THE AGING CUTANEOUS MICROVASCULATURE: ALTERED MECHANISMS AND NOVEL INTERVENTION STRATEGIES

A Dissertation in

Kinesiology

by

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ABSTRACT

An age-related reduction in tetrahydrobiopterin (BH₄) bioavailability is a common mechanistic link that contributes to the decline in reflex cutaneous vasodilation (VD) and vasoconstriction (VC) with age. Intervention strategies that increase the bioavailability of BH₄ may be capable of restoring vessel function in the older cutaneous vasculature. Therefore, the purpose of this series of studies was to examine the efficacy of oral intervention strategies that could potentially increase bioavailable BH₄ in improved reflex cutaneous VD and VC in a healthy, older population. In the first set of studies, we hypothesized that acute oral sapropterin (Kuvan®, shelf-stable pharmaceutical formulation of BH₄) would acutely augment reflex VD and VC in aged skin through nitric oxide (NO)-mediated and adrenergic mechanisms, respectively. Oral sapropterin acutely increased bioavailable BH₄ in aged skin microvasculature sufficiently to (1) increase NO-dependent dilation and restore reflex VD, and (2) increase both reflex and pharmacologically-induced VC through adrenergic mechanisms. In a follow up study, we tested a chronic sapropterin intervention in 4 older adults. The results of this proof-of-concept study suggest that chronic sapropterin augments reflex VD in older human skin. However, there was not sufficient evidence to suggest efficacy in improved reflex VC in the same population. Collectively, these findings suggest that sapropterin acutely restores vessel function in healthy, older adults and that chronic sapropterin may be a viable intervention for improved vascular function in this population. Finally, we hypothesized that chronic folic acid supplementation, which increases endogenous BH₄ bioavailability, would augment reflex VD in aged skin. Folic acid (5mg/daily for 6 weeks) increased reflex VD in healthy, older cutaneous microvasculature through NO-dependent mechanisms. These findings suggest that folic acid treatment may be an effective intervention strategy for improved vascular endothelial function in older adults. Taken
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<td>Body Mass Index</td>
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Chapter 1
INTRODUCTION

Background and Significance

Even in the absence of overt cardiovascular disease, older men and women (>65 years) have a reduced vascular function, evidenced by a marked attenuation in reflex cutaneous vasodilation and vasoconstriction during environmental heat and cold stress, respectively (Kenney and Armstrong 1996; Kenney, Morgan et al. 1997). This attenuated reflex cutaneous vasoreactivity is due to a functional loss of active cholinergic vasodilator co-transmitter and adrenergic vasoconstrictor function. These deficits have negative thermoregulatory and cardiovascular consequences for the aging population and require the identification of novel intervention strategies to improve cutaneous vessel function in older adults.

The human cutaneous circulation is an easily accessible and representative circulation for examining mechanisms of vascular dysfunction in vivo (Holowatz, Thompson-Torgerson et al. 2008). There is a significant relation between endothelial dysfunction measured in the skin and that measured invasively in the coronary and renal circulations, and intervention-induced improvements in vascular function are detectible in the cutaneous circulation prior to improvements in clinical outcome (RG, de Jongh et al. 2003; Abularrage, Sidawy et al. 2005). The use of whole-body thermal stimuli coupled with acute or chronic systemic interventions and localized pharmacological manipulations (intradermal microdialysis) allows for the mechanistic examination of intervention efficacy in improved vessel function during an integrative cardiovascular challenge.
Neural Control of Skin Blood Flow

Human cutaneous blood flow is controlled by dual sympathetic innervation consisting of an adrenergic vasoconstrictor system and a cholinergic active vasodilator system (Grant and Holling 1938; Roddie, Shepherd et al. 1957; Stephens, Aoki et al. 2001). With increasing body temperatures, cutaneous blood flow is first increased through withdrawal of tonic adrenergic tone, followed by activation of the active cholinergic vasodilator system (Roddie, Shepherd et al. 1957). Active vasodilation is mediated by the co-release of acetylcholine and unknown co-transmitter(s) (Kellogg, Pergola et al. 1995) which provoke vasodilation in part through nitric oxide (NO)-dependent mechanisms. NO is required for the full expression of reflex cutaneous vasodilation and mediates ~30-40% of the cutaneous vasodilator response to hyperthermia in young, healthy humans (Kellogg, Crandall et al. 1998; Shastry, Dietz et al. 1998).

In response to whole-body cold exposure, reflex cutaneous vasoconstriction is mediated by an increase in efferent sympathetic nerve activity which stimulates the release of neurotransmitters and cotransmitters from the perivascular nerve terminals. In healthy, young subjects (18 – 30 years) ~60% of the reflex cutaneous vasoconstrictor response is dependent on norepinephrine (NE), with the remaining ~40% mediated by non-adrenergic cotransmitters (Stephens, Aoki et al. 2001; Stephens, Saad et al. 2004; Thompson and Kenney 2004).

Primary Human Aging and Skin Blood Flow

In contrast to young adults, older adults exhibit an attenuated co-transmitter mediated cutaneous vasodilation (Holowatz, Houghton et al. 2003) and a functionally absent co-transmitter mediated cutaneous vasoconstriction (Thompson and Kenney 2004). This is further compounded by altered downstream vascular signaling mechanisms including decreased NO availability and
adrenergic receptor desensitization (Holowatz, Houghton et al. 2003; Thompson and Kenney 2004). Older adults rely primarily on compromised NO-mediated vasodilation and NE-mediated vasoconstriction to control cutaneous blood flow during whole-body heat or cold stress, respectively. Consequently, NO and NE synthesis are important therapeutic targets for potential pharmacological intervention strategies aimed at improving or maintaining vascular health throughout older adulthood.

**Therapeutic Targets and Potential Intervention Strategies**

Because the contributions and identities of the vasodilator co-transmitters in human skin are unclear and many of these co-transmitters converge on the NO pathway, interventions that target NO production and bioavailability may be capable of increasing reflex vasodilation in older human skin. Similarly, because cotransmitter-mediated vasoconstriction is absent in aged skin, noradrenergic mechanisms (such as NE synthesis at the perivascular nerve terminal) are the most viable target for interventions that aim to increase reflex vasoconstriction in the skin of older adults.

The decreased NO bioavailability in aged skin results from a decrease in NO production due, in part, to upregulated vascular arginase and increased NO synthase (NOS) uncoupling with increased oxidant stress (Holowatz, Thompson et al. 2006; Holowatz, Thompson et al. 2006). The age-related decrements in NE- and co-transmitter-mediated constriction are due, in part, to age-related decreases in transmitter synthesis and/or release (Frank, Raja et al. 2000; Connat, Busseuil et al. 2001; Donoso, Gomez et al. 2008) subsequent to elevated oxidative stress and reduced substrate (L-tyrosine) availability for NE synthesis (Lang, Holowatz et al. 2010). Functional data examining the role of substrate availability and oxidant stress in the age-associated decline in reflex vasodilation and vasoconstriction suggests that intervention strategies
that increase substrate availability and/or decrease oxidant stress may be efficacious in improving reflex control of skin blood flow in the older population (Holowatz, Thompson et al. 2006; Holowatz, Thompson et al. 2006; Lang, Holowatz et al. 2010).

In addition to limited substrate availability and elevated oxidative stress, reductions in tetrahydrobiopterin (BH₄) bioavailability also contribute to the decline in reflex cutaneous vasodilation and vasoconstriction mechanisms with age (Kuzkaya, Weissmann et al. 2003; Lang, Holowatz et al. 2010; Stanhewicz, Bruning et al. 2012). BH₄ plays a critical role in NOS dimerization and NO production (Raman, Li et al. 1998) as well as TH synthesis of NE (figure 1-1) (Kumer and Vrana 1996; Thony, Calvo et al. 2008). Consequently, BH₄ bioavailability is required for enzymatic systems in vascular control and is important to both vasodilation and vasoconstriction in the healthy vasculature (Kaufman 1993). The age-associated decline in bioavailable BH₄ may contribute to the vascular dysfunction associated with aging. Indeed: acute, local administration of BH₄ is capable of restoring reflex vasodilation and vasoconstriction in aged skin during whole-body heat and cold stress, respectively (Lang, Holowatz et al. 2010; Stanhewicz, Bruning et al. 2012). These studies, aimed at elucidating the functional role of BH₄ in reflex vasodilation and vasoconstriction, suggest that intervention strategies that increase bioavailability of BH₄ may be capable of restoring functional vasodilation and vasoconstriction in the older cutaneous vasculature.

**Sapropterin**

Sapropterin is a commercially available, shelf-stable, pharmaceutical formulation of R-BH₄ which is prescribed clinically for the treatment of BH₄-responsive phenylketonuria. Sapropterin is currently not available for the on-label use for the treatment or prevention of vascular dysfunction, however phase I clinical trials have suggested that it may be efficacious in specific cardiovascular disease states including hypertension, and aging (Porkert, Sher et al. 2008; Moreau, Meditz et al. 2012; Pierce, Jablonski et al. 2012).
**Folic Acid**

Folic acid and its active metabolite 5-methyltetrahydrofolate (5-MTHF) improve conduit vessel endothelial function in patients with overt cardiovascular and metabolic disease (Verhaar, Wever et al. 1998; Doshi, McDowell et al. 2001; Alian, Hashemipour et al. 2012). 5-MTHF increases vascular BH4 bioavailability by increasing production via BH2 recycling and reducing oxidant stress, and improvements in vessel function may be mediated through restoration of cofactor availability and NO production (Stroes, van Faassen et al. 2000; Antoniades, Shirodaria et al. 2006)

**Summary**

Four separate studies comprising this dissertation were performed to investigate the efficacy of oral intervention strategies for improved reflex control of the cutaneous vasculature in older humans. Because *reduced BH4 bioavailability is a unifying mechanism* by which reflex vasodilation and vasoconstriction are attenuated in aged skin, all four studies aimed to increase BH4 bioavailability in older cutaneous vasculature. The first and second studies examined the efficacy of acute oral sapropterin in improved reflex vasodilation and vasoconstriction, respectively. The third study followed up the first and second studies to examine the efficacy of a chronic sapropterin intervention. The fourth study examined the efficacy of a chronic folic acid intervention in improved NO-dependent reflex vasodilation in older cutaneous vasculature.

**Specific Aims and Hypotheses**

**Specific Aim 1:** The purpose of the study “Oral sapropterin acutely augments reflex vasodilation in older human skin through nitric oxide-dependent mechanisms” was to determine if oral
administration of sapropterin could acutely increase NO-dependent reflex cutaneous vasodilation in healthy older humans. Specifically, by administering oral sapropterin (10mg·kg⁻¹) we aimed to increase bioavailable BH₄ in the aged vasculature and restore NO-dependent reflex cutaneous vasodilation.

**Hypothesis 1a:** Oral sapropterin would acutely increase bioavailable BH₄ in older human vasculature.

**Hypothesis 1b:** Oral sapropterin would acutely augment NO-dependent reflex vasodilation in aged human skin through NOS coupling mechanisms

**Specific Aim 2:** The purpose of the study “Oral sapropterin augments reflex vasoconstriction in aged human skin through noradrenergic mechanisms” was to examine the efficacy of acute oral administration of sapropterin (10mg·kg⁻¹) in improved cutaneous vasoconstriction in healthy older humans. Specifically, by administering sapropterin we aimed to augment catecholamine synthesis and restore reflex and pharmacologically induced cutaneous vasoconstriction in aged human skin.

**Hypothesis 2:** Oral sapropterin would acutely augment reflex and pharmacologically induced cutaneous vasoconstriction in aged human skin through noradrenergic mechanisms.

**Specific Aim 3:** The purpose of the study “Chronic treatment with oral sapropterin: evidence of efficacy in augmented reflex control of blood flow in aged human skin” was to specifically explore the efficacy of a chronic sapropterin intervention (400mg twice-daily for 4 weeks) in improved reflex cutaneous vasodilation and vasoconstriction in aged human skin.

**Hypothesis 3a:** Chronic sapropterin treatment would augment reflex vasodilation in aged human skin through NOS coupling mechanisms.
Hypothesis 3b: Chronic sapropterin treatment would augment reflex vasoconstriction in aged human skin through adrenergic mechanisms.

Specific Aim 4: The purpose of the study “Folic acid supplementation improves cutaneous microvascular function in older humans through nitric oxide-dependent mechanisms” was to determine if exogenous folic acid administration could increase NO-dependent vasodilation in aged human skin.

Hypothesis 4a: Acute, local microperfusion of 5-MTHF directly into the dermal space would increase the cutaneous vasodilation response to an endothelial-NOS (eNOS)-dependent stimulus (local thermal hyperemia) through NO-dependent mechanisms in older adults.

Hypothesis 4b: Chronic, high-dose, oral folic acid (5mg·day for 6 weeks) would increase the magnitude of reflex cutaneous vasodilation through restoration of NO-dependent mechanisms in older adults exposed to passive heat stress.
Figure 1-1. Schematic representation of the role of tetrahydrobiopterin in reflex vasoconstriction and vasodilation. TH, tyrosine hydroxylase; BH$_4$, tetrahydrobiopterin; ·ONO$_2^-$, peroxynitrite, ·O$_2^-$, superoxide; H$_2$O$_2$, hydrogen peroxide; DA, dopamine; NE, norepinephrine; NOS, nitric oxide synthase; NO, nitric oxide.
Chapter 2

REVIEW OF LITERATURE

The human cutaneous circulation is an easily accessible and representative circulation for examining mechanisms of vascular dysfunction in vivo (Holowatz, Thompson-Torgerson et al. 2008). There is a significant relation between endothelial dysfunction measured in the skin and that measured invasively in the coronary and renal circulations, and intervention-induced improvements in vascular function are detectible in the cutaneous circulation prior to improvements in clinical outcome (RG, de Jongh et al. 2003; Abularrage, Sidawy et al. 2005). The studies comprising this dissertation utilize whole-body thermal stimuli coupled with acute or chronic oral interventions and localized pharmacological manipulations (intradermal microdialysis) to mechanistically examine intervention efficacy in improved vessel function during an integrative cardiovascular challenge.

The primary role of the cutaneous circulation during exposure to environmental heat and cold stress is thermoregulation. In humans, increasing blood flow to the cutaneous circulation is one of the primary effector responses critical to the maintenance of body core temperature during hyperthermia. During passive thermal stress in a supine position, blood flow to the skin increases up to 8 liters per minute (or ~60% of cardiac output) allowing for convective heat transfer from the body core to the surface where heat dissipation can occur (Rowell 1993). Conversely, in response to whole-body cold exposure, reflex cutaneous vasoconstriction decreases skin blood flow, increases effective tissue insulation, and minimizes heat loss. Even in the absence of overt cardiovascular disease, older adults (>65 years) exhibit attenuated reflex cutaneous vasodilation and vasoconstriction in response to environmental heat and cold stress, respectively (Kenney and
Armstrong 1996; Kenney, Morgan et al. 1997). These deficits have negative thermoregulatory and cardiovascular consequences for the aging population and require the identification of novel intervention strategies to improve cutaneous vessel function in older adults. This review will focus on the putative mechanisms mediating the age-related decline in reflex control of skin blood flow and potential therapeutic targets and intervention strategies for the restoration of cutaneous vasodilation and vasoconstriction in healthy, older adults.

**Reflex Control of the Cutaneous Circulation: Vasodilation**

Skin blood flow is controlled by dual sympathetic innervation consisting of an adrenergic vasoconstrictor system and a cholinergic active vasodilator system (Grant and Holling 1938). With increasing body temperature, skin blood flow is first increased through withdrawal of tonic adrenergic tone followed by activation of the active cholinergic vasodilator system (Roddie, Shepherd et al. 1957). Cutaneous active vasodilation is mediated by the co-release of acetylcholine and unknown co-transmitter(s) that mediate vasodilation. In support of this hypothesis, muscarinic receptor antagonism only modestly delays the initial rise in skin blood flow with rising core temperature, while pre-synaptic blockade of cholinergic nerves abolishes active vasodilation (Kellogg, Pergola et al. 1995). Evidence from the rabbit ear model of active vasodilation suggests that the cholinergic neurotransmitter(s) mediate cutaneous vascular smooth muscle relaxation through adenylate cyclase-dependent mechanisms (Farrell and Bishop 1997). The specific identity of the unknown neurotransmitter(s) mediating active vasodilation remains unclear, however there is evidence for a role of histamine, vasoactive intestinal peptide, neurokinin 1, and substance P (Bennett, Johnson et al. 2003; Wong, Wilkins et al. 2004; McCord, Cracowski et al. 2006; Wong and Minson 2006) in reflex cutaneous vasodilation.
Functional nitric oxide (NO) is required for full expression of reflex cutaneous vasodilation and mediates ~30-40% of the total vasodilator response to whole body heat stress in young, healthy humans (Kellogg, Crandall et al. 1998; Shastry, Dietz et al. 1998) through the activation of soluble guanylyl cyclase-dependent mechanisms. NO synthesis during active vasodilation is stimulated by a variety of sources, including the putative neurotransmitter(s) involved in active vasodilation. Indeed, acetylcholine (Shibasaki, Wilson et al. 2002), vasoactive intestinal peptide (Wilkins, Chung et al. 2004), substance P (Wong and Minson 2006), and activation of H1 histamine receptors (Wong, Wilkins et al. 2004) have all been shown to contribute to reflex vasodilation through NO-dependent mechanisms. In addition to this direct contribution of NO to active vasodilation, NO is also capable of mediating vasodilation synergistically with the sympathetic co-transmitter(s). The overall reflex cutaneous vasodilation response in the presence of NO and co-transmitter(s) is greater than the sum of the contributions of each of these individual pathways (Wilkins, Holowatz et al. 2003). The precise mechanism of this synergistic interaction is unknown, but may occur downstream considering the potential interactions between neurotransmitter activated cAMP and NO activated cGMP-dependent mechanisms of vasorelaxation (Farrell and Bishop 1997). Despite the fact that the precise interactions of NO and co-transmitter(s) remains unclear, it is apparent that NO plays an important role in mediating the rise in Skin blood flow with hyperthermia, and that NO synthesis is required for the full expression of reflex cutaneous vasodilation.

NO is synthesized through the activity of two constitutively expressed NO synthases (NOS; endothelial-NOS and neuronal-NOS). The NOS enzyme is a dimer that relies on the presence of the essential cofactor tetrahydrobiopterin (BH₄) and available substrate to couple the oxidation of L-arginine to the reduction of molecular oxygen to produce NO and L-citrulline (Liu and Gross 1996). When either substrate or cofactor availability is limited, or under conditions of elevated oxidant stress, the NOS dimer can destabilize and uncouple (Munzel, Daiber et al. 2005).
This uncoupling disrupts the normal flow of electrons through the dimerized complex and results in the production of superoxide radicals rather than NO (Vasquez-Vivar, Kalyanaraman et al. 1998; Xia, Tsai et al. 1998).

In addition to NO, cyclooxygenase (COX)-dependent second messenger cascades also contribute to active vasodilation (McCord, Cracowski et al. 2006). Whether there is a synergistic role of COX and NO-dependent vasodilation is unknown, however combined inhibition of COX and NOS attenuated active vasodilation in an additive fashion, suggesting independent mechanisms. Studies utilizing local delivery of acetylcholine to the cutaneous vasculature suggest that acetylcholine-mediated vasodilation in human skin is both NO and COX-dependent (Kellogg, Zhao et al. 2005). An alternative stimulus for COX-mediated vasodilation is through neurokinin 1 receptor activation of the inositol triphosphate (IP₃) pathway resulting in increased intracellular calcium and activation of both NOS and COX pathways (Wong and Minson 2006). Collectively, the putative neurotransmitter(s) likely mediate the synthesis of both COX-derived vasodilators and NO, which independently contribute to reflex cutaneous vasodilation.

**Reflex Control of the Cutaneous Circulation: Vasoconstriction**

In response to whole-body cold exposure, reflex cutaneous vasoconstriction increases effective tissue insulation and minimizes heat loss. Reflex cutaneous vasoconstriction is mediated by elevated autonomic sympathetic efferent nerve activity traveling to cutaneous sympathetic axon terminals, stimulating the release of norepinephrine (NE) and co-transmitter(s) from the perivascular nerve terminals. Localized administration of antagonists of the adrenergic pathway, bretylium (pre-synaptic blockade of neurotransmitter release) or yohimbine and propranolol (post-synaptic blockade of alpha and beta adrenergic receptors), reveals that reflex cutaneous vasoconstriction relies entirely on adrenergic neurons (Kellogg, Johnson et al. 1989). However,
in healthy, young subjects (older 18-30 years) only ~60% of the reflex cutaneous vasoconstrictor response is dependent on NE, while the remaining ~40% is mediated by non-adrenergic co-transmitters (Thompson and Kenney 2004). There is evidence identifying neuropeptide Y and ATP as putative sympathetic co-transmitters. Both are present with NE in perivascular nerve terminals, and act post-junctionally through Y₁ and P₂X receptors, respectively (Ekblad, Edvinsson et al. 1984; Han, Yang et al. 1998; Bradley, Law et al. 2003; Burnstock 2009). In vivo human evidence suggests a role for neuropeptide Y in reflex cutaneous vasoconstriction in response to whole-body cold exposure (Stephens, Saad et al. 2004). However, no similar studies have been performed investigating the potential role of ATP in the human cutaneous vascular bed. Thus, the identity of the sympathetic co-transmitter(s) that functionally mediate ~40% of reflex cutaneous vasoconstriction remains in question. Despite the fact that the precise co-transmitter(s) remain unclear, it is apparent that NE plays an important role in mediating the decrease in Skin blood flow with whole-body cooling, and that NE synthesis is required for the full expression of reflex cutaneous vasoconstriction.

Cathecholamine synthesis occurs almost exclusively in the cell body of the noradrenergic neuron (Geffen and Rush 1968) from the amino acid precursor L-tyrosine, through the activity of the rate-limiting enzyme tyrosine hydroxylase (TH) and the essential cofactor BH₄ (Levitt, Spector et al. 1965; Sumi-Ichinose, Urano et al. 2001). BH₄ acts as an essential cofactor for TH, reducing the iron moiety of TH, thereby priming TH for catalytic reaction (Kumer and Vrana 1996; Thony, Calvo et al. 2008). Consequently, NE synthesis is reliant on adequate L-tyrosine and BH₄ bioavailability.

NE released from sympathetic adrenergic nerves binds to both pre- and post-junctional adrenoreceptors. In the human cutaneous vascular beds, α₂A- and α₂C- adrenoreceptors function as pre-junctional autoreceptors that decrease NE release from axon terminals when stimulated (Philipp, Brede et al. 2002). Post-junctionally, the α₂A-adrenoceptor subtype primarily elicits the
cutaneous vasoconstriction to NE binding at the cell membrane, while the and α2C subtype is translocated to the cell membrane after direct local cooling of the skin (Ekenvall, Lindblad et al. 1988; Borbujo, Garcia-Villalon et al. 1989; Nielsen, Mortensen et al. 1990; Chotani, Flavahan et al. 2000; Jeyaraj, Chotani et al. 2001). Additionally, α1-adrenoreceptor subtypes likely participate in the vasoconstriction response, but to a lesser extent (Guimaraes and Moura 2001). Although β2-adrenoreceptor subtypes are sparsely found in skin and elicit vasodilation when stimulated, their effect on reflex vasoconstriction remains unclear (Crandall, Etzel et al. 1997).

**Reflex Cutaneous Vasodilation and Vasoconstriction and Primary Human Aging**

Even in the absence of overt pathology, human aging is associated with attenuated reflex cutaneous vasodilation and vasoconstriction in response to whole body heat and cold stress, respectively (Kenney and Armstrong 1996; Kenney, Morgan et al. 1997) rendering them more susceptible to complications during environment heat and cold exposure (Curriero, Heiner et al. 2002; Hajat, Kovats et al. 2007).

**Reflex vasodilation**

Healthy, older adults display a 25–50% reduction in skin blood flow during whole-body heat stress, coupled with a blunted increase in cardiac output and an attenuated redistribution of blood flow from the splanchnic and renal vascular beds (Minson, Wladkowski et al. 1998). The origins of the age-related reduction in skin blood flow during hyperthermia appear to be of peripheral origin due to decreased sensitivity of the active vasodilator system and not age-related alterations in noradrenergic mechanisms (Kenney, Morgan et al. 1997). The direct contribution of cholinergic active vasodilation co-transmitters to reflex vasoconstriction is functionally absent in
aged skin (Holowatz, Houghton et al. 2003). Indeed, older humans require a much greater increase in core temperature ($\Delta T_{\text{core}} > 0.9^\circ\text{C}$) to stimulate significant contributions of non-NO-dependent pathways to active vasodilation (Minson, Holowatz et al. 2002) compared to young, and although NO-dependent vasodilation is reduced overall in aged cutaneous vessels, the elderly rely primarily on this compromised NO-dependent vasodilator signaling to increase thermoregulatory skin blood flow during hyperthermia.

