our SCI subject beyond what exercise training was capable of doing.

By collecting pilot data in SCI subjects, we have demonstrated the feasibility and effectiveness of using heat therapy to improve cardiovascular health in an at-risk clinical population. Our SCI subject tolerated heat therapy well, reporting no adverse effects besides slight light-headedness on the first day, which was also reported by several AB subjects. Heat therapy was also successful at driving profound vascular adaptations, providing strong evidence for its utility for treating cardiovascular risk in patient populations.

CHAPTER IX

CONCLUSIONS

MAIN FINDINGS

The overall purpose of this dissertation was to comprehensively assess the effects of passive heat therapy on vascular function and the associated molecular pathways. To address this goal, we performed one large study in which subjects participated in eight weeks of 4-5x per week heat therapy or thermoneutral water immersion, which served as a sham. Importantly, heat therapy resulted in hallmark adaptations of heat acclimation, including reduced resting core temperature, increased sweating responses to heat stress, plasma volume expansion, and elevated heat shock protein (HSP) expression under non-stressed conditions, as discussed in Chapter IV. Furthermore, no changes were observed in the subjects in the sham group. These observations allow us to conclude that the improvements in vascular function we observed were the result of adaptation to the heat itself (i.e., HSP- and shear stress-mediated).

In Chapter V, we investigated the effects of heat therapy on well-established biomarkers of conduit vessel (macrovascular) function. We observed that passive heat therapy resulted in robust improvements in flow-mediated dilation, arterial stiffness (including superficial femoral dynamic arterial compliance and β-stiffness index, and aortic pulse wave velocity), carotid intima media thickness, and diastolic and mean arterial blood pressure. The magnitude of all of these improvements was on par with improvements typically observed in exercise training studies of similar duration, in some cases greater (e.g., flow-mediated dilation). Furthermore, all of these improvements were great enough to be considered clinically relevant. Based on studies that have correlated these biomarkers with risk of cardiovascular disease and mortality, our subjects were considerably more ‘healthy’ at the end of the 8 weeks.

In Chapter VI, we investigated the effects of heat therapy on microvascular function. To do so, we used the cutaneous circulation as a model of globalized microvascular health and the cutaneous microdialysis technique to pharmacodissect some of the molecular pathways involved with microvascular adaptation to heat therapy. We

observed that heat therapy improved cutaneous thermal hyperemia, a well-established marker of endothelial-dependent microvascular function. Furthermore, we observed that improvements in thermal hyperemia were due to improved nitric oxide (NO)-dependent dilation, as determined by infusing the NO synthase inhibitor L-NNA via microdialysis.

In Chapter VII, we sought to further elucidate the molecular mechanisms behind improvements in vascular function, particularly those associated with the NO pathway. Of the functional improvements we measured in our human subjects, those that showed the most robust improvements were markers of NO-dependent dilation. To do so, we utilized a cell culture model of heat therapy in which we exposed cultured endothelial cells to either 24h of direct heating at the same temperature reached by subjects during heat therapy sessions or to sera collected from human subjects pre- and post-heat therapy in order to isolate the effects of upregulated circulating factors in the blood. We found that heating cells to 39°C increased abundance of Hsp70 and Hsp90 and the anti-oxidative enzyme superoxide dismutase (SOD), and reduced superoxide production, but had no effect on endothelial NO synthase (eNOS). Conversely, we found that exposing cells to sera from heat acclimated subjects increased both eNOS and SOD protein abundance and reduced superoxide production, independent of changes in intracellular HSPs. Additionally, sera from heat acclimated subjects increased endothelial tubule formation, a marker of angiogenesis, in an NO-dependent, but vascular endothelial growth factor (VEGF)-independent manner.

INTEGRATION OF STUDIES

The molecular studies performed in Chapter VII were intended to elucidate the mechanisms behind functional *in vivo* improvements in vascular function observed in Chapters V-VII. In Chapter VII, we isolated the effects of direct heat and circulating factors in the sera. However, *in vivo*, these stimuli act in tandem to improve endothelial function, along with the beneficial effects of intermittent increases in anterograde shear stress, which were reviewed in Chapter II. Based on our molecular data and the known effects of shear stress on endothelial cells, we have developed the working model summarized in Figure 9.1 (next page).

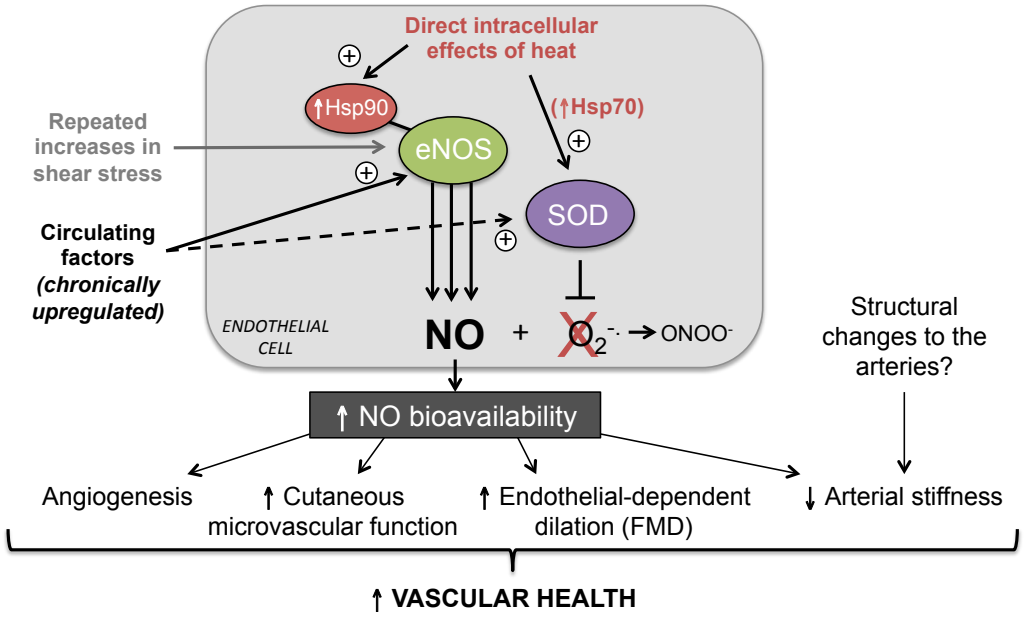

Figure 9.1. Working model of how heat therapy affects the nitric oxide (NO) pathway in endothelial cells, as determined in Chapter VII, resulting in the functional improvements in vascular health observed in human subjects in Chapters V-VII. See text for details. Abbreviations include: Hsp, heat shock protein; eNOS, endothelial NO synthase; SOD, superoxide dismutase; $O_2^{\cdot-}$, superoxide; $ONOO^-$, peroxynitrite; FMD, flow-mediated dilation.

Intermittent increases in body core temperature result in elevated HSP abundance. Hsp70 upregulates SOD [146,147], while Hsp90 stabilizes eNOS [4], playing an important role in all steps necessary for eNOS activation [133,135,136]. Elevated HSP abundance likely had many other cellular effects (e.g., suppression of pro-inflammatory pathways [157,160,165]); however, we chose to focus on the NO pathway for this investigation. Repeated intermittent increases in anterograde shear stress also increases eNOS activation [217,218]. Additionally, shear stress is known to upregulate HSPs [116,117], and thus their effects act in tandem to improve eNOS signaling. Heat therapy also upregulated circulating factors in the blood which increased both eNOS and SOD protein abundance. While determining which circulating factors associated with improved vascular function were upregulated by heat therapy was outside the scope of this investigation, we did determine that vascular endothelial growth factor (VEGF) was unchanged. VEGF concentration is increased in serum following exercise [446,447] and is thought to mediate exercise training-induced angiogenesis [448]. The fact that we observed no change in serum VEGF concentrations suggest that there are some

mechanistic differences between how heat therapy and exercise training improve vascular function. Specifically what these differences are should be investigated in future studies.

The combined effects of direct heat, shear stress, and circulating factors is an increased capacity for NO production (due to increased eNOS protein and presumably increased eNOS activity) and a reduction in superoxide production (due to increased SOD protein). Therefore, not only is NO production increased, but scavenging of NO by superoxide is also reduced, resulting in increased NO bioavailability. Additionally, the damaging effects of superoxide on eNOS activity (uncoupling of eNOS by depletion of tetrahydrobiopterin [143] and reduction in L-arginine by upregulation of arginase activity [144]) will be reduced, further improving eNOS activity and NO bioavailability.

Functionally, this improved NO bioavailability results in angiogenesis, and improved NO-dependent dilation, which we measured *in vivo* both in the cutaneous microvasculature and in the brachial artery using flow-mediated dilation. While we only demonstrated an effect of simulated heat therapy and improved NO bioavailability on serum angiogenic balance, we assume exposure to this pro-angiogenic serum *in vivo* would have also resulted in increased vascularization. Supportive of this, we found that reductions in mean arterial blood pressure were correlated with improvements in serum angiogenic balance, although reductions in blood pressure were likely the result of a combination of factors, including improved endothelial-dependent dilation and reduced vascular resistance [249,250]. Additionally, we did not definitely assess whether angiogenesis occurred *in vivo* in this investigation. Lastly, our observed reductions in arterial stiffness were likely predominantly caused by improved NO-dependent dilation in our disease-free population. However, we did observe some structural improvements in the conduit vessels (e.g., decreased intima media thickness), which may have contributed to the reduction arterial stiffness. The overall result of all of these changes in significantly improved vascular health and reduced risk of cardiovascular disease and mortality.

PASSIVE HEAT THERAPY AS A VIABLE ALTERNATIVE TO EXERCISE FOR PATIENTS WITH LIMTED EXERCISE CAPABILITES

The long-term goal of this line of research is to develop heat therapy as a feasible and effective means of improving cardiovascular health that could be used as an

alternative or adjunctive therapy to exercise training. Many disease states are characterized by impaired vascular function. While exercise is arguably the best 'medicine' for these patients, many of them are unable or unwilling to exercise to an extent great enough to induce protective adaptations (e.g., spinal cord injured, heart failure, and diabetic patients). In the present study, we have demonstrated that heat therapy is capable of inducing robust improvements in vascular function. To confirm that adaptations would also occur in a patient population, we conducted a pilot study in spinal cord injured patients (Chapter VIII), and demonstrated that heat therapy was capable of inducing even greater changes in this population. Our data, combined with the data demonstrating the effectiveness of Waon sauna therapy for treating heart failure and peripheral artery disease [247,252,253], suggest that heat therapy could be used feasibly and effectively for reducing cardiovascular risk across a broad range of patient populations. Additionally, as heat therapy can improve exercise tolerance [23,245], it could be used in some patient populations (e.g., heart failure and peripheral artery disease) to improve health in the meantime before these patients are able to engage in an exercise training program, or as an adjunctive therapy to gain even greater protective benefits.

FUTURE DIRECTIONS

This dissertation comprehensively demonstrated that vascular function is improved in sedentary humans following eight weeks of heat therapy and provided some insight into the cellular mechanisms underlying adaptations. However, the studies described herein are just the beginning. Given the relatively limited research that has been performed thus far, heat therapy is essentially an untapped field.

Just within the realm of vascular function, there is still much to answer. First, there are many more molecular aspects to vascular function than what we investigated in this dissertation. We chose to focus on the NO pathway and oxidative stress as they related more closely with the *in vivo* measures we observed, *and* because we were able to actually detect changes in these pathways in generally healthy subjects and in healthy cultured cells. We did not investigate inflammation, which is a major contributor to cardiovascular disease progression. The primary reason why was because, in pilot data,

we failed to observe any changes in inflammatory markers under non-stressed conditions in young, healthy humans/cells. There are two options to better investigate these pathways: 1) study a population which has elevated baseline inflammation (e.g., older adults, obese/metabolic disease, coronary artery disease patients, etc.) such that it is possible to improve their inflammatory profile with an intervention, or 2) study healthy humans/cells under stimulated pro-inflammatory conditions (e.g., with hypoxia/ischemia, TNFα, lipopolysaccharide). Both options address important, but different, questions related to cardiovascular health. The former option addresses whether a patient could be healthier on a day-to-day basis, hopefully slowing disease progression; whereas, the latter option addresses whether heat therapy confers protection against novel stressors (i.e., is the patient's heart or brain tissue better protected against tissue death *when* they have a heart attack or stroke?).

Even within the NO and oxidative stress pathways, studying a patient population with elevated oxidative stress would better elucidate the extent of cellular improvements that can be attained with heat therapy. For example, we infused the superoxide dismutase mimetic Tempol via microdialysis, but observed no effects in healthy subjects. We did observe an effect in pilot spinal cord injured patients, but studying patient populations with even greater oxidative stress (e.g., older adults, hypertensives) may yield different results. It will also be interesting to determine whether patients with greater vascular dysfunction can still attain the same benefits - when is it too late to reverse their disease progression? Based on the data in heart failure and end-stage peripheral artery disease patients [247,252,449], it seems that heat therapy could be effective at treating even very advanced pathological conditions, but future studies are required to determine if this is the case before heat therapy could be translated to clinical practice.

An interesting disease state to investigate would be atherosclerosis. Many of the biomarkers we measured are supposed to be predictive of the development of atherosclerosis (e.g., intima media thickness [369]). Furthermore, many of the molecular pathways affected by heat therapy are considered to be anti-atherogenic (e.g., shear stress and the anti-inflammatory effects of heat shock proteins). However, while we can conjecture based on our data that heat therapy should be protective against the development of atherosclerosis, we cannot conclude that it would improve symptoms and

risk of mortality in patients who already have clinically-diagnosed atherosclerosis without studying the disease state itself. Several rodent models have been developed to accurately simulate the pathological conditions of atherosclerosis [196,197,450]. Given the potential contraindications of heat therapy for patients with unstable coronary artery disease and angina pectoris [58], performing studies in rodents would be a good place to start, particularly for pairing mechanistic and functional measures.

Another important question to address is dosing – how much heat therapy do you need in order to attain (and maintain) the benefits? In this dissertation, we used a very high 'dose' of heat therapy in order to ensure we would at least see *some* effect. However, 90 min 4-5x per week is likely an unrealistic time commitment for the majority of patients. Can the same benefits be obtained if the duration, frequency, and/or core temperature reached is reduced?

Furthermore, how long do the benefits last if someone stops or reduces the frequency of sessions? We began to investigate the decay of benefits by performing follow-up studies in some subjects for up to 8 weeks after they finished the 8-week heat therapy intervention. In these very preliminary studies, we observed that the FMD response decayed at approximately the same rate at which it improved during heat therapy. Future studies should investigate this decay further. In the real world, patients will not continue to perform heat therapy consistently for the rest of their lives. If they stop for a period of time, how long will they still have benefits and can these benefits return more quickly if they start again? In animal studies, it appears heat acclimation is a 'remembered' phenotype, in that cellular adaptations can be gained more quickly the second time around [451].

Lastly, we only investigated the effects of heat therapy on vascular function. While vascular dysfunction is the primary cause of cardiovascular diseases, there are many other pathological processes that fall under the umbrella of cardiovascular-related diseases, and many other non-cardiovascular-related diseases that could be improved by heat acclimation. In animals, and in some limited studies in humans, there is evidence that heat acclimation could be used to protect against a variety of other pathologies, including myocardial infarction [186], ischemia-reperfusion injury [452], stroke and traumatic brain injury [179], diabetes mellitus [453,454], and metabolic disease [455].

Therefore, future studies should be aimed to undercover the multitude of benefits that could be gleaned from heat therapy.

With any luck, these questions (and many more!) will keep those of us in the heat therapy field busy for a very long time…

APPENDIX

INFORMED CONSENT DOCUMENTS

Group 1: Able-bodied Heat Therapy

TITLE: "Chronic heat exposure and cardiovascular health"

INVESTIGATORS: Vienna E Brunt, M.S., Dr. Christopher T Minson, and colleagues

APPROVED BY INSTITUTIONAL REVIEW BOARD: February 5, 2014

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?

Cardiovascular disease is the number one cause of death in the United States. Exercise is a potent means of improving cardiovascular health, but not all patient populations are able to exercise effectively. There is high demand for novel therapies to better manage cardiovascular risk in these patients. Heat exposure can have many beneficial effects on the cardiovascular system. As such, long-term heat exposure (i.e. sitting in a hot tub 4-5x per week) may provide an alternative means to exercise for improving cardiovascular health. This project will assess the benefits of long-term heat exposure on the health of the vasculature and on the cellular pathways that improve vascular health, which is important as the majority of cardiovascular diseases affect the arteries. We will measure various biomarkers of vascular health before and after 8 weeks of heat exposure in able-bodied individuals and patients with spinal cord injury. SCI patients are a population with elevated cardiovascular risk who have limited exercise capabilities, and who may be able to utilize chronic heat exposure as an alternative to exercise training for improving cardiovascular health. Additionally, prehypertensives represent another patient population with elevated cardiovascular risk whose cardiovascular health may be affected by chronic heat exposure.

We will address the following questions in this study:

- How does 8 weeks of passive heat exposure affect cardiovascular health, as measured by various biomarkers of cardiovascular function?
- How does 8 weeks of passive heat exposure affect levels of factors circulating in the blood and located within muscle tissue that are important for cardiovascular health?
- Do effects differ between healthy normotensive able-bodied individuals and spinal cord injury patients?

What will happen in the study?

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).
2. Additionally during this initial session, you will fill out a health history form and may meet with a physician, so that we can ensure you are healthy enough to participate in the study. In addition, the physician will be available to you to answer any medical questions or concerns you may have throughout the duration of your participation in the study. This visit should last about 60 minutes.
3. We will assign you to a subject group. If you are reading this form, you are in the **able-bodied heating group**.
4. Throughout the study, you will report to the laboratory 4-5 times per week for 8 weeks for hot tub sessions. Additionally, you will participate in 7 experimental days over the course of 8-11 weeks. Experimental Days 1-2 will occur prior to the 8 weeks hot tub sessions, and Days 6-7 will occur immediately following the 8 weeks of hot tub sessions. Days 3, 4, and 5 will occur at Weeks 2, 4, and 6 into hot tub sessions. We will perform the same procedures in Experimental Days 1 and 7, and in Days 3-6. All procedures are explained in detail below.
5. During the screening session, we will schedule your initial experimental days (2 sessions), the start of your hot tub sessions, and tentatively schedule all other sessions. We will give you a hard copy of your schedule to take home with you.

Heating (hot tub) Sessions:

1. You will report to the laboratory 4-5 times per week for 8 weeks, for a total of 36 sessions. Each session will take approximately 2-2.5 hours. You will be asked to bring a swimsuit to wear during the session. If you do not have one, we will provide one for you to use for the duration of the 8 weeks. No other subjects will use the same swimsuit.
2. You will be asked to provide a urine sample so that we can ensure you are properly hydrated before undergoing heat stress. If you are dehydrated, we will give you 5mL/kg body weight (about half a normal sized 20oz bottle of Gatorade®) of fluids to drink prior to getting in the hot tub.
3. Your nude body weight will be measured by a member of the same sex prior to getting in the hot tub. You will stand behind a privacy screen while this measurement is taken. Your nude body weight will also be measured following heat stress. This will be done so that we can quantify the volume of sweat you lose while in the tub.
4. You will be instrumented with a Polar® heart rate monitor chest strap so that we can continuously monitor your heart rate throughout the procedure.
5. We will give you a rectal probe labeled with your subject number. It is made of a thin rubber (flexible) material that is inserted 10 cm (approximately 4 inches) past the anal sphincter. The probe will remain in place throughout the entire study session (up to 2.5 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 anytime. You will be instructed how to self-insert the rectal probe, as well as how to remove it and clean it. If you needed 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.
6. *On the very first and very last heating session (and possibly during one session at about 4wks),* we will place 1 small flexible needle (these are called "intravenous catheters", and are smaller than the lead of a pencil) into a vein near your elbow. The skin will be cleaned before this procedure. This catheter will remain in your vein throughout the heating session. We will take about 40ml of blood, about 2.7

tablespoons, prior to getting into the bathtub, and another 25ml of blood at the end of the heating period so that we can measure various factors in your blood that are affected by the heat exposure. We will remove the catheter after the second blood draw and place a sterile bandage over the site. The vials in which we collect the blood will be coded such that the investigators can determine all samples came from the same subject and the time the sample was taken. No one will be able to determine your identity from the sample.

7. You will then be transferred to a bathtub that will be filled with water at 40°C (104.0°F). The tub is hooked up to a water pump and heater such that water can be circulated and maintained at a desired temperature. You will lay down in the bath tub such that the water level comes up to the level of your collar bone (to the top of your shoulders). You will lay down in this position until your core temperature has reached 38.5°C (101.3°F). This takes approximately 25-35 minutes. At this point, you will sit up such that the water level reaches the middle of your chest (with your shoulders and arms out of the water). You will remain sitting up for another 60 minutes, or until the total time in the hot tub reaches 90 minutes, whichever comes first.
8. We will continuously check in with you throughout the hot tub session. You will be instructed to inform an investigator if you feel any of the following symptoms: light-headedness, dizziness, nausea, headache, or if you feel unbearably warm. If you feel these symptoms, we will have you sit up if you were previously laying down in the bath, or we will have you get out of the bath if you were previously sitting up. If your body temperature gets too high (above 39.5°C, 103°F), we will have you get out of the bath, even if you feel fine.
9. You will be allowed to drink fluids while in the hot tub.
10. At the end of the heating period, you will get out of the hot tub and sit in a recovery chair. We will continue to monitor your body temperature until your core temperature begins to return back to normal. If your body temperature remains high for too long, or if you feel too hot, dizzy, or nauseous, we will cool you down more quickly with cold packs. We will provide you with a towel to dry off during this time.
11. Once you feel fine, you will remove the rectal probe, clean it, and place it in a location allocated to you.

12. If you lost >1% of your body weight while in the hot tub due to sweating, we will ask you to drink fluids prior to leaving the lab in an amount necessary to return you to no more than a 1% loss of body weight.
13. We will also offer you a light snack before leaving the lab.

Experimental Days 1 and 7 (Skin Studies):

1. You will arrive at Dr. Minson's laboratory in Esslinger Hall at the University of Oregon to participate in the experimental protocol. The testing will take approximately 4-5 hours. The day before the study, we will contact you (either by phone or email depending on your preference) to remind you to refrain from all over-the-counter medications, including vitamins and supplements, for 24 hours, alcohol and caffeine for 12 hours, food for 4 hours, and heavy exercise for 24 hours. Additionally, you will be asked to wear a short-sleeved shirt.
2. Upon arrival at the laboratory, your height and weight and resting blood pressure will be measured. Female subjects will 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.
6. We will place 3 small tubes (these are called "microdialysis fibers", and are about the size of sewing thread) in the skin of your forearm. 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 placed inside 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.
7. 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) to go away. During this time, a small probe (laser-Doppler probe) 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.
8. During the study, we may periodically inflate a small cuff that is placed on your middle finger of one of your hands to measure your blood pressure (Portapres device). We will only inflate this cuff 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.

9. Blood pressure will also be measured periodically throughout the study using an inflation cuff on your upper arm.
10. During the protocol we will put some very small doses of drugs through the small tubes in your skin. These drugs will cause the vessels of your skin to either widen 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. You will receive the following drugs:
    a. L-NNA: this stops nitric oxide from being produced and causes the skin vessels to narrow
    b. Tempol: This is a substance that may cause your blood vessels to open.
    c. Sodium nitroprusside: this is a substance that is used to lower blood pressure in patients and causes the skin vessels to open
11. We will heat a small area of your skin with a small heater up to 43.5° Celsius (110 degrees Fahrenheit) to open the vessels in your skin. This is below the temperature where heating becomes painful (about 113 degrees Fahrenheit) and well below the temperature that may burn your skin (about 117 degrees Fahrenheit). If you think the heater is becoming painful, you need to tell the investigator and the temperature will be lowered.
12. 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.
13. Although you will not be allowed food or beverages during the study, you will be given a light snack and fluids to drink before you leave.

Experimental Days 2-6 (Vascular Function):

1. You will arrive at Dr. Minson's laboratory in Esslinger Hall at the University of Oregon to participate in the experimental protocol. This testing will take approximately 3-4 hours. The day before the study, we will contact you (either by phone or email depending on your preference) to remind you to refrain from all over-the-counter medications, including vitamins and supplements, for 24 hours, alcohol

and caffeine for 12 hours, food for 4 hours, and heavy exercise for 24 hours. Additionally, you will be asked to wear a short-sleeved shirt and shorts that can be easily pushed up.

