The Pennsylvania State University

The Graduate School

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SYMPATHETIC NORADRENERGIC MECHANISMS OF THERMOREGULATORY VASOCONSTRICTION IN AGED HUMAN SKIN

A Dissertation in

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by

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ABSTRACT

Reflex vasoconstriction (VC) is attenuated in older human skin, resulting in greater blood flow and convective heat loss during cold exposure, which may in turn increase their risk of developing hypothermia. Central to this impairment may be the effects of elevated oxidative and nitrosative stress on key enzymes that modulate noradrenergic VC such as tyrosine hydroxylase (TH) and rho-kinase (ROCK). The purpose of this series of four studies was to investigate the neural and vascular mechanisms underlying the attenuated VC response in aged skin, and specifically, the role of these enzymes in contributing to this deficit.

The first study tested the hypothesis that compromised noradrenergic VC in aged skin is due to the depletion of an essential cofactor, tetrahydrobiopterin (BH_4), needed in the rate-limited step of norepinephrine (NE) biosynthesis. Functional changes in forearm skin blood flow were assessed with Laser Doppler flowmetry in response to gradual whole-body cooling and tyramine infusion, which evokes NE release from storage vesicles in sympathetic nerve terminals. Four microdialysis (MD) fibers were placed in the forearm skin of eleven young (Y) and eleven older (O) human subjects for infusion of 1) Ringers solution (control), 2) 5 mM BH_4, 3) BH_4 + 10mM ascorbate, and 4) BH_4 + adrenoreceptor blockade (5mM yohimbine + 1mM propranolol). The VC response was lower at the control site in O during both cooling (Y: -34 ± 2, O: -17 ± 2 $\%\Delta CVC_{base}$; $P<0.001$) and tyramine infusion (Y: -33 ± 4, O: -15 ± 3 $\%\Delta CVC_{base}$; $P<0.001$). BH_4 infusion normalized O to Y values during both cooling (Y: -34 ± 4, O: -34 ± 2 $\%\Delta CVC_{base}$; $P<0.001$) and tyramine infusion (Y: -38 ± 4, O: -35 ± 3 $\%\Delta CVC_{base}$; $P<0.001$). The addition of adrenoreceptor blockade abolished VC in aged skin indicating that BH_4 acts through noradrenergic, not cotransmitter, mechanisms. Local BH_4 supplementation augments reflex and tyramine-induced VC in aged skin, suggesting that reduced BH_4 bioavailability may contribute to the attenuated VC during whole-body cooling.

The second study tested the hypothesis that attenuated reflex VC in aged skin may be partly mediated by oxidant-induced reduction in functional substrate and/or cofactor availability required by TH for NE biosynthesis; i.e., tyrosine and BH_4, respectively. Functional changes in skin blood flow were assessed with Laser Doppler flowmetry in response to gradual whole-body cooling and tyramine infusion. Four microdialysis fibers were placed in the forearm skin of 10 Y and 10 O subjects for infusion of 1) Ringer's, 2) 0.5 mM L-tyrosine, 3) 5 mM BH_4, and 4) BH_4 + L-tyrosine. VC was attenuated at the control site in O during whole-body cooling (Y: -39 ± 3, O: -17 ± 3 $\%\Delta CVC_{base}$; $P<0.01$) and tyramine infusion (Y: -41 ± 3, O: -21 ± 4 $\%\Delta CVC_{base}$; $P<0.01$). Similar to BH_4 (cold, Y: -37 ± 3, O: -36 ± 3; tyramine, Y: 41 ± 2, O: -36 ± 3 $\%\Delta CVC_{base}$), tyrosine also augmented the VC in O during cooling (Y: -37 ± 4, O: -34 ± 4 $\%\Delta CVC_{base}$) and tyramine infusion (Y: -40 ± 4, O: -45 ± 4 $\%\Delta CVC_{base}$), but BH_4 + tyrosine did not further augment VC during cooling (Y: -38 ± 4, O: -31 ± 3 $\%\Delta CVC_{base}$) or tyramine infusion (Y: -36 ± 3, O: -36 ± 5 $\%\Delta CVC_{base}$). Individually, both tyrosine and BH_4 infusion augmented VC in aged skin, suggesting that their reduced bioavailability may impair NE synthesis and contribute to the attenuated VC response.