**Reflex vasoconstriction**

Even when matched for adiposity, fat-free mass and aerobic fitness, healthy older adults exhibit reduced peripheral vasoconstriction and attenuated ability to defend core temperature even during mild (22°C) cold exposure (Kenney and Armstrong 1996; Degroot and Kenney 2007). ~60% of the thermoregulatory reflex cutaneous vasoconstrictor response is dependent on NE, while the remaining ~40% is mediated by non-adrenergic cotransmiters (Stephens, Aoki et al. 2001; Stephens, Saad et al. 2004; Thompson and Kenney 2004). However, aged skin exhibits (I) functionally absent cotransmitter-mediated vasoconstriction (Thompson and Kenney 2004), (II) reduced axonal release of NE for a given cold stimulus (Frank, Raja et al. 2000), and (III) diminished adrenoreceptor responsiveness for a given exogenous NE (Thompson, Holowatz et al. 2005) or tyramine dose (Dinenno, Dietz et al. 2002). Consequently, healthy older adults rely primarily on compromised noradrenergic mechanisms to decrease skin blood flow and increase effective tissue insulation during cold exposure.
Mechanisms Mediating the Decline in Reflex Control of Skin Blood Flow

Nitric Oxide

The decreased NO bioavailability in aged skin results from a decrease in NO production by upregulated vascular arginase and increased NO synthase (NOS) uncoupling with increased oxidant stress (Holowatz, Thompson et al. 2006; Holowatz, Thompson et al. 2006). Arginase catalyzes the conversion of L-arginine to L-ornithine in the last step of the urea cycle, limiting substrate availability for NO synthesis by NOS and reciprocally regulating NO production (Berkowitz, White et al. 2003). With aging, upregulated arginase activity results in decreased NO production and attenuated endothelial function (Berkowitz, White et al. 2003). Local L-arginine supplementation or arginase inhibition augments NO-dependent reflex vasodilation in aged skin, demonstrating that increasing available substrate restores vasodilation function in aged cutaneous vessels (Holowatz, Thompson et al. 2006). Similarly, age-associated elevations in oxidant stress due to a combined increase in production and decreased clearance of reactive oxygen species (ROS) (Donato, Eskurza et al. 2007) leads to vascular dysfunction in aged vessels (El Assar, Angulo et al. 2013). In vascular endothelial cells, superoxide can react with NO to form peroxynitrite up to four times faster than what can be metabolized, resulting in decreased NO bioavailability and therefore reduced NO-dependent vasodilation (Beckman 1996). Additionally, NOS itself can uncouple and become a source of ROS in the presence of high oxidant stress and inadequate substrate and/or essential cofactor availability (Munzel, Daiber et al. 2005). Local ascorbate supplementation augments NO-dependent reflex vasodilation in aged skin, pointing to a role for oxidant stress in the age associated attenuation in the reflex vasodilation response to heat stress (Holowatz, Thompson et al. 2006).
In addition to upregulated arginase activity and increased oxidant stress, there are other potential mechanisms associated with impaired downstream signaling and attenuated NO-dependent reflex vasodilation in aged skin. Tetrahydrobiopterin (BH₄) serves as an essential cofactor for pteridine-requiring monoxygenases (Raman, Li et al. 1998). Consequently, BH₄ bioavailability is required for enzymatic systems in vascular control and is important to both vasodilation and vasoconstriction in the healthy vasculature (Kaufman 1993). Specifically, BH₄ plays a critical role in NOS dimerization and NO production (Raman, Li et al. 1998). Reduced BH₄ bioavailability may contribute to several pathologies leading to vascular dysfunction (Higashi, Sasaki et al. 2006; Porkert, Sher et al. 2008; Lang, Holowatz et al. 2009; Schmidt, McNeill et al. 2010). *In vivo* human studies demonstrate that administration of exogenous BH₄ improves NO-dependent vasodilation in conduit vessels of older subjects (Higashi, Sasaki et al. 2006; Pierce, Jablonski et al. 2012). Similarly, local microperfusion of BH₄ augments reflex vasodilation through NO-dependent mechanisms in aged human skin (Stanhewicz, Bruning et al. 2012).

**Catecholamine Synthesis**

The age-related decrements in NE- and co-transmitter-mediated constriction are due, in part, to age-related decreases in transmitter synthesis and/or release (Frank, Raja et al. 2000; Connat, Busseuil et al. 2001; Donoso, Gomez et al. 2008). Although there are limited data available explaining the mechanisms driving the age-related decline in pre-junctional biosynthesis of catecholamines, these impairments may be consequent to elevated oxidative stress and reduced substrate (L-tyrosine) availability for NE synthesis. Animal studies have demonstrated that increasing tyrosine bioavailability in activated neurons augments the concentration of the NE precursor, L-DOPA, indicating that tyrosine concentration in the nerve terminal may be below
saturation (Iuvone, Galli et al. 1978; Fernstrom 1983). In the perivascular neurons of older cutaneous vessels, the bioavailability of tyrosine may be decreased by age-related increases in oxidative stress leading to oxidation of tyrosine to the tyrosyl radical which can further reduce free tyrosine pools and nitrate other proteins (Ischiropoulos, Duran et al. 1995; Reiter, Teng et al. 2000). Indeed, local microperfusion of tyrosine to the cutaneous microvasculature corrects the age-related decline in reflex vasoconstriction through catecholamine synthesis mechanisms (Lang, Holowatz et al. 2010), suggesting that limited tyrosine availability may significantly limit catecholamine synthesis in the aged vasculature.

In addition to elevated oxidant stress and reduced L-tyrosine availability, reduced bioavailability of BH$_4$ also contributes to the attenuated noradrenergic reflex vasoconstriction in aged human skin (Lang, Holowatz et al. 2009). BH$_4$ is found throughout the neural and vascular tissue, and is an essential cofactor for tyrosine hydroxylase, the rate limiting enzyme in catecholamine biosynthesis (Kaufman 1978; Moens and Kass 2007). Mechanistically, BH$_4$ serves as a reducing agent and is required to maintain TH in its active form (Kaufman 1978; Urano, Hayashi et al. 2006). Oxidant-induced depletion of intraneuronal BH$_4$ may deplete newly synthesized or stored pools of NE within the perivascular neurons of aged skin, resulting in a functionally attenuated vasoconstriction during cold-induced sympathetic activation.

**Potential Therapeutic Targets and Intervention Strategies**

Because the contributions and identities of the vasodilator co-transmitters in human skin are unclear and many of these co-transmitters converge on the NO pathway, interventions that target NO production and bioavailability may be capable of increasing reflex vasodilation in aged human skin. Similarly, because cotransmitter-mediated vasoconstriction is absent in aged skin, noradrenergic mechanisms (such as NE synthesis at the perivascular nerve terminal) are the most...
viable target for interventions that aim to increase reflex vasoconstriction in the skin of older humans during whole-body cold exposure. Although there are a number of studies examining the specific mechanisms by which thermoregulatory control of the cutaneous vasculature declines with aging, there are few studies examining the efficacy of potential intervention strategies that may mitigate this decline in aged skin.

Both NO and NE synthesis rely on adequate substrate (L-arginine and L-tyrosine, respectively) availability. Age-associated impairment in downstream vasodilation and vasoconstriction signaling can be attributed to limited L-arginine and L-tyrosine availability due to up regulated arginase and oxidant stress (Berkowitz, White et al. 2003). Local L-arginine supplementation or arginase inhibition augments NO-dependent reflex vasodilation in aged skin, demonstrating that increasing available substrate restores vasodilation function in older cutaneous vessels (Holowatz, Thompson et al. 2006). Similarly, local microperfusion of L-tyrosine augments reflex vasoconstriction in aged human skin (Lang, Holowatz et al. 2010). Taken together, studies examining the functional role of substrate availability in reflex vasodilation and vasoconstriction suggest that increasing bioavailable substrate (L-arginine and L-tyrosine for vasodilation and vasoconstriction, respectively) may be a viable intervention strategy to increase thermoregulatory control of the aged vasculature.

In addition to limited substrate availability, age-associated increases in oxidant stress due to a combined increase in production and decreased clearance of reactive oxygen species (Donato, Eskurza et al. 2007) may also contribute to impairments in reflex vasodilation and vasoconstriction with aging. The administration of intra-arterial ascorbic acid has been shown to improve endothelium-dependent vasodilation in subjects with endothelial dysfunction (Frei 1999; Cross, Donald et al. 2003) and local ascorbate supplementation augments NO-dependent reflex vasodilation in aged skin, pointing to a role for oxidant stress in the age associated attenuation in the reflex vasodilation response to heat stress (Holowatz, Thompson et al. 2006). Intervention
strategies that aim to reduce oxidative stress in the aged cutaneous vasculature may be capable of increasing reflex vasoconstriction and vasodilation in older adults.

BH$_4$ bioavailability is required for enzymatic systems in vascular control and is important to both vasodilation and vasoconstriction in the healthy vasculature (Kaufman 1993). Specifically, BH$_4$ plays a critical role in NOS dimerization and NO production (Raman, Li et al. 1998) as well as TH synthesis of NE (Kumer and Vrana 1996; Thony, Calvo et al. 2008) and reduced BH$_4$ bioavailability may contribute to several pathologies leading to vascular dysfunction (Higashi, Sasaki et al. 2006; Porkert, Sher et al. 2008; Lang, Holowatz et al. 2009; Schmidt, McNeill et al. 2010). In primary aging, high oxidant stress may deplete bioavailable BH$_4$ by (1) direct oxidation of existing BH$_4$ to dihydrobiopterin (BH$_2$) and/or (2) reducing BH$_4$ synthesis in vivo (Vasquez-Vivar 2009). This attenuation may contribute to the vascular dysfunction associated with aging. Indeed: acute, local administration of BH$_4$ is capable of restoring reflex vasodilation and vasoconstriction in aged skin during whole-body heat and cold stress, respectively (Lang, Holowatz et al. 2010; Stanhewicz, Bruning et al. 2012). These studies, aimed at elucidating the functional role of BH$_4$ in reflex vasodilation and vasoconstriction, suggest that intervention strategies that increase bioavailability of BH$_4$ (figure 2-2) in the aged cutaneous vasculature may be capable of restoring functional vasodilation and vasoconstriction in response to whole-body heat and cold stress, respectively.

**Sapropterin**

Sapropterin is a shelf-stable, pharmaceutical formulation of R-BH$_4$ which is commercially available in the EU and the US for the treatment of BH$_4$-responsive phenylketonuria. In BH$_4$ deficiency, its mechanism of action is presumed to be secondary to replacement of endogenous cofactor bioavailability (Sanford and Keating 2009). Pharmacokinetic
analysis of sapropterin shows that it exhibits similar time to peak plasma concentrations (~3 hours) and elimination half life (~4 hours) as BH₄ administration, following a single oral dose (Fiege, Ballhausen et al. 2004; Feillet, Clarke et al. 2008). However, compared to BH₄ powder or capsules, oral sapropterin is commercially available, has superior shelf-stability, and has been shown to have a high tolerability among patients (Sanford and Keating 2009). Sapropterin is currently not available for the on-label use for the treatment or prevention of vascular dysfunction, however phase I clinical trials have suggested that it may be efficacious in specific cardiovascular disease states including hypertension, and aging (Porkert, Sher et al. 2008; Moreau, Meditz et al. 2012; Pierce, Jablonski et al. 2012). Similarly, oral sapropterin may be a viable intervention strategy to restore reflex cutaneous vasodilation and vasoconstriction in older adults.

Folic Acid

Folic acid and its active metabolite 5-methyltetrahydrofolate (5-MTHF) improve conduit vessel endothelial function in patients with overt cardiovascular and metabolic disease (Verhaar, Wever et al. 1998; Doshi, McDowell et al. 2001; Alian, Hashemipour et al. 2012). 5-MTHF increases vascular BH₄ bioavailability by increasing production via BH₂ recycling and reducing oxidant stress, and improvements in vessel function may be mediated though restoration of cofactor availability and NO production (Stroes, van Faassen et al. 2000; Antoniades, Shirodaria et al. 2006). Folic acid supplementation may be a viable intervention strategy to improve vascular health and prevent cardiovascular morbidity in older adults; however few in vivo studies have examined the mechanistic role of folic acid in improved endothelial function in primary older adults.
Because reduced BH$_4$ bioavailability is a unifying mechanism by which reflex vasodilation and vasoconstriction are attenuated in aged skin, focus on examining the efficacy of oral interventions that increase BH$_4$ bioavailability may provide novel strategies for improved thermoregulatory control – both vasodilation and vasoconstriction - of the aged cutaneous vasculature.
Figure 2-1. Schematic representation of the mechanisms mediating reflex cutaneous vasoconstriction (left) and vasodilation (right). TH, tyrosine hydroxylase; BH₄, tetrahydrobiopterin; ·ONO₂⁻, peroxynitrite; ·O₂⁻, superoxide; H₂O₂, hydrogen peroxide; DA, dopamine; NE, norepinephrine; NPY, neuropeptide Y; MLCP, myosin light chain phosphatase; MLCK, myosin light chain kinase; Ach, acetylcholine; ?, unknown co-transmitter(s); eNOS, endothelial nitric oxide synthase; NO, nitric oxide. From reference (Holowatz, Thompson-Torgerson et al. 2010).
Figure 2-2. Schematic representation of the tetrahydrobiopterin synthesis pathways with possible intervention strategies. BH₄, tetrahydrobiopterin; GTPCH, guanosine triphosphate cyclohydrolase.
Chapter 3

ORAL SAPROPTERIN ACUTELY AUGMENTS REFLEX
VASODILATION IN AGED HUMAN SKIN THROUGH NITRIC OXIDE-DEPENDENT MECHANISMS

Introduction

Skin blood flow (SkBF) is controlled by dual sympathetic innervation consisting of an adrenergic vasoconstrictor system and a cholinergic active vasodilator system (Grant and Holling 1938). With increasing body temperature SkBF is first increased through withdrawal of tonic adrenergic tone followed by activation of the active cholinergic vasodilator system (Roddie, Shepherd et al. 1957). Active vasodilation is mediated by the co-release of acetylcholine and unknown co-transmitter(s) (Kellogg, Pergola et al. 1995) that mediate vasodilation in part through nitric oxide (NO)-dependent mechanisms. NO is required for full expression of reflex cutaneous vasodilation and mediates ~30-40% of the total vasodilator response to whole body heat stress in young, healthy humans (Kellogg, Crandall et al. 1998; Shastry, Dietz et al. 1998).

Primary human aging is associated with an attenuated cutaneous vasodilation response to hyperthermia (Kenney, Morgan et al. 1997) due to decreased cotransmitter and attenuated NO-dependent contributions (Holowatz, Houghton et al. 2003). The decreased NO bioavailability in aged skin results from a decrease in NO production by upregulated vascular arginase and increased nitric oxide synthase (NOS) uncoupling with increased oxidant stress (Holowatz, Thompson et al. 2006; Holowatz, Thompson et al. 2006). NOS is a dimeric enzyme that requires functional coupling of the oxygenase and reductase domains for NO production (Raman, Li et al. 1998; Andrew and Mayer 1999). In conditions where substrate (L-arginine)
availability is reduced through increased arginase activity or oxidative stress, the NOS dimer is uncoupled and produces superoxide rather than functional NO (Vasquez-Vivar, Kalyanaraman et al. 1998; Munzel, Daiber et al. 2005).

In addition to upregulated arginase activity and increased oxidant stress, we have recently demonstrated that reduced bioavailability of tetrahydrobiopterin (BH₄) in aged vasculature also contributes to the attenuated NO-dependent reflex cutaneous vasodilation in aged skin (Stanhewicz, Bruning et al. 2012). BH₄ is an essential cofactor for NOS and is required for optimal NO production through NOS (Vasquez-Vivar, Kalyanaraman et al. 1998; Moens and Kass 2006). Mechanistically, BH₄ stabilizes NOS in the coupled conformation and reduces oxidant stress in and around the NOS molecule (Raman, Li et al. 1998; Vasquez-Vivar, Kalyanaraman et al. 1998). In conditions of reduced BH₄ bioavailability, NOS uncouples and produces superoxide which contributes to peroxynitrite formation (Raman, Li et al. 1998). Furthermore, superoxide produced from uncoupled NOS, as well as peroxynitrite, oxidize BH₄, contributing to increased oxidant stress and vascular dysfunction (Vasquez-Vivar, Kalyanaraman et al. 1998; Milstien and Katusic 1999; Kuzkaya, Weissmann et al. 2003) In vivo human studies demonstrate that administration of exogenous BH₄ improves NO-dependent vasodilation in conduit vessels of older subjects (Higashi, Sasaki et al. 2006; Pierce, Jablonski et al. 2012). Similarly, we have recently demonstrated that acute, local microperfusion of BH₄ augments reflex vasodilation through NO-dependent mechanisms in aged human skin (Stanhewicz, Bruning et al. 2012). Collectively, these findings suggest that a systemic BH₄ intervention may be a clinically relevant therapy for improved NO-dependent reflex cutaneous vasodilation in older adults. In this study, we aimed to specifically address the clinical aspect of systemic exogenous BH₄ administration by examining the efficacy of an acute oral dose of sapropterin (pharmaceutical BH₄) in improved NO-dependent reflex vasodilation in aged skin. Sapropterin is a commercially available, shelf-stable, pharmaceutical formulation of R-BH₄ which is prescribed clinically for the
treatment of BH₄-responsive phenylketonuria. Sapropterin is currently not available for the on-label use for the treatment or prevention of vascular dysfunction, however phase I clinical trials have suggested that it may be efficacious in specific cardiovascular disease states including hypertension, and aging (Porkert, Sher et al. 2008; Moreau, Meditz et al. 2012; Pierce, Jablonski et al. 2012). Because sapropterin is commercially available in the US, and oral dosing is more clinically practical than intradermal microdialysis for the delivery of BH₄ to the cutaneous vasculature, the purpose of this study was to determine if oral administration of sapropterin could acutely increase NO-dependent reflex cutaneous vasodilation in healthy older humans. We hypothesized that oral sapropterin would acutely increase bioavailable BH₄ in older human vasculature. We further hypothesized that oral sapropterin would acutely augment NO-dependent reflex vasodilation in aged human skin through NOS coupling mechanisms.

Methods

Subjects. Experimental protocols were approved by the institutional review board of The Pennsylvania State University. Written and verbal consent were obtained voluntarily from all subjects prior to participation according to the Declaration of Helsinki. Studies were performed on nine healthy subjects (76±1 years, 4 men and 5 women). Subjects were screened for neurological, cardiovascular and dermatological diseases and underwent a complete medical screening including resting ECG, physical examination, lipid profile and blood chemistry (Quest Diagnostics, Pittsburgh, PA). All subjects were normally active, non-hypertensive, non-diabetic, healthy non-smokers who were not taking prescription medications with primary or secondary vascular effects (e.g. statins, antihypertensives, anticoagulants, antidepressants, etc). Women taking hormone replacement therapy or who had recently taken hormone replacement therapy were excluded from the study.
Instrumentation. All protocols were performed in a thermoneutral laboratory with the subjects in a semisupine position and the experimental arm supported at heart level. All testing took place in the morning to eliminate diurnal variation in blood flow responses (Aoki, Kondo et al. 1997). Study days were separated by at least 48 hours to ensure adequate washout of sapropterin (Feillet, Clarke et al. 2008). Subjects entered the laboratory between 0800 and 0900 and were instrumented with an intravenous catheter for blood sampling. A fasted blood sample was obtained and then subjects ingested 10mg/kg body weight sapropterin (Kuvan®; BioMarin Pharmaceutical Inc, Novato, CA) or placebo with a standardized breakfast meal in a double-blind, randomized crossover study design. A second blood sample was obtained 3 hours after ingestion of the treatment for analysis of peak plasma BH4 concentrations. Pharmacokinetic analysis of sapropterin shows that plasma BH4 concentrations peak at 3 hours following oral administration (Feillet, Clarke et al. 2008). All blood samples were collected in 4ml tubes containing EDTA and 0.1% w/v dithioerythritol and centrifuged immediately. The plasma was flash frozen in liquid nitrogen and stored at -80°C until further analysis of BH4 concentration by HPLC (Meininger and Wu 2002). Baseline and post treatment blood samples from 3 subjects were not analyzed due to technical difficulties with the samples.

After breakfast, subjects were instrumented with three intradermal microdialysis (MD) fibers (10mm, 20kDa cutoff membrane, MD 2000; Bioanalytical Systems, West Lafayette, IN) placed in the ventral forearm skin using sterile technique. Before MD fiber placement, ice packs were applied to the sites for 5 minutes to temporarily anesthetize the skin (Hodges, Chiu et al. 2009). MD sites were at least 4cm apart to ensure no cross reactivity of the pharmacological agents. For each fiber, a 25-gauge needle was inserted horizontally in the intradermal layers of the skin such that the entry and exit points were ~2.5cm apart. MD fibers were then threaded through the lumen of the needle and the needle was removed leaving the membrane of the MD fiber in place. The MD fibers were randomly assigned to deliver 1) lactated Ringer’s solution to
serve as control, 2) 10mM BH₄ (Sigma, St. Louis, MO) for local BH₄ administration, or 3) 20mM N⁶-nitro-L-arginine (L-NAME; Calbiochem, San Diego, CA) to inhibit NOS. Concentrations of BH₄ and L-NAME were based on previous studies conducted in our laboratory (Stanhewicz, Bruning et al. 2012). Pharmacological agents were mixed just before use, dissolved in lactated Ringer’s solution, sterilized using syringe microfilters (Acrodisc; Pall, Ann Arbor, MI), and wrapped in foil to prevent degradation due to light exposure. During the trauma resolution period (60 – 90 minutes), site-specific pharmacological solutions (10mM BH₄, 20mM L-NAME, or lactated Ringer’s) were perfused through the MD fibers at a rate of 2µL/min (Bee Hive controller and Baby Bee microinfusion pumps; Bioanalytical Systems).

Skin temperature (Tsk) was controlled using a water-perfused suit that covered the entire body except for the head, hands, feet and forearms. Copper-constantan thermocouples were placed on the surface of the skin at six sites (calf, thigh, abdomen, chest, shoulder and back) for continuous measurement of Tsk. Each subject’s heart rate was monitored throughout the protocol (Cardiocap; GE Healthcare) and arterial blood pressure was measured by brachial auscultation every 5 minutes. Oral temperature (Tor) was measured as an index of changes in body temperature using a thermistor placed in the sublingual sulcus throughout baseline and whole body heating. Proper placement of the thermistor was checked based on temperature readings and once verified, was taped in place and closely monitored to ensure that it did not move throughout the protocol. Local Tsk over each MD site was clamped at 33°C throughout baseline and whole body heating (MoorLab, Temperature Monitor, SHO2; Moor Instruments, Devon, UK) to ensure that changes in SkBF were reflex in origin.

To obtain an index of SkBF, cutaneous red blood cell flux was continually measured directly over each MD site with an integrated laser-Doppler flowmetry probe placed in a local heating unit (Moor Instruments SHO2). Cutaneous vascular conductance (CVC) was calculated as red blood cell flux divided by mean arterial pressure (MAP) and expressed as a percent of site-
specific maximal vasodilation [%CVCmax; 28mM sodium nitroprusside (SNP) and local heat to 43°C]. MAP was calculated as diastolic pressure plus one-third pulse pressure. Forearm blood flow (FBF) was measured at baseline and every 0.1°C rise in T_or by venous occlusion plethysmography using a mercury-in-silastic strain gauge (EC6 Plethysmograph, Hokanson, Bellevue, WA) while blood flow to the hand was occluded (Whitney 1949). In contrast to laser-Doppler flowmetry, which images skin blood flow over a limited area (1mm²) above the MD fiber, venous occlusion plethysmography provides an index of skin blood flow over the entire forearm. During whole body heating under resting conditions, increases in FBF are confined to the skin rather than the underlying muscle (Detry, Brengelmann et al. 1972). Forearm vascular conductance (FVC) was calculated as FBF divided by MAP.

**Experimental Protocol.** Figure 3-1 shows a representative tracing of T_or and mean T_sk throughout baseline and whole body heating. After MD fiber placement, the insertion trauma resolution period, and instrumentation, baseline data were collected (~20 min). Throughout baseline mean T_sk was held at thermoneutral by perfusing 33°C water through the suit. Following baseline data collection, warm water was perfused through the suit to clamp mean T_sk at 38°C and cause a gradual rise in T_or. Whole body heating began exactly 3 hours after ingestion of sapropterin or placebo. This timing was chosen to ensure peak plasma concentrations of sapropterin during heating (Feillet, Clarke et al. 2008). At a 1°C rise in T_or, mean body temperature (T_b) was clamped by lowering the water temperature in the suit, such that mean T_sk and T_or ceased to rise. After 5 minutes of steady laser-Doppler flux values, 20mM L-NAME was perfused through the control and BH₄-perfused MD fibers at a rate of 4µL/min to inhibit NOS production of NO and quantify NO-dependent vasodilatation within each site. L-NAME perfusion was discontinued after laser-Doppler flux values decreased to a steady plateau (~40 min). At this time, whole body heat was terminated, the water perfused suit was perfused with 33°C water and subjects were returned to thermoneutral.
After completion of the whole body heating protocol, site specific pharmacological treatments were discontinued and each MD fiber was perfused with 28mM SNP (Nitropress; Abbott Laboratories, Chicago, IL) at a rate of 4µl/min. Simultaneously, the local Tsk over the experimental sites was increased to 43°C to obtain maximal CVC values within each site.

Data Acquisition and Analysis. CVC data from the control, BH₄ and L-NAME-perfused sites were acquired at 40Hz, digitized and stored on a personal computer until further analysis (WinDaq; Dataq Instruments, Akron, OH). CVC values were averaged over a stable period of laser-Doppler flux at baseline, over a stable period for every 0.1°C rise in T₀r during whole body heating, and over a stable plateau during L-NAME perfusion. Maximal CVC values were averaged over a stable plateau in laser-Doppler flux during perfusion of 28mM SNP and local temperature of 43°C. NO-dependent vasodilation within each site was assessed at clamped 1°C rise in T₀r by quantifying the decrease in CVC observed with complete NOS inhibition (20mM L-NAME).

A three-way repeated-measures mixed-model ANOVA was conducted to detect oral treatment and local drug treatment differences over the rise in T₀r. A two-way repeated-measures mixed-model ANOVA was used to detect oral treatment and local drug treatment differences in NO-dependent vasodilation, plasma BH₄ concentration, and maximal CVC (version 9.1.3; SAS, Cary, NC). Post hoc comparisons with Bonferroni corrections were performed when necessary to determine where differences between oral treatments and local drug treatments occurred. The level of significance was set at α = 0.05 for main effects. Values are presented as mean ± SEM.