2. Upon arrival at the laboratory, your height and weight and resting blood pressure will be measured. Female subjects will 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.
3. We will prep you for the study by placing 5 sticky electrodes on your skin and attaching a small wire or lead to each electrode. These leads will be attached to a monitor that will allow us to measure your heart rate and heart rhythm. These electrodes will be placed on your skin by a member of the same sex. The electrodes will be placed on your body in the following locations: 2 electrodes are placed on your upper chest close to your shoulder (one on the left and one on the right); 2 electrodes will be placed on your stomach just above your hip bones (just above where your pants line is) on the left and right side; and one will be placed on your lower ribcage on the left side.
4. After these electrodes are in place, you will lay down on a padded exam table for the remainder of the study.
5. During the study, blood pressure will be measured periodically throughout the study using an inflation cuff on your upper arm.
6. We may also periodically inflate a small cuff that is placed on your middle finger of one of your hands to measure your blood pressure. We will only inflate this cuff 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.
7. We will use an ultrasound probe to image the large arteries in your neck (carotid) and thigh (femoral). We will be measuring the diameter of the arteries, the thickness of the artery walls, and the resting blood flow.
8. You will undergo carotid arterial tonometry with ultrasound. An ultrasound probe will be placed on one carotid artery while a non-invasive tonometer device (looks like a blunt pencil) is placed on the opposite carotid artery which non-invasively measures blood pressure using pressure sensors pressed against the skin over the artery. The

ultrasound probe measures carotid artery diameter and blood velocity, while the tonometer measures the blood pressure wave traveling down the vessel. The two values are compared to give an index of the stiffness of the arteries. This is a non-invasive procedure and should not be uncomfortable, other than some minor pressure of the measurement sites.

9. Your pulse wave will be measured using tonometry. Two tonometers that measure blood pressure waves will be placed on the skin over arteries on your neck and thigh (carotid and femoral) and your arm and foot (brachial and posterior tibial or dorsal pedal). The time between the onset of the pulse waves from the two locations will be measured and compared. This is a non-invasive procedure and should not be uncomfortable, other than some minor pressure at the measurement sites.
10. You will have a blood pressure cuff placed around your forearm, just below your elbow. We will position an ultrasound transducer probe on your upper arm (above your elbow) at the brachial artery. We will occlude blood flow to your arm by inflating the blood pressure cuff on your lower arm to 250 mmHg for 5 min. There is possible risk of discomfort due to the occlusion of the forearm. This discomfort should be mild, and only comparable to the lower portion of the arm falling asleep. This discomfort should subside immediately following deflation of the cuff. However, if this discomfort exceeds a tolerable level, please alert the investigator so that the cuff can be deflated early. We will use the ultrasound transducer probe to image your brachial artery before and after the blood pressure cuff is inflated and released. This test may be repeated 1-2 times. If it is repeated, we will allow a rest period of at least 20 min in between each blood flow occlusion.
11. Next, we will administer one spray of nitroglycerin below your tongue and continue to image your brachial artery for 10 more minutes. This will cause your blood vessels to relax, and will slightly lower your blood pressure. This test causes the smooth muscle of your brachial artery blood vessel to relax and dilate allowing an increase in blood flow, and we call this test ‘endothelium-independent vasodilation’. You will continue to lie supine for at least 15 minutes after nitroglycerin administration to ensure your blood pressure has returned to normal.

12. At the end of the study, we will estimate your total blood volume using a carbon monoxide (CO) uptake test. For this test, you will breathe 100% oxygen for 4 minutes to remove nitrogen from your lungs (this naturally exists). Then, you will start to breathe on a rebreathing system. This essentially means you will inhale and exhale into a bag. The air in this rebreathing system is pre-filled with 100% oxygen. After several minutes you will expire all of the air out that you possibly can through your nose. When you cannot get any more air out, your nose will be closed with noseclips. A dose of CO (1.0 mL CO/kg of body mass) will then be injected into the breathing system and you will inhale deeply to make sure you inhale as much CO as possible. CO is a gas that quickly moves from the air in your lungs to your blood. To further help this you will hold your breath for ~10 seconds after you deeply inhale. You will then breathe 100% oxygen normally for another several minutes. Small blood samples (~5ml) will be taken before you start breathing on the rebreathing system and immediately after you stop breathing on the rebreathing system. These blood samples will be taken from the catheter in your arm.
13. Although you will not be allowed food or beverages during the study, you will be given a light snack and fluids to drink before you leave.

Optional Follow-Up Testing:

If you are interested and if you continue to qualify, you may be asked by the investigators to return to the laboratory after you have completed the 8 weeks of hot water immersion. This is so that we can assess how long any changes in your cardiovascular health last. Follow-up testing would take place 2, 4, 6, and/ 8 weeks after your last hot tub session. If you are interested in participating in follow-up testing, you will be asked to give consent and will sign an additional consent form closer to the end of your 8 weeks of hot water immersion. Importantly, if you are not interested in participating in follow-up testing, it does not affect your participation in the primary study, the 8 weeks of hot water immersion. Also please note that all time frames and compensation amounts in this section of the consent form only include the initial 8 weeks of hot water immersion.

If you are interested in being asked later on about participating in follow-up testing, **please initial here: _____**. You can still change your mind at any time.

How long will I be in the study?

You will be in the study for 8-11 weeks. You will participate in one screening session (about 60 min), seven experimental days (a total of up to 25 hours), and 36 heating sessions (up to 2 hours per session).

What are the risks of the study?

1. Heat exposure: There are some risks associated with heat exposure, including: fatigue, light-headedness, muscle cramps, dehydration, and neurological detriments (i.e. heat stroke). However, these symptoms do not typically occur until core temperature rises above 40°C. Your core temperature will be constantly recorded (rectal probe), and you will be removed from the hot bath immediately if either core temperature reaches 39.5°C or you experience any symptoms of heat-related illness. You will be instructed to notify the investigators immediately if you experience any of these symptoms. All symptoms subside upon lowering core temperature. Ice packs will be on hand for rapid cooling if necessary. Additionally, heat exposure may have detrimental effects on a developing fetus in females and` on sperm counts in males. Thus, subjects who are pregnant, trying to conceive, and/or undergoing treatment to increase sperm counts will be excluded from the study.
2. Rectal temperature probes: The use of rectal probes to measure core body temperature, even during exercise, 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 probe that is designed for this purpose. There is also the risk of infection, either by you not washing your hands properly or exposure to a poorly cleaned probe. The probe has been sterilized before use, and we will instruct you on how to properly clean the probe after each time you use it. 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 how they treat you.
3. Venous blood draws and catheters: There may be some discomfort during the blood draw. Once the catheter is in place, or once the needle is removed, the pain should subside. After the blood draw, the needle will be withdrawn and a sterile dressing will

be applied. Any swelling or redness after the study should subside by a few hours after completion of the study. Although the needles are sterile, there is a slight risk of infection at the site where the needle was placed in your skin. You will be instructed how to keep the area clean for a day or two following the experimental day. The most common complications of inserting a small needle into a vein is a small bruise and pain at the site of the needle location which may last several days after removal of the needle. A small amount of bleeding may occur directly after removal of the catheter. Application of pressure and a gauze dressing will alleviate the bleeding. The maximum amount of blood we will draw across the entire study is 270ml, or about 18 tablespoons, which is well below the volume drawn in a standard blood donation (~450ml). Even so, we will exclude you from the study if you have donated blood within the last two months.

4. 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 or 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.

5. Microdialysis drugs: We will be infusing very small doses of each drug and only into a very small area of your skin. You will not have any systemic (whole body) effects of these drugs, and they will not alter your blood pressure in the small doses given in this study. 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. In the case of an adverse event, the study will be discontinued. Investigators are trained in Advanced Cardiac Life Support and anaphylaxis.
6. 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. If the local heating becomes painful, you should tell the investigator and the temperature of the local heater will be lowered. There is a slight risk of burning the skin at this site, so it is important that you tell the investigators of any pain you are feeling. The heating device may be removed at any time if you experience any discomfort.
7. Laser-Doppler Probes: These probes send a small light into your skin. You will not feel anything except the probe touching your skin. There are no major risks associated with this procedure.
8. 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, you experience any discomfort you may request that the blood pressure cuff be either loosened or removed.

9. Finger Blood Pressure: In some people, this blood pressure cuff becomes 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, let the investigator know and they will turn it off for a few minutes. There are no major risks associated with this device.
10. Nitroglycerin administration: We will administer the standard dose of nitroglycerin under your tongue. This drug has minimal risk when used in low doses. Nitroglycerin is commonly prescribed to prevent and treat angina pectoris (chest pain), a condition occurring from constriction in the arteries of the heart. Nitroglycerin is a potent blood vessel dilator, causing the artery to open up and increase blood flow. You may feel slight light-headedness or dizziness, but this symptom should not last more than a few minutes. Please inform your investigator if these feelings persist during the study and do not simply come and go. You may feel your heart rate slowing as this drug causes a drop in blood pressure (transient hypotension), but this will also last only a few minutes. After nitroglycerin administration, we will take ultrasound images for several minutes to monitor changes in your blood flow and vessel size. You will remain laying down on your back for an additional 15 minutes to ensure that your blood pressure and heart rate are back to their normal values before we allow you to leave the study area. There is a risk that you may experience a headache after this treatment, and if you are predisposed, you may experience the onset of a migraine headache.
11. Blood Volume Measurement: This measurement involves exposure to a small amount of carbon monoxide. Carbon monoxide is a colorless, odorless gas. We typically express CO in the blood as bound to a molecule called hemoglobin (Hb; HbCO). Healthy nonsmoking city dwellers typically have 1.5-2.0% of their Hb as HbCO. This test will increase this level by roughly 6%. Although you do not want to increase your level of HbCO, the levels used for this test are low and present minimal risks. The side effects associated with excessive HbCO typically occur at levels above 8% and may include headache, fatigue, shortness of breath, nausea, cherry-red colored lips, dizziness, and death, the last of which rarely occurs below 15% HbCO in normal

individuals. This method has been in use in research for measuring blood volume for over 100 years without notable complications. Over time the CO bound to Hb in your blood will be removed. This process will occur naturally over the several hours following the test. For every 5 to 6 hours that passes after the test, half of the CO will leave your blood. Thus, after 15-18 hours you would have less than 1% CO in your blood.

In order to assure that subjects remain below the level of %HbCO that is associated with side effects, we will test %HbCO in each individual prior to administration of CO. If an individual's baseline %HbCO is greater than 2.0% we will not administer CO to that individual. Furthermore, if for any reason an individual's post-administration %HbCO rises above 10% or an individual develops side-effects associated with excess %HbCO, we will initiate O_2 therapy to accelerate CO clearance from their system. O_2 will be given using a nasal administration similar to what someone may wear while in the hospital. Administering O_2 reduces the half-life of CO (speeds up the process of getting rid of it) from ~5 hours to ~80 minutes. We will measure %HbCO throughout the O_2 administration and periodically afterwards. We will stop treatment if %HbCO is < 8% or the symptoms go away. If symptoms progress or do not improve, the Department of Public Safety (6-6666) or 911 directly will be called depending on the perceived or real severity of the symptoms. In the unlikely case of an adverse cardiovascular event, the laboratory is equipped with an Automatic Electronic Defibrillator (located in a clearly marked first-aid cabinet), and Vienna Brunt, Brett Ely, and Dr. Minson are trained in Advanced Cardiac Life Support (ACLS).

12. Emergencies: In the event of an emergency, you will be transported by ambulance to a local emergency facility.

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

No. 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 each

experimental day. If the pregnancy test is positive (meaning that you are pregnant), you will no longer be able to take part in the study.

Are there benefits to taking part in this study?

This study will likely not make your health better. This study is being conducted to learn about the effects of chronic heat exposure on cardiovascular health. It is possible the information gathered in this study could be used to better treat patients with elevated cardiovascular risk in the future.

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 receive compensation for completed each session in the study as follows: initial screening session, $10; Experimental Days 1-5, $30 each; Experimental Day 6, $50; Experimental Days 7, $40 each; and $10 per heating session completed. If you complete all parts of the study, you will receive **$630**. This money is for the inconvenience and time you spent in this study, and works out to be approximately $10 per hour of participation in experimental days and $10 per heating session. 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 based on which study sessions you completed. You will receive compensation in the form of a check at the end of the study, or if you prefer, approximately every 2 weeks into the study. If you choose to receive compensation every 2 weeks, you will receive an amount corresponding to which study sessions you completed in each two-week time interval. There will be no difference in the total compensation you will receive if you chose to receive compensation at the end of the study versus every 2 weeks.

Please note, compensation from participation in Human Subjects Research studies may be considered taxable income. Compensation amounts are tracked across all studies in which you participate. If compensation totals $600 or more in a calendar year, the University is required to report the income to the IRS. University departments are required to track participant compensation and may contact you to complete a W9 form

for tax reporting purposes. Because of this, your name will be associated with participation in a research study. 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 his student, Vienna Brunt, M.S. at any time about any question you have on this study. You may contact Dr. Minson by calling (541) 346-4105, (541) 346-4311 or on his cell phone (541) 953-2231, and Vienna Brunt at (541) 346-4507 or on her cell phone at (541) 968-2635.

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
Office of the President
University of Oregon
Eugene, OR 97403
(541) 346-3082

Research Compliance Services
University of Oregon
Eugene, OR 97403
(541) 346-2510

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 stored on a secure computer with double password protection in Dr. Minson's office and only one list with names and identification numbers will exist. This list will be destroyed after all data has been collected and analyzed and for a period of one year after the results from the study have been published. Only coded specimens and data will exist thereafter.

Your blood will be analyzed for such things as heat shock proteins, hormone concentrations, and cytokines (factors released by cells that affect other cells). Your muscle tissue will be analyzed for changes in DNA (**not genetic testing**), RNA, cell signaling, and changes in cell structure. The researchers may store the information gathered during this study indefinitely.

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)

Group 2: Able-bodied thermoneutral water immersion

TITLE: "Chronic heat exposure and cardiovascular health"

INVESTIGATORS: Vienna E Brunt, M.S., Dr. Christopher T Minson, and colleagues

APPROVED BY INSTITUTIONAL REVIEW BOARD: February 5, 2014

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?

Cardiovascular disease is the number one cause of death in the United States. Exercise is a potent means of improving cardiovascular health, but not all patient populations are able to exercise effectively. There is high demand for novel therapies to better manage cardiovascular risk in these patients. Heat exposure can have many beneficial effects on the cardiovascular system. As such, long-term heat exposure (i.e. sitting in a hot tub 4-5x per week) may provide an alternative means to exercise for improving cardiovascular health. This project will assess the benefits of long-term heat exposure on the health of the vasculature and on the cellular pathways that improve vascular health, which is important as the majority of cardiovascular diseases affect the arteries. We will measure various biomarkers of vascular health before and after 8 weeks of heat exposure in able-bodied individuals and patients with spinal cord injury. SCI patients are a population with elevated cardiovascular risk who have limited exercise capabilities, and who may benefit greatly from chronic heat exposure.

We will address the following questions in this study:

- How does 8 weeks of passive heat exposure affect cardiovascular health, as measured by various biomarkers of cardiovascular function?
- How does 8 weeks of passive heat exposure affect levels of factors circulating in the blood and located within muscle tissue that are important for cardiovascular health?
- Do effects differ between healthy able-bodied individuals and spinal cord injury patients?

What will happen in the study?

1. If you meet all the initial subject criteria (based on initial phone and/or email conversations) and 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. Additionally, you will fill out a health history form so that we can ensure you are healthy enough to participate in the study. This visit should last about 60 minutes.
2. We will assign you to a subject group. To do this, we will match you to a spinal cord injury patient who is currently enrolled in the study based on your age, sex, and approximate fitness level. You will be assigned to the same group (heating or thermoneutral/control) as that subject. You will be informed of what group you are in prior to reading this form. If you are reading this form, you are in the **able-bodied thermoneutral group.**
3. Throughout the study, you will report to the laboratory 4-5 times per week for thermoneutral water immersion sessions. You will participate in 7 experimental days over the course of 8-11 weeks. Experimental Days 1-2 will occur prior to the 8 weeks thermoneutral water immersion, and Days 6-7 will occur immediately following the 8 weeks of thermoneutral water immersion. Days 3, 4, and 5 will occur at Weeks 2, 4, and 6 into thermoneutral water immersion. We will perform the same procedures in Experimental Days 1 and 7, and in Days 2-6. All procedures are explained in detail below.
4. During the screening session, we will schedule your initial experimental days (3 sessions), the start of your thermoneutral water immersion sessions, and tentatively schedule all other sessions. We will give you a hard copy of your schedule to take home with you.

<u>Thermoneutral Water Immersion Sessions:</u>

1. You will report to the laboratory 4-5 times per week for 8 weeks, for a total of 36 sessions. Each session will take approximately 2-2.5 hours. You will be asked to bring a swimsuit to wear during the session. If you do not have one, we will provide

one for you to use for the duration of the 8 weeks. No other subjects will use the same swimsuit.

2. You may be asked to provide a urine sample so that we can ensure you are properly hydrated. If you are dehydrated, we will give you 5mL/kg body weight (about half a normal sized 20oz bottle of Gatorade®) of fluids to drink prior to getting in the tub. You will be able to drink fluids throughout the water immersion period.
3. You will be instrumented with a Polar® heart rate monitor chest strap so that we can continuously monitor your heart rate throughout the procedure.
4. We will give you a rectal probe labeled with your subject number. It is made of a thin rubber (flexible) material that is inserted 10 cm (approximately 4 inches) past the anal sphincter. The probe will remain in place throughout the entire study session (up to 2.5 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 anytime. You will be instructed how to self-insert the rectal probe, as well as how to remove it and clean it. If you needed 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.
5. *On the very first and very last heating session (and possibly during one sesson at about 4wks),* we will place 1 small flexible needle (these are called "intravenous catheters", and are smaller than the lead of a pencil) into a vein near your elbow. The skin will be cleaned before this procedure. This catheter will remain in your vein throughout the water immersion session. We will take about 40ml of blood, about 2.7 tablespoons, prior to getting into the bathtub, and another 25ml of blood at the end of the heating period so that we can measure various factors in your blood that may be affected by heat exposure. We will remove the catheter after the second blood draw and place a sterile bandage over the site. The vials in which we collect the blood will be coded such that the investigators can determine all samples came from the same subject and the time the sample was taken. No one will be able to determine your identity from the sample.

6. You will then be transferred to a hot tub. The tub will be filled with water at 36°C (96.8°F). The tub is hooked up to a water pump and heater such that water can be circulated and maintained at a desired temperature. You will lay down in the bath tub such that the water level comes up to the level of your collar bone (to the top of your shoulders). You will lay down in this position for 30 minutes. At this point, you will sit up such that the water level reaches the middle of your chest (with your shoulders and arms out of the water). You will remain sitting up for another 60 minutes.
7. At the end of the water immersion period, you will get out of the tub and sit in a recovery chair. We will give you cool fluids to drink and a towel to dry off, and will offer you a light snack.
8. We will instruct you on how to remove the rectal probe, clean it, and place it in a location allocated to you. You will then be free to leave the lab.

Experimental Days 1 and 7 (Skin Studies):

1. You will arrive at Dr. Minson's laboratory in Esslinger Hall at the University of Oregon to participate in the experimental protocol. The testing will take approximately 4-5 hours. The day before the study, we will contact you (either by phone or email depending on your preference) to remind you to refrain from all over-the-counter medications, including vitamins and supplements, for 24 hours, alcohol and caffeine for 12 hours, food for 4 hours, and heavy exercise for 24 hours. Additionally, you will be asked to wear a short-sleeved shirt.
2. Upon arrival at the laboratory, your height and weight and resting blood pressure will be measured. Female subjects will 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.
3. We will place 3 small tubes (these are called "microdialysis fibers", and are about the size of sewing thread) in the skin of your forearm. 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 placed inside 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.

4. 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) to go away. During this time, a small probe (laser-Doppler probe) 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.
5. During the study, we may periodically inflate a small cuff that is placed on your middle finger of one of your hands to measure your blood pressure (Portapres device). We will only inflate this cuff 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.
6. Blood pressure will also be measured periodically throughout the study using an inflation cuff on your upper arm.
7. During the protocol we will put some very small doses of drugs through the small tubes in your skin. These drugs will cause the vessels of your skin to either widen 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. You will receive the following drugs:
    a. L-NNA: this stops nitric oxide from being produced and causes the skin vessels to narrow
    b. Tempol: This is a substance that may cause your blood vessels to open.
    c. Sodium nitroprusside: this is a substance that is used to lower blood pressure in patients and causes the skin vessels to open
8. We will heat a small area of your skin with a small heater up to 43.5° Celsius (110 degrees Fahrenheit) to open the vessels in your skin. This is below the temperature where heating becomes painful (about 113 degrees Fahrenheit) and well below the temperature that may burn your skin (about 117 degrees Fahrenheit). If you think the heater is becoming painful, you need to tell the investigator and the temperature will be lowered.
9. 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.

Although you will not be allowed food or beverages during the study, you will be given a light snack and fluids to drink before you leave.

Experimental Days 2-6 (Vascular Function):

1. You will arrive at Dr. Minson's laboratory in Esslinger Hall at the University of Oregon to participate in the experimental protocol. This testing will take approximately 3-4 hours. The day before the study, we will contact you (either by phone or email depending on your preference) to remind you to refrain from all over-the-counter medications, including vitamins and supplements, for 24 hours, alcohol and caffeine for 12 hours, food for 4 hours, and heavy exercise for 24 hours. Additionally, you will be asked to wear a short-sleeved shirt and shorts that can be easily pushed up.
2. Upon arrival at the laboratory, your height and weight and resting blood pressure will be measured. Female subjects will 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.
3. We will prep you for the study by placing 5 sticky electrodes on your skin and attaching a small wire or lead to each electrode. These leads will be attached to a monitor that will allow us to measure your heart rate and heart rhythm. These electrodes will be placed on your skin by a member of the same sex. The electrodes will be placed on your body in the following locations: 2 electrodes are placed on your upper chest close to your shoulder (one on the left and one on the right); 2 electrodes will be placed on your stomach just above your hip bones (just above where your pants line is) on the left and right side; and one will be placed on your lower ribcage on the left side.
4. After these electrodes are in place, you will lay down on a padded exam table for the remainder of the study.
5. During the study, blood pressure will be measured periodically throughout the study using an inflation cuff on your upper arm.
6. We may also periodically inflate a small cuff that is placed on your middle finger of one of your hands to measure your blood pressure. We will only inflate this cuff 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.

7. We will use an ultrasound probe to image the large arteries in your neck (carotid) and thigh (femoral). We will be measuring the diameter of the arteries, the thickness of the artery walls, and the resting blood flow.
8. You will undergo carotid arterial tonometry with ultrasound. An ultrasound probe will be placed on one carotid artery while a non-invasive tonometer device (looks like a blunt pencil) is placed on the opposite carotid artery which non-invasively measures blood pressure using pressure sensors pressed against the skin over the artery. The ultrasound probe measures carotid artery diameter and blood velocity, while the tonometer measures the blood pressure wave traveling down the vessel. The two values are compared to give an index of the stiffness of the arteries. This is a non-invasive procedure and should not be uncomfortable, other than some minor pressure of the measurement sites.
9. Your pulse wave will be measured using tonometry. Two tonometers that measure blood pressure waves will be placed on the skin over arteries on your neck and thigh (carotid and femoral) and your arm and foot (brachial and posterior tibial or dorsal pedal). The time between the onset of the pulse waves from the two locations will be measured and compared. This is a non-invasive procedure and should not be uncomfortable, other than some minor pressure at the measurement sites.
10. You will have a blood pressure cuff placed around your forearm, just below your elbow. We will position an ultrasound transducer probe on your upper arm (above your elbow) at the brachial artery. We will occlude blood flow to your arm by inflating the blood pressure cuff on your lower arm to 250 mmHg for 5 min. There is possible risk of discomfort due to the occlusion of the forearm. This discomfort should be mild, and only comparable to the lower portion of the arm falling asleep. This discomfort should subside immediately following deflation of the cuff. However, if this discomfort exceeds a tolerable level, please alert the investigator so that the cuff can be deflated early. We will use the ultrasound transducer probe to image your brachial artery before and after the blood pressure cuff is inflated and

released. This test may be repeated 1-2 times. If it is repeated, we will allow a rest period of at least 20 min in between each blood flow occlusion.

11. Next, we will administer one spray of nitroglycerin below your tongue and continue to image your brachial artery for 10 more minutes. This will cause your blood vessels to relax, and will slightly lower your blood pressure. This test causes the smooth muscle of your brachial artery blood vessel to relax and dilate allowing an increase in blood flow, and we call this test 'endothelium-independent vasodilation'. You will continue to lie supine for at least 15 minutes after nitroglycerin administration to ensure your blood pressure has returned to normal.
12. At the end of the study, we will estimate your total blood volume using a carbon monoxide (CO) uptake test. For this test, you will breathe 100% oxygen for 4 minutes to remove nitrogen from your lungs (this naturally exists). Then, you will start to breathe on a rebreathing system. This essentially means you will inhale and exhale into a bag. The air in this rebreathing system is pre-filled with 100% oxygen. After several minutes you will expire all of the air out that you possibly can through your nose. When you cannot get any more air out, your nose will be closed with noseclips. A dose of CO (1.0 mL CO/kg of body mass) will then be injected into the breathing system and you will inhale deeply to make sure you inhale as much CO as possible. CO is a gas that quickly moves from the air in your lungs to your blood. To further help this you will hold your breath for ~10 seconds after you deeply inhale. You will then breathe 100% oxygen normally for another several minutes. Small blood samples (~5ml) will be taken before you start breathing on the rebreathing system and immediately after you stop breathing on the rebreathing system. These blood samples will be taken from the catheter in your arm.
13. Although you will not be allowed food or beverages during the study, you will be given a light snack and fluids to drink before you leave.