The third study tested the hypothesis that the contribution of ROCK to reflex VC is greater in aged skin. NOS inhibited sites would be used to control for the putative effects Cutaneous VC was assessed with Laser Doppler flowmetry and was elicited by 1) whole-body cooling (T_{sk} = 30.5°C) and 2) infusion of a local physiological concentration of NE ($1x10^{-6}$M). Four microdialysis fibers were placed in the forearm skin of 8 Y and 8 O subjects for infusion of 1) Ringers solution, 2) 3 mM fasudil (ROCK inhibition), 3) 20 mM N^G-L-arginine methyl ester (L-NAME) (NOS inhibition), and 4) both ROCK + NOS inhibitors. VC was reduced at the control site in O during both cooling (Y: -34 ± 3, O: -18 ± 3 $\%\Delta CVC_{baseline}$; $P<0.001$) and NE infusion (Y: -53 ± 4, O: -41 ± 9 $\%\Delta CVC_{baseline}$; $P=0.006$). Fasudil attenuated VC in both age groups during mild cooling; however, this reduction remained only in O but not Y skin during moderate cooling (Y: -30 ± 5, O: -7 ± 1 $\%\Delta CVC_{baseline}$; $P=0.016$), and was not altered by NOS inhibition. Fasudil blunted NE-mediated VC in both age groups (Y: -23 ± 4, O: -7 ± 3 $\%\Delta CVC_{baseline}$; $P<0.01$).

Cumulatively, these data suggest that although ROCK modestly contributes to the VC response in young, it is upregulated in aged skin during moderate cooling and mediates approximately half of the total reflex VC response in aged skin.

The fourth study tested the hypothesis that localized BH_4 supplementation would not affect end-organ VC responsiveness to exogenous NE after localized sympathetic nerve blockade. Two microdialysis fibers were placed in bretylium tosylate-pretreated sites (presynaptically blocks neurotransmitters release from sympathetic adrenergic nerve terminals; iontophoresis, 200 μA for 20 min) on the forearm skin of 10 Y and 10 older O subjects for infusion of 1) Ringer's (control) and 2) 5 mM BH_4. While local skin temperature was clamped at 34°C, 6 concentrations of NE (10^{-12}, 10^{-10}, 10^{-8}, 10^{-6}, 10^{-4}, 10^{-2} M) were randomly infused (except the supraphysiological doses) at each laser Doppler site. Despite prejunctional adrenergic blockade, NE-mediated VC was blunted in aged skin at each NE dose (10^{-12}: -12 ± 2 vs. -21 ± 2, 10^{-10}: -15 ± 2 vs. -27 ± 1, 10^{-8}: -22 ± 2 vs. -32 ± 2, 10^{-6}: -27 ± 2 vs. -38 ± 1, 10^{-4}: -52 ± 3 vs. -66 ± 5, 10^{-2}: -62 ± 3 vs. -75 ± 4 $\%\Delta CVC_{base}$; $P<0.01$), and this response was not affected by pretreatment with BH_4 ($P>0.05$). Localized BH_4 did not affect end-organ responsiveness to exogenous NE suggesting that the effects of BH_4 on cutaneous VC are primarily limited to the NE biosynthetic pathway in prejunctional adrenergic nerve terminals.

The collective results of this series of studies suggest that compromised noradrenergic function in aged skin has both a pre- and postjunctional component. The ability for TH to upregulate during cold exposure is suboptimal in aged skin, which is likely due to reduced tyrosine and BH_4 bioavailability. Furthermore, ~50% of the extant reflex VC response in aged skin relies on ROCK; an enzyme that is also upregulated with various age-related vascular diseases.