Results

Subject characteristics are presented in Table 3-1. There was no effect of acute sapropterin treatment on systolic pressure, diastolic pressure, or MAP. Table 3-2 presents plasma
BH₄ concentration data following oral placebo and sapropterin treatment from 6 subjects. Plasma BH₄ was significantly elevated 3 hours after ingestion of sapropterin (0 hours: 19.1±2 pmol/ml vs. 3 hours: 43.8±3 pmol/ml; p<0.001) but not after ingestion of placebo (0 hours: 15.2±1 pmol/ml vs. 3 hours: 18.6 ± 4 pmol/ml; p=0.40).

Figure 3-2 shows skin blood flow (%CVC_max) as a function of increasing core temperature (ΔT_or) at baseline (ΔT_or = 0) and throughout whole body heating at Ringer’s control, BH₄-perfused, and L-NAME-perfused MD sites with placebo and sapropterin treatment. Local BH₄ administration increased baseline %CVC_max compared to Ringer’s control (control: 11±2%CVC_max vs BH₄: 19±3%CVC_max; p<0.001) with placebo treatment only. Oral sapropterin increased baseline %CVC_max at the Ringer’s control site compared to placebo treatment (placebo control: 11±2%CVC_max vs. sapropterin control: 16±2%CVC_max; p = 0.01). L-NAME perfusion did not alter baseline %CVC_max in either group. Oral sapropterin treatment increased vasodilation in the Ringer’s control site compared to placebo treatment during hyperthermia (all p<0.05). Local administration of BH₄ increased %CVC_max response compared to Ringer’s control with placebo treatment only (all p<0.05). There was no difference in vasodilation between sapropterin treatment and placebo treatment at the BH₄-perfused site. When NOS was inhibited throughout the heating protocol, there was no difference in %CVC_max response between sapropterin treatment and placebo treatment.

Figure 3-3 shows FVC as a function of increasing core temperature (ΔT_or) at baseline and throughout whole body heating with oral sapropterin and placebo treatments. Oral sapropterin treatment increased FVC compared to placebo (p<0.01 main effect of oral treatment).

Figure 3-4 shows the NO-dependent vasodilatation (%CVC_max) response at a 1°C rise in T_or at Ringer’s control and BH₄-perfused MD sites with oral sapropterin and placebo treatments. Oral sapropterin increased NO-dependent vasodilation in the Ringer’s control site (placebo: 14±1%CVC_max vs. sapropterin: 25±4%CVC_max; p =0.004). Local BH₄ perfusion
increased NO-dependent vasodilation compared to Ringer’s control site with placebo treatment only (control: 14±1%CVC_{max} vs. BH4: 24±3%CVC_{max}; p =0.02). There was no difference between NO-dependent vasodilation with oral sapropterin or placebo in the BH4-perfused site (placebo: 24±3%CVC_{max} vs. sapropterin: 27±2%CVC_{max}; p = 0.55). There were no differences between maximal CVC values across MD sites or oral treatments (p>0.05 for all comparisons).

**Discussion**

The principal finding of this study was that oral sapropterin acutely (3-hours post ingestion) increased reflex vasodilation in aged human skin measured by both laser-Doppler flowmetry and venous occlusion plethysmography. Furthermore, it did so through NO-dependent mechanisms. These data agree with our previous conclusions that decreased BH4 contributes to attenuated reflex cutaneous vasodilation in older humans by limiting NO production through uncoupled NOS (Stanhewicz, Bruning et al. 2012), and suggest that with 10mg/kg oral dose of sapropterin, BH4 acutely becomes bioavailable in aged skin microvasculature sufficiently to increase NO synthesis through NOS. Oral sapropterin may be a clinically relevant intervention for improved thermoregulatory skin blood flow in older adults.

In healthy young subjects, ~30-40% of the total reflex vasodilation response is mediated by NO signaling, with the remaining ~60-70% relying on co-released neurotransmitter(s) with downstream vasodilation mediated through other second messenger pathways and activation of cyclo-oxygenase (Bennett, Johnson et al. 2003; Wong, Wilkins et al. 2004; McCord, Cracowski et al. 2006). With aging, the cofactor-mediated contribution to the overall expression of reflex vasodilation is attenuated and COX-dependent signaling favors the production of vasoconstrictors (Holowatz, Jennings et al. 2009). Consequently, healthy older adults rely predominately on a functionally compromised NO-dependent vasodilation to increase
skin blood flow during hyperthermia (Holowatz, Houghton et al. 2003). Because the contributions and identities of the co-transmitters in human skin are unclear and many of these co-transmitters converge on the NO pathway, interventions that target NO production and bioavailability may be capable of increasing reflex vasodilation in aged human skin. We have examined the efficacy of an acute oral sapropterin intervention in older humans because (1) BH4 bioavailability is decreased with age, (2) this decrease contributes to attenuated endothelial dysfunction in older adults, and (3) BH4, as an essential NOS cofactor, is capable of modulating NO synthesis. Our results suggest that oral sapropterin increases the magnitude of reflex cutaneous vasodilation in aged human skin by increasing NOS coupling and subsequent NO synthesis.

NO is synthesized in the cutaneous vasculature by the constitutively expressed NOS isoforms endothelial-NOS (eNOS) and neuronal-NOS (nNOS). Although specific NOS inhibitors have not been utilized to examine the exact contributions of these NOS isoforms to reflex cutaneous vasodilatation in older adults, in healthy young subjects the response appears to be mediated primarily by nNOS (Kellogg, Zhao et al. 2009). BH4 acts as an essential enzymatic cofactor for both eNOS and nNOS isoforms and in the absence of adequate BH4 availability both isoforms uncouple and produce superoxide rather than NO (Raman, Li et al. 1998). Consequently, NOS is highly dependent on BH4 bioavailability for functional NO synthesis. In agreement with our previous findings, in the present study we show that local BH4 perfusion through intradermal microdialysis augments full and NO-dependent reflex vasodilation in aged skin following placebo treatment. However, this localized perfusion did not further increase the magnitude of the full or NO-dependent cutaneous vasodilatation response observed with oral sapropterin treatment. Further, there was no difference in full or NO-dependent reflex vasodilation between the BH4-perfused microdialysis sites across oral treatments. These data could indicate that (1) the 10mg/kg oral sapropterin dose maximized activity through the NOS
pathway such that the enzyme was working at or near $V_{\text{max}}$, and/or (2) we had reached a ceiling effect for the ability of the aged cutaneous vessels to vasodilate during hyperthermia. Collectively, these data suggest that 10mg/kg oral sapropterin increases bioavailable BH$_4$ sufficiently to increase NO production through NOS. In contrast to laser-Doppler flowmetry, which measures a limited area of skin (1mm$^2$) directly over the microdialysis membrane, venous occlusion plethysmography provides an index of skin blood flow over the entire arm at rest. In this study, oral sapropterin treatment increased FVC during hyperthermia. Given the systemic nature of the oral treatment and the clinical significance of demonstrating changes in skin blood flow over large areas of skin, these data further support the finding that oral sapropterin acutely increases the magnitude of reflex vasodilation, and reiterate the clinically relevant application of sapropterin in improved thermoregulatory skin blood flow during hyperthermia. Sapropterin is a shelf-stable, pharmaceutical formulation of R-BH$_4$ which is commercially available in the EU and the US for the treatment of BH$_4$-responsive phenylketonuria. In BH$_4$ deficiency, its mechanism of action is presumed to be secondary to replacement of endogenous cofactor bioavailability (Sanford and Keating 2009). Pharmacokinetic analysis of sapropterin shows that it exhibits similar time to peak plasma concentrations (~3 hours) and elimination half life (~4 hours) as BH$_4$ administration, following a single oral dose (Fiege, Ballhausen et al. 2004; Feillet, Clarke et al. 2008). Prior studies examining oral BH$_4$ as an intervention for improved vascular function in aging or cardiovascular disease have utilized BH$_4$ powder or capsules administered orally (Porkert, Sher et al. 2008; Pierce, Jablonski et al. 2012). In the present study we have chosen to utilize an oral sapropterin intervention because it is commercially available, has superior shelf-stability compared to BH$_4$ powder or capsules, and has been shown to have a high tolerability among patients (Sanford and Keating 2009). Our data suggest that, similar to oral BH$_4$ administration, a single oral dose of sapropterin increases plasma BH$_4$ concentrations in older subjects sufficiently to induce a functional increase in NO production through NOS.
Oral sapropterin and local BH₄ perfusion increased baseline vasodilation measured with laser-Doppler flowmetry compared to oral placebo control. This may indicate that a portion of the increased vasodilation observed in those sites could be due to a baseline shift. Previously we have shown that despite a modest increase in baseline skin blood flow, the same 10mM concentration of BH₄ delivered locally through microdialysis does not increase %CVCₜₐₓ response to hyperthermia in healthy young subjects (Stanhewicz, Bruning et al. 2012). In that study, we concluded that the lack of a continuous upward shift in vasodilation throughout body heating suggested that the differences observed in the older group were not simply due to a baseline shift. Furthermore, we did not observe a baseline effect of oral sapropterin on FVC but we were still able to detect a significant augmentation of the cutaneous blood flow response due to the oral treatment using this method. Considering the putative role of BH₄ in vascular function (Eskurza, Myerburgh et al. 2005; Delp, Behnke et al. 2008; Pierce, Jablonski et al. 2012) and the augmentation of %CVCₜₐₓ and FVC with significantly elevated increases in body core temperatures, oral sapropterin is a potential novel pharmaceutical intervention for augmenting thermoregulatory skin blood flow in older humans.

One alternate explanation for our results is that the augmented reflex cutaneous vasodilation observed with oral sapropterin and/or local BH₄-perfusion is due to the antioxidant properties of BH₄ independent of its role in NOS coupling. Reducing oxidant stress through local ascorbate-perfusion can augment the vasodilator response in aged skin (Holowatz, Thompson et al. 2006) and ascorbate infusion has been utilized to examine the role of oxidant stress in large elastic artery compliance in a number of studies, to mixed results (Eskurza, Monahan et al. 2004; Eskurza, Monahan et al. 2004; Moreau, Gavin et al. 2005). However, ascorbate has been shown to mediate its effects on vasodilation partially through the protection and stabilization of the BH₄ molecule (Huang, Vita et al. 2000). Furthermore, a recent study that aimed to more directly answer the question of antioxidant properties v. NOS coupling mechanisms by utilizing the
stereoisomer S-BH$_4$, which contains the same antioxidant capacity but lacks the NOS-coupling properties of the cofactor, found that exogenous R-BH$_4$ predominately restores vasodilation in the skin of hypercholesterolemic subjects through its NOS coupling mechanisms (Alexander, Kutz et al. 2013). Thus, the observed augmentations in full and NO-dependent reflex cutaneous vasodilation seen here are likely due to the NOS-coupling mechanisms of BH$_4$.

**Limitations.** We did not examine the effects of an oral sapropterin intervention in a healthy, young subject population. Healthy, young men and women are unlikely to have a reduced BH$_4$ bioavailability such as that seen in an older population (Delp, Behnke et al. 2008), suggesting that young subjects would be unlikely to benefit from additional supplementation. Furthermore, previously published data from our lab (Lang, Holowatz et al. 2010; Stanhewicz, Bruning et al. 2012) and others (Eskurza, Myerburgh et al. 2005; Pierce, Jablonski et al. 2012) suggests that exogenous BH$_4$ administration has no effect on cutaneous or conduit vascular function in young subjects aged 18 – 30 years. However, in previous studies utilizing the same methodology that did include a young subject group, we observed that healthy young subject exhibit a reflex cutaneous vasodilation response up to ~55%CVC$_{max}$ at ΔT$_{tot}$ = 1°C with ~23% of that dilation being NO-dependent (Stanhewicz, Bruning et al. 2012). In the present study, oral sapropterin increased the magnitude of reflex vasodilation in aged skin, and normalized the cutaneous vasodilator response to that observed earlier in healthy, young subjects.

**Perspectives.** Our results suggest that an acute 10mg/kg dose of oral sapropterin increases reflex vasodilation in aged human skin through NO-dependent mechanisms and that oral administration of exogenous BH$_4$ may be a clinically relevant intervention for improved thermoregulatory function in older adults during hyperthermia. Oral supplementation with BH$_4$ improves vascular function in animal and human models of vascular disease and dysfunction (Hattori, Hattori et al. 2007; Porkert, Sher et al. 2008), and taken together these data suggest that the specific NOS-coupling mechanisms of BH$_4$ may be an emerging therapy for endothelial
dysfunction. In contrast to our previous study in which we used a localized microperfusion of BH₄ to explore the role of the cofactor in NOS coupling, in this study we utilized a commercially available, pharmaceutical formulation of BH₄ (sapropterin) to explore the clinically relevant efficacy of an acute oral intervention for improved thermoregulatory skin blood flow in healthy older adults exposed to heat stress.

By design, this study excluded subjects who had overt cardiovascular disease and/or were taking medications. Although these subjects were not elite athletes, they represent a specific subset of the population and our results may not be generalizable to older adults who are unhealthy and/or taking a variety of medications. However, exogenous BH₄ therapy has been shown to effectively increase vascular endothelial function in populations with cardiovascular disease (Cosentino, Hurlimann et al. 2008; Porkert, Sher et al. 2008; Alexander, Kutz et al. 2013). Although the subjects in those studies were younger than the subjects in the present study, their results suggest that BH₄ may be efficacious in older adults who do exhibit overt cardiovascular disease. Further research is certainly warranted to investigate the efficacy of this intervention in an older diseased population and other human populations that exhibit attenuated thermoregulatory skin blood flow, and to determine the efficacy of a long-term dosing strategy.

Summary. In summary, acute oral sapropterin treatment increases reflex cutaneous vasodilation in older humans through NO-dependent mechanisms. In addition, there is no additive effect of local BH₄-perfusion, suggesting that the 10mg/kg dose increases bioavailable BH₄ sufficiently to maximally increase NO synthesis through NOS. Considering the putative role of BH₄ in vascular function and the observed increase in the magnitude of reflex cutaneous vasodilation in the present study, oral sapropterin administration is a potential intervention strategy for improved thermoregulatory function in older adults exposed to environmental heat stress.
Table 3-1. Subject Characteristics

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Sex (M,F)</th>
<th>BMI (kg/m²)</th>
<th>LDL (mg/dL)</th>
<th>HDL (mg/dL)</th>
<th>oxLDL (U/mL)</th>
<th>Total cholesterol (mg/dL)</th>
<th>HbA1c (%)</th>
<th>SBP (mmHg)</th>
<th>DBP (mmHg)</th>
<th>MAP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>76±1</td>
<td>4,5</td>
<td>25±1</td>
<td>120±5</td>
<td>64±4</td>
<td>48±3</td>
<td>203±7</td>
<td>5.7±0.1</td>
<td>122±3</td>
<td>72±3</td>
<td>91±3</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. BMI: body mass index, HDL: high density lipoprotein, LDL: low density lipoprotein, oxLDL: oxidized low density lipoprotein, SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure.
Table 3-2. Plasma BH\textsubscript{4} concentrations at arrival (0 hours) and 3 hours after ingestion of placebo or sapropterin.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 Hours</th>
<th>3 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>15.2 ± 1 pmol/ml</td>
<td>18.6 ± 4 pmol/ml</td>
</tr>
<tr>
<td>Sapropterin (10mg/kg)</td>
<td>19.1 ± 2 pmol/ml</td>
<td>43.8 ± 3 pmol/ml*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, n=6. *p<0.05 significant difference from 0 hours.
Figure 3-2. Representative tracing of oral temperature ($T_{or}$) and mean skin temperature ($T_{sk}$) throughout baseline, whole body heating, and clamped $T_{or}$. 
Figure 3-2. Group mean ± SEM of vasodilation response (%CVC_{max}) to increased core temperature (ΔT_{or}) at baseline (T_{or} = 0.0) and during whole body heating in Ringer’s control (A), BH4-perfused (B), and L-NAME-perfused (C) microdialysis sites with oral placebo or sapropterin treatment. * p<0.05 significant difference compared to placebo within site. ‡ p<0.05 significant difference compared to placebo control in panel A.
Figure 3-3. Group mean ± SEM of forearm cutaneous vasodilation response (FVC) to increased core temperature ($\Delta T_{or}$) at baseline ($T_{or} = 0.0$) and during whole body heating with oral placebo or sapropterin treatment. $p<0.01$ main effect of oral treatment.
Figure 3-4. Group mean ± SEM of NO-dependent vasodilation (%$CVC_{\text{max}}$) response at 1°C rise in oral temperature in Ringer’s (control) and BH$_4$-perfused sites with oral placebo or sapropterin treatment. * $p<0.05$ compared to placebo control.
Chapter 4

ORAL SAPROPTERIN AUGMENTS REFLEX VASCONSTRUCTION IN AGED HUMAN SKIN THROUGH NORADRENERGIC MECHANISMS

Introduction

Skin blood flow (SkBF) is controlled by dual sympathetic innervations consisting of an adrenergic vasoconstrictor system and a cholinergic active vasodilator system (Grant and Holling 1938; Stephens, Aoki et al. 2001). In response to whole-body cold exposure, reflex cutaneous vasoconstriction increases effective tissue insulation and minimizes heat loss. Reflex cutaneous vasoconstriction is mediated by elevated efferent sympathetic nerve activity which stimulates the release of neurotransmitters and cotransmitters from the perivascular nerve terminals. In healthy, young subjects (aged 18-30 years) ~60% of the reflex cutaneous vasoconstrictor response is dependent on norepinephrine, while the remaining ~40% is mediated by non-adrenergic cotransmitters, including neuropeptide Y and ATP (Stephens, Aoki et al. 2001; Stephens, Saad et al. 2004; Thompson and Kenney 2004). In contrast, aged skin exhibits (I) functionally absent cotransmitter-mediated vasoconstriction (Thompson and Kenney 2004), (II) reduced axonal release of norepinephrine for a given cold stimulus (Frank, Raja et al. 2000), and (III) diminished adrenoreceptor responsiveness for a given exogenous norepinephrine (Thompson, Holowatz et al. 2005) or tyramine dose (Dineno, Dietz et al. 2002). These impairments may be consequent to elevated oxidative stress and reduced substrate (L-tyrosine) availability for norepinephrine synthesis (Lang, Holowatz et al. 2010), both of which contribute to attenuated reflex vasoconstriction in aged human skin. Collectively, older adults rely entirely on a functionally compromised noradrenergic-mediated vasoconstriction to reduce skin blood flow during cold exposure (Kenney and Armstrong 1996; Degroot and Kenney 2007).
In addition to elevated oxidant stress and reduced L-tyrosine availability, our lab has recently suggested that reduced bioavailability of tetrahydrobiopterin (BH$_4$) also contributes to the attenuated noradrenergic reflex vasoconstriction in aged human skin (Lang, Holowatz et al. 2009). BH$_4$ is found throughout the neural and vascular tissue, and is an essential cofactor for nitric oxide synthase (NOS) as well as tyrosine hydroxylase (TH), the rate limiting enzyme in catecholamine biosynthesis (Kaufman 1978; Moens and Kass 2007). Mechanistically, BH$_4$ serves as a reducing agent and is required to maintain TH in its active form (Kaufman 1978; Urano, Hayashi et al. 2006). Oxidant-induced depletion of intraneuronal BH$_4$ may deplete newly synthesized or stored pools of norepinephrine within the perivascular neurons of aged skin, resulting in a functionally attenuated vasoconstriction during cold-induced sympathetic activation. We recently demonstrated that localized exogenous-BH$_4$ administration through an intradermal microdialysis fiber increases vasoconstriction in response to physiological (whole-body cold exposure) and pharmacological (local tyramine perfusion) stimuli through noradrenergic mechanisms with no effect on cotransmitter-mediated vasoconstriction or end organ responsiveness to norepinephrine (Lang, Holowatz et al. 2009; Lang, Holowatz et al. 2010). These findings open the possibility that a systemic BH$_4$ intervention may be a clinically applicable intervention to increase reflex cutaneous vasoconstriction in older adults during cold exposure.

We have previously examined the role of oral sapropterin (pharmaceutical BH$_4$) administration in improved reflex cutaneous vasodilation in older adults exposed to environmental heat stress (Stanhewicz, Alexander et al. 2013). Sapropterin is a commercially available, shelf stable, pharmaceutical formulation of R-BH$_4$, which is prescribed clinically in the US for the treatment of BH$_4$-responsive phenylketonuria. In that study, an acute oral dose (10mg/kg) of sapropterin increased bioavailable BH$_4$ sufficiently to increase nitric oxide (NO)-dependent reflex vasodilation in aged human skin, presumably though the coupling cofactor
properties of BH₄ on NOS. However the clinical efficacy of a systemic BH₄ intervention on the separate neural catecholamine synthesis mechanisms mediated by TH and its essential cofactor BH₄ has not been examined.

The purpose of the present study was to specifically address the role of oral BH₄ administration in improved reflex cutaneous vasoconstriction in aged human skin exposed to cold stress. Because sapropterin is a commercially available drug that has been shown to increase bioavailable BH₄ in aged cutaneous vessels, and because oral dosing is more clinically practical than intradermal microdialysis for the delivery of BH₄, we chose to utilize an oral sapropterin intervention. We hypothesized that oral sapropterin would acutely augment reflex and pharmacologically induced cutaneous vasoconstriction in aged human skin through noradrenergic mechanisms.

**Methods**

**Subjects.** Experimental protocols were approved by the institutional review board of The Pennsylvania State University. Written and verbal consent were obtained voluntarily from all subjects prior to participation according to the Declaration of Helsinki. Studies were performed on ten healthy subjects (75±2 years, 5 men and 5 women). Subjects were screened for neurological, cardiovascular and dermatological diseases and underwent a complete medical screening including resting ECG, physical examination, lipid profile and blood chemistry (Quest Diagnostics, Pittsburgh, PA). Subject characteristics are presented in Table 4-1. All subjects were normally active, non-hypertensive, non-diabetic, healthy non-smokers who were not taking over the counter or prescription medications or supplements with primary or secondary vascular effects (e.g. statins, antihypertensives, anticoagulants, antidepressants, etc). Women taking
hormone replacement therapy or who had recently taken hormone replacement therapy were excluded from the study.

**Instrumentation.** All protocols were performed in a thermoneutral laboratory with the subjects in a semisupine position and the experimental arm supported at heart level. All testing took place in the morning to eliminate diurnal variation in blood flow responses (Aoki, Kondo et al. 1997). Study days were separated by at least 48 hours to ensure adequate washout of sapropterin (Feillet, Clarke et al. 2008). Subjects entered the laboratory between 0800 and 0900 and were instrumented with an intravenous catheter for blood sampling. A fasted blood sample was obtained and then subjects ingested 10mg/kg body weight sapropterin (Kuvan®; BioMarin Pharmaceutical Inc, Novato, CA) or placebo with a standardized breakfast meal in a double-blind, randomized crossover study design. The 10mg/kg dose was chosen because it has been shown to increase plasma biopterin concentration ~50 fold (Fiege, Ballhausen et al. 2004), and improvements in vascular function have been observed at increases as small as ~4 fold above baseline (Ueda, Matsuoka et al. 2000). A second blood sample was obtained 3 hours after ingestion of the treatment for analysis of peak plasma BH₄ concentrations. Pharmacokinetic analysis of sapropterin shows that plasma BH₄ concentrations peak at 3 hours following oral administration (Feillet, Clarke et al. 2008). All blood samples were collected in 4ml tubes containing EDTA and 0.1% w/v dithioerythritol and centrifuged immediately. The plasma was flash frozen in liquid nitrogen and stored at -80°C until further analysis of BH₄ concentration by HPLC (Meininger and Wu 2002). Baseline and post treatment blood samples from 3 subjects were not analyzed due to technical difficulties with the samples.

After breakfast, subjects were instrumented with three intradermal microdialysis (MD) fibers (10mm, 20kDa cutoff membrane, MD 2000; Bioanalytical Systems, West Lafayette, IN) placed in the ventral forearm skin using sterile technique. Before MD fiber placement, ice packs were applied to the sites for 5 minutes to temporarily anesthetize the skin (Hodges, Chiu et al.
MD sites were at least 4cm apart to ensure no cross reactivity of the pharmacological agents. For each fiber, a 25-gauge needle was inserted horizontally in the intradermal layers of the skin such that the entry and exit points were ~2.5cm apart. The MD fiber was then threaded through the lumen of the needle and the needle was removed leaving the membrane of the MD fiber in place. The MD fibers were randomly assigned to deliver 1) lactated Ringer’s solution to serve as control, 2) 5mM BH$_4$ (Sigma, St. Louis, MO) to test whether additional, local BH$_4$ administration would provide further benefit over systemic sapropterin treatment, or 3) 5mM yohimbine + 1mM propranolol (Sigma, St. Louis, MO) for α- and β-adrenergic blockade. Concentrations of BH$_4$, yohimbine, and propranolol were based on previous studies conducted in our laboratory (Lang, Holowatz et al. 2009). Pharmacological agents were mixed just before use, dissolved in lactated Ringer’s solution, sterilized using syringe microfilters (Acrodisc; Pall, Ann Arbor, MI), and wrapped in foil to prevent degradation due to light exposure. During the trauma resolution period (60 – 90 minutes), pharmacological solutions were perfused through the MD fibers at a rate of 2µL/min (Bee Hive controller and Baby Bee microinfusion pumps; Bioanalytical Systems).