How long will I be in the study?

You will be in the study for 8-11 weeks. You will participate in one screening session (about 60 min), seven experimental days (a total of up to 25 hours), and 36 water immersion sessions (up to 2 hours each).

What are the risks of the study?

1. Rectal temperature probes: The use of rectal probes to measure core body temperature, even during exercise, 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 probe that is designed for this purpose. There is also the risk of infection, either by you not washing your hands properly or exposure to a poorly cleaned probe. The probe has been sterilized before use, and we will instruct you on how to properly clean the probe after each time you use it. 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 how they will treat you.
2. Venous blood draws and catheters: There may be some discomfort during the blood draw. Once the catheter is in place, or once the needle is removed, the pain should subside. After the blood draw, the needle will be withdrawn and a sterile dressing will be applied. Any swelling or redness after the study should subside by a few hours after completion of the study. Although the needles are sterile, there is a slight risk of infection at the site where the needle was placed in your skin. You will be instructed how to keep the area clean for a day or two following the experimental day. The most common complications of inserting a small needle into a vein is a small bruise and pain at the site of the needle location which may last several days after removal of the needle. A small amount of bleeding may occur directly after removal of the catheter. Application of pressure and a gauze dressing will alleviate the bleeding. The maximum amount of blood we will draw across the entire study is 270ml, or about 18 tablespoons, which is well below the volume drawn in a standard blood donation (~450ml). Even so, we will exclude you from the study if you have donated blood within the last two months.
3. 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 or 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.

4. Microdialysis drugs: We will be infusing very small doses of each drug and only into a very small area of your skin. You will not have any systemic (whole body) effects of these drugs, and they will not alter your blood pressure in the small doses given in this study. 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. In the case of an adverse event, the study will be discontinued. Investigators are trained in Advanced Cardiac Life Support and anaphylaxis.
5. 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. If the local heating becomes painful, you should tell the

investigator and the temperature of the local heater will be lowered. There is a slight risk of burning the skin at this site, so it is important that you tell the investigators of any pain you are feeling. The heating device may be removed at any time if you experience any discomfort.

6. Laser-Doppler Probes: These probes send a small light into your skin. You will not feel anything except the probe touching your skin. There are no major risks associated with this procedure.
7. 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, you experience any discomfort you may request that the blood pressure cuff be either loosened or removed.
8. Finger Blood Pressure: In some people, this blood pressure cuff becomes 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, let the investigator know and they will turn it off for a few minutes. There are no major risks associated with this device.
9. Nitroglycerin administration: We will administer the standard dose of nitroglycerin under your tongue. This drug has minimal risk when used in low doses. Nitroglycerin is commonly prescribed to prevent and treat angina pectoris (chest pain), a condition occurring from constriction in the arteries of the heart. Nitroglycerin is a potent blood vessel dilator, causing the artery to open up and increase blood flow. You may feel slight light-headedness or dizziness, but this symptom should not last more than a few minutes. Please inform your investigator if these feelings persist during the study and do not simply come and go. You may feel your heart rate slowing as this drug causes a drop in blood pressure (transient hypotension), but this will also last only a few minutes. After nitroglycerin administration, we will take ultrasound images for several minutes to monitor changes in your blood flow and vessel size. You will

remain laying down on your back for an additional 15 minutes to ensure that your blood pressure and heart rate are back to their normal values before we allow you to leave the study area. There is a risk that you may experience a headache after this treatment, and if you are predisposed, you may experience the onset of a migraine headache.

10. Blood Volume Measurement: This measurement involves exposure to a small amount of carbon monoxide. Carbon monoxide is a colorless, odorless gas. We typically express CO in the blood as bound to a molecule called hemoglobin (Hb; HbCO). Healthy nonsmoking city dwellers typically have 1.5-2.0% of their Hb as HbCO. This test will increase this level by roughly 6%. Although you do not want to increase your level of HbCO, the levels used for this test are low and present minimal risks. The side effects associated with excessive HbCO typically occur at levels above 8% and may include headache, fatigue, shortness of breath, nausea, cherry-red colored lips, dizziness, and death, the last of which rarely occurs below 15% HbCO in normal individuals. This method has been in use in research for measuring blood volume for over 100 years without notable complications. Over time the CO bound to Hb in your blood will be removed. This process will occur naturally over the several hours following the test. For every 5 to 6 hours that passes after the test, half of the CO will leave your blood. Thus, after 15-18 hours you would have less than 1% CO in your blood.

    In order to assure that subjects remain below the level of %HbCO that is associated with side effects, we will test %HbCO in each individual prior to administration of CO. If an individual's baseline %HbCO is greater than 2.0% we will not administer CO to that individual. Furthermore, if for any reason an individual's post-administration %HbCO rises above 10% or an individual develops side-effects associated with excess %HbCO, we will initiate O_2 therapy to accelerate CO clearance from their system. O_2 will be given using a nasal administration similar to what someone may wear while in the hospital. Administering O_2 reduces the half-life of CO (speeds up the process of getting rid of it) from ~5 hours to ~80 minutes. We will measure %HbCO throughout the O_2 administration and periodically afterwards. We will stop treatment if %HbCO is < 8% or the symptoms go away. If

symptoms progress or do not improve, the Department of Public Safety (6-6666) or 911 directly will be called depending on the perceived or real severity of the symptoms. In the unlikely case of an adverse cardiovascular event, the laboratory is equipped with an Automatic Electronic Defibrillator (located in a clearly marked first-aid cabinet), and Vienna Brunt, Brett Ely, and Dr. Minson are trained in Advanced Cardiac Life Support (ACLS).

11. Emergencies: In the event of an emergency, you will be transported by ambulance to a local emergency facility.

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

No. 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 each experimental day. If the pregnancy test is positive (meaning that you are pregnant), you will no longer be able to take part in the study.

Are there benefits to taking part in this study?

This study will likely not make your health better. This study is being conducted to learn about the effects of chronic heat exposure on cardiovascular health. It is possible the information gathered in this study could be used to better treat patients with elevated cardiovascular risk in the future.

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 receive compensation for completed each session in the study as follows: initial screening session, $10; Experimental Days 1-5, $30 each; Experimental Day 6, $50; Experimental Days 7, $40 each; and $10 per heating session completed. If you complete all parts of the study, you will receive **$630**. This money is for the inconvenience and time you spent in this study, and works out to be approximately $10 per hour of participation in experimental days and $10 per heating session. 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 based on which study sessions you completed. You will receive compensation in the form of a check at the end of the study, or if you prefer, approximately every 2 weeks into the study. If you choose to receive compensation every 2 weeks, you will receive an amount corresponding to which study sessions you completed in each two-week time interval. There will be no difference in the total compensation you will receive if you chose to receive compensation at the end of the study versus every 2 weeks.

Please note, compensation from participation in Human Subjects Research studies may be considered taxable income. Compensation amounts are tracked across all studies in which you participate. If compensation totals $600 or more in a calendar year, the University is required to report the income to the IRS. University departments are required to track participant compensation and may contact you to complete a W9 form for tax reporting purposes. Because of this, your name will be associated with participation in a research study. 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 his student, Vienna Brunt, M.S. at any time about any question you have on this study. You may contact Dr. Minson by calling (541) 346-4105, (541) 346-4311 or on his cell phone (541) 953-2231, and Vienna Brunt at (541) 346-4507 or on her cell phone at (541) 968-2635.

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 stored on a secure computer with double password protection in Dr. Minson's office and only one list with names and identification numbers will exist. This list will be destroyed after all data has been collected and analyzed and for a period of one year after the results from the study have been published. Only coded specimens and data will exist thereafter.

Your blood will be analyzed for such things as heat shock proteins, hormone concentrations, and cytokines (factors released by cells that affect other cells). Your muscle tissue will be analyzed for changes in DNA (**not genetic testing**), RNA, cell signaling, and changes in cell structure. The researchers may store the information gathered during this study indefinitely.

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)

Group 3: Spinal cord injury Heat Therapy

TITLE: "Chronic heat exposure and cardiovascular health"

INVESTIGATORS: Vienna E Brunt, M.S., Dr. Christopher T Minson, and colleagues

APPROVED BY INSTITUTIONAL REVIEW BOARD: February 5, 2014

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?

Cardiovascular disease is the number one cause of death in the United States. Exercise is a potent means of improving cardiovascular health, but not all patient populations are able to exercise effectively. There is high demand for novel therapies to better manage cardiovascular risk in these patients. Heat exposure can have many beneficial effects on the cardiovascular system. As such, long-term heat exposure (i.e. sitting in a hot tub 4-5x per week) may provide an alternative means to exercise for improving cardiovascular health. This project will assess the benefits of long-term heat exposure on the health of the vasculature and on the cellular pathways that improve vascular health, which is

important as the majority of cardiovascular diseases affect the arteries. We will measure various biomarkers of vascular health before and after 8 weeks of heat exposure in able-bodied individuals and patients with spinal cord injury. SCI patients are a population with elevated cardiovascular risk who have limited exercise capabilities, and who may benefit greatly from chronic heat exposure.

We will address the following questions in this study:

- How does 8 weeks of passive heat exposure affect cardiovascular health, as measured by various biomarkers of cardiovascular function?
- How does 8 weeks of passive heat exposure affect levels of factors circulating in the blood and located within muscle tissue that are important for cardiovascular health?
- Do effects differ between healthy able-bodied individuals and spinal cord injury patients?

What will happen in the study?

1. If you meet all the initial subject criteria (based on initial phone and/or email conversations) and 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. Additionally, you will fill out a health history form and meet with a physician, so that we can ensure you are healthy enough to participate in the study. In addition, the physician will be available to you to answer any medical questions or concerns you may have throughout the duration of your participation in the study. This visit should last about 60 minutes.
2. If you qualify for the study, we will also give you a letter of participation to take back to your primary medical provider. The letter will include details about all procedures that will be performed over the course of the study. We will ask you to have your primary medical provider read the letter and sign it, and to then return it to us. Their signature will mean they believe you are medically healthy enough to participate in the study. The letter can be emailed, mailed, or brought to us in person. Our email address is minsonlab@gmail.com. If you would like to mail the letter, we will provide you with a stamped addressed envelope. **If it is not possible to return a signed letter, you will still be able to participate in the study, although we recommend that you do consult with your primary medical provider.**

3. We will assign you to a subject group. Theses assignments are random in order to ensure the highest quality of research. You will be informed of what group you are in prior to reading this form. If you are reading this form, you are in the **SCI heating** group.
4. You will report to the laboratory 4-5 times per week over the course of 8 weeks for heat exposure sessions. Additionally, you will participate in 7 experimental days. Experimental Days 1-2 will occur prior to the 8 weeks heat exposure sessions, and Days 6-7 will occur immediately following the 8 weeks of heat exposure sessions. Days 3, 4, and 5 will occur at Weeks 2, 4, and 6 into heat exposure sessions. We will perform the same procedures in Experimental Days 1 and 7, and in Days 2-6. All procedures are explained in detail below.
5. During the screening session, we will schedule your initial experimental days (2 sessions), the start of your heat exposure sessions, and tentatively schedule all other sessions. We will give you a hard copy of your schedule to take home with you.

Heating Sessions:

1. You will report to the laboratory 4-5 times per week for 8 weeks, for a total of 36 sessions. Each session will take approximately 2-2.5 hours. You will be asked to bring a swimsuit to wear during the session. If you do not have one, we will provide one for you to use for the duration of the 8 weeks. No other subjects will use the same swimsuit.
2. You will be asked to provide a urine sample so that we can ensure you are properly hydrated before undergoing heat stress. If you are dehydrated, we will give you 5mL/kg body weight (about half a normal sized 20oz bottle of Gatorade®) of fluids to drink prior to getting in the hot tub.
3. We will ask you if you have had any bouts of autonomic dysreflexia previously that day, or any falls, bumps, or other stressful events. If the answer is yes to any of those questions, we cannot let you participate in the heating session for that day. We will also ask you to void your bladder prior to getting in the hot tub to avoid autonomic dysreflexia.

4. You will be instrumented with a Polar® heart rate monitor chest strap so that we can continuously monitor your heart rate throughout the procedure and a cuff on your upper arm so that we can monitor your blood pressure.
5. We will give you a rectal probe labeled with your subject number. It is made of a thin rubber (flexible) material that is inserted 10 cm (approximately 4 inches) past the anal sphincter. The probe will remain in place throughout the entire study session (up to 2.5 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 anytime. You will be instructed how to self-insert the rectal probe, as well as how to remove it and clean it. If you needed 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.
6. *On the very first and very last heating session (and possibly during one session at about 4wks),* we will place 1 small flexible needle (these are called "intravenous catheters", and are smaller than the lead of a pencil) into a vein near your elbow. The skin will be cleaned before this procedure. This catheter will remain in your vein throughout the heating session. We will take about 40ml of blood, about 2.7 tablespoons, prior to getting into the bathtub, and another 25ml of blood at the end of the heating period so that we can measure various factors in your blood that are affected by the heat exposure. We will remove the catheter after the second blood draw and place a sterile bandage over the site. The vials in which we collect the blood will be coded such that the investigators can determine all samples came from the same subject and the time the sample was taken. No one will be able to determine your identity from the sample.
7. You will then be transferred to a bathtub that will be filled with water at 40°C (104.0°F). The tub is hooked up to a water pump and heater such that water can be circulated and maintained at a desired temperature. You will lay down in the bath tub such that the water level comes up to the level of your collar bone (to the top of your shoulders). You will lay down in this position until your core temperature has reached 38.5°C (101.3°F). This takes approximately 25-35 minutes. At this point, you will sit

up such that the water level reaches the middle of your chest (with your shoulders and arms out of the water). You will remain sitting up for another 60 minutes, or until the total time in the hot tub reaches 90 minutes, whichever comes first.

8. We will continuously check in with you throughout the heating period. You will be instructed to inform an investigator if you feel any of the following symptoms: light-headedness, dizziness, nausea, headache, or if you feel unbearably warm. If you feel these symptoms, we will have you sit up if you were previously laying down in the bath, or we will have you get out of the bath if you were previously sitting up. If your body temperature gets too high (above 39.5°C, 103°F), we will have you get out of the bath, even if you feel fine.
9. If your blood pressure or heart either get too high or too low, or if you experience autonomic dysreflexia, we will remove you from the bath tub. A lift will be available for use if you are unable to transfer yourself out of the bath.
10. You will be asked to drink fluids while in the hot tub in an amount equivalent to ~1% of your body weight (approximately 1-1.5 normal sized bottles of Gatorade®). This is to replace some of the fluid you will lose due to sweating. You will be allowed to drink more than this amount if you want to.
11. At the end of the heating period, you will get out of the hot tub and sit in a recovery chair. We will continue to monitor your body temperature until your core temperature begins to return back to normal. If your body temperature remains high for too long, or if you feel too hot, dizzy, or nauseous, we will cool you down more quickly with cold packs. We will provide you with a towel to dry off during this time.
12. Once you feel fine, you will remove the rectal probe, clean it, and place it in a location allocated to you. Before you leave the lab, we will offer you cool fluids and a light snack.

Experimental Days 1 and 7 (Skin Studies):

1. You will arrive at Dr. Minson's laboratory in Esslinger Hall at the University of Oregon to participate in the experimental protocol. The testing will take approximately 4-5 hours. The day before the study, we will contact you (either by phone or email depending on your preference) to remind you to refrain from all over-

the-counter medications, including vitamins and supplements, for 24 hours, alcohol and caffeine for 12 hours, food for 4 hours, and heavy exercise for 24 hours. Additionally, you will be asked to wear a short-sleeved shirt.

2. Upon arrival at the laboratory, your height and weight and resting blood pressure will be measured. Female subjects will 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.
3. We will place 3 small tubes (these are called "microdialysis fibers", and are about the size of sewing thread) in the skin of your forearm. 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 placed inside 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.
4. 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) to go away. During this time, a small probe (laser-Doppler probe) 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.
5. During the study, we may periodically inflate a small cuff that is placed on your middle finger of one of your hands to measure your blood pressure (Portapres device). We will only inflate this cuff 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.
6. Blood pressure will also be measured periodically throughout the study using an inflation cuff on your upper arm.
7. During the protocol we will put some very small doses of drugs through the small tubes in your skin. These drugs will cause the vessels of your skin to either widen 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. You will receive the following drugs:

a. L-NNA: this stops nitric oxide from being produced and causes the skin vessels to narrow
b. Tempol: This is a substance that may cause your blood vessels to open.
c. Sodium nitroprusside: this is a substance that is used to lower blood pressure in patients and causes the skin vessels to open

8. We will heat a small area of your skin with a small heater up to 43.5° Celsius (110 degrees Fahrenheit) to open the vessels in your skin. This is below the temperature where heating becomes painful (about 113 degrees Fahrenheit) and well below the temperature that may burn your skin (about 117 degrees Fahrenheit). If you think the heater is becoming painful, you need to tell the investigator and the temperature will be lowered.
9. 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.
10. Although you will not be allowed food or beverages during the study, you will be given a light snack and fluids to drink before you leave.

Experimental Days 2-6 (Vascular Function):

1. You will arrive at Dr. Minson's laboratory in Esslinger Hall at the University of Oregon to participate in the experimental protocol. This testing will take approximately 3-4 hours. The day before the study, we will contact you (either by phone or email depending on your preference) to remind you to refrain from all over-the-counter medications, including vitamins and supplements, for 24 hours, alcohol and caffeine for 12 hours, food for 4 hours, and heavy exercise for 24 hours. Additionally, you will be asked to wear a short-sleeved shirt and shorts that can be easily pushed up.
2. Upon arrival at the laboratory, your height and weight and resting blood pressure will be measured. Female subjects will 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.
3. We will prep you for the study by placing 5 sticky electrodes on your skin and attaching a small wire or lead to each electrode. These leads will be attached to a

monitor that will allow us to measure your heart rate and heart rhythm. These electrodes will be placed on your skin by a member of the same sex. The electrodes will be placed on your body in the following locations: 2 electrodes are placed on your upper chest close to your shoulder (one on the left and one on the right); 2 electrodes will be placed on your stomach just above your hip bones (just above where your pants line is) on the left and right side; and one will be placed on your lower ribcage on the left side.

4. After these electrodes are in place, you will lay down on a padded exam table for the remainder of the study.
5. During the study, blood pressure will be measured periodically throughout the study using an inflation cuff on your upper arm.
6. We may also periodically inflate a small cuff that is placed on your middle finger of one of your hands to measure your blood pressure. We will only inflate this cuff 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.
7. We will use an ultrasound probe to image the large arteries in your neck (carotid) and thigh (femoral). We will be measuring the diameter of the arteries, the thickness of the artery walls, and the resting blood flow.
8. You will undergo carotid arterial tonometry with ultrasound. An ultrasound probe will be placed on one carotid artery while a non-invasive tonometer device (looks like a blunt pencil) is placed on the opposite carotid artery which non-invasively measures blood pressure using pressure sensors pressed against the skin over the artery. The ultrasound probe measures carotid artery diameter and blood velocity, while the tonometer measures the blood pressure wave traveling down the vessel. The two values are compared to give an index of the stiffness of the arteries. This is a non-invasive procedure and should not be uncomfortable, other than some minor pressure of the measurement sites.
9. Your pulse wave will be measured using tonometry. Two tonometers that measure blood pressure waves will be placed on the skin over arteries on your neck and thigh (carotid and femoral) and your arm and foot (brachial and posterior tibial or dorsal pedal). The time between the onset of the pulse waves from the two locations will be

measured and compared. This is a non-invasive procedure and should not be uncomfortable, other than some minor pressure at the measurement sites.

10. You will have a blood pressure cuff placed around your forearm, just below your elbow. We will position an ultrasound transducer probe on your upper arm (above your elbow) at the brachial artery. We will occlude blood flow to your arm by inflating the blood pressure cuff on your lower arm to 250 mmHg for 5 min. There is possible risk of discomfort due to the occlusion of the forearm. This discomfort should be mild, and only comparable to the lower portion of the arm falling asleep. This discomfort should subside immediately following deflation of the cuff. However, if this discomfort exceeds a tolerable level, please alert the investigator so that the cuff can be deflated early. We will use the ultrasound transducer probe to image your brachial artery before and after the blood pressure cuff is inflated and released. This test may be repeated 1-2 times. If it is repeated, we will allow a rest period of at least 20 min in between each blood flow occlusion.
11. Next, we will administer one spray of nitroglycerin below your tongue and continue to image your brachial artery for 10 more minutes. This will cause your blood vessels to relax, and will slightly lower your blood pressure. This test causes the smooth muscle of your brachial artery blood vessel to relax and dilate allowing an increase in blood flow, and we call this test 'endothelium-independent vasodilation'. You will continue to lie supine for at least 15 minutes after nitroglycerin administration to ensure your blood pressure has returned to normal.
12. At the end of the study, we will estimate your total blood volume using a carbon monoxide (CO) uptake test. For this test, you will breathe 100% oxygen for 4 minutes to remove nitrogen from your lungs (this naturally exists). Then, you will start to breathe on a rebreathing system. This essentially means you will inhale and exhale into a bag. The air in this rebreathing system is pre-filled with 100% oxygen. After several minutes you will expire all of the air out that you possibly can through your nose. When you cannot get any more air out, your nose will be closed with noseclips. A dose of CO (1.0 mL CO/kg of body mass) will then be injected into the breathing system and you will inhale deeply to make sure you inhale as much CO as possible. CO is a gas that quickly moves from the air in your lungs to your blood. To

further help this you will hold your breath for ~10 seconds after you deeply inhale. You will then breathe 100% oxygen normally for another several minutes. Small blood samples (~5ml) will be taken before you start breathing on the rebreathing system and immediately after you stop breathing on the rebreathing system. These blood samples will be taken from the catheter in your arm.

13. Although you will not be allowed food or beverages during the study, you will be given a light snack and fluids to drink before you leave.

Optional Follow-Up Testing:

If you are interested and if you continue to qualify, you may be asked by the investigators to return to the laboratory after you have completed the 8 weeks of hot water immersion. This is so that we can assess how long any changes in your cardiovascular health last. Follow-up testing would take place 2, 4, 6, and/ 8 weeks after your last hot tub session. If you are interested in participating in follow-up testing, you will be asked to give consent and will sign an additional consent form closer to the end of your 8 weeks of hot water immersion. Importantly, if you are not interested in participating in follow-up testing, it does not affect your participation in the primary study, the 8 weeks of hot water immersion. Also please note that all time frames and compensation amounts in this section of the consent form only include the initial 8 weeks of hot water immersion.

If you are interested in being asked later on about participating in follow-up testing, **please initial here**: _____. You can still change your mind at any time.

How long will I be in the study?

You will be in the study for 8-11 weeks. You will participate in one screening session (about 60 min), seven experimental days (a total of up to 25 hours), and 36 heating sessions.

What are the risks of the study?

1. Heat exposure: There are some risks associated with heat exposure, including: fatigue, light-headedness, muscle cramps, dehydration, and neurological detriments (i.e. heat stroke). However, these symptoms do not typically occur until core temperature rises above 40°C. Your core temperature will be constantly recorded (rectal probe), and you will be removed from the hot bath immediately if either core

temperature reaches 39.5°C or you experience any symptoms of heat-related illness. You will be instructed to notify the investigators immediately if you experience any of these symptoms. All symptoms subside upon lowering core temperature. Ice packs will be on hand for rapid cooling if necessary. Additionally, heat exposure may have detrimental effects on a developing fetus in females and on sperm counts in males. Thus, subjects who are pregnant, trying to conceive, and/or undergoing treatment to increase sperm counts will be excluded from the study.

2. Rectal temperature probes (heating group only): The use of rectal probes to measure core body temperature, even during exercise, 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 probe that is designed for this purpose. There is also the risk of infection, either by you not washing your hands properly or exposure to a poorly cleaned probe. The probe has been sterilized before use, and we will instruct you on how to properly clean the probe after each time you use it. 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 how they will treat you.
3. Venous blood draws and catheters: There may be some discomfort during the blood draw. Once the catheter is in place, or once the needle is removed, the pain should subside. After the blood draw, the needle will be withdrawn and a sterile dressing will be applied. Any swelling or redness after the study should subside by a few hours after completion of the study. Although the needles are sterile, there is a slight risk of infection at the site where the needle was placed in your skin. You will be instructed how to keep the area clean for a day or two following the experimental day. The most common complications of inserting a small needle into a vein is a small bruise and pain at the site of the needle location which may last several days after removal of the needle. A small amount of bleeding may occur directly after removal of the catheter. Application of pressure and a gauze dressing will alleviate the bleeding. The maximum amount of blood we will draw across the entire study is 270ml, or about 18 tablespoons, which is well below the volume drawn in a standard blood donation

(~450ml). Even so, we will exclude you from the study if you have donated blood within the last two months.

4. 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 or 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.
5. Microdialysis drugs: We will be infusing very small doses of each drug and only into a very small area of your skin. You will not have any systemic (whole body) effects of these drugs, and they will not alter your blood pressure in the small doses given in this study. 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. In the case of an adverse event, the study will be discontinued. Investigators are trained in Advanced Cardiac Life Support and anaphylaxis.

6. 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. If the local heating becomes painful, you should tell the investigator and the temperature of the local heater will be lowered. There is a slight risk of burning the skin at this site, so it is important that you tell the investigators of any pain you are feeling. The heating device may be removed at any time if you experience any discomfort.
7. Laser-Doppler Probes: These probes send a small light into your skin. You will not feel anything except the probe touching your skin. There are no major risks associated with this procedure.
8. 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, you experience any discomfort you may request that the blood pressure cuff be either loosened or removed.
9. Finger Blood Pressure: In some people, this blood pressure cuff becomes 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, let the investigator know and they will turn it off for a few minutes. There are no major risks associated with this device.
10. Nitroglycerin administration: We will administer the standard dose of nitroglycerin under your tongue. This drug has minimal risk when used in low doses. Nitroglycerin is commonly prescribed to prevent and treat angina pectoris (chest pain), a condition occurring from constriction in the arteries of the heart. Nitroglycerin is a potent blood vessel dilator, causing the artery to open up and increase blood flow. You may feel

slight light-headedness or dizziness, but this symptom should not last more than a few minutes. Please inform your investigator if these feelings persist during the study and do not simply come and go. You may feel your heart rate slowing as this drug causes a drop in blood pressure (transient hypotension), but this will also last only a few minutes. After nitroglycerin administration, we will take ultrasound images for several minutes to monitor changes in your blood flow and vessel size. You will remain laying down on your back for an additional 15 minutes to ensure that your blood pressure and heart rate are back to their normal values before we allow you to leave the study area. There is a risk that you may experience a headache after this treatment, and if you are predisposed, you may experience the onset of a migraine headache.

11. Blood Volume Measurement: This measurement involves exposure to a small amount of carbon monoxide. Carbon monoxide is a colorless, odorless gas. We typically express CO in the blood as bound to a molecule called hemoglobin (Hb; HbCO). Healthy nonsmoking city dwellers typically have 1.5-2.0% of their Hb as HbCO. This test will increase this level by roughly 6%. Although you do not want to increase your level of HbCO, the levels used for this test are low and present minimal risks. The side effects associated with excessive HbCO typically occur at levels above 8% and may include headache, fatigue, shortness of breath, nausea, cherry-red colored lips, dizziness, and death, the last of which rarely occurs below 15% HbCO in normal individuals. This method has been in use in research for measuring blood volume for over 100 years without notable complications. Over time the CO bound to Hb in your blood will be removed. This process will occur naturally over the several hours following the test. For every 5 to 6 hours that passes after the test, half of the CO will leave your blood. Thus, after 15-18 hours you would have less than 1% CO in your blood.

    In order to assure that subjects remain below the level of %HbCO that is associated with side effects, we will test %HbCO in each individual prior to administration of CO. If an individual's baseline %HbCO is greater than 2.0% we will not administer CO to that individual. Furthermore, if for any reason an individual's post-administration %HbCO rises above 10% or an individual develops

side-effects associated with excess %HbCO, we will initiate O_2 therapy to accelerate CO clearance from their system. O_2 will be given using a nasal administration similar to what someone may wear while in the hospital. Administering O_2 reduces the half-life of CO (speeds up the process of getting rid of it) from ~5 hours to ~80 minutes. We will measure %HbCO throughout the O_2 administration and periodically afterwards. We will stop treatment if %HbCO is < 8% or the symptoms go away. If symptoms progress or do not improve, the Department of Public Safety (6-6666) or 911 directly will be called depending on the perceived or real severity of the symptoms. In the unlikely case of an adverse cardiovascular event, the laboratory is equipped with an Automatic Electronic Defibrillator (located in a clearly marked first-aid cabinet), and Vienna Brunt, Brett Ely, and Dr. Minson are trained in Advanced Cardiac Life Support (ACLS).

12. Emergencies: In the event of an emergency, you will be transported by ambulance to a local emergency facility.

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

No. 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 each experimental day. If the pregnancy test is positive (meaning that you are pregnant), you will no longer be able to take part in the study.

Are there benefits to taking part in this study?

This study will likely not make your health better. This study is being conducted to learn about the effects of chronic heat exposure on cardiovascular health. It is possible the information gathered in this study could be used to better treat patients with elevated cardiovascular risk in the future.

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 receive compensation for completed each session in the study as follows: initial screening session, $10; Experimental Days 1-5, $30 each; Experimental Day 6, $50; Experimental Day 7, $40 each; and $10 per heating session completed. If you complete all parts of the study, you will receive **$630**. This money is for the inconvenience and time you spent in this study, and works out to be approximately $10 per hour of participation in experimental days and $10 per heating session. 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 based on which study sessions you completed. You will receive compensation in the form of a check at the end of the study, or if you prefer, approximately every 2 weeks into the study. If you choose to receive compensation every 2 weeks, you will receive an amount corresponding to which study sessions you completed in each two-week time interval. There will be no difference in the total compensation you will receive if you chose to receive compensation at the end of the study versus every 2 weeks.

Please note, compensation from participation in Human Subjects Research studies may be considered taxable income. Compensation amounts are tracked across all studies in which you participate. If compensation totals $600 or more in a calendar year, the University is required to report the income to the IRS. University departments are required to track participant compensation and may contact you to complete a W9 form for tax reporting purposes. Because of this, your name will be associated with participation in a research study. 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 his student, Vienna Brunt, M.S. at any time about any question you have on this study. You may contact Dr. Minson by calling (541) 346-4105, (541) 346-4311 or on his cell phone (541) 953-2231, and Vienna Brunt at (541) 346-4507 or on her cell phone at (541) 968-2635.

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 stored on a secure computer with double password protection in Dr. Minson's office and only one list with names and identification numbers will exist. This list will be destroyed after all data has been collected and analyzed and for a period of one year after the results from the study have been published. Only coded specimens and data will exist thereafter.

Your blood will be analyzed for such things as heat shock proteins, hormone concentrations, and cytokines (factors released by cells that affect other cells). Your muscle tissue will be analyzed for changes in DNA (**not genetic testing**), RNA, cell signaling, and changes in cell structure. The researchers may store the information gathered during this study indefinitely.

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)

<u>Group 4: Spinal cord injury Time Control</u>

TITLE: "Chronic heat exposure and cardiovascular health"

INVESTIGATORS: Vienna E Brunt, M.S., Dr. Christopher T Minson, and colleagues

APPROVED BY INSTITUTIONAL REVIEW BOARD: February 5, 2014

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?

Cardiovascular disease is the number one cause of death in the United States. Exercise is a potent means of improving cardiovascular health, but not all patient populations are able to exercise effectively. There is high demand for novel therapies to better manage cardiovascular risk in these patients. Heat exposure can have many beneficial effects on the cardiovascular system. As such, long-term heat exposure (i.e. sitting in a hot tub 4-5x per week) may provide an alternative means to exercise for improving cardiovascular health. This project will assess the benefits of long-term heat exposure on the health of the vasculature and on the cellular pathways that improve vascular health, which is important as the majority of cardiovascular diseases affect the arteries. We will measure various biomarkers of vascular health before and after 8 weeks of heat exposure in able-bodied individuals and patients with spinal cord injury. SCI patients are a population with elevated cardiovascular risk who have limited exercise capabilities, and who may benefit greatly from chronic heat exposure.

We will address the following questions in this study:

- How does 8 weeks of passive heat exposure affect cardiovascular health, as measured by various biomarkers of cardiovascular function?
- How does 8 weeks of passive heat exposure affect levels of factors circulating in the blood and located within muscle tissue that are important for cardiovascular health?
- Do effects differ between healthy able-bodied individuals and spinal cord injury patients?

What will happen in the study?

1. If you meet all the initial subject criteria (based on initial phone and/or email conversations) and 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. Additionally, you will fill out a health history form and meet with a physician, so that we can ensure you are healthy enough to participate in the study. In addition, the physician will be available to you to answer any medical questions or concerns you may have throughout the duration of your participation in the study. This visit should last about 60 minutes.

2. If you qualify for the study, we will also give you a letter of participation to take back to your primary medical provider. The letter will include details about all procedures that will be performed over the course of the study. We will ask you to have your primary medical provider read the letter and sign it, and to then return it to us. Their signature will mean they believe you are medically healthy enough to participate in the study. The letter can be emailed, mailed, or brought to us in person. Our email address is minsonlab@gmail.com. If you would like to mail the letter, we will provide you with a stamped addressed envelope. **If it is not possible to return a signed letter, you will still be able to participate in the study, although we recommend that you do consult with your primary medical provider.**
3. We will assign you to a subject group. Theses assignments are random in order to ensure the highest quality of research. You will be informed of what group you are in prior to reading this form. If you are reading this form, you are assigned to the **control group**.
4. You will participate in 7 experimental days over the course of 8-11 weeks. Experimental Days 1-2 will occur within a 2 week time period. Days 3, 4, and 5 will occur 2, 4, and 6 weeks after the initial testing. Days 6-7 will occur 8 weeks after the initial testing. We will perform the same procedures in Experimental Days 1 and 9, and in Days 2-6. All procedures are explained in detail below. We will check in with you regularly via phone or email in between experimental days.
5. During the screening session, we will schedule your initial experimental days (2 sessions), and tentatively schedule all other sessions. We will give you a hard copy of your schedule to take home with you.

Experimental Days 1 and 7 (Skin Studies):

1. You will arrive at Dr. Minson's laboratory in Esslinger Hall at the University of Oregon to participate in the experimental protocol. The testing will take approximately 4-5 hours. The day before the study, we will contact you (either by phone or email depending on your preference) to remind you to refrain from all over-the-counter medications, including vitamins and supplements, for 24 hours, alcohol

and caffeine for 12 hours, food for 4 hours, and heavy exercise for 24 hours. Additionally, you will be asked to wear a short-sleeved shirt.

2. Upon arrival at the laboratory, your height and weight and resting blood pressure will be measured. Female subjects will 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.
3. We will place 3 small tubes (these are called "microdialysis fibers", and are about the size of sewing thread) in the skin of your forearm. 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 placed inside 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.
4. 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) to go away. During this time, a small probe (laser-Doppler probe) 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.
5. During the study, we may periodically inflate a small cuff that is placed on your middle finger of one of your hands to measure your blood pressure (Portapres device). We will only inflate this cuff 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.
6. Blood pressure will also be measured periodically throughout the study using an inflation cuff on your upper arm.
7. During the protocol we will put some very small doses of drugs through the small tubes in your skin. These drugs will cause the vessels of your skin to either widen 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. You will receive the following drugs:
   a. L-NNA: this stops nitric oxide from being produced and causes the skin vessels to narrow
   b. Tempol: This is a substance that may cause your blood vessels to open.

c. Sodium nitroprusside: this is a substance that is used to lower blood pressure in patients and causes the skin vessels to open

8. We will heat a small area of your skin with a small heater up to 43.5° Celsius (110 degrees Fahrenheit) to open the vessels in your skin. This is below the temperature where heating becomes painful (about 113 degrees Fahrenheit) and well below the temperature that may burn your skin (about 117 degrees Fahrenheit). If you think the heater is becoming painful, you need to tell the investigator and the temperature will be lowered.
9. 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.
10. Although you will not be allowed food or beverages during the study, you will be given a light snack and fluids to drink before you leave.

Experimental Days 2-6 (Vascular Function):

1. You will arrive at Dr. Minson's laboratory in Esslinger Hall at the University of Oregon to participate in the experimental protocol. This testing will take approximately 3-4 hours. The day before the study, we will contact you (either by phone or email depending on your preference) to remind you to refrain from all over-the-counter medications, including vitamins and supplements, for 24 hours, alcohol and caffeine for 12 hours, food for 4 hours, and heavy exercise for 24 hours. Additionally, you will be asked to wear a short-sleeved shirt and shorts that can be easily pushed up.
2. Upon arrival at the laboratory, your height and weight and resting blood pressure will be measured. Female subjects will 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.
3. We will prep you for the study by placing 5 sticky electrodes on your skin and attaching a small wire or lead to each electrode. These leads will be attached to a monitor that will allow us to measure your heart rate and heart rhythm. These electrodes will be placed on your skin by a member of the same sex. The electrodes will be placed on your body in the following locations: 2 electrodes are placed on

your upper chest close to your shoulder (one on the left and one on the right); 2 electrodes will be placed on your stomach just above your hip bones (just above where your pants line is) on the left and right side; and one will be placed on your lower ribcage on the left side.

4. After these electrodes are in place, you will lay down on a padded exam table for the remainder of the study.
5. We will place 1 small flexible needle (these are called "intravenous catheters", and are smaller than the lead of a pencil) into a vein near your elbow. The skin will be cleaned before this procedure. This catheter will remain in your vein throughout the session. We may take about 40ml of blood, about 2.7 tablespoons, so that we can measure various factors in your blood that may be affected by heat exposure. We will remove the catheter at the end of the session and place a sterile bandage over the site. The vials in which we collect the blood will be coded such that the investigators can determine all samples came from the same subject and the time the sample was taken. No one will be able to determine your identity from the sample.
6. During the study, blood pressure will be measured periodically throughout the study using an inflation cuff on your upper arm.
7. We may also periodically inflate a small cuff that is placed on your middle finger of one of your hands to measure your blood pressure. We will only inflate this cuff 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.
8. We will use an ultrasound probe to image the large arteries in your neck (carotid) and thigh (femoral). We will be measuring the diameter of the arteries, the thickness of the artery walls, and the resting blood flow.
9. You will undergo carotid arterial tonometry with ultrasound. An ultrasound probe will be placed on one carotid artery while a non-invasive tonometer device (looks like a blunt pencil) is placed on the opposite carotid artery which non-invasively measures blood pressure using pressure sensors pressed against the skin over the artery. The ultrasound probe measures carotid artery diameter and blood velocity, while the tonometer measures the blood pressure wave traveling down the vessel. The two values are compared to give an index of the stiffness of the arteries. This is a non-

invasive procedure and should not be uncomfortable, other than some minor pressure of the measurement sites.

10. Your pulse wave will be measured using tonometry. Two tonometers that measure blood pressure waves will be placed on the skin over arteries on your neck and thigh (carotid and femoral) and your arm and foot (brachial and posterior tibial or dorsal pedal). The time between the onset of the pulse waves from the two locations will be measured and compared. This is a non-invasive procedure and should not be uncomfortable, other than some minor pressure at the measurement sites.
11. You will have a blood pressure cuff placed around your forearm, just below your elbow. We will position an ultrasound transducer probe on your upper arm (above your elbow) at the brachial artery. We will occlude blood flow to your arm by inflating the blood pressure cuff on your lower arm to 250 mmHg for 5 min. There is possible risk of discomfort due to the occlusion of the forearm. This discomfort should be mild, and only comparable to the lower portion of the arm falling asleep. This discomfort should subside immediately following deflation of the cuff. However, if this discomfort exceeds a tolerable level, please alert the investigator so that the cuff can be deflated early. We will use the ultrasound transducer probe to image your brachial artery before and after the blood pressure cuff is inflated and released. This test may be repeated 1-2 times. If it is repeated, we will allow a rest period of at least 20 min in between each blood flow occlusion.
12. Next, we will administer one spray of nitroglycerin below your tongue and continue to image your brachial artery for 10 more minutes. This will cause your blood vessels to relax, and will slightly lower your blood pressure. This test causes the smooth muscle of your brachial artery blood vessel to relax and dilate allowing an increase in blood flow, and we call this test ‘endothelium-independent vasodilation’. You will continue to lie supine for at least 15 minutes after nitroglycerin administration to ensure your blood pressure has returned to normal.
13. At the end of the study, we will estimate your total blood volume using a carbon monoxide (CO) uptake test. For this test, you will breathe 100% oxygen for 4 minutes to remove nitrogen from your lungs (this naturally exists). Then, you will start to breathe on a rebreathing system. This essentially means you will inhale and

exhale into a bag. The air in this rebreathing system is pre-filled with 100% oxygen. After several minutes you will expire all of the air out that you possibly can through your nose. When you cannot get any more air out, your nose will be closed with noseclips. A dose of CO (1.0 mL CO/kg of body mass) will then be injected into the breathing system and you will inhale deeply to make sure you inhale as much CO as possible. CO is a gas that quickly moves from the air in your lungs to your blood. To further help this you will hold your breath for ~10 seconds after you deeply inhale. You will then breathe 100% oxygen normally for another several minutes. Small blood samples (~5ml) will be taken before you start breathing on the rebreathing system and immediately after you stop breathing on the rebreathing system. These blood samples will be taken from the catheter in your arm.

14. Although you will not be allowed food or beverages during the study, you will be given a light snack and fluids to drink before you leave.

How long will I be in the study?

You will be in the study for 8-11 weeks. You will participate in one screening session (about 60 min) and seven experimental days (a total of up to 25 hours).

What are the risks of the study?

1. Venous blood draws and catheters: There may be some discomfort during the blood draw. Once the catheter is in place, or once the needle is removed, the pain should subside. After the blood draw, the needle will be withdrawn and a sterile dressing will be applied. Any swelling or redness after the study should subside by a few hours after completion of the study. Although the needles are sterile, there is a slight risk of infection at the site where the needle was placed in your skin. You will be instructed how to keep the area clean for a day or two following the experimental day. The most common complications of inserting a small needle into a vein is a small bruise and pain at the site of the needle location which may last several days after removal of the needle. A small amount of bleeding may occur directly after removal of the catheter. Application of pressure and a gauze dressing will alleviate the bleeding. The maximum amount of blood we will draw across the entire study is 195ml, or about 13 tablespoons, which is well below the volume drawn in a standard blood donation (~450ml). Even so, we will exclude you from the study if you have donated blood

within the last two months.

2. 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 or 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.
3. Microdialysis drugs: We will be infusing very small doses of each drug and only into a very small area of your skin. You will not have any systemic (whole body) effects of these drugs, and they will not alter your blood pressure in the small doses given in this study. 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. In the case of an adverse event,

the study will be discontinued. Investigators are trained in Advanced Cardiac Life Support and anaphylaxis.

4. 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. If the local heating becomes painful, you should tell the investigator and the temperature of the local heater will be lowered. There is a slight risk of burning the skin at this site, so it is important that you tell the investigators of any pain you are feeling. The heating device may be removed at any time if you experience any discomfort.
5. Laser-Doppler Probes: These probes send a small light into your skin. You will not feel anything except the probe touching your skin. There are no major risks associated with this procedure.
6. 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, you experience any discomfort you may request that the blood pressure cuff be either loosened or removed.
7. Finger Blood Pressure: In some people, this blood pressure cuff becomes 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, let the investigator know and they will turn it off for a few minutes. There are no major risks associated with this device.
8. Nitroglycerin administration: We will administer the standard dose of nitroglycerin under your tongue. This drug has minimal risk when used in low doses. Nitroglycerin is commonly prescribed to prevent and treat angina pectoris (chest pain), a condition occurring from constriction in the arteries of the heart. Nitroglycerin is a potent blood vessel dilator, causing the artery to open up and increase blood flow. You may feel slight light-headedness or dizziness, but this symptom should not last more than a few

minutes. Please inform your investigator if these feelings persist during the study and do not simply come and go. You may feel your heart rate slowing as this drug causes a drop in blood pressure (transient hypotension), but this will also last only a few minutes. After nitroglycerin administration, we will take ultrasound images for several minutes to monitor changes in your blood flow and vessel size. You will remain laying down on your back for an additional 15 minutes to ensure that your blood pressure and heart rate are back to their normal values before we allow you to leave the study area. There is a risk that you may experience a headache after this treatment, and if you are predisposed, you may experience the onset of a migraine headache.

9. Blood Volume Measurement: This measurement involves exposure to a small amount of carbon monoxide. Carbon monoxide is a colorless, odorless gas. We typically express CO in the blood as bound to a molecule called hemoglobin (Hb; HbCO). Healthy nonsmoking city dwellers typically have 1.5-2.0% of their Hb as HbCO. This test will increase this level by roughly 6%. Although you do not want to increase your level of HbCO, the levels used for this test are low and present minimal risks. The side effects associated with excessive HbCO typically occur at levels above 8% and may include headache, fatigue, shortness of breath, nausea, cherry-red colored lips, dizziness, and death, the last of which rarely occurs below 15% HbCO in normal individuals. This method has been in use in research for measuring blood volume for over 100 years without notable complications. Over time the CO bound to Hb in your blood will be removed. This process will occur naturally over the several hours following the test. For every 5 to 6 hours that passes after the test, half of the CO will leave your blood. Thus, after 15-18 hours you would have less than 1% CO in your blood.

   In order to assure that subjects remain below the level of %HbCO that is associated with side effects, we will test %HbCO in each individual prior to administration of CO. If an individual's baseline %HbCO is greater than 2.0% we will not administer CO to that individual. Furthermore, if for any reason an individual's post-administration %HbCO rises above 10% or an individual develops side-effects associated with excess %HbCO, we will initiate O_2 therapy to accelerate

CO clearance from their system. O_2 will be given using a nasal administration similar to what someone may wear while in the hospital. Administering O_2 reduces the half-life of CO (speeds up the process of getting rid of it) from ~5 hours to ~80 minutes. We will measure %HbCO throughout the O_2 administration and periodically afterwards. We will stop treatment if %HbCO is < 8% or the symptoms go away. If symptoms progress or do not improve, the Department of Public Safety (6-6666) or 911 directly will be called depending on the perceived or real severity of the symptoms. In the unlikely case of an adverse cardiovascular event, the laboratory is equipped with an Automatic Electronic Defibrillator (located in a clearly marked first-aid cabinet), and Vienna Brunt, Brett Ely, and Dr. Minson are trained in Advanced Cardiac Life Support (ACLS).

10. Emergencies: In the event of an emergency, you will be transported by ambulance to a local emergency facility.

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

No. 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 each experimental day. If the pregnancy test is positive (meaning that you are pregnant), you will no longer be able to take part in the study.

Are there benefits to taking part in this study?

This study will likely not make your health better. This study is being conducted to learn about the effects of chronic heat exposure on cardiovascular health. It is possible the information gathered in this study could be used to better treat patients with elevated cardiovascular risk in the future.

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 receive compensation for completing each session in the study as

follows: initial screening session, $10; Experimental Days 1-5, $30 each; Experimental Day 6, $50; Experimental Day 7, $40 each. If you complete all parts of the study, you will receive **$260**. This money is for the inconvenience and time you spent in this study, and works out to be approximately $10 per hour of participation. 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 based on which study sessions you completed. You will receive compensation in the form of a check at the end of the study, or if you prefer, approximately every 2 weeks into the study. If you choose to receive compensation every 2 weeks, you will receive an amount corresponding to which study sessions you completed in each two-week time interval. There will be no difference in the total compensation you will receive if you chose to receive compensation at the end of the study versus every 2 weeks.

Please note, compensation from participation in Human Subjects Research studies may be considered taxable income. Compensation amounts are tracked across all studies in which you participate. If compensation totals $600 or more in a calendar year, the University is required to report the income to the IRS. University departments are required to track participant compensation and may contact you to complete a W9 form for tax reporting purposes. Because of this, your name will be associated with participation in a research study. 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 his student, Vienna Brunt, M.S. at any time about any question you have on this study. You may contact Dr. Minson by calling (541) 346-4105, (541) 346-4311 or on his cell phone (541) 953-2231, and Vienna Brunt at (541) 346-4507 or on her cell phone at (541) 968-2635.

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 stored on a secure computer with double password protection in Dr. Minson's office and only one list with names and identification numbers will exist. This list will be destroyed after all data has been collected and analyzed and for a period of one year after the results from the study have been published. Only coded specimens and data will exist thereafter.

Your blood will be analyzed for such things as heat shock proteins, hormone concentrations, and cytokines (factors released by cells that affect other cells). Your muscle tissue will be analyzed for changes in DNA (**not genetic testing**), RNA, cell signaling, and changes in cell structure. The researchers may store the information gathered during this study indefinitely.

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. Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, et al. Heart Disease and Stroke Statistics--2015 Update: A Report From the American Heart Association. Circulation. 2015 Jan 17;131(4):e29–322.

2. Van Duijnhoven NTL, Janssen TWJ, Green DJ, Minson CT, Hopman MTE, Thijssen DHJ. Effect of functional electrostimulation on impaired skin vasodilator responses to local heating in spinal cord injury. J Appl Physiol. 2009 Apr;106(4):1065–71.

3. Thijssen DH, Ellenkamp R, Smits P, Hopman MT. Rapid vascular adaptations to training and detraining in persons with spinal cord injury. Arch Phys Med Rehabil. 2006 Apr;87(4):474–81.

4. Pritchard KA, Ackerman AW, Gross ER, Stepp DW, Shi Y, Fontana JT, et al. Heat shock protein 90 mediates the balance of nitric oxide and superoxide anion from endothelial nitric-oxide synthase. J Biol Chem. 2001 May 25;276(21):17621–4.

5. Baek SH, Min JN, Park EM, Han MY, Lee YS, Lee YJ, et al. Role of small heat shock protein HSP25 in radioresistance and glutathione-redox cycle. J Cell Physiol. 2000 Apr;183(1):100–7.

6. Kim I, Shin H-M, Baek W. Heat-shock response is associated with decreased production of interleukin-6 in murine aortic vascular smooth muscle cells. Naunyn Schmiedebergs Arch Pharmacol. 2005 Jan;371(1):27–33.