Whole body mean skin temperature ($T_{sk}$) was controlled using a water-perfused suit that covered the entire body except for the head, hands, feet and forearms. Copper-constantan thermocouples were placed on the surface of the skin at six sites (calf, thigh, abdomen, chest, shoulder and back) and an unweighted average of these sites was taken for continuous measurement of $T_{sk}$. Each subject’s heart rate was monitored throughout the protocol (Cardiocap; GE Healthcare) and arterial blood pressure was measured by brachial auscultation every 5 minutes. Local skin temperature over each MD site was clamped at 33°C throughout baseline and whole body cooling (MoorLab, Temperature Monitor, SHO2; Moor Instruments, Devon, UK) to ensure that changes in SkBF were reflex in origin.
To obtain an index of SkBF, cutaneous red blood cell flux was continually measured directly over each MD site with a laser-Doppler flowmetry probe placed in a local heating unit (Moor Instruments SHO2). Cutaneous vascular conductance (CVC) was calculated as red blood cell flux divided by mean arterial pressure (MAP). MAP was calculated as diastolic pressure plus one-third pulse pressure. Three to five measures of forearm blood flow (FBF) were collected and averaged at baseline and every 0.5°C decrease in $T_{sk}$ by venous occlusion plethysmography. Using a mercury-in-silastic strain gauge (EC6 Plethysmograph, Hokanson, Bellevue, WA), FBF was measured over 5 seconds, while venous flow out of the arm was occluded by a brachial cuff inflated to 50mmHg and blood flow to the hand was occluded at 200mmHg (Whitney 1953). There was an 8 second period between measurements in which the brachial cuff deflated and venous blood flow returned to baseline before the next measurement. In contrast to laser-Doppler flowmetry, which images skin blood flow over a limited area (1mm²) above the MD fiber, venous occlusion plethysmography provides an index of blood flow over the entire forearm. Under thermoneutral resting conditions, changes in FBF are confined to the skin rather than the underlying muscle (Detry, Brengelmann et al. 1972). During whole body cooling, changes in FBF may include reductions in skeletal muscle blood flow which serves to increase effective tissues insulation (Pendergast and Lundgren 2009; Gregson, Black et al. 2011). Forearm vascular conductance (FVC) was calculated as FBF divided by MAP.

**Experimental Protocol.** After MD fiber placement, the insertion trauma resolution period, and instrumentation, baseline data were collected (~20 min). Throughout baseline $T_{sk}$ was held at thermoneutral by perfusing 33°C water through the suit. Following baseline data collection, cool water was perfused through the suit to gradually lower $T_{sk}$ from 34°C to 30.5°C over 30 minutes, followed by ~10 minutes where $T_{sk}$ was clamped at 30.5°C. Cooling began exactly 3 hours after ingestion of sapropterin or placebo. This timing was chosen to ensure peak plasma concentrations of sapropterin during cooling (Feillet, Clarke et al. 2008). Following cooling, warm water was...
perfused through the suit to return $T_{sk}$ to 34°C. Following rewarming, exogenous norepinephrine (1 X $10^{-6}$ M) was perfused at the yohimbine + propranolol perfused site in order to test the $\alpha$- and $\beta$-adrenergic blockade at that site. This norepinephrine dose has been used previously to effectively assess noradrenergic vasoconstriction following whole-body cooling in control and pharmacologically treated MD sites (Lang, Jennings et al. 2009).

After completion of the cooling protocol, site specific pharmacological treatments were discontinued and each MD fiber was perfused with 1mM tyramine to pharmacologically evoke endogenous norepinephrine release. Exogenous norepinephrine (1 X $10^{-2}$ M) was then perfused through each fiber to elicit further noradrenergically-mediated vasoconstriction. Full resolution of the robust vasoconstrictor responses to tyramine and norepinephrine prevented the randomization of these steps with whole-body cooling. Lastly, 28mM sodium nitroprusside (SNP) was perfused through each fiber at a rate of 4µL/min while the local temperature of the skin was increased to 43°C in order to induce a vasodilation response to ensure that vascular responsivity remained intact post-cooling.

Data Acquisition and Analysis. CVC data from the control, BH$_{sk}$, and yohimbine + propranolol-perfused sites were acquired at 40Hz, digitized and stored on a personal computer until further analysis (WinDaq; Dataq Instruments, Akron, OH). CVC values were averaged over a stable 5 minute period of laser-Doppler flux at baseline, over stable periods of flux for every 0.5°C decrease in $T_{sk}$ during whole body cooling (~2-3 minutes), and over a stable plateau during tyramine and norepinephrine perfusion.

A three-way repeated-measures mixed-model ANOVA was conducted to detect oral treatment and local drug treatment differences over the decrease in $T_{sk}$. A two-way repeated-measures mixed-model ANOVA was used to detect oral treatment differences in FVC over the decrease in $T_{sk}$. A two-way repeated-measures mixed-model ANOVA was used to detect oral treatment and local drug treatment differences in vasoconstriction during tyramine and
norepinephrine perfusion, plasma BH₄ concentration, and baseline CVC (version 9.1.3; SAS, Cary, NC). Post hoc comparisons with Bonferroni corrections were performed when necessary to determine where differences between oral treatments and local drug treatments occurred. The level of significance was set at α = 0.05 for main effects. Values are presented as mean ± SEM.

**Results**

Table 4-2 presents plasma BH₄ concentrations following oral placebo and sapropterin treatment from 6 subjects. Plasma BH₄ was significantly elevated 3 hours after ingestion of sapropterin but not after placebo ingestion.

Table 4-3 presents baseline CVC values for all MD sites across oral placebo and sapropterin treatments. There was a significant main effect of MD treatment (p<0.001) on baseline CVC. Accordingly, we have represented changes in skin blood flow as absolute changes from site-specific baseline (ΔCVC).

Table 4-4 presents MAP at baseline, and during whole body cooling with oral placebo and sapropterin treatments. There was no effect of oral treatment on MAP.

Figure 4-1 shows changes in skin blood flow (ΔCVC) from baseline ($\bar{T}_{sk} = 34°C$) and throughout whole-body cooling as a function of decreasing $\bar{T}_{sk}$ at Ringer’s control, BH₄-perfused, and yohimbine + propranolol (Y+P) -perfused MD sites with placebo and sapropterin treatment. Oral sapropterin increased vasoconstriction at the Ringer’s control site compared to placebo at $T_{sk}$ ≤ 32.5°C (p<0.05 for all comparisons at $T_{sk}$ ≤ 32.5°C). Local administration of BH₄ increased vasoconstriction at $\bar{T}_{sk}$ ≤ 31.5°C compared to Ringer’s control with placebo treatment only (p<0.05 for all comparisons at $T_{sk}$ ≤ 31.5°C). There was no difference in vasoconstriction between sapropterin treatment and placebo treatment at the BH₄-perfused site. When
noradrenergic vasoconstriction was inhibited throughout the protocol (Y+P-perfused site) there was no difference in ΔCVC between oral sapropterin and placebo treatment.

Figure 4-2 shows FVC as a function of decreasing $T_{sk}$ at baseline and throughout whole body cooling with oral sapropterin and placebo treatments. Oral sapropterin treatment decreased FVC compared to placebo ($p=0.02$ main effect of oral treatment).

Figure 4-3 shows the vasoconstrictor (ΔCVC) response to 1mM tyramine treatment at the Ringer’s control, BH₄-perfused, and Y+P-perfused MD sites with oral sapropterin and placebo treatments. Oral sapropterin increased vasoconstriction in the Ringer’s control site (placebo: -0.08±0.02 ΔCVC vs. sapropterin: -0.19±0.03 ΔCVC; p=0.01). There was no difference in vasoconstriction between oral placebo or sapropterin treatments at the BH₄-perfused site (placebo: -0.16±0.04 ΔCVC vs. sapropterin: -0.14±0.03 ΔCVC; p=0.60) or the Y+P-perfused site (placebo: -0.05±0.02 ΔCVC vs. sapropterin: -0.06±0.02 ΔCVC; p=0.79). There were no differences between ΔCVC values across MD sites or oral treatments following exogenous norepinephrine ($1 \times 10^{-2}$ M) perfusion through each MD fiber ($p>0.05$ for all comparisons).

**Discussion**

The principal finding of this study was that oral sapropterin acutely (3-hours post ingestion) increased reflex vasoconstriction in aged human skin, as measured by both laser-Doppler flowmetry and venous occlusion plethysmography. Further, it did so through alterations in noradrenergic mechanisms. These data substantiate our previous conclusions that decreased BH₄ contributes to attenuated reflex vasoconstriction in older humans by limiting TH function (Lang, Holowatz et al. 2009), and suggest that with a 10mg/kg oral dose of sapropterin, BH₄ becomes bioavailable in aged skin microvasculature sufficiently to increase norepinephrine
synthesize. Oral sapropterin may be a clinically relevant intervention for improved reflex vasoconstriction in older adults during cold exposure.

In healthy young subjects, ~60% of the total reflex vasoconstriction response to whole-body cooling is mediated by norepinephrine, with the remaining ~40% mediated by cotransmitters such as ATP and neuropeptide Y (Stephens, Saad et al. 2004; Thompson and Kenney 2004). With aging, cotransmitter-mediated reflex vasoconstriction is functionally absent (Thompson and Kenney 2004) and as a result, older humans rely predominately on a compromised norepinephrine-mediated vasoconstriction to decrease skin blood flow and increase tissue insulation during environmental cold exposure. Because cotransmitter-mediated vasoconstriction is absent in aged skin, noradrenergic mechanisms (such as norepinephrine synthesis at the perivascular nerve terminal) are the most viable target for pharmacological interventions that aim to increase axonal release of norepinephrine and reflex vasoconstriction in the skin of older humans during whole-body cold exposure. We examined the efficacy of an acute oral sapropterin intervention in older humans because (I) BH$_4$ bioavailability is reduced with advanced age, (II) this decrease contributes to the attenuated noradrenergic-mediated vasoconstriction in older adults, and (3) BH$_4$, as an essential cofactor for TH, plays a central role in norepinephrine biosynthesis. Our results suggest that oral sapropterin increases the magnitude of reflex cutaneous vasoconstriction in aged human skin by increasing norepinephrine synthesis at the perivascular nerve terminal.

Norepinephrine synthesis requires the functional activity of TH, the rate limiting enzyme in the biosynthesis of catecholamines. BH$_4$ acts as an essential cofactor for TH, reducing the iron moiety of TH, thereby priming TH for catalytic reaction (Kumer and Vrana 1996; Thony, Calvo et al. 2008). Consequently, norepinephrine synthesis is reliant on adequate BH$_4$, and reduced BH$_4$ bioavailability in the older population may contribute to attenuated norepinephrine synthesis and release for a given cold stimulus. Presumably, exogenous BH$_4$ administration enhances functional
vasoconstriction by augmenting norepinephrine biosynthesis and storage in the perivascular nerve terminals, allowing for greater norepinephrine release during sympathetic stimulation (Lang, Holowatz et al. 2010).

In agreement with previous findings from our laboratory, local BH₄ administration through intradermal microdialysis augments reflex (whole-body cold exposure) and pharmacological (tyramine perfusion) vasoconstriction in aged skin following placebo treatment. However, this localized administration did not further increase the magnitude of the vasoconstriction response to either stimulus after oral sapropterin. Further, there was no difference in reflex or pharmacological vasoconstriction between the BH₄-administered microdialysis sites across oral treatments. These data could indicate that (I) the 10mg/kg dose of oral sapropterin maximized activity through TH such that the enzyme was working at or near $V_{\text{max}}$ and/or (II) we had reached a ceiling effect for the ability of the cutaneous vasculature to vasoconstrict under these conditions. Exogenous, locally administered BH₄ does not affect the ability of the vessel to respond to noradrenergic vasoconstrictor stimuli (Lang, Holowatz et al. 2009). Similarly, we found no differences in the vasoconstrictor response to an exogenous norepinephrine perfusion between microdialysis sites or across oral treatments. Collectively, these data suggest that 10mg/kg oral sapropterin increases bioavailable BH₄ sufficiently to increase norepinephrine synthesis within the perivascular nerve terminal.

In contrast to laser-Doppler flowmetry, which measures a limited area of skin (1mm²) directly over the microdialysis membrane, venous occlusion plethysmography provides an index of blood flow over the entire forearm. At rest in thermoneutral to warm environments, changes in blood flow observed with venous occlusion plethysmography are confined to the skin, and do not reflect changes in muscle blood flow (Detry, Brengelmann et al. 1972). During whole-body cold exposure, reductions in FBF may reflect changes in both skin and skeletal muscle blood flow that serve to increase tissue insulation (Pendergast and Lundgren 2009; Gregson, Black et al. 2011). In
this study, oral sapropterin treatment decreased FVC during cold exposure. Given the systemic
nature of the oral treatment and the clinical significance of demonstrating changes in blood flow,
these data further support the finding that oral sapropterin acutely increases the magnitude of
reflex vasoconstriction, and reiterate the clinically relevant application of sapropterin in improved
vascular control mechanisms in older humans.

Sapropterin is a shelf-stable, pharmaceutical formulation of R-BH4 which is
commercially available in the EU and the US for the treatment of BH4-responsive
phenylketonuria. In BH4 deficiency, it’s mechanism of action is presumed to be secondary to
replacement of endogenous cofactor bioavailability (Sanford and Keating 2009). Pharmacokinetic
analysis of sapropterin shows that it exhibits similar time to peak plasma concentrations (~3
hours) and elimination half life (~4 hours) as BH4 administration, following a single oral dose
(Fiege, Ballhausen et al. 2004; Feillet, Clarke et al. 2008). Prior studies examining oral BH4 as an
intervention for improved vascular function in aging or cardiovascular disease have focused on
endothelial NO, and have utilized BH4 powder or capsules administered orally (Porkert, Sher et
al. 2008; Pierce, Jablonski et al. 2012). Recent results from our lab examining the role of
sapropterin in NO-dependent vasodilation suggest that oral sapropterin acutely increases NO-
dependent reflex vasodilation in aged human skin (Stanhewicz, Alexander et al. 2013). In the
present study we utilized an oral sapropterin intervention because it is commercially available,
has superior shelf-stability compared to BH4 powder or capsules, increases bioavailable BH4 in
the microvasculature of older humans (Stanhewicz, Alexander et al. 2013), and has a high
tolerability among patients (Sanford and Keating 2009). Our data suggest that a single oral dose
of sapropterin increases plasma BH4 concentrations in older subjects sufficiently to increase
norepinephrine synthesis though TH and induce a functional increase in the magnitude of reflex
vasoconstriction.
It is of clinical relevance to question whether an intervention that increases vasoconstrictor capacity should be recommended in a population at greater risk for cardiovascular disease. With aging, several signaling mechanisms converge on the vasculature which induce vessel remodeling and endothelial dysfunction, promoting a pro-constrictor status (Csiszar, Wang et al. 2008; Goel, Su et al. 2010; Seals, Jablonski et al. 2011). Reduced BH$_4$ bioavailability is one proposed contributor to this age related vascular dysfunction (Blackwell, Sorenson et al. 2004; Pierce and Larocca 2008) and clinical studies utilizing BH$_4$ as an intervention in aging have found that restoring bioavailable BH$_4$ improves measures of endothelial function in aged vessels (Pierce, Jablonski et al. 2012; Stanhewicz, Bruning et al. 2012). Along these lines, we have shown that oral sapropterin increases NO-dependent vasodilation in aged human skin (Stanhewicz, Alexander et al. 2013). Few studies, if any, have examined the effects of systemic exogeneous BH$_4$ administration on vasoconstrictor mechanisms in vivo. In the present study, we did not see any evidence that oral sapropterin increased mean arterial pressure or heart rate during thermoneutral or whole-body cooling conditions compared to placebo. Similarly, clinical trials of sapropterin have not reported adverse hemodynamic results (Vernon, Koerner et al. 2010; Utz, Lorentz et al. 2012). In context, the observed restoration of a physiological vasoconstriction response to a whole body cold stimulus is not maladaptive, and taken together with our previous finding that oral sapropterin increases NO-dependent reflex vasodilation in a similar subject cohort, these data suggest that exogenous BH$_4$ administration may improve functional vascular control.

**Limitations.** For research purposes, we utilized an oral sapropterin dose standardized to body weight. While scientifically sound, this practice is not commonly used in a medical setting and it is possible that this may affect the clinical validity of our results. However, the results of this study suggest that 10mg/kg sapropterin administered orally, increases plasma BH$_4$ sufficiently to increase functional reflex vasoconstriction in aged skin during whole-body cold
exposure. In a clinical setting, these findings could be of assistance when determining dosing strategies on a patient-to-patient basis. Further research is warranted to determine if there is a standard dose that could be prescribed generally, and to determine the efficacy of a chronic dosing strategy.

It is also unclear when tissues concentrations of BH$_4$ peak following oral administration. Animal models suggest that tissue concentrations peak at the same time blood concentrations peak (Sawabe, Wakasugi et al. 2004), however these time-course data are not available in human models. Despite this uncertainty, our results suggest that three hours was sufficient to increase tissue BH$_4$ in the perivascular nerve terminals of aged cutaneous vessels.

We did not examine the effects of an oral sapropterin intervention in a healthy, young subject population. Healthy young men and women are unlikely to have a reduction in BH$_4$ bioavailability such as that exhibited by an older population (Delp, Behnke et al. 2008). This suggests that young subjects would be unlikely to benefit from added BH$_4$ administration. Furthermore, findings from our lab (Lang, Holowatz et al. 2010; Stanhewicz, Bruning et al. 2012) and others (Eskurza, Myerburgh et al. 2005; Pierce, Jablonski et al. 2012) suggest that exogenous BH$_4$ has no effect on cutaneous or conduit vascular function in young subjects aged 18 – 30 years.

Aside from its roles as an essential cofactor for TH, BH$_4$ also increases NO-dependent vasodilation in aged human skin through its putative role as an essential cofactor for the constitutively expressed NOS (Stanhewicz, Bruning et al. 2012). Similarly, oral sapropterin increases NO-dependent vasodilation in aged human skin (Stanhewicz, Alexander et al. 2013). In the human cutaneous circulation, NO is capable of inhibiting sympathetic adrenergic vasoconstriction; however the exact mechanism by which this inhibition occurs remains unclear (Durand, Davis et al. 2005; Shibasaki, Durand et al. 2007; Shibasaki, Low et al. 2008). Lang et al. utilized the NOS-inhibitor L-NAME to demonstrate that locally administered 5mM BH$_4$ does not
induce NO-mediated effects on the expression of cold- or tyramine-induced vasoconstriction in aged skin (Lang, Holowatz et al. 2009). In the present study we observed a positive effect of the local BH4 and oral sapropterin treatments on absolute and noradrenergic-mediated vasoconstriction despite the role of BH4 in NO synthesis. However, we did not specifically utilize NOS-inhibitors in this study.

**Perspectives.** Our results suggest that an acute 10mg/kg dose of oral sapropterin increases reflex vasoconstriction in aged human skin through noradrenergic mechanisms and that oral administration of exogenous BH4 may be a clinically relevant intervention for improving vasoconstrictor function in older adults exposed to environmental cold. Oral supplementation with BH4 and/or sapropterin improves measures of endothelial function in aging and vascular disease (Hattori, Hattori et al. 2007; Porkert, Sher et al. 2008; Stanhewicz, Alexander et al. 2013), suggesting that NOS-coupling mechanisms of BH4 may be an emerging therapy for endothelial dysfunction. In the present study, we suggest that oral sapropterin, the safe, commercially available, pharmaceutical formulation of BH4, improves thermoregulatory vasoconstriction in older adults through noradrenergic mechanisms in the perivascular nerve terminal. Taken into perspective with the endothelial effects of BH4, these data suggest that oral BH4 administration may improve vascular control across a wide range of requirements, from vasoconstriction to vasodilation. Further research is warranted to investigate the efficacy of this intervention in other human populations that exhibit attenuated cutaneous vasoconstriction, and to determine the efficacy of a long-term dosing strategy.

**Summary.** In summary, acute oral sapropterin increases reflex and pharmacologically-induced cutaneous vasoconstriction in older humans by influencing the norepinephrine synthesis pathway. There is no additive effect of local BH4-perfusion, suggesting that the 10mg/kg dose increases bioavailable BH4 sufficiently to maximally increase norepinephrine synthesis through TH in the perivascular nerve terminal. In addition, there is no effect of locally-administered
exogenous BH4 or oral sapropterin on end-organ responsiveness to norepinephrine. Considering the role of BH4 in norepinephrine synthesis and the observed increase in the magnitude of reflex vasoconstriction in the present study, oral sapropterin is a clinically relevant potential intervention strategy for improving vascular control in older adults.
Table 4-1. Subject Characteristics

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Sex (M,F)</th>
<th>BMI (kg/m²)</th>
<th>LDL (mg/dL)</th>
<th>HDL (mg/dL)</th>
<th>oxLDL (U/mL)</th>
<th>Total cholesterol (mg/dL)</th>
<th>HbA1c (%)</th>
<th>MAP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>75±2</td>
<td>5,5</td>
<td>25±1</td>
<td>119±5</td>
<td>64±4</td>
<td>46±4</td>
<td>200±7</td>
<td>5.7±0.1</td>
<td>90±2</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. BMI: body mass index, HDL: high density lipoprotein, LDL: low density lipoprotein, oxLDL: oxidized low density lipoprotein, MAP: mean arterial pressure.
Table 4-2. Plasma BH$_4$ concentrations at baseline (0 hours) and 3 hours after ingestion of placebo or sapropterin.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 Hours</th>
<th>3 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>15.2 ± 1 pmol/ml</td>
<td>18.6 ± 4 pmol/ml</td>
</tr>
<tr>
<td>Sapropterin (10mg/kg)</td>
<td>19.1 ± 2 pmol/ml</td>
<td>43.8 ± 3 pmol/ml *</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, n=6. *p<0.05 significant difference from 0 hours
Table 4-3. Baseline CVC at Ringer’s (control), yohimbine + propranolol-perfused, and BH$_4$-perfused microdialysis sites with placebo or sapropterin treatments.

<table>
<thead>
<tr>
<th>Placebo</th>
<th>CVC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ringer’s</strong></td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>Yohimbine + Propranolol</td>
<td>0.49 ± 0.12 *</td>
</tr>
<tr>
<td><strong>BH$_4$</strong></td>
<td>0.29 ± 0.05 *</td>
</tr>
<tr>
<td><strong>Sapropterin</strong></td>
<td>CVC</td>
</tr>
<tr>
<td><strong>Ringer’s</strong></td>
<td>0.29 ± 0.06</td>
</tr>
<tr>
<td>Yohimbine + Propranolol</td>
<td>0.50 ± 0.05 *</td>
</tr>
<tr>
<td><strong>BH$_4$</strong></td>
<td>0.29 ± 0.04</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. *p<0.001 main effect of local drug treatment
Table 4-4. Mean arterial pressure (mmHg) at baseline ($\bar{T}_{sk} = 34.0^\circ$C) and throughout whole-body cooling with oral placebo and sapropterin treatment.

<table>
<thead>
<tr>
<th>$\bar{T}_{sk}$ (°C)</th>
<th>Placebo</th>
<th>Sapropterin</th>
</tr>
</thead>
<tbody>
<tr>
<td>34.0</td>
<td>86±2</td>
<td>85±3</td>
</tr>
<tr>
<td>33.5</td>
<td>88±3</td>
<td>84±3</td>
</tr>
<tr>
<td>33.0</td>
<td>90±3</td>
<td>85±3</td>
</tr>
<tr>
<td>32.5</td>
<td>89±3</td>
<td>87±3</td>
</tr>
<tr>
<td>32.0</td>
<td>89±3</td>
<td>87±3</td>
</tr>
<tr>
<td>31.5</td>
<td>92±4</td>
<td>91±3</td>
</tr>
<tr>
<td>31.0</td>
<td>92±4</td>
<td>90±3</td>
</tr>
<tr>
<td>30.5</td>
<td>93±4</td>
<td>91±3</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. There was no effect of sapropterin on mean arterial pressure (all p>0.05)
Figure 4-1. Group mean ± SEM of vasoconstriction response (ΔCVC) to decreasing (x-axis decreases left to right) skin temperature (T_{sk}) at baseline (T_{sk} = 34.0) and during whole body cooling in Ringer’s control (A), BH4-perfused (B), and yohimbine + propranolol (Y+P)-perfused (C) microdialysis sites with oral placebo or sapropterin treatment. * p < 0.05 significant difference compared to placebo within site
Figure 4-2. Group mean ± SEM of forearm cutaneous vasoconstriction response (FVC) to decreased mean skin temperature at baseline ($T_{sk} = 34.0$) and during whole body cooling with oral placebo or sapropterin treatment. $p=0.02$ main effect of oral treatment.
Figure 4-3. Group mean ± SEM of vasoconstriction response (ΔCVC) to 1mM tyramine perfusion in Ringer’s (control), BH₄-perfused, and yohimbine + propranolol (Y+P)-perfused microdialysis sites with oral placebo or sapropterin treatment. *p < 0.05 compared to placebo within site.
Chapter 5

CHRONIC TREATMENT WITH ORAL SAPROPTERIN: EVIDENCE OF EFFICACY IN AUGMENTED REFLEX CONTROL OF BLOOD FLOW IN AGED HUMAN SKIN

Introduction

Primary human aging is associated with an attenuated cutaneous vasodilator and vasoconstrictor response to whole-body heat and cold stress, respectively (Kenney and Armstrong 1996; Kenney, Morgan et al. 1997). This attenuated reflex cutaneous vasoreactivity is due to a functional loss of active cholinergic vasodilator co-transmitter and adrenergic vasoconstrictor function. In contrast to young, healthy subjects older adults exhibit an attenuated co-transmitter mediated vasodilation (Holowatz, Houghton et al. 2003) and a functionally absent co-transmitter mediated vasoconstriction (Thompson and Kenney 2004). This is further compounded by altered downstream vascular signaling mechanisms including decreased NO availability and adrenergic receptor desensitization (Holowatz, Houghton et al. 2003; Thompson and Kenney 2004). Consequently, older adults rely primarily on compromised NO-mediated vasodilation and norepinephrine-mediated vasoconstriction to control thermoregulatory cutaneous blood flow during whole-body heat or cold stress, respectively.