7. Johnson JM, Proppe DW. Cardiovascular adjustments to heat stress. In: Fregly MJ, Blatteis CM, editors. Handbook of Physiology: Environmental Physiology. Bethesda, MD: American Physiological Society; 2011.

8. Locke M, Noble EG, Atkinson BG. Exercising mammals synthesize stress proteins. Am J Physiol Heart Circ Physiol. 1990 Apr;258(4 Pt 1):C723–9.

9. Rhind SG, Gannon GA, Shephard RJ, Buguet A, Shek PN, Radomski MW. Cytokine induction during exertional hyperthermia is abolished by core temperature clamping: neuroendocrine regulatory mechanisms. Int J Hyperthermia. 2004 Jan;20(5):503–16.

10. Joyner MJ, Green DJ. Exercise protects the cardiovascular system: effects beyond traditional risk factors. J Physiol. 2009 Dec 1;587(Pt 23):5551–8.

11. Green DJ, O'Driscoll G, Joyner MJ, Cable NT. Exercise and cardiovascular risk reduction: time to update the rationale for exercise? J Appl Physiol. 2008 Aug;105(2):766–8.

12. Beever R. The Effects of Repeated Thermal Therapy on Quality of Life in Patients with Type II Diabetes Mellitus. J Alter Comp Med. 2010 Jun;16(6):677–81.

13. Laukkanen T, Khan H, Zaccardi F, Laukkanen JA. Association Between Sauna Bathing and Fatal Cardiovascular and All-Cause Mortality Events. JAMA Intern Med. 2015 Apr 23;175(4):542–8.

14. Horowitz M. Heat acclimation and cross-tolerance against novel stressors: genomic–physiological linkage. In: Progress in Brain Research. Elsevier; 2007. pp. 373–92. (Progress in Brain Research).

15. Horowitz M, Assadi H. Heat acclimation-mediated cross-tolerance in cardioprotection. Ann N Y Acad Sci. 2010 Feb 23;1188(1):199–206.

16. Carter HH, Spence AL, Atkinson CL, Pugh CJA, Cable NT, Thijssen DHJ, et al. Distinct Effects of Blood Flow and Temperature on Cutaneous Microvascular Adaptation. Med Sci Sports Exerc. 2014 Nov;46(11):2113–21.

17. Carter HH, Spence AL, Atkinson CL, Pugh CJA, Naylor LH, Green DJ. Repeated core temperature elevation induces conduit artery adaptation in humans. Eur J Appl Physiol. 2014 Jan 8;114(4):859–65.

18. Tinken TM, Thijssen DHJ, Hopkins N, Dawson EA, Cable NT, Green DJ. Shear Stress Mediates Endothelial Adaptations to Exercise Training in Humans. Hypertension. 2010 Jan 20;55(2):312–8.

19. Arborelius M, Ballidin UI, Lilja B, Lundgren CE. Hemodynamic changes in man during immersion with the head above water. Aerosp Med. 1972 Jun;43(6):592–8.

20. Carter HH, Spence AL, Pugh CJA, Ainslie P, Naylor LH, Green DJ. Cardiovascular responses to water immersion in humans: impact on cerebral perfusion. Am J Physiol Regul Integr Comp Physiol. 2014 May;306(9):R636–40.

21. Hannuksela ML, Ellahham S. Benefits and risks of sauna bathing. Am J Med. 2001 Feb 1;110(2):118–26.

22. Taylor NAS. Human Heat Adaptation. Comprehensive Physiology. Hoboken, NJ, USA: John Wiley & Sons, Inc; 2014 Jan 17;4:325–65.

23. Ohori T, Nozawa T, Ihori H, Shida T, Sobajima M, Matsuki A, et al. Effect of repeated sauna treatment on exercise tolerance and endothelial function in patients with chronic heart failure. Am J Cardiol. 2012 Jan 1;109(1):100–4.

24. Tei C, Horikiri Y, Park JC, Jeong JW, Chang KS, Toyama Y, et al. Acute hemodynamic improvement by thermal vasodilation in congestive heart failure. Circulation. 1995 May 15;91(10):2582–90.

25. Kellogg DL, Pergola PE, Piest KL, Kosiba WA, Crandall CG, Grossmann M, et al. Cutaneous active vasodilation in humans is mediated by cholinergic nerve cotransmission. Circ Res. 1995 Dec;77(6):1222–8.

26. 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. 2003 Oct 1;552(1):223–32.

27. Kellogg DL, Zhao JL, Wu Y, Johnson JM. VIP/PACAP receptor mediation of cutaneous active vasodilation during heat stress in humans. J Appl Physiol. 2010 Jul;109(1):95–100.

28. Wong BJ. Neurokinin-1 receptor desensitization to consecutive microdialysis infusions of substance P in human skin. J Physiol. 2005 Jul 7;568(3):1047–56.

29. Kellogg DL, Crandall CG, Liu Y, Charkoudian N, Johnson JM. Nitric oxide and cutaneous active vasodilation during heat stress in humans. J Appl Physiol. 1998 Sep;85(3):824–9.

30. 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. 1998 Sep;85(3):830–4.

31. Wilkins BW, Holowatz LA, Wong BJ, Minson CT. Nitric oxide is not permissive for cutaneous active vasodilatation in humans. J Physiol. 2003 May 1;548(3):963–9.

32. Kellogg DL, Zhao JL, Wu Y. Neuronal nitric oxide synthase control mechanisms in the cutaneous vasculature of humans in vivo. J Physiol. 2007 Nov 22;586(3):847–57.

33. 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. 2009 Nov;107(5):1438–44.

34. Johnson JM, Park MK. Reflex control of skin blood flow by skin temperature: role of core temperature. J Appl Physiol. 1979 Dec;47(6):1188–93.

35. Rowell LB, Brengelmann GL, Murray JA. Cardiovascular responses to sustained high skin temperature in resting man. J Appl Physiol. 1969 Nov;27(5):673–80.

36. Kiss D, Popp W, Wagner C, Zwick H, Sertl K. Effects of the sauna on diffusing capacity, pulmonary function and cardiac output in healthy subjects. Respiration. 1994;61(2):86–8.

37. Vuori I. Sauna bather's circulation. Ann Clin Res. 1988;20(4):249–56.

38. Kauppinen K, Vuori I. Man in the sauna. Ann Clin Res. 1986;18(4):173–85.

39. Rowell LB, Detry JR, Profant GR, Wyss C. Splanchnic vasoconstriction in hyperthermic man--role of falling blood pressure. J Appl Physiol. 1971 Dec;31(6):864–9.

40. Minson CT, Wladkowski SL, Cardell AF, Pawelczyk JA, Kenney WL. Age alters the cardiovascular response to direct passive heating. J Appl Physiol. 1998 Apr;84(4):1323–32.

41. Cooper T, Willman VL, Hanlon CR. Cardiac and peripheral vascular responses to hyperthermia induced by blood stream heating. J Thorac Cardiovasc Surg. 1962 Nov;44:667–73.

42. Moore FT, Marable SA, Ogden E. Contractility of the heart in abnormal temperatures. Ann Thorac Surg. 1966 May;2(3):446–50.

43. Murray RH. Cardiopulmonary effects of brief, intense thermal exposures. J Appl Physiol. 1966 Nov;21(6):1717–24.

44. Rowell LB, Brengelmann GL, Blackmon JR, Murray JA. Redistribution of blood flow during sustained high skin temperature in resting man. J Appl Physiol. 1970 Apr;28(4):415–20.

45. Sancetta SM, Kramer J, Husni E. The effects of dry heat on the circulation of man. I. General hemodynamics. Am Heart J. 1958 Aug;56(2):212–21.

46. Koroxenidis GT, Shepherd JT, Marshall RJ. Cardiovascular response to acute heat stress. J Appl Physiol. 1961 Sep;16:869–72.

47. Fritzsche RG, Switzer TW, Hodgkinson BJ, Coyle EF. Stroke volume decline during prolonged exercise is influenced by the increase in heart rate. J Appl Physiol. 1999 Mar;86(3):799–805.

48. Coyle EF, González-Alonso J. Cardiovascular drift during prolonged exercise: new perspectives. Exerc Sport Sci Rev. 2001 Apr;29(2):88–92.

49. Tei C, Horikiri Y, Park JC, Jeong JW, Chang KS, Tanaka N, et al. [Effects of hot water bath or sauna on patients with congestive heart failure: acute hemodynamic improvement by thermal vasodilation]. J Cardiol. 1994 May;24(3):175–83.

50. Wilson TE, Tollund C, Yoshiga CC, Dawson EA, Nissen P, Secher NH, et al. Effects of heat and cold stress on central vascular pressure relationships during orthostasis in humans. J Physiol. 2007 Nov 15;585(1):279–85.

51. Wilson TE, Brothers RM, Tollund C, Dawson EA, Nissen P, Yoshiga CC, et al. Effect of thermal stress on Frank-Starling relations in humans. J Physiol. 2009 Jul 1;587(13):3383–92.

52. el-Sherif N, Shahwan L, Sorour AH. The effect of acute thermal stress on general and pulmonary hemodynamics in the cardiac patient. Am Heart J. 1970 Mar;79(3):305–17.

53. Faithfull NS, Reinhold HS, van den Berg AP, van Rhoon GC, van der Zee J, Wike-Hooley JL. Cardiovascular changes during whole body hyperthermia treatment of advanced malignancy. Eur J Appl Physiol Occup Physiol. 1984;53(3):274–81.

54. Carlsten A, Gustafson A, Werko L. Hemodynamic influence of warm and dry environment in man with and without rheumatic heart disease. Acta Med Scand. 1961 Apr;169:411–7.

55. Damato AN, Lau SH, Stein E, Haft JI, Kosowsky B, Cohen SI. Cardiovascular response to acute thermal stress (hot dry environment) in unacclimatized normal subjects. Am Heart J. 1968 Dec;76(6):769–74.

56. Löllgen H, Nieding von G, Koppenhagen K, Kersting F, Just H. Hemodynamic response to graded water immersion. Klin Wochenschr. 1981 Jun 15;59(12):623–8.

57. Kukkonen-Harjula K, Kauppinen K. Health effects and risks of sauna bathing. Int J Circumpolar Health. 2006 Jun;65(3):195–205.

58. Eisalo A, Luurila OJ. The Finnish sauna and cardiovascular diseases. Ann Clin Res. 1988;20(4):267–70.

59. Keast ML, Adamo KB. The Finnish sauna bath and its use in patients with cardiovascular disease. J Cardiopulm Rehabil. 2000 Jul;20(4):225–30.

60. Jokinen E, Gregory EL, Välimäki I. The sauna and children. Ann Clin Res. 1988;20(4):283–6.

61. Markkola L, Mattila KJ, Koivikko MJ. Sauna habits and related symptoms in Finnish children. Eur J Pediatr. 1989 Dec;149(3):221–2.

62. Tikkanen J, Heinonen OP. Maternal hyperthermia during pregnancy and cardiovascular malformations in the offspring. Eur J Epidemiol. 1991 Nov;7(6):628–35.

63. Wähä-Eskeli K, Erkkola R. The sauna and pregnancy. Ann Clin Res. 1988;20(4):279–82.

64. Brown-Woodman PD, Post EJ, Gass GC, White IG. The effect of a single sauna exposure on spermatozoa. Arch Androl. 1984;12(1):9–15.

65. Saikhun J, Kitiyanant Y, Vanadurongwan V, Pavasuthipaisit K. Effects of sauna on sperm movement characteristics of normal men measured by computer-assisted sperm analysis. Int J Androl. 1998 Dec;21(6):358–63.

66. Vierula M, Niemi M, Keiski A, Saaranen M, Saarikoski S, Suominen J. High and unchanged sperm counts of Finnish men. Int J Androl. 1996 Feb;19(1):11–7.

67. Joffe M. Decreased fertility in Britain compared with Finland. Lancet. 1996 Jun 1;347(9014):1519–22.

68. Luurila OJ. Cardiac arrhythmias, sudden death and the Finnish sauna bath. Adv Cardiol. 1978;25:73–81.

69. Singer K, Lundberg WB. Ventricular arrhythmias associated with the ingestion of alcohol. Ann Intern Med. 1972 Aug;77(2):247–8.

70. Luurila OJ. The sauna and the heart. J Intern Med. 1992 Apr;231(4):319–20.

71. Ritossa F. A new puffing pattern induced by temperature shock and DNP in drosophila. Experientia. 1962 Oct 7;18:571–3.

72. Zhong N, Zhang Y, Fang QZ, Zhou ZN. Intermittent hypoxia exposure-induced heat-shock protein 70 expression increases resistance of rat heart to ischemic injury. Acta Pharmacol Sin. 2000 May;21(5):467–72.

73. Won E-J, Han J, Lee Y, Kumar KS, Shin K-H, Lee S-J, et al. In vivo effects of UV radiation on multiple endpoints and expression profiles of DNA repair and heat shock protein (Hsp) genes in the cycloid copepod Paracyclopina nana. Aquat Toxicol. 2015 Aug;165:1–8.

74. Saklatvala J, Kaur P, Guesdon F. Phosphorylation of the small heat-shock protein is regulated by interleukin 1, tumour necrosis factor, growth factors, bradykinin and ATP. Biochem J. 1991 Aug 1;277 (3):635–42.

75. Freshney NW, Rawlinson L, Guesdon F, Jones E, Cowley S, Hsuan J, et al. Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. Cell. 1994 Sep 23;78(6):1039–49.

76. Airaksinen S, Jokilehto T, Råbergh CMI, Nikinmaa M. Heat- and cold-inducible regulation of HSP70 expression in zebrafish ZF4 cells. Comp Biochem Physiol B, Biochem Mol Biol. 2003 Oct;136(2):275–82.

77. Reichsman F, Scordilis SP, Clarkson PM, Evans WJ. Muscle protein changes following eccentric exercise in humans. Eur J Appl Physiol Occup Physiol. 1991;62(4):245–50.

78. Puntschart A, Vogt M, Widmer HR, Hoppeler H, Billeter R. Hsp70 expression in human skeletal muscle after exercise. Acta Physiologica Scandinavica. 1996 Aug;157(4):411–7.

79. Lindquist S, Craig EA. The heat-shock proteins. Annu Rev Genet. 1988;22:631–77.

80. Marber MS, Mestril R, Chi SH, Sayen MR, Yellon DM, Dillmann WH. Overexpression of the rat inducible 70-kD heat stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. J Clin Invest. 1995 Apr;95(4):1446–56.

81. Jones Q, Voegeli TS, Li G, Chen Y, Currie RW. Heat shock proteins protect against ischemia and inflammation through multiple mechanisms. Inflamm Allergy Drug Targets. 2011 Aug;10(4):247–59.

82. Barbe MF, Tytell M, Gower DJ, Welch WJ. Hyperthermia protects against light damage in the rat retina. Science. 1988 Sep 30;241(4874):1817–20.

83. Jäättelä M, Wissing D. Heat-shock proteins protect cells from monocyte cytotoxicity: possible mechanism of self-protection. J Exp Med. 1993 Jan 1;177(1):231–6.

84. Mizzen LA, Welch WJ. Characterization of the thermotolerant cell. I. Effects on protein synthesis activity and the regulation of heat-shock protein 70 expression. J Cell Biol. 1988 Apr;106(4):1105–16.

85. Lavoie JN, Gingras-Breton G, Tanguay RM, Landry J. Induction of Chinese hamster HSP27 gene expression in mouse cells confers resistance to heat shock. HSP27 stabilization of the microfilament organization. J Biol Chem. 1993 Feb 15;268(5):3420–9.

86. Chirico WJ, Waters MG, Blobel G. 70K heat shock related proteins stimulate protein translocation into microsomes. Nature. 1988 Apr 28;332(6167):805–10.

87. Brugge J, Yonemoto W, Darrow D. Interaction between the Rous sarcoma virus transforming protein and two cellular phosphoproteins: analysis of the turnover and distribution of this complex. Mol Cell Biol. 1983 Jan;3(1):9–19.

88. Courtneidge SA, BISHOP JM. Transit of pp60v-src to the plasma membrane. Proc Natl Acad Sci USA. 1982 Dec;79(23):7117–21.

89. Sanchez ER, Meshinchi S, Tienrungroj W, Schlesinger MJ, Toft DO, Pratt WB. Relationship of the 90-kDa murine heat shock protein to the untransformed and transformed states of the L cell glucocorticoid receptor. J Biol Chem. 1987 May 25;262(15):6986–91.

90. Baulieu EE. Steroid hormone antagonists at the receptor level: a role for the heat-shock protein MW 90,000 (hsp 90). J Cell Biochem. 1987 Oct;35(2):161–74.

91. Welch WJ, Suhan JP. Morphological study of the mammalian stress response: characterization of changes in cytoplasmic organelles, cytoskeleton, and nucleoli, and appearance of intranuclear actin filaments in rat fibroblasts after heat-shock treatment. J Cell Biol. 1985 Oct;101(4):1198–211.

92. Paek KH, Walker GC. Escherichia coli dnaK null mutants are inviable at high temperature. J Bacteriol. 1987 Jan;169(1):283–90.

93. Welch WJ, Feramisco JR. Nuclear and nucleolar localization of the 72,000-dalton heat shock protein in heat-shocked mammalian cells. J Biol Chem. 1984 Apr 10;259(7):4501–13.

94. Milarski KL, Morimoto RI. Expression of human HSP70 during the synthetic phase of the cell cycle. Proc Natl Acad Sci USA. 1986 Dec;83(24):9517–21.

95. Munro S, Pelham HR. An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. Cell. 1986 Jul 18;46(2):291–300.

96. Lee AS, Bell J, Ting J. Biochemical characterization of the 94- and 78-kilodalton glucose-regulated proteins in hamster fibroblasts. J Biol Chem. 1984 Apr 10;259(7):4616–21.

97. Deshaies RJ, Koch BD, Werner-Washburne M, Craig EA, Schekman R. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature. 1988 Apr 28;332(6167):800–5.

98. Eilers M, Oppliger W, Schatz G. Both ATP and an energized inner membrane are required to import a purified precursor protein into mitochondria. EMBO J. 1987 Apr;6(4):1073–7.

99. Chappell TG, Welch WJ, Schlossman DM, Palter KB, Schlesinger MJ, Rothman JE. Uncoating ATPase is a member of the 70 kilodalton family of stress proteins. Cell. 1986 Apr 11;45(1):3–13.

100. Welch WJ, Feramisco JR. Rapid purification of mammalian 70,000-dalton stress proteins: affinity of the proteins for nucleotides. Mol Cell Biol. 1985 Jun;5(6):1229–37.

101. Verner K, Schatz G. Import of an incompletely folded precursor protein into isolated mitochondria requires an energized inner membrane, but no added ATP. EMBO J. 1987 Aug;6(8):2449–56.

102. Velazquez JM, Lindquist S. hsp70: nuclear concentration during environmental stress and cytoplasmic storage during recovery. Cell. 1984 Mar;36(3):655–62.

103. Pelham HR. Hsp70 accelerates the recovery of nucleolar morphology after heat shock. EMBO J. 1984 Dec 20;3(13):3095–100.

104. Blake MJ, Gershon D, Fargnoli J, Holbrook NJ. Discordant expression of heat shock protein mRNAs in tissues of heat-stressed rats. J Biol Chem. 1990 Sep 5;265(25):15275–9.

105. Cairo G, Bardella L, Schiaffonati L, Bernelli-Zazzera A. Synthesis of heat shock proteins in rat liver after ischemia and hyperthermia. Hepatology. 1985 May;5(3):357–61.

106. Currie RW, White FP. Characterization of the synthesis and accumulation of a 71-kilodalton protein induced in rat tissues after hyperthermia. Can J Biochem Cell Biol. 1983 Jun;61(6):438–46.

107. Nowak TS, Bond U, Schlesinger MJ. Heat shock RNA levels in brain and other tissues after hyperthermia and transient ischemia. J Neurochem. 1990 Feb;54(2):451–8.

108. Flanagan SW, Ryan AJ, Gisolfi CV, Moseley PL. Tissue-specific HSP70 response in animals undergoing heat stress. Am J Physiol Heart Circ Physiol. 1995 Jan;268:R28–32.

109. Cosgrove JW, Brown IR. Heat shock protein in mammalian brain and other organs after a physiologically relevant increase in body temperature induced by D-lysergic acid diethylamide. Proc Natl Acad Sci USA. 1983 Jan;80(2):569–73.

110. Nowak TS. Effects of amphetamine on protein synthesis and energy metabolism in mouse brain: role of drug-induced hyperthermia. J Neurochem. 1988 Jan;50(1):285–94.

111. Uney JB, Leigh PN, Marsden CD, Lees A, Anderton BH. Stereotaxic injection of kainic acid into the striatum of rats induces synthesis of mRNA for heat shock protein 70. FEBS Lett. 1988 Aug 1;235:215–8.

112. Milne KJ, Wolff S, Noble EG. Myocardial accumulation and localization of the inducible 70-kDa heat shock protein, Hsp70, following exercise. J Appl Physiol. 2012 Sep;113(6):853–60.

113. Whyte JJ, Laughlin MH. The effects of acute and chronic exercise on the vasculature. Acta Physiol. 2010 Aug;199(4):441–50.

114. Ryan AJ, Gisolfi CV, Moseley PL. Synthesis of 70K stress protein by human leukocytes: effect of exercise in the heat. J Appl Physiol. 1991 Jan;70(1):466–71.

115. Sonna LA, Wenger CB, Flinn S, Sheldon HK, Sawka MN, Lilly CM. Exertional heat injury and gene expression changes: a DNA microarray analysis study. J Appl Physiol. 2004 May;96(5):1943–53.

116. Li S, Piotrowicz RS, Levin EG, Shyy YJ, Chien S. Fluid shear stress induces the phosphorylation of small heat shock proteins in vascular endothelial cells. Am J Physiol. 1996 Sep;271:C994–1000.

117. Brooks AR, Lelkes PI, Rubanyi GM. Gene expression profiling of human aortic endothelial cells exposed to disturbed flow and steady laminar flow. Physiol Genomics. 2002;9(1):27–41.

118. Arrigo AP, Welch WJ. Characterization and purification of the small 28,000-dalton mammalian heat shock protein. J Biol Chem. 1987 Nov 15;262(32):15359–69.

119. Arrigo AP, Pauli D. Characterization of HSP27 and three immunologically related polypeptides during Drosophila development. Exp Cell Res. 1988 Mar;175(1):169–83.

120. Jerius H, Karolyi DR, Mondy JS, Beall A, Wootton D, Ku D, et al. Endothelial-dependent vasodilation is associated with increases in the phosphorylation of a small heat shock protein (HSP20). J Vasc Surg. 1999 Apr;29(4):678–84.

121. Rembold CM, Foster DB, Strauss JD, Wingard CJ, Eyk JE. cGMP-mediated phosphorylation of heat shock protein 20 may cause smooth muscle relaxation without myosin light chain dephosphorylation in swine carotid artery. J Physiol. 2000 May 1;524 Pt 3:865–78.

122. Harris MB, Blackstone MA, Ju H, Venema VJ, Venema RC. Heat-induced increases in endothelial NO synthase expression and activity and endothelial NO release. Am J Physiol Heart Circ Physiol. 2003 Jul;285(1):H333–40.

123. Djurhuus R, Nossum V, Lundsett N, Hovin W, Svardal AM, Havnes MB, et al. Simulated diving after heat stress potentiates the induction of heat shock protein 70 and elevates glutathione in human endothelial cells. Cell Stress Chaperones. 2009 Nov 19;15(4):405–14.

124. Oishi Y, Taniguchi K, Matsumoto H, Ishihara A, Ohira Y, Roy RR. Muscle type-specific response of HSP60, HSP72, and HSC73 during recovery after elevation of muscle temperature. J Appl Physiol. 2002 Mar;92(3):1097–103.

125. Oishi Y, Taniguchi K, Matsumoto H, Ishihara A, Ohira Y, Roy RR. Differential responses of HSPs to heat stress in slow and fast regions of rat gastrocnemius muscle. Muscle Nerve. 2003 Oct 7;28:587–94.

126. Khassaf M, Child RB, McArdle A, Brodie DA, Esanu C, Jackson MJ. Time course of responses of human skeletal muscle to oxidative stress induced by nondamaging exercise. J Appl Physiol. 2001 Mar;90(3):1031–5.

127. Morton JP. Time course and differential responses of the major heat shock protein families in human skeletal muscle following acute nondamaging treadmill exercise. J Appl Physiol. 2006 May 11;101(1):176–82.

128. Morton JP, MacLaren D, Cable NT, Campbell IT, Evans L, Bongers T, et al. Elevated core and muscle temperature to levels comparable to exercise do not increase heat shock protein content of skeletal muscle of physically active men. Acta Physiologica. 2007 May 6;190:319–27.

129. Deguchi Y, Negoro S, Kishimoto S. Heat-shock protein synthesis by human peripheral mononuclear cells from SLE patients. Biochem Biophys Res Commun. 1987 Nov 13;148(3):1063–8.

130. Song Y, Zweier JL, Xia Y. Heat-shock protein 90 augments neuronal nitric oxide synthase activity by enhancing Ca2+/calmodulin binding. Biochem J. 2001 Apr 15;355(2):357–60.