The decreased NO bioavailability in aged skin results from a decrease in NO production by upregulated vascular arginase and increased nitric oxide synthase (NOS) uncoupling with increased oxidant stress (Holowatz, Thompson et al. 2006; Holowatz, Thompson et al. 2006). The age-related decrements in norepinephrine- and co-transmitter-mediated constriction are due, in part, to age-related decreases in transmitter synthesis and/or release (Frank, Raja et al. 2000; Connat, Busseuil et al. 2001; Donoso, Gomez et al. 2008) consequent to elevated oxidative stress
and reduced substrate (L-tyrosine) availability for norepinephrine synthesis (Lang, Holowatz et al. 2010). As a result, both reflex vasodilation and vasoconstriction are attenuated, in part, by reductions in substrate availability and increases in oxidant stress.

In addition to limited substrate and elevated oxidative stress, reductions in tetrahydrobiopterin (BH₄) bioavailability also contribute to the decline in reflex cutaneous VD and VC mechanisms (Lang, Holowatz et al. 2010; Stanhewicz, Bruning et al. 2012). BH₄ is a critical enzymatic cofactor required for the synthesis of the adrenergic neurotransmitter NE as well as the gasotransmitter NO (Werner, Blau et al. 2011). Consequently, BH₄ is required for full expression of reflex cutaneous vasoconstriction and vasodilation. Human aging is associated with a decrease in BH₄ bioavailability, primarily through increased degradation by age-associated increases in oxidant radicals (Kuzkaya, Weissmann et al. 2003).

Acute, localized microperfusion of exogenous BH₄ directly into the skin increases reflex vasodilation and vasoconstriction through NO-dependent and adrenergic mechanisms, respectively, in older humans (Lang, Holowatz et al. 2010; Stanhewicz, Bruning et al. 2012; Stanhewicz, Alexander et al. 2013). Similarly, we have also recently demonstrated that a single oral dose of sapropterin (pharmaceutical BH₄) restores reflex cutaneous vasodilation and vasoconstriction in older (>65 years) adults (Stanhewicz, Alexander et al. 2013; Stanhewicz, Alexander et al. 2013). These data suggest that sapropterin may be an effective intervention; however no data exist to support the role of chronic sapropterin treatment in improved reflex control of the cutaneous vascular bed in older humans. In this pilot study, we aimed to specifically explore the efficacy of a chronic sapropterin intervention. We hypothesized that chronic sapropterin (400mg twice daily for four weeks) (Cosentino, Hurlimann et al. 2008; Porkert, Sher et al. 2008) treatment would augment reflex vasodilation and vasoconstriction in older human skin through NOS coupling and adrenergic mechanisms, respectively.
Methods

Subjects. Experimental protocols were approved by the institutional review board of The Pennsylvania State University. Written and verbal consent were obtained voluntarily from all subjects prior to participation according to the Declaration of Helsinki. Studies were performed on four healthy subjects (74±4 years, 2 men and 2 women). Subjects were screened for neurological, cardiovascular and dermatological diseases and underwent a complete medical screening including resting ECG, physical examination, lipid profile and blood chemistry (Quest Diagnostics, Pittsburgh, PA). All subjects were normally active, non-hypertensive, non-diabetic, healthy non-smokers who were not taking prescription medications with primary or secondary vascular effects (e.g. statins, antihypertensives, anticoagulants, antidepressants, or hormone replacement therapy, etc). Subjects ingested 400mg sapropterin (Kuvan®, BioMarinPharmaceutical Inc, Novato, CA) or placebo twice daily for four weeks before returning to the laboratory for testing. Treatment periods were separated by at least two weeks to ensure adequate washout of sapropterin (Feillet, Clarke et al. 2008).

Instrumentation. All protocols were performed in a thermoneutral laboratory with the subjects in a semisupine position and the experimental arm supported at heart level. All testing took place in the morning to eliminate diurnal variation in blood flow responses (Aoki, Kondo et al. 1997). Subjects were instrumented with one intradermal microdialysis (MD) fiber (10mm, 20kDa cutoff membrane, MD 2000; Bioanalytical Systems, West Lafayette, IN) placed in the ventral forearm skin as previously described (Stanhewicz 2013). During the trauma resolution period (60 – 90 minutes) lactated Ringer’s was perfused through the MD fiber at a rate of 2μL/min (Bee Hive controller and Baby Bee microinfusion pumps; Bioanalytical Systems).

Skin temperature (Tsk) was controlled using a water-perfused suit that covered the entire body except for the head, hands, feet and forearms. Copper-constantan thermocouples were
placed on the surface of the skin at six sites (calf, thigh, abdomen, chest, shoulder and back) for continuous measurement of Tsk. Each subject’s heart rate was monitored throughout the protocol (Cardiocap; GE Healthcare) and arterial blood pressure was measured by brachial auscultation every 5 minutes. Oral temperature (Tora) was measured as an index of changes in body temperature using a thermistor placed in the sublingual sulcus throughout baseline and whole body heating. Proper placement of the thermistor was checked based on temperature readings and once verified, was taped in place and closely monitored to ensure that it did not move throughout the protocol. Local Tsk over each MD site was clamped at 33°C throughout the protocol(MoorLab, Temperature Monitor, SHO2; Moor Instruments, Devon, UK) to ensure that changes in SkBF were reflex in origin.

To obtain an index of SkBF, cutaneous red blood cell flux was continually measured directly over each MD site with an integrated laser-Doppler flowmetry probe placed in a local heating unit (Moor Instruments SHO2). Cutaneous vascular conductance (CVC) was calculated as red blood cell flux divided by mean arterial pressure (MAP) and expressed as a percent of site-specific maximal vasodilation [%CVCmax; 28mM sodium nitroprusside (SNP) and local heat to 43°C] for heating studies and as a change in CVC from baseline [ΔCVC] for cooling studies.

**Whole-Body Heating Experimental Protocol.** Following MD fiber placement, the insertion trauma resolution period, and instrumentation, baseline data were collected (~20 min). Throughout baseline mean Tsk was held at thermoneutral by perfusing 33°C water through the suit. Following baseline data collection, warm water was perfused through the suit to clamp mean Tsk at 38°C and cause a gradual rise in Tora. At a 1°C rise in Tora, mean body temperature (Tb) was clamped by lowering the water temperature in the suit, such that mean Tsk and Tora ceased to rise. After 5 minutes of steady laser-Doppler flux values, 20mM N^G^-nitro-L-arginine (L-NAME; Calbiochem, San Diego, CA) was perfused through the fiber at a rate of 4µL/min to inhibit NOS production of NO and quantify NO-dependent vasodilatation within the site. Concentrations of
L-NAME were based on previous studies conducted in our laboratory (Stanhewicz, Bruning et al. 2012; Stanhewicz, Alexander et al. 2013). L-NAME was mixed just before use, dissolved in lactated Ringer’s solution, sterilized using syringe microfilters (Acrodisc; Pall, Ann Arbor, MI), and wrapped in foil to prevent degradation due to light exposure. L-NAME perfusion was discontinued after laser-Doppler flux values decreased to a steady plateau (~40 min). At this time, whole body heating was terminated, the water perfused suit was perfused with 33°C water and subjects were returned to thermoneutral.

After completion of the whole body heating protocol, the MD fiber was perfused with 28mM SNP (Nitropress; Abbott Laboratories, Chicago, IL) at a rate of 4µl/min. Simultaneously, the local $T_{sk}$ over the experimental site was increased to 43°C to obtain maximal CVC values.

**Whole-Body Cooling Experimental Protocol.** After MD fiber placement, the insertion trauma resolution period, and instrumentation, baseline data were collected (~20 min). Throughout baseline $T_{sk}$ was held at thermoneutral by perfusing 33°C water through the suit. Following baseline data collection, cool water was perfused through the suit to gradually lower $T_{sk}$ from 34°C to 30.5°C over 30 minutes, followed by ~10 minutes where $T_{sk}$ was clamped at 30.5°C. Following cooling, warm water was perfused through the suit to return $T_{sk}$ to 34°C.

After completion of the cooling protocol, the MD fiber was perfused with 1mM tyramine to pharmacologically evoke endogenous norepinephrine release. Exogenous norepinephrine (1 X $10^{-2}$ M) was then perfused through the fiber to elicit further noradrenergically-mediated vasoconstriction. Full resolution of the robust vasoconstrictor responses to tyramine and norepinephrine prevented the randomization of these steps with whole-body cooling. Lastly, 28mM sodium nitroprusside (SNP) was perfused through each fiber at a rate of 4µL/min while the local temperature of the skin was increased to 43°C in order to induce a vasodilation response to ensure that vascular responsivity remained intact post-cooling.
Data Acquisition and Analysis. CVC data were acquired at 40Hz, digitized and stored on a personal computer until further analysis (WinDaq; Dataq Instruments, Akron, OH). CVC values were averaged over a stable period of laser-Doppler flux at each point of interest (baseline, ΔT_{or} = 1°C, L-NAME plateau, T_{sk} = 30.5°C, tyramine-plateau).

A repeated-measures ANOVA with Bonferroni correction was used to detect oral treatment differences at baseline and at a 1°C rise in T_{or} for the heating study and baseline and at T_{sk} = 30.5°C for the cooling study. One-tailed t-tests were used to detect treatment differences in NO-dependent vasodilation and maximal CVC, as well as tyramine-induced VC (version 9.1.3; SAS, Cary, NC). The level of significance was set at α = 0.05 for main effects. Values are presented as mean ± SEM.

Results

Subject characteristics are presented in Table 5-1. There was no effect of chronic sapropterin treatment on systolic pressure, diastolic pressure, MAP, or HR.

Figure 5-1 shows individual and mean skin blood flow (%CVC_{max}) at baseline and at a 1°C rise in T_{or} (ΔT_{or} = 1°C) with placebo and sapropterin treatment. Sapropterin increased mean %CVC_{max} at a 1°C rise in T_{or} (sapropterin: 57±4 vs. placebo: 46±4 %CVC_{max}; p<0.001).

Figure 5-2 shows mean NO-dependent vasodilation (%CVC_{max}) at a 1°C rise in T_{or}. NO-dependent vasodilation was greater following sapropterin treatment however the difference was not statistically significant (sapropterin: 26±8 vs. placebo: 10±5%; p = 0.07).

Figure 5-3 shows individual and mean skin blood flow (ΔCVC) at baseline and at T_{sk} = 30.5°C with placebo and sapropterin treatment. There was no difference between treatments (sapropterin: -0.06±0.01 vs. placebo: -0.06±0.01 ΔCVC; p=0.60).
Figure 5-4 shows mean vasoconstriction response to 1mM tyramine infusion following placebo and saproterin treatment. There was no difference between treatments (saproterin: -0.09±0.02 vs. placebo: -0.08±0.03ΔCVC; p=0.70).

**Discussion**

The findings of this pilot study provide preliminary evidence that chronic supplementation with oral saproterin (400mg twice daily for 4 weeks) increases reflex vasodilation in aged human skin. Furthermore, it may do so through NO-dependent mechanisms. These findings agree with our previous conclusions that decreased BH₄ bioavailability contributes to age-related attenuations in reflex vasodilation (Stanhewicz, Bruning et al. 2012), and that acute oral administration of saproterin increases BH₄ bioavailability and increases NO-dependent reflex vasodilation in older adults (Stanhewicz, Alexander et al. 2013). Oral saproterin may be a clinically relevant intervention for improved thermoregulatory skin blood flow in older adults during hyperthermia. Specifically, the findings of this preliminary study suggest that chronic saproterin administration is efficacious in increasing reflex vasodilation in healthy, older adults. However, there is not sufficient evidence to suggest that chronic saproterin increases reflex vasoconstriction in the skin of healthy, older adults.

In healthy young subjects, ~30-40% of the total reflex vasodilation response is mediated by NO signaling, with the remaining ~60-70% relying on co-released neurotransmitter(s) with downstream vasodilation mediated through other second messenger pathways and activation of cyclo-oxygenase (Bennett, Johnson et al. 2003; Wong, Wilkins et al. 2004; McCord, Cracowski et al. 2006). With aging, the cofactor-mediated contribution to the overall expression of reflex vasodilation is attenuated and cyclo-oxygenase-dependent signaling favors the production of vasoconstrictors (Holowatz, Jennings et al. 2009). Consequently, healthy older adults rely
predominately on a functionally compromised NO-dependent vasodilation to increase skin blood flow during hyperthermia (Holowatz, Houghton et al. 2003) and interventions that target NO production and bioavailability may be capable of increasing reflex vasodilation in aged human skin.

We have shown that both local microperfusion of BH₄, and a single 10mg·kg⁻¹ oral dose of sapropterin (pharmaceutical BH₄) restore reflex cutaneous vasodilation through NO-dependent dilation in aged human skin (Stanhewicz, Bruning et al. 2012; Stanhewicz, Alexander et al. 2013). Mechanistically, BH₄ stabilizes NOS in the coupled conformation and reduces oxidant stress in and around the NOS molecule (Raman, Li et al. 1998; Vasquez-Vivar, Kalyanaraman et al. 1998). In conditions of reduced BH₄ bioavailability, NOS uncouples and produces superoxide rather than NO (Raman, Li et al. 1998). Because BH₄ bioavailability is reduced with aging, and because our previous data suggested that sapropterin may be a clinically relevant intervention strategy for improved cutaneous vasodilation in aging, we conducted this pilot study to examine the efficacy of a chronic dosing strategy in improved thermoregulatory skin blood flow in older adults.

The results of this pilot study do not support our hypothesis that chronic oral sapropterin treatment restores reflex cutaneous vasoconstriction in older adults exposed to whole-body cold stress. Previously, we have shown that localized microperfusion of exogenous BH₄ as well as acute oral doses of sapropterin augment reflex vasoconstriction through noradrenergic mechanisms (Lang, Holowatz et al. 2010; Stanhewicz, Alexander et al. 2013). Mechanistically, BH₄ acts as an essential cofactor for TH, reducing the iron moiety of TH, thereby priming TH for catalytic reaction (Kumer and Vrana 1996; Thony, Calvo et al. 2008). Consequently, norepinephrine synthesis is reliant on adequate BH₄, and reduced BH₄ bioavailability in the older population may contribute to attenuated norepinephrine synthesis and release for a given cold stimulus.
Initial power analysis (power = 0.80, α=0.05) suggested that 12 subjects would be required to detect a meaningful physiological difference due to treatment. Unfortunately, the cost of the chronic sapropterin treatment (~$8,000 per subject) was prohibitive and we were unable to test more than four subjects. It is possible that with such a small sample size we were unable to detect a difference due to treatment.

It is also possible that chronically, sapropterin treatment is unable to restore adrenergic constrictor mechanisms in aged cutaneous vasculature at the dose utilized in this study (400mg twice daily). Presumably, BH₄ bioavailability needs to increase in the perivascular nerve terminal for TH-dependent synthesis of NE to be restored in aging. It is possible that with the lower, chronic doses (400mg twice daily compared to 10mg·kg⁻¹ acute dose) sapropterin did not accumulate in the perivascular nerve terminal at high enough concentrations to affect TH function. In this study, we did not examine the distribution of sapropterin to the various tissues, however lower sapropterin concentrations in the perivascular nerve terminal could explain the failure of the treatment to work in these subjects. Further study of the tissue specific distribution and/or accumulation of orally administered sapropterin, as well as investigations of different dosing strategies, may shed light on the issue and explain the possible discrepancies between acute and chronic efficacy of oral sapropterin in improved reflex vasoconstriction in aged human skin.

Sapropterin is a commercially available, shelf-stable, pharmaceutical formulation of R-BH₄ which is prescribed clinically for the treatment of BH₄-responsive phenylketonuria. Sapropterin is not indicated for the treatment or prevention of vascular dysfunction; however, phase I clinical trials have suggested that it may be efficacious in specific cardiovascular disease states including hypertension, dyslipidemia, and aging (Porkert, Sher et al. 2008; Moreau, Meditz et al. 2012; Pierce, Jablonski et al. 2012). Presumably, the chronic sapropterin intervention used in the current study exerted its effects through the NOS-coupling properties of BH₄, thereby
increasing NO-dependent vasodilation. NO-dependent dilation assessed in this study was
increased following sapropterin treatment; however, these results were not statistically significant
(p = 0.07) with such a small sample size. Given the putative role of BH₄ in NOS coupling and
endothelial function (Eskurza, Myerburgh et al. 2005; Higashi, Sasaki et al. 2006) and the
mechanistic data from our previous studies utilizing BH₄ and sapropterin in older subjects
(Stanhewicz, Alexander et al. 2013), we speculate that the improvement in reflex cutaneous
vasodilation in this study was the result of improved NO-dependent vasodilation, rather than
alterations in vessel wall structure.

By design this study excluded subjects who had overt cardiovascular disease and/or were
taking medications. For that reason our results may not be generalizable to older adults who are
unhealthy and/or taking a variety of medications. However, exogenous BH₄ therapy has been
shown to effectively increase vascular endothelial function in populations with cardiovascular
disease (Cosentino, Hurlimann et al. 2008; Porkert, Sher et al. 2008; Alexander, Kutz et al. 2013).
These findings, combined with the results of the present pilot study, suggest that chronic BH₄ or
sapropterin treatment may be efficacious in older adults who do exhibit overt cardiovascular
disease.

In the present pilot study we did not report values for plasma BH₄ in these subjects.
However, we have previously shown that plasma BH₄ increases significantly following oral
administration in healthy, older adults (Stanhewicz, Alexander et al. 2013). Similarly, Pierce et.al.
report that plasma BH₄ increased in healthy older men who had ingested BH₄ (Pierce, Jablonski et
al. 2012). Based on these previous data, as well as the positive results from the current study, we
are confident that our chronic dosing strategy increased BH₄ bioavailability in these subjects.

Perspectives. Our results suggest that chronic sapropterin (400mg twice daily for 4
weeks) increases reflex vasodilation in aged human skin and that chronic oral administration of
sapropterin may be a clinically relevant intervention for improved thermoregulatory skin blood
flow in older adults exposed to heat stress. Taken in the context of our previous findings that acute sapropterin increases reflex vasodilation in aged skin through NO-dependent mechanisms (Stanhewicz, Alexander et al. 2013) and given the role of BH₄ in improved endothelial function in aging conduit vessels (Pierce, Jablonski et al. 2012), this pilot study suggests that chronic sapropterin treatment may improve endothelial function in healthy older adults. These preliminary results provide the basis for larger investigations aimed at confirming the mechanistic role of sapropterin in improved thermoregulatory vasodilation in aged skin, as well as examining the broader application of chronic sapropterin in improved endothelial function and vascular health with aging.
Table 5-1. Subject characteristics at baseline and following placebo and sapropterin treatments

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Placebo</th>
<th>Sapropterin</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>74 ± 4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMI (kg·m⁻²)</td>
<td>24 ± 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Resting HR (bpm)</td>
<td>66 ± 3</td>
<td>65 ± 2</td>
<td>63 ± 3</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>120 ± 4</td>
<td>122 ± 3</td>
<td>120 ± 4</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>75 ± 6</td>
<td>71 ± 3</td>
<td>75 ± 2</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>89 ± 5</td>
<td>88 ± 3</td>
<td>90 ± 3</td>
</tr>
<tr>
<td>Total cholesterol (mg·dl⁻¹)</td>
<td>202 ± 18</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LDL cholesterol (mg·dl⁻¹)</td>
<td>123 ± 11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HDL cholesterol (mg·dl⁻¹)</td>
<td>59 ± 8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.7 ± 0.1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

n = 4. Data are mean ± SEM. BMI, body mass index; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; LDL, low density lipoprotein; HDL, high density lipoprotein; HbA1c, hemoglobin A1c.
Figure 5-1. Individual (top) and group mean ± SEM (bottom) vasodilation response (%CVC max) at baseline and at 1°C rise in oral temperature (ΔT or = 1°C) following placebo and sapropterin treatments. * p<0.001 significant difference compared to placebo.
Figure 5-2. Group mean ± SEM of NO-dependent vasodilation (\%CVC_{max}) response at 1°C rise in oral temperature following placebo and sapropterin treatments.
Figure 5-3. Individual (top) and group mean ± SEM (bottom) vasoconstriction response ($\DeltaCVC$) at $T_{sk} = 30.5^\circ C$ following placebo and sapropterin treatments.
Figure 5-4. Group mean ± SEM of vasoconstriction response (ΔCVC) to 1mM tyramine perfusion following placebo and sapropterin treatments.
Chapter 6

FOLIC ACID SUPPLEMENTATION IMPROVES CUTANEOUS MICROVASCULAR FUNCTION IN OLDER HUMANS THROUGH NITRIC OXIDE-DEPENDENT MECHANISMS

Introduction

Healthy older (>65 years) men and women demonstrate an attenuated vascular endothelial function evidenced by a reduced cutaneous vasodilation response to local skin heating and whole body heat stress (Kenney, Morgan et al. 1997; Minson, Holowatz et al. 2002; Holowatz, Houghton et al. 2003). This compromised vascular function can be attributed to an age-related decrease in dilator mechanisms, such that older adults rely primarily on an attenuated nitric oxide (NO)-dependent vasodilation to increase blood flow (Kenney, Morgan et al. 1997; Holowatz, Houghton et al. 2003). Consequently, the NO pathway is an important molecular target for potential pharmacological intervention strategies aimed at improving or maintaining vascular health and function throughout older adulthood.

Age-related impairments in NO-dependent vasodilation are due, in part, to decreases in tetrahydrobiopterin (BH₄) bioavailability (Delp, Behnke et al. 2008; Stanhewicz, Bruning et al. 2012), and increases in oxidant stress (Lu, Lee et al. 1999; Holowatz, Thompson et al. 2006). BH₄ is required to maintain the functional conformation of the nitric oxide synthase (NOS) dimer for NO production and is reduced with aging due to decreased synthesis and increased oxidation by free radicals (Crabtree and Channon 2011; Werner, Blau et al. 2011). In the absence of adequate cofactor (BH₄) availability, the NOS dimer becomes functionally uncoupled, and produces superoxide, rather than NO (Vasquez-Vivar, Kalyanaraman et al. 1998). Increases in reactive
oxygen species, including superoxide ($O_2^-$), with advancing age further contribute to reduced BH$_4$ bioavailability by oxidizing BH$_4$ to dihydrobiopterin (BH$_2$), a competitive inhibitor which lacks the cofactor properties of BH$_4$ (Sugiyama, Levy et al. 2009). Collectively, these age-related increases in oxidant stress and subsequent decreases in BH$_4$ bioavailability contribute to endothelial dysfunction and attenuated NO-dependent vasodilation in primary aging.

Recently published data from our lab demonstrates that (A) local perfusion of BH$_4$ directly into the dermal space, and (B) oral administration of sapropterin (pharmaceutical BH$_4$) acutely reverse vascular dysfunction in aged human skin through NO-dependent mechanisms (Stanhewicz, Bruning et al. 2012; Stanhewicz, Alexander et al. 2013). However, the high cost and relative inaccessibility of oral formulations of BH$_4$ underscores the need for a readily available, inexpensive intervention strategy to effectively improve vascular health in older adults.

Folic acid and its active metabolite 5-methyltetrahydrofolate (5-MTHF) improve conduit vessel endothelial function in patients with overt cardiovascular and metabolic disease (Verhaar, Wever et al. 1998; Doshi, McDowell et al. 2001; Alian, Hashemipour et al. 2012). 5-MTHF increases vascular BH$_4$ bioavailability by increasing production via BH$_2$ recycling and reducing oxidant stress, and improvements in vessel function may be mediated though restoration of NO production (Stroes, van Faassen et al. 2000; Antoniades, Shirodaria et al. 2006). The purpose of this study was to determine if exogenous folic acid administration could increase NO-dependent vasodilation in aged human skin. Folic acid supplementation may be a viable intervention strategy to improve vascular health and prevent cardiovascular morbidity in older adults; however, few in vivo studies have examined the mechanistic role of folic acid in improved endothelial function in primary aged adults. We hypothesized that acute, local microperfusion of 5-MTHF directly into the dermal space would increase the cutaneous vasodilation response to an endothelial-NOS (eNOS)-dependent stimulus (local thermal hyperemia) through NO-dependent
mechanisms in older adults (study 1). We further hypothesized that a chronic, high-dose, oral folic acid intervention (5mg daily for 6 weeks) would increase the magnitude of reflex cutaneous vasodilation through restoration of NO-dependent mechanisms in older adults exposed to passive heat stress (study 2).

**Methods**

*Subjects.* Subject characteristics are presented in Table 6-1. Older subjects had significantly higher LDL and total cholesterol compared to young. However, these values were within normal range defined by the American Heart Association. Experimental protocols were approved by the institutional review board of The Pennsylvania State University. Written and verbal consent were obtained voluntarily from all subjects prior to participation according to the Declaration of Helsinki. Subjects were screened for neurological, cardiovascular and dermatological diseases and underwent a complete medical screening including resting ECG, physical examination, lipid profile and blood chemistry (Quest Diagnostics, Pittsburgh, PA). All subjects were normally active, non-hypertensive, non-diabetic, healthy non-smokers who were not taking prescription medications with primary or secondary vascular effects (e.g. statins, antihypertensives, anticoagulants, antidepressants, etc). Women taking hormonal birth control, hormone replacement therapy, or who had recently taken hormone replacement therapy were excluded from the study. All pre-menopausal women were normally menstruating and were studied during the early follicular phase (days 1 – 7) of their menstrual cycle.

**Study 1: Local Heating**

All experiments took place in a thermoneutral laboratory. Two intradermal microdialysis fibers (10mm, 20kDa cutoff membrane, MD 2000; Bioanalytical Systems, West Lafayette, IN) were placed into the dermal layer of the ventral left forearm for the local delivery of
pharmacological agents (Bruning, Santhanam et al. 2012). Microdialysis sites were randomly assigned to receive 1) 5mM 5-MTHF (USP, Rockville, MD) for local delivery of the folic acid metabolite; or 3) lactated Ringer’s solution to serve as control. Pharmacological agents were mixed just before use, dissolved in lactated Ringer’s solution, sterilized using syringe microfilters (Acrodisc; Pall, Ann Arbor, MI), and wrapped in foil to prevent degradation due to light exposure. Site-specific pharmacological solutions were perfused through the MD fibers at a rate of 2µL/min (Bee Hive controller and Baby Bee microinfusion pumps; Bioanalytical Systems).

Sixty to ninety minutes were allowed for hyperemia to cease before a standard local heating protocol to induce eNOS-dependent vasodilation (Minson, Berry et al. 2001; Bruning, Santhanam et al. 2012). Local heater temperature was increased from the baseline clamped temperature of 33°C to 42°C at a rate of 0.1°C every second and then clamped at 42°C for the remainder of the heating protocol. After ~30–40 min, when skin blood flow reached an established plateau, 20mML-NAME was perfused at a rate of 4µL/min to quantify NO-dependent vasodilation at all sites. After infusion of L-NAME and subsequent stabilization of a post-L-NAME plateau in skin blood flow, 28mM sodium nitroprusside (Nitropress; Abbott Laboratories, Chicago, IL) was perfused and local temperature increased to 43°C to elicit CVCmax (Johnson, O’Leary et al. 1986).

Cutaneous red blood cell flux was continually measured directly over each MD site with an integrated laser-Doppler flowmetry probe placed in a local heating unit (Moor Instruments SHO2). Cutaneous vascular conductance (CVC) was calculated as red blood cell flux divided by mean arterial pressure (MAP) and expressed as a percent of site-specific maximal vasodilation (%CVCmax).

A two-way repeated-measures mixed-model ANOVA was used to detect age and local treatment differences in local heating plateau, NO-dependent vasodilation, and maximal CVC (version 9.1.3; SAS, Cary, NC). Post hoc comparisons with Bonferroni corrections were
performed when necessary. The level of significance was set at $\alpha = 0.05$ for main effects. Values are presented as mean $\pm$ SEM.

**Study 2: Whole-Body Heating**

Before each experiment, subjects ingested 5mg folic acid (BioTech Pharmacal) or placebo once daily for 6 weeks in a double-blind, crossover study design. Treatment periods were separated by at least 2 weeks to ensure adequate washout of folic acid. Subjects did not ingest their treatment on the day of the experiment. Upon arrival to the laboratory, a blood sample was obtained for the measurement of plasma folic acid and homocysteine.

All protocols were performed in a thermoneutral laboratory with the subjects in a semisupine position and the experimental arm supported at heart level. All repeated measures took place at the same time of day to eliminate diurnal variation in blood flow responses (Aoki, Kondo et al. 1997). Subjects were instrumented with three intradermal MD fibers placed in the ventral forearm skin for the local delivery of pharmacological agents (Stanhewicz 2013). MD sites were randomly assigned 1) 5mM 5-MTHF for local delivery of the folic acid metabolite; 2) 0.1mM BH$_4$ for local delivery of the essential NOS cofactor; or 3) lactated Ringer’s to serve as control. During the trauma resolution period (60 – 90 minutes) site specific pharmacological agents were perfused through the MD fibers at a rate of 2µL/min.

Skin temperature ($T_{sk}$) was controlled using a water-perfused suit that covered the entire body except for the head, hands, feet and forearms. Copper-constantan thermocouples were placed on the surface of the skin at six sites (calf, thigh, abdomen, chest, shoulder and back) for continuous measurement of $T_{sk}$. Each subject’s heart rate was monitored throughout the protocol (Cardiocap; GE Healthcare) and arterial blood pressure was measured by brachial auscultation every 5 minutes. Oral temperature ($T_{oral}$) was measured as an index of changes in body temperature using a thermistor placed in the sublingual sulcus throughout baseline and whole body heating. Proper placement of the thermistor was checked based on temperature readings and once verified,
was taped in place and closely monitored to ensure that it did not move throughout the protocol. Local T_{sk} over each MD site was clamped at 33°C throughout baseline and whole body heating (MoorLab, Temperature Monitor, SHO2; Moor Instruments, Devon, UK) to ensure that changes in SkBF were reflex in origin.

Cutaneous red blood cell flux was continually measured directly over each MD site with an integrated laser-Doppler flowmetry probe placed in a local heating unit (Moor Instruments SHO2). Cutaneous vascular conductance (CVC) was calculated as red blood cell flux divided by mean arterial pressure (MAP) and expressed as a percent of site-specific maximal vasodilation.

Following MD fiber placement, the insertion trauma resolution period, and instrumentation, baseline data were collected (~20 min). Throughout baseline mean T_{sk} was held at thermoneutral by perfusing 33°C water through the suit. Following baseline data collection, warm water was perfused through the suit to clamp mean T_{sk} at 38°C and cause a gradual rise in T_{or}. At a 1°C rise in T_{or}, mean body temperature (T_{b}) was clamped by lowering the water temperature in the suit, such that mean T_{sk} and T_{or} ceased to rise. After 5 minutes of steady laser-Doppler flux values, 20mM NG-nitro-L-arginine (L-NAME; Calbiochem, San Diego, CA) was perfused through the fiber at a rate of 4µL/min to inhibit NOS production of NO and quantify NO-dependent vasodilatation within the site (Stanhewicz, Bruning et al. 2012; Stanhewicz, Alexander et al. 2013). L-NAME perfusion was discontinued after laser-Doppler flux values decreased to a steady plateau (~40 min). At this time, whole body heat was terminated, the water perfused suit was perfused with 33°C water and subjects were returned to thermoneutral.

After completion of the whole body heating protocol, the MD fiber was perfused with 28mM SNP at a rate of 4µl/min. Simultaneously, the local T_{sk} over the experimental site was increased to 43°C to obtain CVC_{max}. 

Paired t-tests were conducted to detect significant differences in skin blood flow at Δ1°C rise in oral temperature and NO-dependent vasodilation between groups and across treatments. The level of significance was set at α = 0.05. Values are presented as mean ± SEM.

Results

Figure 6-1 shows skin blood flow (%CVC<sub>max</sub>) at the local heating plateau and following NOS-inhibition in control and MTHF treated microdialysis sites in young and older subjects. Older subjects had a significantly attenuated local heating plateau (older: 84±2 vs. younger: 94±2 %CVC<sub>max</sub>; p=0.002) and NO-dependent vasodilation (older: 26±6 vs. younger: 49±5%; p=0.03) compared to young. Local MTHF administration increased the magnitude of the local heating plateau compared to the control site in older (MTHF: 93±2 vs. control: 84±2 %CVC<sub>max</sub>; p=0.03) but not younger (MTHF: 96±1 vs. control: 94±2 %CVC<sub>max</sub>; p=0.8) subjects. Furthermore, local MTHF increased NO-dependent vasodilation compared to the control site in older (MTHF: 43±4 vs. control: 26±6%; p=0.04) but not younger (MTHF: 43±9 vs. control: 49±5%; p=0.4) subjects.

Table 6-2 presents values for plasma folate and homocysteine following placebo and folic acid treatments in young and older subjects. Folic acid treatment significantly increased plasma folic acid in the older subjects (folic acid: 23.7±0.3 vs. placebo: 18.5±1.4 ng/ml; p=0.02) but not the young subjects (folic acid: 24.8±1.4 vs. placebo: 21.2±1.4 ng/ml; p>0.05). Folic acid treatment had no effect on plasma homocysteine in either group.

Figure 6-2 shows skin blood flow (%CVC<sub>max</sub>) response to a 1°C rise in oral temperature at control (Ringer’s) and MTHF-treated microdialysis sites in older and young subjects following placebo and folic acid oral treatments. Following placebo treatment, older subjects had an attenuated reflex vasodilation compared to young at the control microdialysis site (older: 37±4 vs.
young: 49±9 %CVC\textsubscript{max}; p=0.04). Folic acid treatment increased vasodilation in the control sites of older (folic acid: 53±8 vs. placebo: 37±4 %CVC\textsubscript{max}; p=0.06) but not young (folic acid: 49±6 vs. placebo: 49±9 %CVC\textsubscript{max}; p>0.05) subjects. There was no difference between oral treatments at the MTHF-treated sites in older (folic acid: 47±5 vs. placebo: 49±7 %CVC\textsubscript{max}; p>0.05) and young (folic acid: 48±5 vs. placebo: 51±3 %CVC\textsubscript{max}; p>0.05) subjects.

Figure 6-3 shows the NO-dependent vasodilation (%CVC\textsubscript{max}) response at a 1°C rise in oral temperature at control (Ringer’s) and MTHF-treated microdialysis sites in older and young subjects following placebo and folic acid oral treatments. Older subjects had an attenuated NO-dependent vasodilation compared to young in the control microdialysis site (older: 6±3 vs. younger: 23±3%; p=0.003). Folic acid treatment increased NO-dependent vasodilation in the control site of older (folic acid: 23±5 vs. placebo: 6±3%; p=0.02) but not young (folic acid: 28±3 vs. placebo: 23±3%; p>0.05) subjects. There was no difference between oral treatments at the MTHF-treated sites in older (folic acid: 17±3 vs. placebo: 16±6%; p>0.05) and young (folic acid: 21±4 vs. placebo: 29±4 %CVC\textsubscript{max}; p>0.05) subjects.

**Discussion**

The primary findings of this study are that 1) local perfusion of 5-MTHF directly into the dermal space increases cutaneous vasodilation in response to an eNOS-dependent stimulus through NO-dependent mechanisms, and 2) systemically increasing plasma folate through a high-dose oral folic acid intervention increases NO-dependent reflex cutaneous vasodilation in healthy, older adults. Our older subjects had a significantly attenuated cutaneous vasodilation response to both local and whole-body heat stress. Local 5-MTHF administration, as well as chronic folic acid treatment, restored cutaneous vasodilator function in healthy older humans through NO-
dependent mechanisms. Clinically, folic acid treatment may be a relevant intervention strategy for improved vascular endothelial function in older adulthood.

The human cutaneous circulation is an easily accessible and representative circulation for examining mechanisms of vascular dysfunction in vivo (Holowatz, Thompson-Torgerson et al. 2008). There is a significant relation between endothelial dysfunction measured in the skin and that measured invasively in the coronary and renal circulations; and intervention-induced improvements in vascular function are detectible in the cutaneous circulation prior to improvements in clinical outcome (RG, de Jongh et al. 2003; Abularrage, Sidawy et al. 2005). The use of whole-body thermal stimuli coupled with systemic interventions and localized pharmacological manipulations (intradermal microdialysis) allows for the mechanistic examination of intervention efficacy in improved vessel function during an integrative cardiovascular challenge. Here, we have examined the efficacy of a high-dose folic acid intervention because folic acid and its active metabolite 5-MTHF have been shown to increase NO production in recombinant eNOS and in cultured endothelial cells and, as such, may be capable of modulating NO synthesis in the vasculature of older adults (Stroes, van Faassen et al. 2000). Our results suggest that folic acid increases the magnitude of reflex cutaneous vasodilation in aged human skin by increasing NOS coupling and subsequent NO synthesis.

The mechanisms mediating cutaneous vasodilation in response to local skin heating and whole-body heat stress are distinctly different. Local skin heating induces a biphasic increase in skin blood flow that is mediated by two independent mechanisms (Minson, Berry et al. 2001). The initial rapid rise in skin blood flow is caused by a sensory axon reflex and is followed by a brief nadir. The secondary phase consists of a more slowly developing rise to a stable plateau that is approximately 60-70% reliant on NO production by eNOS in healthy young skin (Minson, Berry et al. 2001; Bruning, Santhanam et al. 2012). With aging, there is a significant attenuation in the initial sensory axon reflex as well as a reduction in the NO-dependent plateau. Although
the mechanisms mediating this decline are undefined in the aging microvasculature, age-related changes in oxidant stress as well as substrate and cofactor availability contribute to endothelial dysfunction with aging and are likely contributing factors (Holowatz, Thompson et al. 2006; Delp, Behnke et al. 2008; Stanhewicz, Bruning et al. 2012). Because the skin blood flow response to local heating is predominantly NO-dependent, local heating is a valuable method to noninvasively assess endothelium dependent vasodilation in vivo in the human vasculature.

In healthy young subjects, ~30-40% of the total reflex vasodilation response is mediated by NO signaling, with the remaining ~60-70% relying on co-released neurotransmitter(s) with downstream vasodilation mediated through other second messenger pathways and activation of cyclo-oxygenase (Bennett, Johnson et al. 2003; Wong, Wilkins et al. 2004; McCord, Cracowski et al. 2006). With aging, the cofactor-mediated contribution to the overall expression of reflex vasodilation is attenuated and COX-dependent signaling favors the production of vasoconstrictors (Holowatz, Jennings et al. 2009). Consequently, healthy older adults rely predominately on a functionally compromised NO-dependent vasodilation to increase skin blood flow during hyperthermia (Holowatz, Houghton et al. 2003). The decreased NO bioavailability in aged skin results from a decrease in NO production by upregulated vascular arginase and increased NOS uncoupling with increased oxidant stress and reduced cofactor bioavailability (Holowatz, Thompson et al. 2006; Holowatz, Thompson et al. 2006; Stanhewicz, Bruning et al. 2012; Stanhewicz, Alexander et al. 2013). Because the contributions and identities of the co-transmitters in human skin are unclear and many of these co-transmitters converge on the NO pathway, interventions that target NO production and bioavailability may be capable of increasing reflex vasodilation in aged human skin. In the present study, we utilized whole-body heating coupled with localized pharmacological manipulations to mechanistically examine folic acid efficacy in improved vessel function and NO-dependent vasodilation during an integrative cardiovascular challenge.
We have chosen to utilize both local and whole-body heating to test the efficacy of 5-MTHF and folic acid in improved NO-dependent vasodilation because both methods provide applied and mechanistic data about the role of NO in vascular endothelial function. Collectively, the data from studies 1 and 2 suggest that folic acid, and its active metabolite 5-MTHF, improve cutaneous vessel reactivity to vasodilator stimuli. In both studies we demonstrate that increasing 5-MTHF and/or folic acid fully restores total and NO-dependent vasodilation in older adults.

In study 1 we examined the efficacy of local, microperfusion of 5-MTHF in augmented NO-dependent vasodilation response to an eNOS-specific stimulus (Bruning, Santhanam et al. 2012). 5-MTHF (1) increases vascular BH4 bioavailability by increasing production via BH2 recycling and reducing oxidant stress, and (2) improves NO-dependent vasodilation in ex vivo human vessels through eNOS coupling mechanisms (Antoniades, Shirodaria et al. 2006). In agreement with these data, our findings suggest that local delivery of 5-MTHF acutely restores NO-dependent vasodilation in aged cutaneous vessels to the level of that observed in our young subjects.

In study 2 we sought to determine the efficacy of a systemic, high-dose folic acid intervention on improved vascular endothelial function in older humans. In the past, our laboratory group has demonstrated that the effects of systemic treatments can be detected in the cutaneous vasculature (Holowatz, Santhanam et al. 2011; Bruning, Dahmus et al. 2013). Specifically, we have recently shown that systemically increasing BH4 bioavailability through oral administration of sapropterin restores NO-dependent vasodilation in the cutaneous vessels of older adults (Stanhewicz, Alexander et al. 2013). Prior studies examining the use of folic acid for improved vascular function suggest that chronic folic acid treatment is capable of improving markers of vessel health in populations with overt cardiovascular disease (Verhaar, Wever et al. 1998; Lekakis, Papamichael et al. 2004; Alian, Hashemipour et al. 2012). In agreement with these findings, our results suggest that 5mg folic acid daily for 6 weeks restores reflex vasodilation in
aged cutaneous vessels to the level observed in young, healthy individuals. Furthermore, our results suggest that this increase is mediated through NO-dependent mechanisms.

Our results from study 2 demonstrated that local 5-MTHF perfusion through intradermal microdialysis augments full and NO-dependent reflex vasodilation in aged skin following placebo treatment. These results are in agreement with the findings from study 1, which suggest that local 5-MTHF increases NO synthesis in the aged cutaneous vasculature. However, this localized perfusion did not further increase the magnitude of the full or NO-dependent cutaneous vasodilatation response observed with folic acid treatment in study 2. Further, there was no difference in full or NO-dependent reflex vasodilation between the 5-MTHF-perfused microdialysis sites across oral treatments. These data could indicate that (1) the systemic folic acid treatment maximized activity through the NOS pathway such that the enzyme was working at or near $V_{\text{max}}$, and/or (2) we had reached a ceiling effect for the ability of the aged cutaneous vessels to vasodilate during hyperthermia. Collectively, these data suggest that systemic folic acid treatment increases plasma folate and/or 5-MTHF sufficiently to increase NO production through NOS.

**Summary.** In summary, folic acid and its metabolite 5-MTHF increase cutaneous vasodilation in older humans through NO-dependent mechanisms. Considering the putative role of NO in vascular function and the observed increase in the magnitude of cutaneous vasodilation in the present study, folic acid administration may be a clinically relevant intervention strategy for improved vascular health and reduced cardiovascular morbidity and mortality in older adults.
Table 6-1. Subject Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Age (years)</th>
<th>Sex (M,F)</th>
<th>BMI (kg/m²)</th>
<th>LDL (mg/dL)</th>
<th>HDL (mg/dL)</th>
<th>Total cholesterol (mg/dL)</th>
<th>HbA1c (%)</th>
<th>SBP (mmHg)</th>
<th>DBP (mmHg)</th>
<th>MAP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Older</td>
<td>77±3</td>
<td>4,2</td>
<td>25±1</td>
<td>126±6 *</td>
<td>74±4</td>
<td>208±11*</td>
<td>5.7±0.1</td>
<td>127±4</td>
<td>75±3</td>
<td>92±3</td>
</tr>
<tr>
<td>Young</td>
<td>22±1</td>
<td>3,3</td>
<td>21±1</td>
<td>98±10</td>
<td>68±6</td>
<td>173±12</td>
<td>5.3±0.1</td>
<td>118±4</td>
<td>71±4</td>
<td>86±4</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. BMI: body mass index, HDL: high density lipoprotein, LDL: low density lipoprotein, oxLDL: oxidized low density lipoprotein, SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure. *p<0.05 significant difference from young.
Table 6-2. Plasma folate and homocysteine in young and older subjects following placebo and folic acid treatment.

<table>
<thead>
<tr>
<th></th>
<th>Plasma folate (ng/mL)</th>
<th>Plasma Homocysteine (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Older</td>
<td></td>
<td></td>
</tr>
<tr>
<td>placebo</td>
<td>18.5±1.4*</td>
<td>11.7±1.3</td>
</tr>
<tr>
<td>folic acid</td>
<td>23.7±0.3</td>
<td>11.0±1.4</td>
</tr>
<tr>
<td>Young</td>
<td></td>
<td></td>
</tr>
<tr>
<td>placebo</td>
<td>21.2±1.4</td>
<td>7.2±0.6</td>
</tr>
<tr>
<td>folic acid</td>
<td>24.8±1.4</td>
<td>7.1±0.4</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. * p = 0.02 significant difference from folic acid
Figure 6-1. Group mean ± SEM skin blood flow (%CVC\textsubscript{max}) at the local heating plateau and following NOS-inhibition in control and MTHF-perfused microdialysis sites in young and older subjects. *p<0.05 significant difference compared to young control.
Figure 6-2. Group mean ± SEM vasodilation response (%CVC max) vasodilation response to 1°C rise in oral temperature in control and MTHF-perfused microdialysis sites in young and older subjects following placebo and folic acid treatment. * p = 0.04 compared to young placebo control. † p=0.06 compared to older placebo control.
Figure 6-3. Group mean ± SEM %NO-dependent vasodilation at 1°C rise in oral temperature in control and MTHF-perfused microdialysis sites in young and older subjects following placebo and folic acid treatment. * p= 0.003 compared to young placebo control. † p=0.02 compared to older placebo control.
Chapter 7
CONCLUSIONS AND FUTURE DIRECTIONS

The four studies comprising this dissertation were performed to investigate the efficacy of oral intervention strategies for improved reflex control of the cutaneous vasculature in older humans. Because reduced BH₄ bioavailability is a unifying mechanism by which reflex vasodilation and vasoconstriction are attenuated in aged skin, all four studies aimed to increase BH₄ bioavailability in aged cutaneous vasculature. Specifically, these studies investigated the efficacy of (1) acute and chronic oral sapropterin in improved reflex vasodilation and vasoconstriction, and (2) folic acid and its active metabolite 5-MTHF in improved NO-dependent reflex vasodilation, in aged cutaneous vasculature. Collectively, these studies suggest that sapropterin and folic acid are novel intervention strategies that restore vessel function in the aged cutaneous vasculature.

This chapter is intended to summarize the results of these studies with respect to intervention strategies and microvascular aging. Finally, future directions of research that may provide further insight into mechanistic targets and intervention strategies for age-associated vascular dysfunction will be discussed.

Oral Sapropterin Augments Reflex Vasodilation in Aged Human Skin

The principal finding of this study was that oral sapropterin acutely (3-hours post ingestion) increased reflex vasodilation in aged human skin measured by both laser-Doppler flowmetry and venous occlusion plethysmography. Furthermore, it did so through NO-dependent
mechanisms. These data agree with our previous conclusions that decreased BH₄ contributes to attenuated reflex cutaneous vasodilation in older humans by limiting NO production through uncoupled NOS (Stanhewicz, Bruning et al. 2012), and suggest that with 10mg/kg oral dose of sapropterin, BH₄ acutely becomes bioavailable in aged skin microvasculature sufficiently to increase NO synthesis through NOS.

**Implications**

Oral sapropterin may be a clinically relevant intervention for improved thermoregulatory skin blood flow in older adults. The results of this study suggest that an acute 10mg/kg dose of oral sapropterin increases reflex vasodilation in aged human skin through NO-dependent mechanisms and that oral administration of exogenous BH₄ may be a clinically relevant intervention for improved thermoregulatory function in older adults during hyperthermia. Oral supplementation with BH₄ improves vascular function in animal and human models of vascular disease and dysfunction (Hattori, Hattori et al. 2007; Porkert, Sher et al. 2008), and taken together these data suggest that the specific NOS-coupling mechanisms of BH₄ may be an emerging therapy for endothelial dysfunction.

**Oral Sapropterin Augments Reflex Vasoconstriction in Aged Human Skin**

The principal finding of this study was that oral sapropterin acutely (3-hours post ingestion) increased reflex vasoconstriction in aged human skin. Further, it did so through alterations in noradrenergic mechanisms. These data substantiate our previous conclusions that decreased BH₄ contributes to attenuated reflex vasoconstriction in older humans by limiting TH function (Lang, Holowatz et al. 2009), and suggest that with a 10mg/kg oral dose of sapropterin,
BH₄ becomes bioavailable in aged skin microvasculature sufficiently to increase norepinephrine synthesis.

**Implications**

Oral sapropterin may be a clinically relevant intervention for improved reflex vasoconstriction in older adults during cold exposure. The results of this study suggest that an acute 10mg/kg dose of oral sapropterin increases reflex vasoconstriction in aged human skin through noradrenergic mechanisms and that oral administration of exogenous BH₄ may be a clinically relevant intervention for improving vasoconstrictor function in older adults exposed to environmental cold. We suggest that oral sapropterin, the safe, commercially available, pharmaceutical formulation of BH₄, improves thermoregulatory vasoconstriction in older adults through noradrenergic mechanisms in the perivascular nerve terminal. Taken into perspective with the endothelial effects of BH₄, these data suggest that oral BH₄ administration may improve vascular control across a wide range of requirements, from vasoconstriction to vasodilation.

**Chronic Treatment with Oral Sapropterin: Evidence of Efficacy in Augmented Reflex Control of Blood Flow in Aged Human Skin**

This study provides evidence to suggest that chronic oral sapropterin treatment may be a viable intervention strategy for improved reflex control of thermoregulatory skin blood flow in aged skin. In particular, chronic supplementation with oral sapropterin (400mg twice daily for 4 weeks) increases reflex vasodilation in older human skin. Furthermore, it may do so through NO-dependent mechanisms. Oral sapropterin may be a clinically relevant intervention for improved thermoregulatory skin blood flow in older adults during hyperthermia. However, there is not
sufficient evidence to suggest that chronic sapropterin increases reflex vasoconstriction in the skin of healthy, older adults.

Implications

Our results suggest that chronic oral administration of sapropterin may be a clinically relevant intervention for improved thermoregulatory skin blood flow in older adults exposed to heat stress. Taken in the context of our previous findings that acute sapropterin increases reflex vasodilation in aged skin through NO-dependent mechanisms (Stanhewicz, Alexander et al. 2013) and given the role of BH4 in improved endothelial function in aging conduit vessels (Pierce, Jablonski et al. 2012), this pilot study suggests that chronic sapropterin treatment may improve endothelial function in healthy older adults. However, the results of this pilot study do not support our hypothesis that chronic oral sapropterin treatment restores reflex cutaneous vasoconstriction in older adults exposed to whole-body cold stress.

Overall, the preliminary results from this pilot study provide the basis for larger investigations aimed at confirming the mechanistic role of sapropterin in improved thermoregulatory vasodilation in aged skin, as well as examining the broader application of chronic sapropterin in improved endothelial function and vascular health with aging.

Folic Acid Augments Nitric Oxide-Dependent Vasodilation in Aged Human Skin

The principal finding of this study was that folic acid, and its active metabolite 5-MTHF, augments vasodilation in aged human skin through NO-dependent mechanisms. Specifically, the results of this study suggest that 5-MTHF increases NO-dependent vasodilation in aged skin in response to an eNOS-dependent stimulus (local thermal hyperemia) as well as during whole-body
heat stress. Chronic folic acid treatment (5mg daily for 6 weeks) augmented reflex cutaneous vasodilation through NO-dependent mechanisms in older, but not young, subjects.

Implications

The results of this study suggest that chronic folic acid treatment may be a viable intervention strategy for improved reflex cutaneous vasodilation in healthy, older adults. The previous studies in this dissertation suggest that oral sapropterin restores reflex vasodilation in aged skin. However, the high cost and relative inaccessibility of oral formulations of BH4 underscores the need for a readily available, inexpensive intervention strategy to effectively improve vascular health in older adults. Folic acid and its active metabolite 5-MTHF improve conduit vessel endothelial function in patients with overt cardiovascular and metabolic disease (Verhaar, Wever et al. 1998; Doshi, McDowell et al. 2001; Alian, Hashemipour et al. 2012). The results of this study provide in vivo evidence that high-dose folic acid is efficacious for improving vascular endothelial function in a healthy older cohort.