131. Harris MB, Ju H, Venema VJ, Blackstone M, Venema RC. Role of heat shock protein 90 in bradykinin-stimulated endothelial nitric oxide release. Gen Pharmacol. 2000 Sep;35(3):165–70.

132. Garcia-Cardena G, Fan R, Shah V, Sorrentino R, Cirino G, Papapetropoulos A, et al. Dynamic activation of endothelial nitric oxide synthase by Hsp90. Nature. 1998 Apr 23;392(6678):821–4.

133. Gratton JP, Fontana J, O'Connor DS, Garcia-Cardena G, McCabe TJ, Sessa WC. Reconstitution of an endothelial nitric-oxide synthase (eNOS), hsp90, and caveolin-1 complex in vitro. Evidence that hsp90 facilitates calmodulin stimulated displacement of eNOS from caveolin-1. J Biol Chem. 2000 Jul 21;275(29):22268–72.

134. Takahashi S, Mendelsohn ME. Calmodulin-dependent and -independent activation of endothelial nitric-oxide synthase by heat shock protein 90. J Biol Chem. 2003 Mar 14;278(11):9339–44.

135. Brouet A, Sonveaux P, Dessy C, Balligand JL, Feron O. Hsp90 ensures the transition from the early Ca2+-dependent to the late phosphorylation-dependent activation of the endothelial nitric-oxide synthase in vascular endothelial growth factor-exposed endothelial cells. J Biol Chem. 2001 Aug 31;276(35):32663–9.

136. Sato S, Fujita N, Tsuruo T. Modulation of Akt kinase activity by binding to Hsp90. Proc Natl Acad Sci USA. 2000 Sep 26;97(20):10832–7.

137. Harris MB, Mitchell BM, Sood SG, Webb RC, Venema RC. Increased nitric oxide synthase activity and Hsp90 association in skeletal muscle following chronic exercise. Eur J Appl Physiol. 2008 Nov;104(5):795–802.

138. Bedard K, Krause K-H. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev. 2007 Jan;87(1):245–313.

139. Wenzel P, Knorr M, Kossmann S, Stratmann J, Hausding M, Schuhmacher S, et al. Lysozyme M-positive monocytes mediate angiotensin II-induced arterial hypertension and vascular dysfunction. Circulation. 2011 Sep 20;124(12):1370–81.

140. Kim Y-W, Byzova TV. Oxidative stress in angiogenesis and vascular disease. Blood. 2014 Mar 8;123:625–31.

141. Ushio-Fukai M, Alexander RW. Reactive oxygen species as mediators of angiogenesis signaling: role of NAD(P)H oxidase. Mol Cell Biochem. 2004 Sep;264(1-2):85–97.

142. Vásquez-Vivar J, Kalyanaraman B, Martásek P, Hogg N, Masters BS, Karoui H, et al. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. Proc Natl Acad Sci USA. 1998 Aug 4;95(16):9220–5.

143. Kuzkaya N, Weissmann N, Harrison DG, Dikalov S. Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric-oxide synthase. J Biol Chem. 2003 Jun 20;278(25):22546–54.

144. Chandra S, Romero MJ, Shatanawi A, Alkilany AM, Caldwell RB, Caldwell RW. Oxidative species increase arginase activity in endothelial cells through the RhoA/Rho kinase pathway. Br J Pharmacol. 2011 Dec 16;165(2):506–19.

145. Turrens JF. Mitochondrial formation of reactive oxygen species. J Physiol. 2003 Oct 15;552(Pt 2):335–44.

146. Ohtsuki T, Matsumoto M, Kuwabara K, Kitagawa K, Suzuki K, Taniguchi N, et al. Influence of oxidative stress on induced tolerance to ischemia in gerbil hippocampal neurons. Brain Res. 1992 Dec 25;599(2):246–52.

147. Choi S, Park KA, Lee HJ, Park MS, Lee JH, Park KC, et al. Expression of Cu/Zn SOD protein is suppressed in hsp 70.1 knockout mice. J Biochem Mol Biol. 2005 Jan 31;38(1):111–4.

148. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation. 2002 Mar 5;105(9):1135–43.

149. Fichtlscherer S, Rosenberger G, Walter DH, Breuer S, Dimmeler S, Zeiher AM. Elevated C-reactive protein levels and impaired endothelial vasoreactivity in patients with coronary artery disease. Circulation. 2000 Aug 29;102(9):1000–6.

150. Brevetti G, Silvestro A, Di Giacomo S, Bucur R, Di Donato A, Schiano V, et al. Endothelial dysfunction in peripheral arterial disease is related to increase in plasma markers of inflammation and severity of peripheral circulatory impairment but not to classic risk factors and atherosclerotic burden. J Vasc Surg. 2003 Aug;38(2):374–9.

151. de Winther MPJ, Kanters E, Kraal G, Hofker MH. Nuclear factor kappaB signaling in atherogenesis. Arterioscler Thromb Vasc Biol. 2005 May;25(5):904–14.

152. Guzik TJ, Harrison DG. Endothelial NF-kappaB as a mediator of kidney damage: the missing link between systemic vascular and renal disease? Circ Res. 2007 Aug 3;101(3):227–9.

153. Neish AS, Williams AJ, Palmer HJ, Whitley MZ, Collins T. Functional analysis of the human vascular cell adhesion molecule 1 promoter. J Exp Med. 1992 Dec 1;176(6):1583–93.

154. Osborn L. Leukocyte adhesion to endothelium in inflammation. Cell. 1990 Jul 13;62(1):3–6.

155. Chen Y, Arrigo A-P, Currie RW. Heat shock treatment suppresses angiotensin II-induced activation of NF-kappaB pathway and heart inflammation: a role for IKK depletion by heat shock? Am J Physiol Heart Circ Physiol. 2004 Sep;287(3):H1104–14.

156. Frossard JL, Pastor CM, Hadengue A. Effect of hyperthermia on NF-kappaB binding activity in cerulein-induced acute pancreatitis. Am J Physiol Gastrointest Liver Physiol. 2001 Jun;280(6):G1157–62.

157. Nakabe N, Kokura S, Shimozawa M, Katada K, Sakamoto N, Ishikawa T, et al. Hyperthermia attenuates TNF-alpha-induced up regulation of endothelial cell adhesion molecules in human arterial endothelial cells. Int J Hyperthermia. 2015 Mar 6;23(3):217–24.

158. Ensor JE, Wiener SM, McCrea KA, Viscardi RM, Crawford EK, Hasday JD. Differential effects of hyperthermia on macrophage interleukin-6 and tumor necrosis factor-alpha expression. Am J Physiol. 1994 Apr;266:C967–74.

159. Snyder YM, Guthrie L, Evans GF, Zuckerman SH. Transcriptional inhibition of endotoxin-induced monokine synthesis following heat shock in murine peritoneal macrophages. J Leukoc Biol. 1992 Feb;51(2):181–7.

160. Kluger MJ, Rudolph K, Soszynski D, Conn CA, Leon LR, Kozak W, et al. Effect of heat stress on LPS-induced fever and tumor necrosis factor. Am J Physiol. 1997 Sep;273:R858–63.

161. Rakonczay Z, Takács T, Mándi Y, Iványi B, Varga S, Pápai G, et al. Water immersion pretreatment decreases pro-inflammatory cytokine production in cholecystokinin-octapeptide-induced acute pancreatitis in rats: possible role of HSP72. Int J Hyperthermia. 2001 Nov;17(6):520–35.

162. Landry J, Chrétien P, Lambert H, Hickey E, Weber LA. Heat shock resistance conferred by expression of the human HSP27 gene in rodent cells. J Cell Biol. 1989 Jul;109(1):7–15.

163. Kohn G, Wong HR, Bshesh K, Zhao B, Vasi N, Denenberg A, et al. Heat shock inhibits tnf-induced ICAM-1 expression in human endothelial cells via I kappa kinase inhibition. Shock. 2002 Feb;17(2):91–7.

164. Gao L, Wang F, Wang B, Gong B, Zhang J, Zhang X, et al. Cilostazol protects diabetic rats from vascular inflammation via nuclear factor-kappa B-dependent down-regulation of vascular cell adhesion molecule-1 expression. J Pharmacol Exp Ther. 2006 Jul;318(1):53–8.

165. Wieten L, Berlo SE, Brink Ten CB, van Kooten PJ, Singh M, van der Zee R, et al. IL-10 is critically involved in mycobacterial HSP70 induced suppression of proteoglycan-induced arthritis. PLoS ONE. 2009;4(1):e4186.

166. Treinin M, Shliar J, Jiang H, Powell-Coffman JA, Bromberg Z, Horowitz M. HIF-1 is required for heat acclimation in the nematode Caenorhabditis elegans. Physiol Genomics. 2003 Jun 24;14(1):17–24.

167. Bayley M, Petersen SO, Knigge T, Köhler HR, Holmstrup M. Drought acclimation confers cold tolerance in the soil collembolan Folsomia candida. J Insect Physiol. 2001 Sep;47(10):1197–204.

168. Todgham AE, Schulte PM, Iwama GK. Cross-tolerance in the tidepool sculpin: the role of heat shock proteins. Physiol Biochem Zool. 2005 Mar;78(2):133–44.

169. McClung JP, Hasday JD, He J-R, Montain SJ, Cheuvront SN, Sawka MN, et al. Exercise-heat acclimation in humans alters baseline levels and ex vivo heat inducibility of HSP72 and HSP90 in peripheral blood mononuclear cells. Am J Physiol Regul Integr Comp Physiol. 2008 Jan;294(1):R185–91.

170. Mirit E, Palmon A, Hasin Y, Horowitz M. Heat acclimation induces changes in cardiac mechanical performance: the role of thyroid hormone. Am J Physiol. 1999 Feb;276(2):R550–8.

171. Cohen O, Kanana H, Zoizner R, Gross C, Meiri U, Stern MD, et al. Altered Ca2+ handling and myofilament desensitization underlie cardiomyocyte performance in normothermic and hyperthermic heat-acclimated rat hearts. J Appl Physiol. 2007 Jul;103(1):266–75.

172. Horowitz M. Matching the heart to heat-induced circulatory load: heat-acclimatory responses. News Physiol Sci. 2003 Dec;18:215–21.

173. Maloyan A, Palmon A, Horowitz M. Heat acclimation increases the basal HSP72 level and alters its production dynamics during heat stress. Am J Physiol. 1999 May;276(5 Pt 2):R1506–15.

174. Lyashko VN, Vikulova VK, Chernicov VG, Ivanov VI, Ulmasov KA, Zatsepina OG, et al. Comparison of the heat shock response in ethnically and ecologically different human populations. Proc Natl Acad Sci USA. 1994 Dec 20;91(26):12492–5.

175. Horowitz M, Maloyan A, Shlaier J. HSP 70 kDa dynamics in animals undergoing heat stress superimposed on heat acclimation. Ann N Y Acad Sci. 1997 Mar 15;813:617–9.

176. Ulmasov KA, Shammakov S, Karaev K, Evgen'ev MB. Heat shock proteins and thermoresistance in lizards. Proc Natl Acad Sci USA. 1992 Mar 1;89(5):1666–70.

177. Gehring WJ, Wehner R. Heat shock protein synthesis and thermotolerance in Cataglyphis, an ant from the Sahara desert. Proc Natl Acad Sci USA. 1995 Mar 28;92(7):2994–8.

178. Levi E, Vivi A, Hasin Y, Tassini M, Navon G, Horowitz M. Heat acclimation improves cardiac mechanics and metabolic performance during ischemia and reperfusion. J Appl Physiol. 1993 Aug;75(2):833–9.

179. Yacobi A, Bach YS, Horowitz M. The protective effect of heat acclimation from hypoxic damage in the brain involves changes in the expression of glutamate receptors. Temperature. 2014;1:57–65.

180. Arieli Y, Eynan M, Gancz H, Arieli R, Kashi Y. Heat acclimation prolongs the time to central nervous system oxygen toxicity in the rat. Possible involvement of HSP72. Brain Res. 2003 Feb 7;962(1-2):15–20.

181. Horowitz M, Meiri U. Thermoregulatory activity in the rat: effects of hypohydration, hypovolemia and hypertonicity and their interaction with short-term heat acclimation. Comp Biochem Physiol A Comp Physiol. 1985;82(3):577–82.

182. Assayag M, Gerstenblith G, Stern MD, Horowitz M. Long- but not short-term heat acclimation produces an apoptosis-resistant cardiac phenotype: a lesson from heat stress and ischemic/reperfusion insults. Cell Stress Chaperones. 2010 Sep;15(5):651–64.

183. Gaffney DK, Lundquist M, Warters RL, Rosley R. Effects of modifying topoisomerase II levels on cellular recovery from radiation damage. Radiat Res. 2000 Oct;154(4):461–6.

184. Kültz D, Chakravarty D. Maintenance of genomic integrity in mammalian kidney cells exposed to hyperosmotic stress. Comp Biochem Physiol, Part A Mol Integr Physiol. 2001 Oct;130(3):421–8.

185. Beit-Yannai E, Trembovler V, Horowitz M, Lazarovici P, Kohen R, Shohami E. Neuroprotection against oxidative stress by serum from heat acclimated rats. Neurosci Lett. 1998 Sep 25;254(2):89–92.

186. Eynan M, Knubuvetz T, Meiri U, Navon G, Gerstenblith G, Bromberg Z, et al. Heat acclimation-induced elevated glycogen, glycolysis, and low thyroxine improve heart ischemic tolerance. J Appl Physiol. 2002 Dec;93(6):2095–104.

187. O'Keeffe LM, Muir G, Piterina AV, McGloughlin T. Vascular cell adhesion molecule-1 expression in endothelial cells exposed to physiological coronary wall shear stresses. J Biomech Eng. 2009 Aug;131(8):081003.

188. Hastings NE, Simmers MB, McDonald OG, Wamhoff BR, Blackman BR. Atherosclerosis-prone hemodynamics differentially regulates endothelial and smooth muscle cell phenotypes and promotes pro-inflammatory priming. Am J Physiol Cell Physiol. 2007 Dec;293(6):C1824–33.

189. Chappell DC, Varner SE, Nerem RM, Medford RM, Alexander RW. Oscillatory shear stress stimulates adhesion molecule expression in cultured human endothelium. Circ Res. 1998 Mar 23;82(5):532–9.

190. Ziegler T, Bouzourène K, Harrison VJ, Brunner HR, Hayoz D. Influence of oscillatory and unidirectional flow environments on the expression of endothelin and nitric oxide synthase in cultured endothelial cells. Arterioscler Thromb Vasc Biol. 1998 May;18(5):686–92.

191. Hwang J, Ing MH, Salazar A, Lassègue B, Griendling K, Navab M, et al. Pulsatile versus oscillatory shear stress regulates NADPH oxidase subunit expression: implication for native LDL oxidation. Circ Res. 2003 Dec 12;93(12):1225–32.

192. McNally JS, Davis ME, Giddens DP, Saha A, Hwang J, Dikalov S, et al. Role of xanthine oxidoreductase and NAD(P)H oxidase in endothelial superoxide production in response to oscillatory shear stress. Am J Physiol Heart Circ Physiol. 2003 Dec;285(6):H2290–7.

193. Lawrence MB, McIntire LV, Eskin SG. Effect of flow on polymorphonuclear leukocyte/endothelial cell adhesion. Blood. 1987 Nov;70(5):1284–90.

194. Davies PF, Remuzzi A, Gordon EJ, Dewey CF, Gimbrone MA. Turbulent fluid shear stress induces vascular endothelial cell turnover in vitro. Proc Natl Acad Sci USA. 1986 Apr;83(7):2114–7.

195. Gambillara V, Chambaz C, Montorzi G, Roy S, Stergiopulos N, Silacci P. Plaque-prone hemodynamics impair endothelial function in pig carotid arteries. Am J Physiol Heart Circ Physiol. 2006 Jun;290(6):H2320–8.

196. Nam D, Ni C-W, Rezvan A, Suo J, Budzyn K, Llanos A, et al. Partial carotid ligation is a model of acutely induced disturbed flow, leading to rapid endothelial dysfunction and atherosclerosis. Am J Physiol Heart Circ Physiol. 2009 Oct;297(4):H1535–43.

197. Cheng C, Tempel D, van Haperen R, van der Baan A, Grosveld F, Daemen MJAP, et al. Atherosclerotic lesion size and vulnerability are determined by patterns of fluid shear stress. Circulation. 2006 Jun 13;113(23):2744–53.

198. Dinenno FA, Jones PP, Seals DR, Tanaka H. Limb blood flow and vascular conductance are reduced with age in healthy humans: relation to elevations in sympathetic nerve activity and declines in oxygen demand. Circulation. 1999 Jul 13;100(2):164–70.

199. Credeur DP, Dobrosielski DA, Arce-Esquivel AA, Welsch MA. Brachial artery retrograde flow increases with age: relationship to physical function. Eur J Appl Physiol. 2009 Sep;107(2):219–25.

200. Cardillo C, Campia U, Iantorno M, Panza JA. Enhanced vascular activity of endogenous endothelin-1 in obese hypertensive patients. Hypertension. 2004 Jan;43(1):36–40.

201. Taddei S, Virdis A, Ghiadoni L, Sudano I, Notari M, Salvetti A. Vasoconstriction to endogenous endothelin-1 is increased in the peripheral circulation of patients with essential hypertension. Circulation. 1999 Oct 19;100(16):1680–3.

202. Padilla J, Simmons GF, Fadel PF, Laughlin HM, Joyner MJ, Casey DP. Impact of Aging on Conduit Artery Retrograde and Oscillatory Shear at Rest and During Exercise Role of Nitric Oxide. Hypertension. 2011 Apr 4;:1–6.

203. Casey DP, Padilla J, Joyner MJ. α-adrenergic vasoconstriction contributes to the age-related increase in conduit artery retrograde and oscillatory shear. Hypertension. 2012 Oct;60(4):1016–22.

204. Cardillo C, Campia U, Kilcoyne CM, Bryant MB, Panza JA. Improved endothelium-dependent vasodilation after blockade of endothelin receptors in patients with essential hypertension. Circulation. 2002 Jan 29;105(4):452–6.

205. Schinzari F, Iantorno M, Campia U, Mores N, Rovella V, Tesauro M, et al. Vasodilator responses and endothelin-dependent vasoconstriction in metabolically healthy obesity and the metabolic syndrome. Am J Physiol Endocrinol Metab. 2015 Nov 1;309(9):E787–92.

206. Seals DR, DeSouza CA, Donato AJ, Tanaka H. Habitual exercise and arterial aging. J Appl Physiol. 2008 Jul 10;105(4):1323–32.

207. Zeiher AM, Drexler H, Wollschläger H, Just H. Modulation of coronary vasomotor tone in humans. Progressive endothelial dysfunction with different early stages of coronary atherosclerosis. Circulation. 1991 Feb;83(2):391–401.

208. Zeiher AM, Drexler H, Wollschläger H, Just H. Endothelial dysfunction of the coronary microvasculature is associated with coronary blood flow regulation in patients with early atherosclerosis. Circulation. 1991 Nov;84(5):1984–92.

209. Thijssen DHJ, Dawson EA, Tinken TM, Cable NT, Green DJ. Retrograde Flow and Shear Rate Acutely Impair Endothelial Function in Humans. Hypertension. 2009 May 20;53(6):986–92.

210. Jenkins NT, Padilla J, Boyle LJ, Credeur DP, Laughlin MH, Fadel PJ. Disturbed Blood Flow Acutely Induces Activation and Apoptosis of the Human Vascular Endothelium. Hypertension. 2013 Feb 13;61(3):615–21.

211. Jimenez JJ, Jy W, Mauro LM, Soderland C, Horstman LL, Ahn YS. Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. Thromb Res. 2003 Feb 15;109(4):175–80.

212. Habersberger J, Strang F, Scheichl A, Htun N, Bassler N, Merivirta R-M, et al. Circulating microparticles generate and transport monomeric C-reactive protein in patients with myocardial infarction. Cardiovasc Res. 2012 Oct 1;96(1):64–72.

213. Diehl P, Fricke A, Sander L, Stamm J, Bassler N, Htun N, et al. Microparticles: major transport vehicles for distinct microRNAs in circulation. Cardiovasc Res. 2012 Mar 15;93(4):633–44.

214. Agouni A, Lagrue-Lak-Hal AH, Ducluzeau PH, Mostefai HA, Draunet-Busson C, Lefthériotis G, et al. Endothelial dysfunction caused by circulating microparticles from patients with metabolic syndrome. Am J Pathol. 2008 Oct;173(4):1210–9.

215. Dignat-George F, Boulanger CM. The many faces of endothelial microparticles. Arterioscler Thromb Vasc Biol. 2011 Jan;31(1):27–33.

216. Boulanger CM, Amabile N, Guérin AP, Pannier B, Leroyer AS, Mallat CNZ, et al. In vivo shear stress determines circulating levels of endothelial microparticles in end-stage renal disease. Hypertension. 2007 Apr;49(4):902–8.

217. Dimmeler S, Hermann C, Galle J, Zeiher AM. Upregulation of superoxide dismutase and nitric oxide synthase mediates the apoptosis-suppressive effects of shear stress on endothelial cells. Arterioscler Thromb Vasc Biol. 1999 Mar;19(3):656–64.

218. Uematsu M, Ohara Y, Navas JP, Nishida K, Murphy TJ, Alexander RW, et al. Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress. Am J Physiol. 1995 Dec;269(6):C1371–8.

219. Ranjan V, Xiao Z, Diamond SL. Constitutive NOS expression in cultured endothelial cells is elevated by fluid shear stress. Am J Physiol. 1995 Aug;269(2):H550–5.

220. Widder JD, Chen W, Li L, Dikalov S, Thöny B, Hatakeyama K, et al. Regulation of tetrahydrobiopterin biosynthesis by shear stress. Circ Res. 2007 Oct 12;101(8):830–8.

221. Jin Z-G, Ueba H, Tanimoto T, Lungu AO, Frame MD, Berk BC. Ligand-independent activation of vascular endothelial growth factor receptor 2 by fluid shear stress regulates activation of endothelial nitric oxide synthase. Circ Res. 2003 Aug 22;93(4):354–63.

222. Boo YC, Hwang J, Sykes M, Michell BJ, Kemp BE, Lum H, et al. Shear stress stimulates phosphorylation of eNOS at Ser(635) by a protein kinase A-dependent mechanism. Am J Physiol Heart Circ Physiol. 2002 Nov;283(5):H1819–28.

223. Kuchan MJ, Frangos JA. Shear stress regulates endothelin-1 release via protein kinase C and cGMP in cultured endothelial cells. Am J Physiol. 1993 Jan;264(1):H150–6.

224. Malek A, Izumo S. Physiological fluid shear stress causes downregulation of endothelin-1 mRNA in bovine aortic endothelium. Am J Physiol. 1992 Aug;263(2):C389–96.

225. Tsao PS, Buitrago R, Chan JR, Cooke JP. Fluid flow inhibits endothelial adhesiveness. Nitric oxide and transcriptional regulation of VCAM-1. Circulation. 1996 Oct 1;94(7):1682–9.

226. Himburg HA, Dowd SE, Friedman MH. Frequency-dependent response of the vascular endothelium to pulsatile shear stress. Am J Physiol Heart Circ Physiol. 2007 Jul;293(1):H645–53.

227. Woodman CR, Muller JM, Rush JW, Laughlin MH, Price EM. Flow regulation of ecNOS and Cu/Zn SOD mRNA expression in porcine coronary arterioles. Am J Physiol. 1999 Mar;276(3):H1058–63.

228. Woodman CR, Price EM, Laughlin MH. Shear stress induces eNOS mRNA expression and improves endothelium-dependent dilation in senescent soleus muscle feed arteries. J Appl Physiol. 2005 Mar;98(3):940–6.

229. Green DJ, Carter HH, Fitzsimons MG, Cable NT, Thijssen DHJ, Naylor LH. Obligatory role of hyperaemia and shear stress in microvascular adaptation to repeated heating in humans. J Physiol. 2010 Apr 30;588(9):1571–7.

230. Birk GK, Dawson EA, Atkinson C, Haynes A, Cable NT, Thijssen DHJ, et al. Brachial artery adaptation to lower limb exercise training: role of shear stress. J Appl Physiol. 2012 May 15;112(10):1653–8.

231. Thijssen DHJ, Dawson EA, van den Munckhof ICL, Tinken TM, Drijver den E, Hopkins N, et al. Exercise-mediated changes in conduit artery wall thickness in humans: role of shear stress. Am J Physiol Heart Circ Physiol. 2011 Jun 29;301(1):H241–6.

232. Niebauer J, Cooke JP. Cardiovascular effects of exercise: role of endothelial shear stress. J Am Coll Cardiol. 1996 Dec;28(7):1652–60.

233. Laughlin MH, Newcomer SC, Bender SB. Importance of hemodynamic forces as signals for exercise-induced changes in endothelial cell phenotype. J Appl Physiol. 2008 Mar;104(3):588–600.

234. Tuttle JL, Nachreiner RD, Bhuller AS, Condict KW, Connors BA, Herring BP, et al. Shear level influences resistance artery remodeling: wall dimensions, cell density, and eNOS expression. Am J Physiol Heart Circ Physiol. 2001 Sep;281(3):H1380–9.