Future Directions

1.) The first three studies presented in this dissertation provide insight into the efficacy of oral sapropterin in improved reflex control of thermoregulatory skin blood flow in primary aged adults. Our results suggest that acute (10mg·kg⁻¹) and chronic (400mg twice daily for 4 weeks) sapropterin treatment improves reflex vasodilation through NO-dependent mechanisms. Acute dosing with sapropterin was also efficacious for improving reflex vasoconstriction through noradrenergic mechanisms. However, we were unable to find evidence that chronic dosing improved reflex vasoconstriction in an older subject group. Further study of the tissue specific
distribution and/or accumulation of orally administered sapropterin, as well as investigations of
different dosing strategies, may shed light on the issue and explain the possible discrepancies
between acute and chronic efficacy of oral sapropterin in improved reflex vasoconstriction in
aged human skin. Additionally, we did not test alternative doses of sapropterin. Given the high
cost (~$36.00 per 100mg) of treatment it is necessary to test the efficacy of lower doses in reflex
vasodilation, and possible higher doses for functionally improved reflex vasoconstriction.
2.) By design, the sapropterin studies excluded subjects who had overt cardiovascular disease
and/or were taking medications. Although these subjects were not elite athletes, they represent a
specific subset of the population and our results may not be generalizable to older adults who are
unhealthy and/or taking a variety of medications. However, exogenous BH₄ therapy has been
shown to effectively increase vascular endothelial function in populations with cardiovascular
disease (Cosentino, Hurlimann et al. 2008; Porkert, Sher et al. 2008; Alexander, Kutz et al. 2013),
suggesting that BH₄ may be efficacious in older adults who do exhibit overt cardiovascular
disease. Further research is warranted to investigate the efficacy of oral sapropterin intervention
in an older diseased population and other human populations that exhibit attenuated vascular
function.
3.) We have demonstrated that local microperfusion of 5-MTHF directly into the dermal space
improves reflex vasodilation in aged human skin through NO-dependent mechanisms. Further,
chronic high-dose folic acid treatment improves NO-dependent reflex vasodilation, possibly
through systemic increases in circulating 5-MTHF and/or increases in bioavailable BH₄. Because
folic acid is readily available and relatively inexpensive, further research is warranted to
investigate the broader application of high-dose folic acid in improved vessel function in
populations that exhibit vascular dysfunction and overt cardiovascular disease.
4) The collection of studies that comprise this dissertation have focused on interventions that increase bioavailable BH₄ for improved vascular control mechanisms in aging. Although BH₄ is a common mechanistic link to reduced cutaneous vasodilation and vasoconstriction, there are additional contributing factors (i.e. reduced substrate and elevated oxidative stress). Novel intervention strategies that target more than one limitation may be more viable treatments for reduced vascular function with aging and vascular disease. For example, treatment with HMG-CoA reductase inhibitors has been shown to reduce oxidant stress, increase BH₄ bioavailability (Antoniades, Bakogiannis et al. 2011), and inhibit arginase, thereby increasing substrate (L-arginine) availability (Holowatz, Santhanam et al. 2011). Further investigation into therapies that target more than one component are warranted to determine if they are more efficacious in vessel dysfunction in aging and vascular disease.


Pierce, G. L. and T. J. Larocca (2008). "Reduced vascular tetrahydrobiopterin (BH4) and endothelial function with ageing: is it time for a chronic BH4 supplementation trial in middle-aged and older adults?" J Physiol 586(Pt 11): 2673-2674.


Appendix

Informed Consent Forms

INFORMED CONSENT FORM FOR CLINICAL RESEARCH STUDY
The Pennsylvania State University

Title of Project: Cutaneous Vascular Effects of Acute and Chronic Oral BH4 supplementation in the older

Principal Investigator: Lacy Alexander Holowatz, Ph.D.
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Research Assistants: Anna Stanhewicz
Phone 845-551-3869

Susan Slimak, RN
Phone: 814-863-8556

Jane Pierzga, M.S., Research Assistant
Phone: 814-865-1236

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

1. **Purpose of the study:** When you are exposed to the heat, nerves in your skin make natural chemicals that cause the skin’s blood vessels to get bigger. This increases the amount of blood flowing through those vessels. This increased flow helps to cool your body. When you are exposed to the cold, nerves in your skin make natural chemicals that cause the skin’s blood vessels to get smaller. This decreases the amount of blood flowing through those vessels. This decreased flow helps to retain your body’s heat. As you age, you cannot control the blood flow in your skin as well as when you are younger. So, aging can make you more prone to illness in extreme heat or cold. **BH4** is a natural substance found in your cells. Researchers have shown that **BH4** helps your body to control skin blood flow. They also have shown that your **BH4**
declines as you age. This study examines the role of BH₄ further. We look at whether adding BH₄ to your skin and to your body helps to restore the control of skin blood flow.

This research study has 2 basic experiments that you repeat several times. One experiment looks at the role BH₄ in the control of skin blood flow during whole body heating. The other looks at the role of BH₄ in the control of skin blood flow during whole body cooling.

In this study, we use “microdialysis” (MD). This technique involves placing very thin plastic tubing between the layers of your skin. The largest part of the tubing is about 6x the diameter of a human hair. We pump fluid like that found in your body’s tissues through the tubing. The tubing acts like very small blood vessels in your skin by allowing some substances to pass between the fluid in the tubing and the fluid in your skin. During the experiment, we will add substances to the fluid in the tubing. The substances can only reach a 2.5 cm² (0.4 inch²), nickel-sized area of skin at each tube. Some of these substances are like some natural chemicals found in your body. Some of these substances block the action of several of the natural chemicals found in your body.

The substances used for these experiments are:

1. BH₄ – ((6R)-5,6,7,8-Tetrahydrobiopterin dihydrochloride) – a natural substance found in your cells. BH₄ shuts down waste products that produce reactive oxygen molecules in cells. It may also help blood vessels to get bigger or smaller.
2. L-NAME (N⁶-nitro-L-arginine methyl ester) – like a natural protein found in your cells. It stops chemical reactions that involve that protein.
3. SNP (sodium nitroprusside) causes your blood vessels to get as large as they can.
4. Norepinephrine - is a natural chemical made by the nerves in the skin.
5. Vitamin C – Vitamin C occurs naturally in foods. We use Vitamin C to preserve norepinephrine.
6. Yohimbine - stops the action of your body’s natural chemicals upon the blood vessels in the skin
7. Propranolol - stops the action of your body’s natural chemicals upon the blood vessels in the skin
8. Tyramine - causes the nerves in your skin to release norepinephrine
9. Lactated Ringers

We use BH₄ in two ways. 1) We put BH₄ directly into your skin with microdialysis. 2) We put BH₄ into your whole body by having you take BH₄ pills (“Kuvan”). Sometimes during the study you will take fake BH₄ pills (placebo). Each time, you will not know whether the pill is real BH₄ or a placebo. We will tell you which pills you were taking when you complete the project.

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

2. Procedures: Please read the descriptions of the days. Then write your initials by the days.

You could be asked to repeat a trial, procedure, or test. This could include blood draws and inserting a catheter in you vein with your OK. This could happen for many reasons such as equipment failure, power outage, inconclusive test results, etc. You do not have to repeat a trial, procedure, and/or test if you do not wish to do so.
**initial Screening Day:** You do not eat or drink after midnight before your exam. You report to the Noll Lab for your appointment. The research and/or Clinical Research Center staff will perform screening procedures. When you arrive, the staff draws about 21 ml (less than 2 Tbsp) of blood from a vein in your arm. You have a screening by medical staff that includes blood pressure, heart rate, height, waist circumference, weight, medical history, and 12-lead resting ECG. We send the blood to labs that test it for blood cells, fats in the blood, blood chemistry, and proteins in which we are interested. If you are taking thyroid hormone, we will draw an extra 3.5 ml (0.2 Tbsp) to check the level of thyroid hormone. We may test the blood for other substances of interest. We do not perform genetic tests on the blood. We do not test the blood for the presence of disease (e.g. HIV). We may measure the thickness of folds of skin at several places on your body to determine your percent body fat. Women who are not post-menopausal will submit urine samples for pregnancy tests.

**initial Visit 2 Non-invasive experiments:**

We give you printed and verbal instructions listing what you need to do before you arrive at the lab. Please follow the instructions carefully and arrive prepared. If you have questions, please contact us right away.

When you arrive, we measure your heart rate and blood pressure. The nurse draws a 14 ml (< 1 Tbsp) blood sample.

**FLPI Experiment: Local Heating / Reactive Hyperemia (LH/RH)**

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

**Reactive Hyperemia:** You gently wash your arm with soap and water. You lie on a bed or recliner. The researchers place two ring-shaped local heaters 35mm diameter (about 1.5 inches) on your forearm with sticky rings. The researchers label each site with a marker pen. They fill the inner well of each heater with 2 ml (about 1/2 teaspoon) of water. They place a clear cover over the well. They set the temperature of the local heaters to a comfortable 34°C (93°F). The researchers position and focus the FLPI camera 8-10 inches above your arm. They place a blood pressure cuffs on each of your arms. They record blood pressure every 5-7 minutes on the arm that does not have the local heaters. They record baseline data for about 20 minutes with the FLPI. The researchers inflate the cuff on the arm sporting the local heaters for 5 minutes so that blood does not enter or exit the arm (occlusion). Then they rapidly deflate the cuff and blood flows back into your arm while they record data with the FLPI. They wait at least 20 minutes and repeat the occlusion. They wait at least 20 more minutes and repeat the occlusion a third time. The researchers record data until the skin blood flow becomes stable (about 10 minutes).

**Local Heating:** The researchers continue to record blood pressure every 5-7 minutes on the arm that does not have the local heaters. They record skin blood flow with the FLPI. They collect baseline data for about 20 minutes. They increase the temperature to 42°C (107°F) and wait 40-50 minutes for the skin blood flow to become stable. Then they raise the temperature to 43°C (109°F). The researchers collect data for about 30 more minutes. Then they remove the local heaters. Your blood pressure and heart rate are measured before you leave.
Pulse Wave Velocity (PWV) and Flow Mediated Dilation (FMD)

PWV measures the health of blood vessels. You lie on a bed with blood pressure cuffs on your ankles and upper arms. The researchers place sensors on each wrist. These sensors attach to an ECG machine. The researchers place a small plastic sensor on the side of your neck over the carotid artery. To perform a reading, the cuffs inflate to stop blood flow to your arms and feet, and then slowly deflate. Each reading takes two minutes. The sensors in the cuffs and on the neck measure how fast each pulse of blood travels through your blood vessels. The data allows us to estimate the stiffness of your arteries. Also, it allows us to determine how well the blood flows through your leg’s arteries. We perform the measure about 2-4 times.

FMD measures the health of blood vessels, too. The researchers place a blood pressure cuff around your forearm. They put gel on your upper arm just above your elbow. Then they place a Doppler ultrasound probe on the gel. The ultrasound makes sound waves to measure the size of blood vessels and the speed of your blood. They make a “resting” measurement before they inflate the cuff. Then they inflate the cuff for 5 minutes to stop blood flowing to and from your forearm. After they deflate the cuff, they perform a second reading for 3 minutes.

**initial Experiment Visits 3-6 “Acute” BH4 / Placebo - Whole Body Heating and Cooling Experiments (maximum of 6 hours each):**

We conduct heating and cooling experiments on separate days. They may occur in any order. You will have a heating experiment and a cooling experiment with BH4. You will have a heating experiment and a cooling experiment with a placebo. This yields 4 “acute” experiments.

**initial Preparation for each experiment:** We give you printed and verbal instructions listing what you need to do before you arrive at the lab. Please follow the instructions carefully and arrive prepared. If you have questions, please contact us right away.

During the experiment, men wear shorts. Women wear shorts and a sports bra. We can provide this clothing. When you arrive at the laboratory, a staff member measures your vitals. Women who are not postmenopausal will submit urine for a pregnancy test.

The nurse uses a needle to insert a tube in your vein (see details under “Discomforts and risks”). Then the nurse draws a 6 ml (< 1 Tbsp) blood sample. You swallow the BH4 or placebo pills with water.

The amount of BH4 you take is 10mg/kg bodyweight (0.00076oz/ lb bodyweight). If you weighed 68 kg (150 lbs), you would take 680mg (0.114 oz) of BH4. We take a 6 ml (< 1 Tbsp) blood sample every 30 minutes after you take the BH4 until the end of the experiment. The total amount of blood drawn that day is no more than 72 ml (< 5 Tbsp). You wash your forearm and pat it dry. You don a suit that has tubing lining the inside and lie down. We tape 6 wires to your skin to measure skin temperatures. Also, we tape three ECG leads to your chest to measure heart rate. Water that is a comfortable 33°C (91.4°F) flows through the suit’s tubing. Then we prepare the MD sites on your arm.

Microdialysis (MD): We place a tight band around your upper arm so we can easily see your veins. For each MD site, we make pairs of pen-marks on your arm 2.5 cm (1 inch) apart and away from veins. The MD tubing will enter and exit your skin at the marks. We remove the tight band. We clean your arm with an orange-colored povidone iodine fluid and alcohol. We place
an ice bag on your arm for 5 minutes to numb your skin. Then we insert a thin needle into your skin at each entry mark. The needle’s tip travels between the layers of skin for 2.5 cm (1 inch) and leaves your skin at the matching exit mark. The tubing is threaded through the needle. Next, we withdraw the needle leaving the tubing in your skin. Any redness of your skin subsides in about 60 – 120 minutes.

We tape a thin probe and its holder over each site where there is MD tubing in your skin. The thin probe measures skin blood flow with a weak laser light. We can control the temperature of the holders. The holders will start at 33°C (91.4°F). During the experiment, we use two methods to measure blood pressure. One method uses a cuff that inflates on your upper arm while the researcher listens with a stethoscope at the inside of your elbow. Another method, Finapres, uses a small cuff that fits on your finger. Finapres also measures heart rate. Throughout the experiment, we measure skin blood flow, skin temperature, and inner body temperature. We also measure ECG, heart rate, and blood pressure.

When the redness from placing the MD tubing in your skin is gone, the experiment begins.

We start the Lactated Ringer’s, a solution similar to your body’s fluid, flowing through the tubing in your skin. After 10 minutes, we add the test-substances to the Ringer’s until the end of the protocol.

Initial Cooling Experiment: When the experiment begins, you will rest for 20 minutes. Then we add the test-substances to the plain fluid running through the tubing.

Probe 1. Lactated Ringer’s only
Probe 2. Lactated Ringer’s + BH4
Probe 3. Lactated Ringer’s+ BH4 + Yohimbine + Propranolol

After 60 minutes, we perform another 20-minute baseline and set of measurements. We place a wire under your tongue to measure your temperature. Then we pump cold water through the tubing of the suit for the cooling phase of the experiment. You may decide to end the cooling phase at any time. After about 40 minutes of cooling, we re-warm you. When your skin blood flow readings are stable, we add Norepinephrine to site #3 for 10 minutes. Next, we add Tyramine to all sites for 20 minutes. Then we add Norepinephrine to all sites for 20 minutes. We stop the flow of test substances through the MD tubing. Lastly, Lactated Ringer’s + SNP flows through all tubing, and we heat the laser probes’ holders to 43°C (109.4°F) for 30 – 45 minutes. This creates the greatest amount of blood flow possible. Then the study ends. The nurse removes the tube from your vein. We clean the places where the tubing enters and exits your skin with alcohol, and pull the tubing from your skin. We place a sterile bandage over the sites where the tubing was in your skin. We may place a bag of ice on your arm for 10 minutes to reduce any bruising that may occur. The staff measures your blood pressure and heart rate before you leave.

Initial Heating Experiment: When the experiment begins, you will rest for 20 minutes. Then we add the test-substances to the plain fluid running through the tubing.

Probe 1. Lactated Ringer’s only
Probe 2. Lactated Ringer’s + L-NAME
Probe 3. Lactated Ringer’s + BH4

After 60 minutes, we perform another 20-minute baseline and set of measurements. We place a wire under your tongue to measure your temperature. To start the heating phase, we increase the
temperature of the water flowing through the suit to about 48°C (118.4°F). In this way, we raise your body’s temperature 1.0°C (1.8°F). We reduce the temperature of the water flowing through the suit to 45°C (113°F) to hold your body’s temperature at the desired level for 30-45 minutes. You may decide to stop the heating phase at any time. During heating, your skin blood flow at the MD sites rises and then stays at the higher level. When the skin blood flow is stable at the new level, we add L-NAME to the fluid running through probes 1 and 3. Heating ends when the skin blood flow at probes 1 and 3 becomes stable at a new level. When heating ends, cooler water 22°C (71.6°F) flows through the suit’s tubing to cool you quickly. Then 33°C (91.4°F) flows through the suit’s tubing to keep your comfortable. Also, we stop the flow of test substances through the MD tubing. Lastly, Lactated Ringer’s + SNP flow through all tubing, and we heat the laser probes’ holders to 43°C (109.4°F) for 30 – 45 minutes. This creates the greatest amount of blood flow possible. Then the study ends. The nurse removes the tube from your vein. We clean the places where the tubing enters and exits your skin with alcohol, and pull the tubing from your skin. We place a sterile bandage over the sites where the tubing was in your skin. We may place a bag of ice on your arm for 10 minutes to reduce any bruising that may occur. The staff measures your blood pressure and heart rate before you leave.

initial Experiment Visits 7-12 “Chronic” BH₄ / Placebo – Non-Invasive, Whole Body Heating, and Cooling Experiments:

initial Pretreatment 1: Before you do the experiments, you have a pretreatment with BH₄ or Placebo: You take BH₄ or a placebo for 4 weeks. You visit the lab weekly (4 visits) to pick up the pills, get your blood pressure measured, and repeat the PWV test. Women who are not postmenopausal will submit urine for a pregnancy test before receiving the pills. After the 4 weeks, you continue taking the BH₄ or placebo until you repeat the non-invasive, cooling, and heating experiments. We expect to conduct the three experiments during the 5th week.

Dose:
BH₄: 400 mg twice daily (AM and PM) for a total of 800 mg for about 4 weeks and continue until you repeat the three experiments.
Or
Placebo: You take the placebo twice daily (AM and PM) for about 4 weeks and continue until you repeat the three experiments.

We will give you verbal and written instructions about how to store and use the BH₄ and placebo, information on side effects, and what actions to take if you suspect problems.

Preparation for each experiment: We prepare for these experiments in the same manner as the “Acute” experiments described above except as noted below.
We do not insert a venous catheter nor collect blood during these experiments.
You do not take a 10mg/kg bodyweight (0.00076oz/ lb bodyweight) dose of BH₄ or placebo when you arrive at the lab.

initial Non-invasive Experiments: The nurse draws a 19.5 ml (< 2 Tbsp) blood sample. We conduct these experiments in the same manner described above.

initial Cooling Experiment: We perform these experiments in the same manner as
the “Acute” experiments described above.

______ initial Heating Experiment: We perform these experiments in the same manner as the “Acute” experiments described above.

______ initial Washout Period: You wait for 2 weeks to allow the BH$_4$ you have taken, if any, to leave your body.

______ initial Pretreatment 2: Before you do the experiments, you have a pretreatment with BH$_4$ or Placebo:
You take whichever pill you have not taken yet (placebo or BH$_4$) for 4 weeks using the same method you used during the first 4 weeks. You visit the lab weekly (4 visits) to pick up the pills, get your blood pressure measured, and repeat the PWV test. After the 4 weeks, you continue taking the BH$_4$ or placebo until you repeat the non-invasive, cooling, and heating experiments. We expect to conduct the three experiments during the 5th week.

Once again, we give you verbal and written instructions about how to store and use the BH$_4$ and placebo, information on side effects, and what actions to take if you suspect problems.

Preparation for each experiment: We prepare for these experiments in the same manner as the “Acute” experiments described above except as noted below.

We do not insert a venous catheter nor collect blood during these experiments.

You do not take a 10mg/kg bodyweight (0.00076oz/ lb bodyweight) dose of BH$_4$ or placebo when you arrive at the lab.

______ initial Non-invasive Experiments: The nurse draws a 19.5 ml (< 2 Tbsp) blood sample. We conduct these experiments in the same manner described above.

______ initial Cooling Experiment: We perform these experiments in the same manner as the “Acute” experiments described above.

______ initial Heating Experiment: We perform these experiments in the same manner as the “Acute” experiments described above.

3. Discomforts and risks:

Microdialysis: The risks are less than that for a blood draw because microdialysis uses only a small, localized area of skin. In contrast, a blood draw involves not only skin, but also large blood vessels and blood. You will probably experience some pain and bruising like that from a blood draw. However, we use ice to numb your arm during the insertion of the tubing. Also, the small needle reduces pain during placement of the tubing. You will probably not have pain after
the tubing is in place. You may feel a little pain when the tubing is removed from your skin. You may be nervous about needles. If so, your blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick to your stomach, or may faint. Sometimes the tubing can break during removal from your skin. Then we remove the tubing by pulling on the other end of it. This produces no additional risk for you. The tubing could break so that a small piece is left under your skin. This has not occurred in any of our studies. If this happened, we would treat any tubing remaining in your skin like a splinter. In this case, the thin layer of skin over the tubing may have to be cut to allow removal. Mild pressure with sterile gauze stops any slight bleeding that may occur. Infection is possible, but has never occurred in our lab or others that we know of. Sterile techniques and supplies like those used in hospital keep the risk minimal. We apply a sterile bandage after the experiment. We tell you how to take care of the site.

Fluid flowing through the tubing: The substances flowing through the tubing only go to a 2.5 cm² (0.4 inch²) area of skin at each tubing site. The amount that enters the skin is very small. However, there is a chance of your having a bad reaction to the substances. This reaction could produce redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, blood pressure change and/or fainting. If a bad reaction should occur, medical help will be summoned right away.

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

*L-NAME, BH₄, Norepinephrine, Vitamin C, Yohimbine, Propranolol, Tyramine, and SNP:* Only minute amounts of these substances enter the nickel-sized area of skin around the MD tubing. We and other researchers have used these substances in human skin. There have been no reports of bad reactions.

BH₄ (oral, “Kuvan”): Your body’s cells make BH₄. Researchers have shown that BH₄ in your body helps your body to control your skin blood flow. BH₄ can protect against reactive oxygen as does Vitamin C. BH₄ has other uses. Researchers worldwide have been using oral BH₄ in studies looking at such diverse issues as blood flow in heart muscle, diseases that cause low BH₄, treatments for Phenylketonuria (PKU), and Vitamin C’s effects on BH₄ levels. Doctors in the United States use a BH₄ pill called “Kuvan” to treat PKU. PKU is caused by a missing liver enzyme. This serious disease causes phenylalanine in the blood to be too high. Phenylalanine is one of the building blocks of proteins. There are no known conditions that would suggest that you should not take Kuvan. Researchers did not observe any severe allergic reactions when they tested Kuvan in patients. However, mild to moderate allergic reactions could include redness, itching, rash, and/or swelling. Researchers observed mild to moderate fall in the number of white blood cells during Kuvan-use 4% of PKU patients. The most common problems (≥4% of PKU patients) were headache, dizziness, diarrhea, vomiting, and tummy pain. Other common problems are runny or stuffy nose, cough, sore throat, and nausea. The most serious bad reactions during Kuvan-use in patients with PKU were stomach problems, spinal cord injury, infection, testicular cancer, and urinary tract infection. However, these serious problems may not have been related to treatment. The number of bad reactions in PKU patients using Kuvan was like that in patients receiving placebo. We give you verbal and written instructions on how to store and use the Kuvan (and Placebo). We include a list of these and other possible side effects and what to do if you think that you are having problems.
Placebo: The placebo contains Vitamin C like that contained in Kuvan tablets. Vitamin C is an essential vitamin often contained in foods. Bad reactions are not common. Bad reactions could include feeling sick to your stomach, vomiting, and/or heartburn. You could have fatigue, flushing, and/or headache. You could have trouble getting to sleep or sleepiness. You could have diarrhea, bloating from gas, short-term abdominal pain and/or cramps.

Blood Draw: Blood draws often cause mild pain, bruising, swelling, or bleeding. There is also a slight chance of infection or a small clot. You may be nervous about needles. If so, your blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick to your stomach, or may faint. To keep the chance of infection minimal, the staff uses the same techniques used in hospitals. Do not exercise hard for 24 hours before a blood draw.

Special note about blood draws for the acute experiments: A trained nurse inserts a thin tube into a vein in your arm or hand through which she can draw blood. To do this, the nurse inserts a needle with the tube around it into your vein. Then the nurse removes the needle. The tube stays in the vein during the whole experiment. The tube allows the nurse to take more than one 6 ml (<1 Tbsp) blood sample without sticking you with a needle each time. If the first attempt to insert the tube does not work, the nurse may need to try again. She will try again only if you allow. The nurse uses sterile saline to flush the blood out of the tube between blood draws. Sometimes the tube can stop letting the nurse draw blood through it. If this occurs, the nurse removes the tube. You may stop the experiment if you wish or you may proceed. If you allow, you may have tube put back in your vein. Or you may have the nurse stick you with a needle for each of the rest of the blood draws. The tube does not stay in your vein longer than 6 hours. At the end of the experiment, the nurse removes the tube from your vein. The maximum, total volume of blood drawn over the four acute experiments is 288 ml. This is just a little over ½ pint. You are likely to complete the four experiments within a 2-week period. A typical Red Cross blood donation is 500 ml (1 pint) drawn in less than 15 minutes.

ECG: This machine measures the electrical activity of your heart. You will have 2-12 wires from the machine taped to spots on your body. There have been no adverse effects. The tape may irritate your skin.

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

Medical Screening: You may feel shy about giving health information. The staff collects the information in a private and professional manner. You may feel shy about being measured. If you request someone of the same sex to conduct the screening, we will make our best effort to provide one.

Phone screening form: Only the researcher uses this form. We use the form to help decide whether you are a good candidate for the study. You may feel shy about answering questions. You may request someone of the same sex to ask you the questions. We collect the information in a private and professional manner. The completed form is kept confidential and secure.
**Laser Doppler Flowmetry:** Weak lasers can hurt your eye if you stare into the light for a long time. We do not turn on the laser until the probes are taped to a surface. The tape may irritate your skin.

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

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

**Whole Body Cooling:** You may feel cold, have “goose-bumps” and may shiver. You may stop the cooling at any time.

**Body Temperature:** We place a plastic tube has a wire that measures temperature under your tongue. You may become tired from holding the wire in place. We can tape the wire to your face to help you. We can also give you a short break at some times to let you rest. The wires taped to your skin at 6 sites are not harmful, but the tape may irritate.

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

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

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

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

**PWV Test:** During the short time the researchers inflate the cuffs, your arms and feet may feel numb or tingly. The cuffs could cause mild bruising. Minor redness may occur where the probe was placed on the skin of the neck. This is temporary.

**FMD Test / Doppler Ultrasound:** There is a small chance the probe could irritate your skin. Minor redness may occur at the site where the probe is placed against the arm. This is temporary. While the cuffs are inflated, your arms and feet may feel numb or tingly, and the color of your
arm may change slightly. The cuffs could cause mild bruising. The gel is the same as that used with medical ultrasound tests. The gel may feel cool or cold on your skin. A bad reaction to the gel is highly unlikely.

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

b. **Potential benefits to society:** Recent data suggests that reduced BH₄ in the body may lead to age-related reduction in the control of body temperature. This project could not only increase our knowledge about the reasons for age-related vascular problems, but also may suggest oral BH₄ as a possible treatment. Also, as more people grow older, their health concerns have a greater impact on society. The projects provide valuable experience, education and partial fulfillment of degree-work for graduate and undergraduate students of The Pennsylvania State University.

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

2. **Time duration of the procedures and study:** You will need to visit Noll Lab for the following:
   - Day 1: Screening: 1.5 hour
   - Day 2: “Non-invasive experiments”: 4 hours
   - Days 3-6: “Acute Experiments”: 6 hours each (total: 24 hours)
   - Days 7-12: “Chronic Experiments” (total: 40 hours) the experiments are:
     - “Non-invasive Experiments”: 4 hours each day (2 days)
     - “Heating / Cooling Experiments”: 6 hours each day (4 days)
   - Pick up pills, blood pressure, PWV: 1 hour each visit (8 Visits)
   
   Total: **69.5 hours**

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

8. **Right to ask questions:** Please contact Lacy Alexander Holowatz (W: 814-867-1781, H: 814-880-9217), Anna Stanhewicz (W: 814-865-2432, M: 845-551-3869), Susan Slimak (W: 814-863-8556, H: 814-237-4618), or Jane Pierzga (W: 814-865-1236, H: 814-692-4720) with questions, complaints or concerns about this research. You can also call this number if you feel this study has harmed you. If there are findings during the research that could relate to you wanting to help with the study, you will be told of the findings. If you have any questions,
concerns, or problems about your rights as a research participant or would like to offer input, please contact Penn State University’s Office for Research Protections (ORP) at (814) 865-1775. The ORP cannot answer questions about research procedures. All questions about research procedures can only be answered by the research team.

9. **Compensation:** You receive a lab T-shirt or other item of like value.

   a. **Non-Invasive Experiments:** You receive $40.00 for each experiment-day.

   b. **Acute MD Experiments:** You receive $15.00 for each of the 3 MD probes inserted in your arm (maximum $45.00) in each experiment. You receive $55.00 more for completing each experiment ($100.00 total / experiment). (4 experiments x $100.00 = $400.00)

   c. **Chronic Experiments:**
      - Non-Invasive Experiments: 2 experiments x $40.00 = $80.00
      - MD Experiments: 4 experiments x $100.00 = $400.00
      - **Pill Pick-up Visits:** ($10.00 / visit) 8 visits x $10.00 = $80.00

   d. **If you complete the whole study:** You receive an additional $100.00.

   **TOTAL:** $1,100.00

For each experiment, you are paid an amount of money equal to the part of the trial that you complete. For instance, if you complete only half of an acute experiment, you will be paid for each probe that was inserted plus $27.50 for that experiment. This is because $27.50 is one half of $55.00. Another example: If you complete ½ of a non-invasive experiment-day, you receive $20.00 because $20.00 is ½ of $40.00. You may be asked to repeat an experiment. If you agree to repeat an experiment, you will be paid for the repeated experiment as stated above.

Total payments within one calendar year that exceed $600 will require the University to annually report these payments to the IRS. This may require you to claim the compensation that you receive for participation in this study as taxable income.

10. **Injury Clause:** In the unlikely event you become injured as a result of your participation in this study, medical care is available. Please call Lacy Alexander Holowatz (W: 814-867-1781, 814-880-9217), Anna Stanhewicz (W: 814-865-2432, M: 845-551-3869), Susan Slimak (W: 814-863-8556, H: 814-237-4618), and Jane Pierzga (W: 814-865-1236, H: 814-692-4720). It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

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

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

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

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

Volunteer  Date

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

Investigator  Date
Title of Project: Cutaneous Vascular Effects of Chronic Folic Acid Supplementation in the older

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Research Assistants: Susan Slimak, RN
Phone: 814-863-8556

Jane Pierzga, M.S., Research Assistant
Phone: 814-865-1236

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

1. Purpose of the study: When you are exposed to the heat, nerves in your skin make natural chemicals that cause the skin’s blood vessels to get bigger. This increases the amount of blood flowing through those vessels. This increased flow helps to cool your body. As you age, you cannot control the blood flow in your skin as well as when you are younger. So, aging can make you more prone to illness in extreme heat. Folic acid (vitamin B9) is a natural substance found in many common foods. Researchers have shown that folic acid helps your body to control blood flow. This research looks at whether adding folic acid to your skin and to your body helps to restore the control of skin blood flow when you are exposed to the heat.

This research study has 1 main experiment that you repeat twice. The experiment looks at the role of folic acid in the control of skin blood flow during whole body heating.

In this study, the researchers use “microdialysis” (MD). This technique involves placing very thin plastic tubing between the layers of the skin. The largest part of the tubing is about 6x the diameter of a human hair. They pump fluid like that found in the body’s tissues through the tubing. The tubing acts like very small blood vessels in the skin by allowing some substances to
pass between the fluid in the tubing and the fluid in the skin. During the experiment, they will add substances to the fluid in the tubing. The substances can only reach a 2.5 cm² (0.4 inch²), nickel-sized area of skin at each tube. Some of these substances are like natural chemicals found in the body. Some of these substances block the actions of natural chemicals found in the body.

The substances used for these experiments are:

\[ BH_4 \] – \((6R)-5,6,7,8\text{-Tetrahydrobiopterin dihydrochloride}\) – a natural substance found in cells. BH$_4$ shuts down waste products that produce reactive oxygen molecules in cells. It also helps blood vessels to get bigger (dilate).

\[ \text{L-NAME}\left(\text{N}^\text{G}-\text{nitro-L-arginine methyl ester}\right) \] – like a natural protein found in cells. It stops chemical actions that involve that protein.

\[ \text{SNP} \left(\text{sodium nitroprusside}\right) \] causes your blood vessels to dilate as much as they can.

\[ \text{Ca-5MTHF} \left(\text{D calcium L-5-methyltetrahydrofolate}\right) \] – the metabolite of folic acid as a calcium salt. This is a natural substance found in your blood after you ingest folic acid.

\[ \text{Na-5MTHF} \left(\text{5-Methyltetrahydrofolic acid disodium salt}\right) \] - the metabolite of folic acid as a sodium salt. This is a natural substance found in your blood after you ingest folic acid.

\[ \text{Lactated Ringer’s} \] – like the plain fluid that bathes cells in the body

The researchers use folic acid in two ways. 1) They put 5MTHF, the metabolite of folic acid, directly into the skin with microdialysis. 2) They put folic acid into the whole body by having you take folic acid pills. Sometimes during the study you will take fake folic acid pills (placebo). You will not know which pills you are taking, but the research nurse who gives you the pills will know in case you have a question.

Also, the researchers use weak laser light to measure blood flow in small vessels in the skin.

2. Procedures: Please read the descriptions of the days. Then write your initials by the days.

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

________ initial Screening Day: Drink only water and do not eat after 10 PM the evening before your visit. Go to the Noll Lab for your appointment. The research and/or Clinical Research Center staff perform the screening procedures. When you arrive, the nurse draws about 30 ml (2 Tbsp) of blood from a vein in your arm. Your screening by medical staff includes blood pressure, heart rate, height, waist circumference, weight, medical history, and 12-lead resting ECG. They send the blood to labs that test it for blood cells, fats in the blood, blood chemistry, and proteins in which they are interested. If you take thyroid hormone, they draw an extra 3.5 ml (0.2 Tbsp) to check the level of thyroid hormone. They may test the blood for other substances of interest. They do not perform genetic tests on the blood. They do not test the blood for the presence of disease (e.g. HIV). Women who are not post-menopausal will submit urine samples for pregnancy tests.
**initial Visit 2 Pilot experiment:**

You receive printed and verbal instructions listing what you need to do before coming to the lab. Please follow the instructions carefully and arrive prepared. If you have questions, please contact the researchers right away.

When you arrive, the researcher measures your heart rate and blood pressure. Women who are not postmenopausal submit urine for a pregnancy test before the experiment begins.

**Microdialysis (MD):** We place a tight band around your upper arm so we can easily see your veins. For each MD site, we make pairs of pen-marks on your arm 2.5 cm (1 inch) apart and away from veins. We remove the tight band. The MD tubing will enter and exit your skin at the marks. We clean your arm with an orange-colored povidone iodine fluid and alcohol. We place an ice bag on your arm for 5 minutes to numb your skin. Then we insert a thin needle into your skin at each entry mark. The needle’s tip travels between the layers of skin for 2.5 cm (1 inch) and leaves your skin at the matching exit mark. We thread the tubing through the needle. Next, we withdraw the needle leaving the tubing in your skin. Any redness of your skin subsides in about 60 – 120 minutes. The treatments at the three MD sites are:

1. Lactated Ringer's
2. Lactated Ringer’s + Ca-5MTHF or Na-5MTHF
3. Lactated Ringer’s + BH4 + Ca-5MTHF or Na-5MTHF

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

**Local Heating:** The researchers continue to record blood pressure every 5-7 minutes on the arm that does not have the local heaters. They record baseline skin blood flow for about 20 minutes. They increase the temperature to 42°C (107°F) and wait 40-50 minutes for the skin blood flow to become stable. After about 50 minutes, we replace the fluid flowing through the fibers so that all sites receive lactated Ringer’s + L-NAME. After about 30 minutes, we replace the lactated Ringer’s + L-NAME at all sites with lactated Ringers + SNP and increase the local heat to 43°C (109°F) for about 40 minutes to produce the maximal skin blood flow. The researchers collect data for about 30 more minutes. Then they remove the local heaters. They measure your blood pressure and heart rate before you leave.

**initial Experiment Visits 3-4 Whole Body Heating Experiments (maximum of 6 hours each):**

**Initial Pretreatment 1:** Before you do the experiments, you have a pretreatment with folic acid or Placebo. You take folic acid or a placebo for 6 weeks. You visit the lab once to pick up the pills. Women who are not postmenopausal submit urine for a pregnancy test before getting the folic acid or placebo pills. You take the folic acid or placebo while you repeat the heating experiment during the 7th week.
Dose:
Folic acid: 5 mg once daily about 6 weeks and continue until you repeat the heating experiment.

Or
Placebo: You take the placebo once daily for about 6 weeks and continue until you repeat the heating experiment.

You receive verbal and written instructions about how to store and use the folic acid and placebo. Also, they tell you about side effects and what actions to take if you suspect problems.

Initial Washout Period: You wait for 2 weeks to allow the folic acid you have taken, if any, to leave your body.

Initial Pretreatment 2: Before you do the experiments, you have a pretreatment with folic acid or placebo. You take folic acid or a placebo for 6 weeks. You visit the lab once to pick up the pills. Women who are not postmenopausal submit urine for a pregnancy test before getting the folic acid or placebo pills. You take the folic acid or placebo while you repeat the heating experiment during the 7th week.

Dose:
Folic acid: 5 mg once daily about 6 weeks and continue until you repeat the heating experiment.

Or
Placebo: You take the placebo once daily for about 6 weeks and continue until you repeat the heating experiment.

Again, you will receive verbal and written instructions.

Initial Preparation for each experiment: The researchers give you printed and verbal instructions listing what you need to do before you come to the lab. Please follow the instructions carefully and arrive prepared. If you have questions, please contact them right away.

During the experiment, men wear shorts. Women wear shorts and a sports bra. The researchers can provide this clothing. When you come to the lab, a staff member measures your heart rate, blood pressure, and oral temperature. Women who are not postmenopausal submit urine for a pregnancy test.

The nurse draws a 30ml (2 Tbsp) blood sample.

You wash your forearm and pat it dry. You don a suit that has tubing lining the inside. You lie down. The researchers tape 6 wires to your skin to measure temperatures. They tape three ECG leads to your chest to measure heart rate. Comfortable 33°C (91.4°F) water flows through the suit’s tubing. Then they prepare the MD sites on your arm.

Microdialysis (MD): The researchers place a tight band around your upper arm so they can easily see the veins. For each MD site, they make pairs of pen-marks on the forearm 2.5 cm (1 inch) apart and away from veins. The MD tubing will enter and exit the skin at the marks. They
remove the tight band. They clean the arm with an orange-colored povidone iodine fluid and alcohol. They place an ice bag on the arm for 5 minutes to numb the skin. Then they insert a thin needle into the skin at each entry mark. The needle’s tip travels between the layers of skin for 2.5 cm (1 inch) and leaves the skin at the matching exit mark. The tubing is threaded through the needle. Next, they withdraw the needle leaving the tubing in the skin. Any redness of the skin subsides in about 60 – 120 minutes. The treatments at the four MD sites are:
- Probe 1. Lactated Ringer’s only
- Probe 2. Lactated Ringer’s + Ca-5MTHF or Na-5MTHF
- Probe 3. Lactated Ringer’s + BH₄
- Probe 4. Lactated Ringer’s + BH₄ + Ca-5MTHF or Na-5MTHF

They tape a thin probe and its holder over each site where there is MD tubing in the skin. The thin probe measures skin blood flow with a weak laser light. The researchers can control the temperature of the holders. The holders will start at 33°C (91.4°F). During the experiment, they use two methods to measure blood pressure. One method uses a cuff that inflates on the upper arm while the researcher listens with a stethoscope at the inside of the elbow. Another method, Cardiocap, also uses a cuff that fits on an upper arm. Cardiocapmeasures heart rate, too. Throughout the experiment, the researchers measure skin blood flow, skin temperature, and inner-body temperature. They also measure ECG, heart rate, and blood pressure.

When the redness from placing the MD tubing in the skin is gone, the experiment begins. The researchers start the Lactated Ringer’s flowing through the tubing in the skin.

**initial Heating Experiment:** When the experiment begins, you rest for a 20 minute baseline. Then the researchers place a wire under your tongue to measure temperature. To start the heating phase, they increase the temperature of the water flowing through the suit to about 48°C (118.4°F). This raises your body’s temperature 1.0°C (1.8°F). Then they reduce the temperature of the water to 45°C (113°F) to hold the body’s temperature at that level for 30-45 minutes. You may end the heating phase at any time. During heating, the skin blood flow at the MD sites rises. When the skin blood flow is stable, they add L-NAME to the fluid of the probes. Heating ends when the skin blood flow becomes stable again. When heating ends, cooler water 22°C (71.6°F) flows through the suit’s tubing to cool you quickly. Then 33°C (91.4°F) flows through the suit’s tubing to keep your comfortable. Also, they stop the flow of test substances through the MD tubing. Lastly, Lactated Ringer’s + SNPreplaces the fluid in all probes. The researchers heat the laser probes’ holders to 43°C (109.4°F) for 30 – 45 minutes. This creates the greatest amount of blood flow possible. Then the study ends. The researchers use alcohol to clean the MD sites. They pull the tubing from the skin and apply a sterile bandage. They may place a bag of ice on the arm for 10 minutes to reduce any bruising. They measure your blood pressure and heart rate before you leave.

### 3. Discomforts and risks:

**Microdialysis (MD):** The risks are less than that for a blood draw because MD uses only a small, local area of skin. In contrast, a blood draw involves not only skin, but also large blood vessels and blood. MD is likely to cause some pain and bruising like that of a blood draw. However, the researchers use ice to numb the arm before they insert the tubing. Also, the small needle helps to reduce pain. Most people do not feel pain after the tubing is in place. You may feel a little pain when they remove the tubing at the end of the experiment. If you are nervous about needles,
blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick
to your stomach, or may faint. Sometimes the tubing can break during removal from the skin.
Then the researchers remove the tubing by pulling on the other end of it. This produces no added
risk for you. The tubing could break so that a small piece is left under the skin. This has not
occurred in any of the studies in this lab. If this happened, they would treat any tubing remaining
in the skin like a splinter. In this case, they would cut the thin layer of skin over the tubing to
remove the tubing. Mild pressure with sterile gauze stops any slight bleeding that may occur.
Aseptic technique and sterile supplies like those used in hospitals keep the risk of infection
minimal. Infection has not occurred with MD in this lab or others that the researchers know of.
They apply a sterile bandage after the experiment. They tell you how to take care of the site.

Fluid flowing through the tubing: The substances flowing through the tubing only go to a 2.5 cm²
(0.4 inch²) area of skin at each tubing site. The amount that enters the skin is very small.
However, there is a chance of a bad reaction to the substances. This reaction could produce
redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing
problems, changes in pulse, convulsions, blood pressure change and/or fainting. If a bad reaction
should occur, medical help is summoned right away.

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

L-NAME, BH₄, MTHF, and SNP: Only minute amounts of these substances enter the nickel-sized
area of skin around the MD tubing. These and other researchers have used the substances in
human skin before. There have been no reports of bad reactions.

Folic Acid (vitamin B9): Folic acid is a natural vitamin contained in many foods that you eat.
Folic acid is needed for the proper development of the human body. It is involved in producing
the genetic material called DNA and in numerous other bodily functions. Folic acid helps the body
to control blood flow. Researchers have used folic acid in studies looking at blood flow in large
blood vessels, in cardiovascular diseases, and diseases that cause low folic acid. Doctors in the
United States recommend folic acid pills for patients that do not get enough folic acid in their diet
and to patients that need extra (e.g. pregnant women). You should not take folic acid if you have
a deficiency of vitamin B12. We will test your blood for vitamin B12 before we give you folic
acid to make sure it is safe for you to take. Patients with kidney disease and patients taking
chloramphenicol, cholestyramine, medication for seizures, methotrexate, nitrofurantoin,
tetracycline, barbiturates, or pyrimethamine should not take folic acid. If you are taking any of
these medications tell the researchers and do not take folic acid. Researchers and doctors did not
observe any severe allergic reactions when they tested folic acid in patients. High doses of folic
acid might cause abdominal cramps, diarrhea, rash, sleep disorders, irritability, confusion, nausea,
stomach upset, behavior changes, skin reactions, seizures, gas, excitability, and other side effects.
However, most adults do not experience any side effects when taking folic acid. You receive
verbal and written instructions on how to store and use the folic acid. They include a list of side
effects and what to do if you think that you are having problems.

Placebo: The placebo contains cellulose like that contained in folic acid tablets. Reactions could
include nausea, diarrhea, bloating from gas, short-term abdominal pain and/or cramps.

Blood Draw: Blood draws often cause mild pain, bruising, swelling, or bleeding. There is also a
slight chance of infection or a small clot. If you are nervous about needles, blood pressure and
heart rate may increase for a little while. You may also feel lightheaded, sick to your stomach, or
may faint. Using the same techniques used in hospitals keeps the chance of infection minimal.
Do not exercise hard for 24 hours before a blood draw.

Tape and adhesive disks: You could be sensitive to the adhesive of the tape and double-sided
adhesive disks used in the study causing redness, rash, tenderness, and/or itching.

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

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

Medical Screening: You may feel shy about giving health information. The staff collects the
information in a private and professional manner. You may feel shy about being measured. If
you request someone of the same sex to conduct the screening, the researchers will make their
best effort to provide one.

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

Thermoregulation Lab Website: You may enter data into the screening form via the Qualtrics
website. You may be concerned about the data’s security. Qualtrics is a secure website and
survey application designed to support data capture for research studies. The data is kept
encrypted and your confidentiality and security are protected.

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

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

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

Body Temperature: The researchers place a plastic tube has a wire that measures temperature under your tongue. You may become tired from holding the wire in place. They can tape the wire to your face to help you. They can also give you a short break at some times to let you rest. The wires taped to the skin at 6 sites are not harmful, but the tape may irritate.

Povidine Iodine: Hospitals and researchers use this orange-colored fluid to clean and sterilize the skin. You could have a bad reaction to povidone iodine if you are allergic to iodine. You inform the researchers if you have this allergy so that they use only alcohol instead. A bad reaction could cause redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or blood pressure change and/or fainting.

Latex: Some gloves and medical materials are made of latex rubber. Inform the researchers if you are allergic to latex and decline to participate in the study.

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

b. Potential benefits to society: As more people grow older, their health concerns have a greater impact on society. Recent data suggests that aging can reduce folic acid in the body. In turn, this may lead to reduced control of blood flow in skin and other organs. Reduced control can impair organ and system health and function. The knowledge gained could help us to learn the causes for some of the vascular problems that occur with aging. The results may suggest new beneficial effects of folic acid that could bear more study. The projects provide valuable experience, education and partial fulfillment of degree-work for graduate and undergraduate students of The Pennsylvania State University.

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

4. Time duration of the procedures and study: You will need to visit the Noll Lab for the following:
   Day 1: Screening: 1.5 hour
   Day 2: “Pilot experiments” 5 hours
   Days 3-6: “Heating Experiments”: 6 hours each (total: 12 hours)

   Total: 18.5 hours

7. Statement of confidentiality: Volunteers are coded by an identification number for statistical analyses. All records are kept in a secure location. All records associated with your participation in the study will be subject to the usual confidentiality standards applicable to medical records (e.g., such as records maintained by physicians, hospitals, etc.), and in the event of any publication resulting from the research no personally identifiable information is disclosed. The Office of Human Research Protections in the U.S. Department of Health and Human Services, the U.S. Food and Drug Administration (FDA), The Penn State University Office for Research Protections (ORP) and The Penn State University Institutional Review Board may review records related to this project.
8. **Right to ask questions:** Please contact Anna Stanhewicz (W: 814-865-2432, M: 845-551-3869), Susan Slimak (W: 814-863-8556, H: 814-237-4618), or Jane Pierzga (W: 814-865-1236, H: 814-692-4720) with questions, complaints or concerns about this research. You can also call these numbers if you feel this study has harmed you. If there are findings during the research that could relate to you wanting to help with the study, you will be told of the findings. If you have any questions, concerns, or problems about your rights as a research participant or would like to offer input, please contact Penn State University’s Office for Research Protections (ORP) at (814) 865-1775. The ORP cannot answer questions about research procedures. All questions about research procedures can only be answered by the research team.

9. **Compensation:** lab T-shirt or other item of like value.

- **Pilot Experiment:** $15.00 for each of the 3 MD probes inserted in the arm (maximum $45.00) in each experiment. $30.00 more for completing each experiment ($75.00 total / experiment).

- **Heating Experiments:** $15.00 for each of the 4 MD probes inserted in the arm (maximum $60.00) in each experiment. $55.00 more for completing each experiment ($115.00 total / experiment). (2 experiments x $115.00 = $230.00)

If you complete the whole study: an additional $100.00. You do not have to complete the pilot study to receive the additional $100.00.

**TOTAL:** $405.00 (with the pilot study) or $330.00 (without the pilot study)

For each experiment, you are paid an amount of money equal to the part of the trial that you complete. For instance, if you complete only half of a heating experiment, you will be paid for each probe that was inserted plus $27.50 for that experiment. This is because $27.50 is one half of $55.00. If you agree to repeat an experiment, you will be paid for the repeated experiment as stated above.

Total payments within one calendar year that exceed $600 will require the University to annually report these payments to the IRS. This may require you to claim the compensation that you receive for participation in this study as taxable income.

10. **Injury Clause:** In the unlikely event you become injured as a result of your participation in this study, medical care is available. Please call Anna Stanhewicz (W: 814-865-2432, M: 845-551-3869), Susan Slimak (W: 814-863-8556, H: 814-237-4618), and Jane Pierzga (W: 814-865-1236, H: 814-692-4720). It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

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

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

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

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

Volunteer Date

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

Investigator Date
VITA
Anna E. Stanhewicz

Education

Ph.D. The Pennsylvania State University, Department of Kinesiology 2014
M.S. The Pennsylvania State University, ICGDP Physiology 2011
B.S. University of Rhode Island, Department of Kinesiology 2009

Honors and Awards

Virginia S. Shirley Graduate Scholarship, ICGDP Physiology, Pennsylvania State University 2009
University Graduate Fellowship, The Graduate School, Pennsylvania State University 2009
Caroline tum Suden Professional Opportunity Award, American Physiological Society 2011
Graduate Research Award, Department of Kinesiology, Pennsylvania State University 2012
Doctoral Student Research Award, ACSM Mid-Atlantic Regional Chapter 2012
Rigel Pharmaceutical Pre-Doctoral Award, EEP Section, American Physiological Society 2013
Gatorade Pre-Doctoral Award, EEP Section, American Physiological Society 2014

Peer Reviewed Publications