235. Hambrecht R, Adams V, Erbs S, Linke A, Kränkel N, Shu Y, et al. Regular physical activity improves endothelial function in patients with coronary artery disease by increasing phosphorylation of endothelial nitric oxide synthase. Circulation. 2003 Jul 1;107(25):3152–8.

236. Touati S, Montezano ACI, Meziri F, Riva C, Touyz RM, Laurant P. Exercise training protects against atherosclerotic risk factors through vascular NADPH oxidase, extracellular signal-regulated kinase 1/2 and stress-activated protein kinase/c-Jun N-terminal kinase downregulation in obese rats. Clin Exp Pharmacol Physiol. 2015 Feb;42(2):179–85.

237. Dinenno FA, Tanaka H, Monahan KD, Clevenger CM, Eskurza I, DeSouza CA, et al. Regular endurance exercise induces expansive arterial remodelling in the trained limbs of healthy men. J Physiol. 2001 Jul 1;534(Pt 1):287–95.

238. Miyachi M, Tanaka H, Yamamoto K, Yoshioka A, Takahashi K, Onodera S. Effects of one-legged endurance training on femoral arterial and venous size in healthy humans. J Appl Physiol. 2001 Jun;90(6):2439–44.

239. Johnson LR, Laughlin MH. Chronic exercise training does not alter pulmonary vasorelaxation in normal pigs. J Appl Physiol. 2000 Jun;88(6):2008–14.

240. Oltman CL, Parker JL, Adams HR, Laughlin MH. Effects of exercise training on vasomotor reactivity of porcine coronary arteries. Am J Physiol. 1992 Aug;263(2):H372–82.

241. Oltman CL, Parker JL, Laughlin MH. Endothelium-dependent vasodilation of proximal coronary arteries from exercise-trained pigs. J Appl Physiol. 1995 Jul;79(1):33–40.

242. Tang BT, Cheng CP, Draney MT, Wilson NM, Tsao PS, Herfkens RJ, et al. Abdominal aortic hemodynamics in young healthy adults at rest and during lower limb exercise: quantification using image-based computer modeling. Am J Physiol Heart Circ Physiol. 2006 Aug;291(2):H668–76.

243. Green DJ, Maiorana A, O'Driscoll G, Taylor R. Effect of exercise training on endothelium-derived nitric oxide function in humans. J Physiol. 2004 Nov 15;561(1):1–25.

244. Lutjemeier BJ, Miura A, Scheuermann BW, Koga S, Townsend DK, Barstow TJ. Muscle contraction-blood flow interactions during upright knee extension exercise in humans. J Appl Physiol. 2005 Apr;98(4):1575–83.

245. Sobajima M, Nozawa T, Ihori H, Shida T, Ohori T, Suzuki T, et al. Repeated sauna therapy improves myocardial perfusion in patients with occluded coronary artery-related ischemia. Int J Cardiol. Elsevier Ireland Ltd; 2013 Jul 15;167(1):237–43.

246. Tei C, Tanaka N. Thermal vasodilation as a treatment of congestive heart failure: a novel approach. J Cardiol. 1996 Jan;27(1):29–30.

247. Kihara T, Biro S, Ikeda Y, Fukudome T, Shinsato T, Masuda A, et al. Effects of repeated sauna treatment on ventricular arrhythmias in patients with chronic heart failure. Circ J. 2004 Dec;68(12):1146–51.

248. Kuwahata S, Miyata M, Fujita S, Kubozono T, Shinsato T, Ikeda Y, et al. Improvement of autonomic nervous activity by Waon therapy in patients with chronic heart failure. J Cardiol. 2011 Jan;57(1):100–6.

249. Kihara T, Biro S, Imamura M, Yoshifuku S, Takasaki K, Ikeda Y, et al. Repeated sauna treatment improves vascular endothelial and cardiac function in patients with chronic heart failure. J Am Coll Cardiol. 2002 Mar 6;39(5):754–9.

250. Akasaki Y, Miyata M, Eto H, Shirasawa T, Hamada N, Ikeda Y, et al. Repeated thermal therapy up-regulates endothelial nitric oxide synthase and augments angiogenesis in a mouse model of hindlimb ischemia. Circ J. 2006 Apr;70(4):463–70.

251. Winterfeld HJ, Siewert H, Strangfeld D, Warnke H, Kruse J, Engelmann U. [Potential use of the sauna in the long-term treatment of hypertensive cardiovascular circulation disorders--a comparison with kinesiotherapy]. Schweiz Rundsch Med Prax. 1992 Aug 25;81(35):1016–20.

252. Tei C, Shinsato T, Miyata M, Kihara T, Hamasaki S. Waon therapy improves peripheral arterial disease. J Am Coll Cardiol. 2007 Nov 27;50(22):2169–71.

253. Tei C, Shinsato T, Kihara T, Miyata M. Successful thermal therapy for end-stage peripheral artery disease. J Cardiol. 2006 Apr;47(4):163–4.

254. Winterfeld HJ, Siewert H, Strangfeld D, Bohm J, Aurisch R, Engelmann U, et al. [Sauna therapy in coronary heart disease with hypertension after bypass operation, in heart aneurysm operation and in essential hypertension]. Z Gesamte Inn Med. 1993 May;48(5):247–50.

255. Siewert C, Siewert H, Winterfeld HJ, Strangfeld D. [The behavior of central and peripheral hemodynamics in isometric and dynamic stress in hypertensive patients treatment with regular sauna therapy]. Z Kardiol. 1994 Sep;83(9):652–7.

256. Laitinen LA, Lindqvist A, Heino M. Lungs and ventilation in sauna. Ann Clin Res. 1988;20(4):244–8.

257. Isomäki H. The sauna and rheumatic diseases. Ann Clin Res. 1988;20(4):271–5.

258. Cox NJ, Oostendorp GM, Folgering HT, van Herwaarden CL. Sauna to transiently improve pulmonary function in patients with obstructive lung disease. Arch Phys Med Rehabil. 1989 Dec;70(13):911–3.

259. Ernst E, Pecho E, Wirz P, Saradeth T. Regular sauna bathing and the incidence of common colds. Ann Med. 1990;22(4):225–7.

260. Minson CT, Halliwill JR, Young TM, Joyner MJ. Influence of the menstrual cycle on sympathetic activity, baroreflex sensitivity, and vascular transduction in young women. Circulation. 2000 Feb 29;101(8):862–8.

261. Minson CT, Halliwill JR, Young TM, Joyner MJ. Sympathetic activity and baroreflex sensitivity in young women taking oral contraceptives. Circulation. 2000 Sep 26;102(13):1473–6.

262. Gagge AP, Gonzalez RR. Mechanisms of heat exchange: biophysics and physiology. In: Fregly MJ, Blatteis CM, editors. Handbook of Physiology: Environmental Physiology. Bethesda, MD: American Physiological Society; 1996.

263. Fox RH, Goldsmith R, Kidd DJ, Lewis HE. Acclimatization to heat in man by controlled elevation of body temperature. J Physiol. 1963 May;166:530–47.

264. Kenny GP, Giesbrecht GG, Thoden JS. A comparison of human thermoregulatory response following dynamic exercise and warm-water immersion. Eur J Appl Physiol Occup Physiol. 1996;74(4):336–41.

265. Thrush A, Hartshorne T. Vascular ultrasound: How, why and when. Thrush A, Hartshorne T, editors. London: Elsevier; 2010.

266. Potter K, Reed CJ, Green DJ, Hankey GJ, Arnolda LF. Ultrasound settings significantly alter arterial lumen and wall thickness measurements. Cardiovasc Ultrasound. 2008;6:6.

267. Kelly R, Hayward C, Ganis J, Daley J, Avolio A, O'Rourke M. Noninvasive registration of the arterial pressure pulse waveform using high-fidelity applanation tonometry. J Vasc Med Biol. 1989 Mar 10;1:142–9.

268. Armentano R, Megnien JL, Simon A, Bellenfant F, Barra J, Levenson J. Effects of hypertension on viscoelasticity of carotid and femoral arteries in humans. Hypertension. 1995 Jul;26(1):48–54.

269. Tanaka H, Dinenno FA, Monahan KD, Clevenger CM, DeSouza CA, Seals DR. Aging, habitual exercise, and dynamic arterial compliance. Circulation. 2000 Sep 12;102(11):1270–5.

270. Meyer B, Mörtl D, Strecker K, Hülsmann M, Kulemann V, Neunteufl T, et al. Flow-mediated vasodilation predicts outcome in patients with chronic heart failure: comparison with B-type natriuretic peptide. J Am Coll Cardiol. 2005 Sep 20;46(6):1011–8.

271. Shechter M, Issachar A, Marai I, Koren-Morag N, Freinark D, Shahar Y, et al. Long-term association of brachial artery flow-mediated vasodilation and cardiovascular events in middle-aged subjects with no apparent heart disease. Int J Cardiol. 2009 May 1;134(1):52–8.

272. Yeboah J, Crouse JR, Hsu F-C, Burke GL, Herrington DM. Brachial flow-mediated dilation predicts incident cardiovascular events in older adults: the Cardiovascular Health Study. Circulation. 2007 May 8;115(18):2390–7.

273. Anderson TJ, Uehata A, Gerhard MD, Meredith IT, Knab S, Delagrange D, et al. Close relation of endothelial function in the human coronary and peripheral circulations. J Am Coll Cardiol. 1995 Nov 1;26(5):1235–41.

274. Joannides R, Haefeli WE, Linder L, Richard V, Bakkali EH, Thuillez C, et al. Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. Circulation. 1995 Mar 1;91(5):1314–9.

275. Doshi SN, Naka KK, Payne N, Jones CJ, Ashton M, Lewis MJ, et al. Flow-mediated dilatation following wrist and upper arm occlusion in humans: the contribution of nitric oxide. Clin Sci. 2001 Dec;101(6):629–35.

276. Bellien J, Iacob M, Gutierrez L, Isabelle M, Lahary A, Thuillez C, et al. Crucial Role of NO and Endothelium-Derived Hyperpolarizing Factor in Human Sustained Conduit Artery Flow-Mediated Dilatation. Hypertension. 2006 Oct 23;48(6):1088–94.

277. Black MA, Cable NT, Thijssen DHJ, Green DJ. Importance of Measuring the Time Course of Flow-Mediated Dilatation in Humans. Hypertension. 2008 Jan 2;51(2):203–10.

278. Thijssen DHJ, Black MA, Pyke KE, Padilla J, Atkinson G, Harris RA, et al. Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. Am J Physiol Heart Circ Physiol. 2011 Jan 3;300(1):H2–H12.

279. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, et al. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. J Am Coll Cardiol. 2002 Jan 16;39(2):257–65.

280. Woodman RJ, Playford DA, Watts GF, Cheetham C, Reed C, Taylor RR, et al. Improved analysis of brachial artery ultrasound using a novel edge-detection software system. J Appl Physiol. 2001 Aug;91(2):929–37.

281. Pyke KE, Tschakovsky ME. The relationship between shear stress and flow-mediated dilatation: implications for the assessment of endothelial function. J Physiol. 2005 Oct 15;568(2):357–69.

282. Padilla J, Johnson BD, Newcomer SC, Wilhite DP, Mickleborough TD, Fly AD, et al. Adjusting flow-mediated dilation for shear stress stimulus allows demonstration of endothelial dysfunction in a population with moderate cardiovascular risk. J Vasc Res. 2009;46(6):592–600.

283. Bank AJ, Sih R, Mullen K, Osayamwen M, Lee PC. Vascular ATP-dependent potassium channels, nitric oxide, and human forearm reactive hyperemia. Cardiovasc Drugs Ther. 2000 Feb;14(1):23–9.

284. Carlsson I, Sollevi A, Wennmalm A. The role of myogenic relaxation, adenosine and prostaglandins in human forearm reactive hyperaemia. J Physiol. 1987 Aug;389:147–61.

285. Meredith IT, Currie KE, Anderson TJ, Roddy MA, Ganz P, Creager MA. Postischemic vasodilation in human forearm is dependent on endothelium-derived nitric oxide. Am J Physiol. 1996 Apr;270(4):H1435–40.

286. Anderson TJ, Charbonneau F, Title LM, Buithieu J, Rose MS, Conradson H, et al. Microvascular function predicts cardiovascular events in primary prevention: long-term results from the Firefighters and Their Endothelium (FATE) study. Circulation. 2011 Jan 18;123(2):163–9.

287. Huang AL, Silver AE, Shvenke E, Schopfer DW, Jahangir E, Titas MA, et al. Predictive value of reactive hyperemia for cardiovascular events in patients with peripheral arterial disease undergoing vascular surgery. Arterioscler Thromb Vasc Biol. 2007 Oct;27(10):2113–9.

288. Lind L, Berglund L, Larsson A, Sundström J. Endothelial function in resistance and conduit arteries and 5-year risk of cardiovascular disease. Circulation. 2011 Apr 12;123(14):1545–51.

289. Patterson GC, Whelan RF. Reactive hyperaemia in the human forearm. Clin Sci. 1955 May;14(2):197–211.

290. Thijssen DHJ, Green DJ, Hopman MTE. Blood vessel remodeling and physical inactivity in humans. J Appl Physiol. 2011 Dec;111(6):1836–45.

291. Takeshita A, Mark AL. Decreased vasodilator capacity of forearm resistance vessels in borderline hypertension. Hypertension. 1980 Sep;2(5):610–6.

292. Agewall S, Whalley GA, Doughty RN, Sharpe N. Handgrip exercise increases postocclusion hyperaemic brachial artery dilatation. Heart. 1999 Jul;82(1):93–5.

293. O'Rourke MF, Vlachopoulos C, Graham RM. Spurious systolic hypertension in youth. Vasc Med. 2000;5(3):141–5.

294. Janner JH, Godtfredsen NS, Ladelund S, Vestbo J, Prescott E. Aortic augmentation index: reference values in a large unselected population by means of the SphygmoCor device. Am J Hypertens. 2010 Feb;23(2):180–5.

295. Fronek K, Bloor CM, Amiel D, Chvapil M. Effect of long-term sympathectomy on the arterial wall in rabbits and rats. Exp Mol Pathol. 1978 Jun;28(3):279–89.

296. Laurent S, Boutouyrie P, Asmar R, Gautier I, Laloux B, Guize L, et al. Aortic stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients. Hypertension. 2001 May;37(5):1236–41.

297. Tu S-T, Wang I-W, Lin H-F, Liao Y-C, Lin R-T, Liu C-S, et al. Carotid intima-media thickness and stiffness are independent risk factors for atherosclerotic diseases. J Investig Med. 2010 Aug;58(6):786–90.

298. Boutouyrie P, Tropeano AI, Asmar R, Gautier I, Benetos A, Lacolley P, et al. Aortic stiffness is an independent predictor of primary coronary events in hypertensive patients: a longitudinal study. Hypertension. 2002 Jan;39(1):10–5.

299. Levy D, Garrison RJ, Savage DD. Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. N Engl J Med. 1990;322(22):1561–6.

300. O'Rourke MF, Mancia G. Arterial stiffness. J Hypertens. 1999 Jan;17(1):1–4.

301. van Popele NM, Grobbee DE, Bots ML, Asmar R, Topouchian J, Reneman RS, et al. Association between arterial stiffness and atherosclerosis: the Rotterdam Study. Stroke. 2001 Feb;32(2):454–60.

302. Blacher J, Asmar R, Djane S, London GM, Safar ME. Aortic pulse wave velocity as a marker of cardiovascular risk in hypertensive patients. Hypertension. 1999 May;33(5):1111–7.

303. Reneman RS, Van Merode T, Hick P, Muytjens AM, Hoeks AP. Age-related changes in carotid artery wall properties in men. Ultrasound Med Biol. 1986 Jun;12(6):465–71.

304. Giannattasio C, Failla M, Mangoni AA, Scandola L, Fraschini N, Mancia G. Evaluation of arterial compliance in humans. Clin Exp Hypertens. 1996 Apr;18(3-4):347–62.

305. Kawasaki T, Sasayama S, Yagi S, Asakawa T, Hirai T. Non-invasive assessment of the age related changes in stiffness of major branches of the human arteries. Cardiovasc Res. 1987 Sep;21(9):678–87.

306. Lakatta EG, Levy D. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part I: aging arteries: a “set up” for vascular disease. Circulation. 2003 Jan 7;107(1):139–46.

307. Mohammed M, Zito C, Cusmà-Piccione M, Di Bella G, Antonini-Canterin F, Taha NM, et al. Arterial stiffness changes in patients with cardiovascular risk factors but normal carotid intima-media thickness. J Cardiovasc Med. 2013 Sep;14(9):622–8.

308. Jurasic M-J, Josef-Golubic S, Sarac R, Lovrencic-Huzjan A, Demarin V. Beta stiffness - setting age standards. Acta Clin Croat. 2009 Sep 23;48:253–8.

309. Adji A, O’nRourke MF, Namasivayam M. Arterial stiffnes, its assessment, prognostic value, and implications for treatment. Am J Hypertens. Nature Publishing Group; 2009 Nov 13;24(1):5–17.

310. Laurent S, Cockcroft J, Van Bortel L, Boutouyrie P, Giannattasio C, Hayoz D, et al. Expert consensus document on arterial stiffness: methodological issues and clinical applications. Eur Heart J. 2006 Nov;27(21):2588–605.

311. Mitchell GF, Hwang S-J, Vasan RS, Larson MG, Pencina MJ, Hamburg NM, et al. Arterial stiffness and cardiovascular events: the Framingham Heart Study. Circulation. 2010 Feb 2;121(4):505–11.

312. Mancia G, De Backer G, Dominiczak A, Cifkova R, Fagard R, Germano G, et al. 2007 Guidelines for the management of arterial hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). Eur Heart J. 2007 Jun;28(12):1462–536.

313. Huybrechts SAM, Devos DG, Vermeersch SJ, Mahieu D, Achten E, de Backer TLM, et al. Carotid to femoral pulse wave velocity: a comparison of real travelled aortic path lengths determined by MRI and superficial measurements. J Hypertens. 2011 Aug;29(8):1577–82.

314. O'Rourke MF, Staessen JA, Vlachopoulos C, Duprez D, Plante GE. Clinical applications of arterial stiffness; definitions and reference values. Am J Hypertens. 2002 May;15(5):426–44.

315. Mitchell GF, Wang N, Palmisano JN, Larson MG, Hamburg NM, Vita JA, et al. Hemodynamic correlates of blood pressure across the adult age spectrum: noninvasive evaluation in the Framingham Heart Study. Circulation. 2010 Oct 5;122(14):1379–86.

316. Hayashi K, Sugawara J, Komine H, Maeda S, Yokoi T. Effects of aerobic exercise training on the stiffness of central and peripheral arteries in middle-aged sedentary men. Jpn J Physiol. 2005 Aug;55(4):235–9.

317. Potter K, Green DJ, Reed CJ, Woodman RJ, Watts GF, McQuillan BM, et al. Carotid intima-medial thickness measured on multiple ultrasound frames: evaluation of a DICOM-based software system. Cardiovasc Ultrasound. 2007;5:29.

318. van den Munckhof ICL, Scholten R, Cable NT, Hopman MT, Green DJ, Thijssen DHJ. Impact of age and sex on carotid and peripheral arterial wall thickness in humans. Acta Physiol. 2012 Feb 6;206:220–8.

319. Agarwal SC, Allen J, Murray A, Purcell IF. Laser Doppler assessment of dermal circulatory changes in people with coronary artery disease. Microvas res. 2012 Feb 15;84:55–9.

320. Kruger A, Stewart J, Sahityani R, O'Riordan E, Thompson C, Adler S, et al. Laser Doppler flowmetry detection of endothelial dysfunction in end-stage renal disease patients: correlation with cardiovascular risk. Kidney Int. 2006 Jul;70(1):157–64.

321. Minson CT, Holowatz LA, Wong BJ, Kenney WL, Wilkins BW. Decreased nitric oxide- and axon reflex-mediated cutaneous vasodilation with age during local heating. J Appl Physiol. 2002 Nov;93(5):1644–9.

322. 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. 2011 Nov;58(5):935–42.

323. Holowatz LA, Thompson-Torgerson CS, Kenney WL. The human cutaneous circulation as a model of generalized microvascular function. J Appl Physiol. 2008 Jul;105(1):370–2.

324. Brunt VE, Minson CT. Cutaneous thermal hyperemia: more than skin deep. J Appl Physiol. 2011 May 5;111:5–7.

325. Minson CT. Thermal provocation to evaluate microvascular reactivity in human skin. J Appl Physiol. 2010 Oct;109(4):1239–46.

326. Fujii N, Brunt VE, Minson CT. Tempol improves cutaneous thermal hyperemia through increasing nitric oxide bioavailability in young smokers. Am J Physiol Heart Circ Physiol. 2014 Mar 28;306:H1507–11.

327. Hodges GJ, Nawaz S, Tew GA. Evidence that reduced nitric oxide signal contributes to cutaneous microvascular dysfunction in peripheral arterial disease. Clin Hemorheol Microcirc. 2015;59(1):83–95.

328. Boignard A, Salvat-Melis M, Carpentier PH, Minson CT, Grange L, Duc C, et al. Local hyperemia to heating is impaired in secondary Raynaud's phenomenon. Arthritis Res Ther. 2005;7(5):R1103–12.

329. Stewart JM, Medow MS, Minson CT, Taneja I. Cutaneous neuronal nitric oxide is specifically decreased in postural tachycardia syndrome. Am J Physiol Heart Circ Physiol. 2007 Oct;293(4):H2161–7.

330. Colberg SR, Parson HK, Nunnold T, Holton DR, Swain DP, Vinik AI. Change in cutaneous perfusion following 10 weeks of aerobic training in Type 2 diabetes. J Diabetes Complicat. 2005 Aug;19(5):276–83.

331. Minson CT, Berry LT, Joyner MJ. Nitric oxide and neurally mediated regulation of skin blood flow during local heating. J Appl Physiol. 2001 Apr 17;91:1619–26.

332. Wong BJ, Fieger SM. Transient receptor potential vanilloid type-1 (TRPV-1) channels contribute to cutaneous thermal hyperaemia in humans. J Physiol. 2010 Nov 1;588(21):4317–26.

333. 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. 1987;67(3):185–92.

334. Kellogg DL, Liu Y, Kosiba IF, O'Donnell D. Role of nitric oxide in the vascular effects of local warming of the skin in humans. J Appl Physiol. 1999 Apr;86(4):1185–90.

335. Fieger SM, Wong BJ. Adenosine receptor inhibition with theophylline attenuates the skin blood flow response to local heating in humans. Exp Physiol. 2010 Sep;95(9):946–54.

336. Medow MS, Bamji N, Clarke D, Ocon AJ, Stewart JM. Reactive Oxygen Species (ROS) from NADPH and Xanthine Oxidase Modulate the Cutaneous Local Heating Response in Healthy Humans. J Appl Physiol. 2011 Mar 24;111:20–6.

337. McCord GR, Cracowski J-L, Minson CT. Prostanoids contribute to cutaneous active vasodilation in humans. Am J Physiol Regul Integr Comp Physiol. 2006 Sep;291(3):R596–602.

338. Brunt VE, Minson CT. KCa channels and epoxyeicosatrienoic acids: major contributors to thermal hyperaemia in human skin. J Physiol. 2012 Jul 9;590(15):3523–34.

339. Hillig T, Krustrup P, Fleming I, Osada T, Saltin B, Hellsten Y. Cytochrome P450 2C9 plays an important role in the regulation of exercise-induced skeletal muscle blood flow and oxygen uptake in humans. J Physiol. 2003 Jan 1;546(1):307–14.

340. Ozkor MA, Murrow JR, Rahman AM, Kavtaradze N, Lin J, Manatunga A, et al. Endothelium-derived hyperpolarizing factor determines resting and stimulated forearm vasodilator tone in health and in disease. Circulation. 2011 May 24;123(20):2244–53.

341. Choi PJ, Brunt VE, Fujii N, Minson CT. New approach to measure cutaneous microvascular function: an improved test of NO-mediated vasodilation by thermal hyperemia. J Appl Physiol. 2014 Aug 1;117(3):277–83.

342. Medow MS, Minson CT, Stewart JM. Decreased microvascular nitric oxide-dependent vasodilation in postural tachycardia syndrome. Circulation. 2005 Oct 25;112(17):2611–8.

343. Sawka MN, Convertino VA, Eichner ER, Schnieder SM, Young AJ. Blood volume: importance and adaptations to exercise training, environmental stresses, and trauma/sickness. Med Sci Sports Exerc. 2000 Feb;32(2):332–48.

344. Schmidt W, Prommer N. The optimised CO-rebreathing method: a new tool to determine total haemoglobin mass routinely. Eur J Appl Physiol. 2005 Sep 29;:1–10.

345. Gregerson MI, Schiro H. The behavior of the dye T 1824 with respect to its absorption by red blood cells and its fate in blood undergoing coagulation. Am J Physiol. 1938 Jun 4;121:284.

346. Ebert RV, Stead EA. Demonstration that the cell plasma ratio of blood contained in minute vessels is lower than that of venous blood. J Clin Invest. 1941 May;20(3):317–21.

347. Kubota Y, Kleinman HK, Martin GR, Lawley TJ. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. J Cell Biol. 1988 Oct;107(4):1589–98.

348. Banek CT, Bauer AJ, Gingery A, Gilbert JS. Timing of ischemic insult alters fetal growth trajectory, maternal angiogenic balance, and markers of renal oxidative stress in the pregnant rat. Am J Physiol Regul Integr Comp Physiol. 2012 Sep 15;303(6):R658–64.

349. Goodwin AM. In vitro assays of angiogenesis for assessment of angiogenic and anti-angiogenic agents. Microvas res. 2007 Jul 12;74:172–83.

350. Donovan D, Brown NJ, Bishop ET, Lewis CE. Comparison of three in vitro human “angiogenesis” assays with capillaries formed in vivo. Angiogenesis. 2001 Jul 12;4(2):113–21.

351. Ziche M, Morbidelli L, Masini E, Amerini S, Granger HJ, Maggi CA, et al. Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P. J Clin Invest. 1994 Nov;94(5):2036–44.

352. Périard JD, Racinais S, Sawka MN. Adaptations and mechanisms of human heat acclimation: Applications for competitive athletes and sports. Scand J Med Sci Sports. 2015 Jun;25 Suppl 1:20–38.

353. Zuhl M, Dokladny K, Mermier C, Schneider S, Salgado R, Moseley P. The effects of acute oral glutamine supplementation on exercise-induced gastrointestinal permeability and heat shock protein expression in peripheral blood mononuclear cells. Cell Stress Chaperones. 2014 Jul 26;20(1):85–93.

354. Wyndham CH. Effect of acclimatization on the sweat rate-rectal temperature relationship. J Appl Physiol. 1967 Jan;22(1):27–30.

355. Bunn HF. Erythropoietin. Cold Spring Harb Perspect Med. 2013 Mar;3:a011619.

356. Bass DE, Buskirk ER, Iampietro PF, Mager M. Comparison of blood volume during physical conditioning, heat acclimatization and sedentary living. J Appl Physiol. 1958 Mar;12(2):186–8.

357. Fortney SM, Senay LC. Effect of training and heat acclimation on exercise responses of sedentary females. J Appl Physiol Respir Environ Exerc Physiol. 1979 Nov;47(5):978–84.

358. Amorim F, Yamada P, Robergs R, Schneider S, Moseley P. Effects of whole-body heat acclimation on cell injury and cytokine responses in peripheral blood mononuclear cells. Eur J Appl Physiol. 2010 Dec 30;111(8):1609–18.

359. Sareh H, Tulapurkar ME, Shah NG, Singh IS, Hasday JD. Response of mice to continuous 5-day passive hyperthermia resembles human heat acclimation. Cell Stress Chaperones. 2011 May;16(3):297–307.

360. Shastry S, Toft DO, Joyner MJ. HSP70 and HSP90 expression in leucocytes after exercise in moderately trained humans. Acta Physiologica Scandinavica. 2002 Jun;175(2):139–46.

361. Mackay RS, Marg E, Oeschsli R. Automatic tonometer with exact theory: various biological applications. Science. 1960 Jun 3;131(3414):1668–9.

362. Asmar R, Benetos A, Topouchian J, Laurent P, Pannier B, Brisac AM, et al. Assessment of arterial distensibility by automatic pulse wave velocity measurement. Validation and clinical application studies. Hypertension. 1995 Sep;26(3):485–90.

363. Imamura M, Biro S, Kihara T, Yoshifuku S, Takasaki K, Otsuji Y, et al. Repeated thermal therapy improves impaired vascular endothelial function in patients with coronary risk factors. J Am Coll Cardiol. 2001 Oct;38(4):1083–8.

364. Yeboah J, Folsom AR, Burke GL, Johnson C, Polak JF, Post W, et al. Predictive value of brachial flow-mediated dilation for incident cardiovascular events in a population-based study: the multi-ethnic study of atherosclerosis. Circulation. 2009 Aug 11;120(6):502–9.

365. Tinken TM, Thijssen DHJ, Black MA, Cable NT, Green DJ. Time course of change in vasodilator function and capacity in response to exercise training in humans. J Physiol. 2008 Oct 15;586(Pt 20):5003–12.

366. Green DJ, Spence A, Halliwill JR, Cable NT, Thijssen DHJ. Exercise and vascular adaptation in asymptomatic humans. Exp Physiol. 2010 Jul 13;96.2:57–70.

367. Miyauchi T, Miyata M, Ikeda Y, Akasaki Y, Hamada N, Shirasawa T, et al. Waon Therapy Upregulates Hsp90 and Leads to Angiogenesis Through the Akt-Endothelial Nitric Oxide Synthase Pathway in Mouse Hindlimb Ischemia. Circ J. 2012;76(7):1712–21.

368. Sun J, Liao JK. Induction of Angiogenesis by Heat Shock Protein 90 Mediated by Protein Kinase Akt and Endothelial Nitric Oxide Synthase. Arterioscler Thromb Vasc Biol. 2004 Dec 1;24(12):2238–44.

369. Agabiti-Rosei E, Muiesan ML. Carotid atherosclerosis, arterial stiffness and stroke events. Adv Cardiol. 2007;44:173–86.

370. Ganio MS, Brothers RM, Shibata S, Hastings JL, Crandall CG. Effect of passive heat stress on arterial stiffness. Exp Physiol. 2011 Sep;96(9):919–26.

371. Hunter SD, Dhindsa MS, Cunningham E, Tarumi T, Alkatan M, Nualnim N, et al. The Effect of Bikram Yoga on Arterial Stiffness in Young and Older Adults. J Altern Complement Med. 2013 Jun 5;19(12):930–4.

372. Oliver JJ, Webb DJ. Noninvasive assessment of arterial stiffness and risk of atherosclerotic events. Arterioscler Thromb Vasc Biol. 2003 Apr 1;23(4):554–66.

373. Connolly EM, Kelly CJ, Chen G, O'grady T, Kay E, Leahy A, et al. Pharmacological induction of HSP27 attenuates intimal hyperplasia in vivo. Eur J Vasc Endovasc Surg. 2003 Jan;25(1):40–7.

374. Zheng Y, Im C-N, Seo J-S. Inhibitory effect of Hsp70 on angiotensin II-induced vascular smooth muscle cell hypertrophy. Exp Mol Med. 2006 Oct 31;38(5):509–18.

375. Wilkinson IB, MacCallum H, Cockcroft JR, Webb DJ. Inhibition of basal nitric oxide synthesis increases aortic augmentation index and pulse wave velocity in vivo. Br J Clin Pharmacol. 2002 Feb;53(2):189–92.

376. Boutouyrie P, Lacolley P, Girerd X, Beck L, Safar M, Laurent S. Sympathetic activation decreases medium-sized arterial compliance in humans. Am J Physiol. 1994 Oct;267(4 Pt 2):H1368–76.

377. Niimi Y, Matsukawa T, Sugiyama Y, Shamsuzzaman AS, Mano T. Comparison of sympathetic nerve response to head-up tilt in summer and winter. J Gravit Physiol. 1999 Jul;6(1):P43–4.

378. Bank AJ, Wilson RF, Kubo SH, Holte JE, Dresing TJ, Wang H. Direct effects of smooth muscle relaxation and contraction on in vivo human brachial artery elastic properties. Circ Res. 1995 Nov;77(5):1008–16.

379. Lewington S, Clarke R, Qizilbash N, Peto R, Collins R, Prospective Studies Collaboration. Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. Lancet. 2002 Dec 14;360(9349):1903–13.

380. Boussuges A. Immersion in thermoneutral water: effects on arterial compliance. Aviat Space Environ Med. 2006 Nov;77(11):1183–7.

381. Cameron JD, Dart AM. Exercise training increases total systemic arterial compliance in humans. Am J Physiol. 1994 Feb;266(2 Pt 2):H693–701.

382. Clarkson P, Montgomery HE, Mullen MJ, Donald AE, Powe AJ, Bull T, et al. Exercise training enhances endothelial function in young men. J Am Coll Cardiol. 1999 Apr;33(5):1379–85.

383. Abularrage CJ, Sidawy AN, Aidinian G, Singh N, Weiswasser JM, Arora S. Evaluation of the microcirculation in vascular disease. J Vasc Surg. 2005 Sep;42(3):574–81.

384. Hodges GJ, Kosiba WA, Zhao K, Johnson JM. The involvement of norepinephrine, neuropeptide Y, and nitric oxide in the cutaneous vasodilator response to local heating in humans. J Appl Physiol. 2008 Jul;105(1):233–40.

385. Stewart JM, Ocon AJ, Clarke D, Taneja I, Medow MS. Defects in Cutaneous Angiotensin-Converting Enzyme 2 and Angiotensin-(1-7) Production in Postural Tachycardia Syndrome. Hypertension. 2009 Apr 22;53(5):767–74.

386. 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. 2006 Sep;27(9):503–8.

387. Houghton BL, Meendering JR, Wong BJ, Minson CT. Nitric oxide and noradrenaline contribute to the temperature threshold of the axon reflex response to gradual local heating in human skin. J Physiol. 2006 May 1;572(Pt 3):811–20.

388. Kaldur T, Kals J, Ööpik V, Zilmer M, Zilmer K, Eha J, et al. Effects of heat acclimation on changes in oxidative stress and inflammation caused by endurance capacity test in the heat. Oxid Med Cell Longev. 2014;2014:107137.

389. Medow MS, Glover JL, Stewart JM. Nitric oxide and prostaglandin inhibition during acetylcholine-mediated cutaneous vasodilation in humans. Microcirculation. 2008 Aug;15(6):569–79.

390. Jia Z, Zhu H, Li J, Wang X, Misra H, Li Y. Oxidative stress in spinal cord injury and antioxidant-based intervention. Spinal Cord. 2012 Apr;50(4):264–74.

391. Shastry S, Joyner MJ. Geldanamycin attenuates NO-mediated dilation in human skin. Am J Physiol Heart Circ Physiol. 2002 Jan;282(1):H232–6.

392. Zarling JA, Brunt VE, Vallerga AK, Li W, Tao A, Zarling DA, et al. Nitroxide pharmaceutical development for age-related degeneration and disease. Front Genet. 2015 Nov 6;6(e76766):574.

393. Holowatz LA, Thompson CS, Kenney WL. Acute ascorbate supplementation alone or combined with arginase inhibition augments reflex cutaneous vasodilation in aged human skin. Am J Physiol Heart Circ Physiol. 2006 Dec;291(6):H2965–70.

394. Holowatz LA, Kenney WL. Local ascorbate administration augments NO- and non-NO-dependent reflex cutaneous vasodilation in hypertensive humans. Am J Physiol Heart Circ Physiol. 2007 Aug;293(2):H1090–6.

395. 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. 2012 Mar;112(5):791–7.

396. Holowatz LA, Kenney WL. Up-regulation of arginase activity contributes to attenuated reflex cutaneous vasodilatation in hypertensive humans. J Physiol. 2007 Jun 1;581(Pt 2):863–72.

397. DuPont JJ, Ramick MG, Farquhar WB, Townsend RR, Edwards DG. NADPH oxidase-derived reactive oxygen species contribute to impaired cutaneous microvascular function in chronic kidney disease. Am J Physiol Renal Physiol. 2014 Jun 15;306(12):F1499–506.

398. Groothuis JT, Thijssen DHJ, Rongen GA, Deinum J, Danser AHJ, Geurts ACH, et al. Angiotensin II contributes to the increased baseline leg vascular resistance in spinal cord-injured individuals. J Hypertens. 2010 Oct;28(10):2094–101.

399. Chan SHH, Hsu K-S, Huang C-C, Wang L-L, Ou C-C, Chan JYH. NADPH oxidase-derived superoxide anion mediates angiotensin II-induced pressor effect via activation of p38 mitogen-activated protein kinase in the rostral ventrolateral medulla. Circ Res. 2005 Oct 14;97(8):772–80.

400. Pryor WA, Stone K. Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. Ann N Y Acad Sci. 1993 May 28;686:12–27–discussion27–8.

401. Yamauchi K, Stone AJ, Stocker SD, Kaufman MP. Blockade of ATP-sensitive potassium channels prevents the attenuation of the exercise pressor reflex by tempol in rats with ligated femoral arteries. Am J Physiol Heart Circ Physiol. 2012 Aug 1;303(3):H332–40.

402. Barlow RS, White RE. Hydrogen peroxide relaxes porcine coronary arteries by stimulating BKCa channel activity. Am J Physiol. 1998 Oct;275(4 Pt 2):H1283–9.

403. Feng L, Stern DM, Pile-Spellman J. Human endothelium: endovascular biopsy and molecular analysis. Radiology. 1999 Sep;212(3):655–64.

404. Rattan SIS, Sejerson H, Fernandes RA, Luo W. Stress-Mediated Hormetic Modulation of Aging, Wound Healing, and Angiogenesis in Human Cells. Ann N Y Acad Sci. 2007 Nov 1;1119(1):112–21.

405. Papapetropoulos A, Garcia-Cardena G, Madri JA, Sessa WC. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. J Clin Invest. 1997 Dec 15;100(12):3131–9.

406. MD TS, Masaaki Miyata MD F, MD TK, MD YI, MD SF, MD SK, et al. Waon therapy mobilizes CD34+ cells and improves peripheral arterial disease. J Cardiol. Japanese College of Cardiology; 2010 Nov 1;56(3):361–6.

407. Inoue N, Venema RC, Sayegh HS, Ohara Y, Murphy TJ, Harrison DG. Molecular regulation of the bovine endothelial cell nitric oxide synthase by transforming growth factor-beta 1. Arterioscler Thromb Vasc Biol. 1995 Aug;15(8):1255–61.

408. Babaei S, Teichert-Kuliszewska K, Monge JC, Mohamed F, Bendeck MP, Stewart DJ. Role of nitric oxide in the angiogenic response in vitro to basic fibroblast growth factor. Circ Res. 1998 May 18;82(9):1007–15.

409. Sun L, Lamont SJ, Cooksey AM, McCarthy F, Tudor CO, Vijay-Shanker K, et al. Transcriptome response to heat stress in a chicken hepatocellular carcinoma cell line. Cell Stress Chaperones. 2015 Nov;20(6):939–50.

410. Yang J, Zhu T, Liu X, Zhang L, Yang Y, Zhang J, et al. Heat shock protein 70 protects rat peritoneal mesothelial cells from advanced glycation end-products-induced epithelial-to-mesenchymal transition through mitogen-activated protein kinases/extracellular signal-regulated kinases and transforming growth factor-β/Smad pathways. Mol Med Rep. 2015 Jun;11(6):4473–81.

411. Zhou Y, Cao S, Li H, Peng X, Wang Y, Fan J, et al. Heat Shock Protein 72 Antagonizes STAT3 Signaling to Inhibit Fibroblast Accumulation in Renal Fibrogenesis. Am J Pathol. 2016 Apr;186(4):816–28.

412. Erdös G, Lee YJ, Cho JM, Corry PM. Heat-induced bFGF gene expression in the absence of heat shock element correlates with enhanced AP-1 binding activity. J Cell Physiol. 1995 Aug;164(2):404–13.

413. Jones N, Master Z, Jones J, Bouchard D, Gunji Y, Sasaki H, et al. Identification of Tek/Tie2 binding partners. Binding to a multifunctional docking site mediates cell survival and migration. J Biol Chem. 1999 Oct 22;274(43):30896–905.

414. Hwang B, Lee S-H, Kim J-S, Moon JH, Jeung IC, Lee NG, et al. Stimulation of angiogenesis and survival of endothelial cells by human monoclonal Tie2 receptor antibody. Biomaterials. 2015 May;51:119–28.

415. Mofarrahi M, McClung JM, Kontos CD, Davis EC, Tappuni B, Moroz N, et al. Angiopoietin-1 enhances skeletal muscle regeneration in mice. Am J Physiol Regul Integr Comp Physiol. 2015 Apr 1;308(7):R576–89.

416. Li M, Fuchs S, Böse T, Schmidt H, Hofmann A, Tonak M, et al. Mild heat stress enhances angiogenesis in a co-culture system consisting of primary human osteoblasts and outgrowth endothelial cells. Tissue Eng Part C Methods. 2014 Apr;20(4):328–39.

417. Mackie AR, Losordo DW. CD34-positive stem cells: in the treatment of heart and vascular disease in human beings. Tex Heart Inst J. 2011 Oct 11;38(5):474–85.

418. Gong B, Asimakis GK, Chen Z, Albrecht TB, Boor PJ, Pappas TC, et al. Whole-body hyperthermia induces up-regulation of vascular endothelial growth factor accompanied by neovascularization in cardiac tissue. Life Sci. 2006 Oct 4;79(19):1781–8.

419. Ihori H, Nozawa T, Sobajima M, Shida T, Fukui Y, Fujii N, et al. Waon therapy attenuates cardiac hypertrophy and promotes myocardial capillary growth in hypertensive rats: a comparative study with fluvastatin. Heart Vessels. 2015 Dec 19;31:1–9.

420. Sobajima M, Nozawa T, Shida T, Ohori T, Suzuki T, Matsuki A, et al. Repeated sauna therapy attenuates ventricular remodeling after myocardial infarction in rats by increasing coronary vascularity of noninfarcted myocardium. Am J Physiol Heart Circ Physiol. 2011 Aug;301(2):H548–54.

421. 2015 NSCISC Annual Statistical Report. United States National Spinal Cord Injury Statistical Center.

422. Soden RJ, Walsh J, Middleton JW, Craven ML, Rutkowski SB, Yeo JD. Causes of death after spinal cord injury. Spinal Cord. 2000 Oct;38(10):604–10.

423. Garshick E, Kelley A, Cohen SA, Garrison A, Tun CG, Gagnon D, et al. A prospective assessment of mortality in chronic spinal cord injury. Spinal Cord. 2005 Jul;43(7):408–16.

424. Wu J-C, Chen Y-C, Liu L, Chen T-J, Huang W-C, Cheng H, et al. Increased risk of stroke after spinal cord injury: a nationwide 4-year follow-up cohort study. Neurology. 2012 Apr 3;78(14):1051–7.

425. Yang T-Y, Chen H-J, Sung F-C, Kao C-H. The association between spinal cord injury and acute myocardial infarction in a nationwide population-based cohort study. Spine. 2015 Feb 1;40(3):147–52.

426. Wahman K, Nash MS, Lewis JE, Seiger A, Levi R. Increased cardiovascular disease risk in Swedish persons with paraplegia: The Stockholm spinal cord injury study. J Rehabil Med. 2010 May;42(5):489–92.

427. Liang H, Chen D, Wang Y, Rimmer JH, Braunschweig CL. Different risk factor patterns for metabolic syndrome in men with spinal cord injury compared with able-bodied men despite similar prevalence rates. Arch Phys Med Rehabil. 2007 Sep;88(9):1198–204.

428. de Groot PC, Bleeker MW, van Kuppevelt DH, van der Woude LH, Hopman MT. Rapid and extensive arterial adaptations after spinal cord injury. Arch Phys Med Rehabil. 2006 May;87(5):688–96.

429. De Groot PCE, Van Kuppevelt DHJM, Pons C, Snoek G, Van Der Woude LHV, Hopman MTE. Time course of arterial vascular adaptations to inactivity and paralyses in humans. Med Sci Sports Exerc. 2003 Dec;35(12):1977–85.

430. Thijssen DHJ, Groot PCE, Bogerd A, Veltmeijer M, Cable NT, Green DJ, et al. Time course of arterial remodelling in diameter and wall thickness above and below the lesion after a spinal cord injury. Eur J Appl Physiol. 2012 Apr 17;112(12):4103–9.

431. Miyatani M, Masani K, Oh PI, Miyachi M, Popovic MR, Craven BC. Pulse wave velocity for assessment of arterial stiffness among people with spinal cord injury: a pilot study. J Spinal Cord Med. 2009;32(1):72–8.

432. Wecht JM, Weir JP, DeMeersman RE, Spungen AM, Bauman WA. Arterial stiffness in persons with paraplegia. J Spinal Cord Med. 2004;27(3):255–9.

433. Hopman MTE, Groothuis JT, Flendrie M, Gerrits KHL, Houtman S. Increased vascular resistance in paralyzed legs after spinal cord injury is reversible by training. J Appl Physiol. 2002 Dec;93(6):1966–72.

434. Hall ED. Antioxidant therapies for acute spinal cord injury. Neurotherapeutics. 2011 Apr;8(2):152–67.

435. Thijssen DHJ, Ellenkamp R, Kooijman M, Pickkers P, Rongen GA, Hopman MTE, et al. A causal role for endothelin-1 in the vascular adaptation to skeletal muscle deconditioning in spinal cord injury. Arterioscler Thromb Vasc Biol. 2007 Feb;27(2):325–31.

436. Olive JL, Dudley GA, McCully KK. Vascular remodeling after spinal cord injury. Med Sci Sports Exerc. 2003 Jun;35(6):901–7.

437. Nicotra A, asahina M, Mathias CJ. Skin vasodilator response to local heating in human chronic spinal cord injury. Eur J Neurol. 2004 Dec;11(12):835–7.

438. Raymond J, Schoneveld K, Van Kemenade CH, Davis GM. Onset of electrical stimulation leg cycling in individuals with paraplegia. Med Sci Sports Exerc. 2002 Oct;34(10):1557–62.

439. Hopman MT. Circulatory responses during arm exercise in individuals with paraplegia. Int J Sports Med. 1994 Apr;15(3):126–31.

440. Saunders LL, Clarke A, Tate DG, Forchheimer M, Krause JS. Lifetime prevalence of chronic health conditions among persons with spinal cord injury. Arch Phys Med Rehabil. 2015 Apr;96(4):673–9.

441. Piatt JA, Nagata S, Zahl M, Li J, Rosenbluth JP. Problematic secondary health conditions among adults with spinal cord injury and its impact on social participation and daily life. J Spinal Cord Med. 2015 Dec 15;:1–6.

442. Sheerin F, Gillick A, Doyle B. Pressure ulcers and spinal-cord injury: incidence among admissions to the Irish national specialist unit. J Wound Care. 2005 Mar;14(3):112–5.

443. Chen D, Apple DF, Hudson LM, Bode R. Medical complications during acute rehabilitation following spinal cord injury--current experience of the Model Systems. Arch Phys Med Rehabil. 1999 Nov;80(11):1397–401.

444. Chung S-B, Lee S-H, Kim ES, Eoh W. Incidence of deep vein thrombosis after spinal cord injury: a prospective study in 37 consecutive patients with traumatic or nontraumatic spinal cord injury treated by mechanical prophylaxis. J Trauma. 2011 Oct;71(4):867–70–discussion870–1.

445. de Groot P, Crozier J, Rakobowchuk M, Hopman M, MacDonald M. Electrical stimulation alters FMD and arterial compliance in extremely inactive legs. Med Sci Sports Exerc. 2005 Aug;37(8):1356–64.

446. Czarkowska-Paczek B, Bartlomiejczyk I, Przybylski J. The serum levels of growth factors: PDGF, TGF-beta and VEGF are increased after strenuous physical exercise. J Physiol Pharmacol. 2006 Jun;57(2):189–97.

447. Ross MD, Wekesa AL, Phelan JP, Harrison M. Resistance exercise increases endothelial progenitor cells and angiogenic factors. Med Sci Sports Exerc. 2014 Jan;46(1):16–23.

448. Wagner PD. The critical role of VEGF in skeletal muscle angiogenesis and blood flow. Biochem Soc Trans. 2011 Dec;39(6):1556–9.

449. Tei C. Thermal therapy for congestive heart failure: estimation by TEI index. J Cardiol. 2001;37 Suppl 1:155–9.

450. Willems Van Dijk K, Hofker MH, Havekes LM. Use of transgenic mice to study the role of apolipoprotein E in lipid metabolism and atherosclerosis. Int J Tissue React. 2000;22(2-3):49–58.

451. Horowitz M. Heat acclimation, epigenetics, and cytoprotection memory. [Internet]. Vol. 4, Comprehensive Physiology. Hoboken, NJ, USA: John Wiley & Sons, Inc; 2011. 32 p. Available from: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=24692139&retmode=ref&cmd=prlinks

452. Horowitz M, Eli-Berchoer L, Wapinski I, Friedman N, Kodesh E. Stress-related genomic responses during the course of heat acclimation and its association with ischemic-reperfusion cross-tolerance. J Appl Physiol. 2004 Oct;97(4):1496–507.

453. Hunter SD, Dhindsa M, Cunningham EM, Tarumi T, Alkatan M, Tanaka H. Improvements in glucose tolerance with Bikram yoga in older obese adults: A pilot study. Journal of Bodywork & Movement Therapies. Elsevier Ltd; 2013 Oct 1;17(4):404–7.

454. Hooper PL. Hot-tub therapy for type 2 diabetes mellitus. N Engl J Med. 1999 Sep 16;341(12):924–5.

455. Henstridge DC, Whitham M, Febbraio MA. Chaperoning to the metabolic party: The emerging therapeutic role of heat-shock proteins in obesity and type 2 diabetes. Molecular Metabolism. Elsevier GmbH; 9999 Jan 1;3(8):781–93.