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LIST OF ABBREVIATIONS

ANOVA	Analaysis of variance
AR	Adrenergic receptor
ATP	Adenosine triphosphate
BH_4	Tetrahydrobiopterin
BMI	Body mass index
BT	Bretylium tosylate
Ca^{2+}	Calcium
cGMP	Cyclic guanosine monophosphate
CVC	Cutaneous vascular conductance
Fe^{2+}	Iron (ferrous form)
Fe^{3+}	Iron (ferric form)
GTP	Guanosine triphosphate
kg	Kilograms
LDF	Laser Doppler flowmetry
L-NAME	N^G-nitro-L-arginine methyl ester
m	Meters
mL	Milliliters
mM	Millimolar
MAP	Mean arterial pressure
MD	Microdialysis
MLC	Myosin light chain
NA*	Noradrenaline
NE	Norepinephrine
NF-κB	Nuclear factor kappa-beta
NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y

ROCK	Rho-kinase
T_{sk}	Mean skin temperature
TH	Tyrosine hydroxylase
VC	Vasoconstriction
VD	Vasodilation
VSM	Vascular smooth muscle
Y+P	Yohimbine + propranolol

* Preferred designation by *The Journal of Physiology*

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Chapter 1

INTRODUCTION

Background and Significance

Figure **1.1**

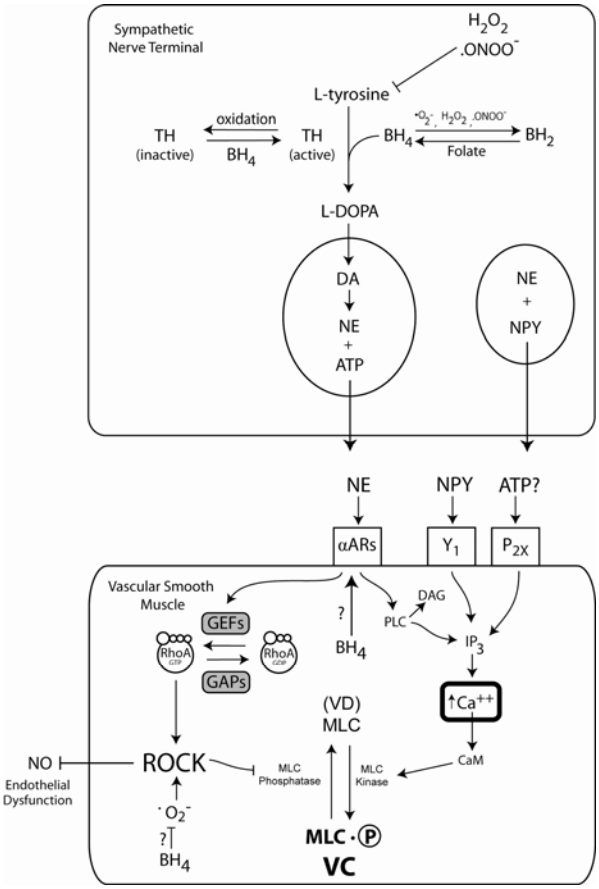

Figure **1.1**: Schematic overview of the mechanism of reflex vasoconstriction in humans. TH, tyrosine hydroxylase; BH_4, tetrahydrobiopterin; $ONOO^-$, peroxynitrite; $•O_2^-$, superoxide; DA, dopamine; NE, norepinephrine; NPY, neuropeptide Y; αARs, α-adrenoreceptors; MLC, myosin light chain; ROCK, rho kinase; GEFs, guanine nucleotide exchange factors; GAPs, GTPase activating proteins; NO, nitric oxide; CaM, calmodulin; DAG, diacylglycerol; PLC, phospholipase C; IP_3, inositol triphosphate; Ca^{++}, calcium; VD, vasodilation; VC, vasoconstriction

Human skin is a primary thermoregulatory effector organ that helps maintain body core temperature through distinct local and reflex vascular mechanisms. The control of blood flow to the skin importantly modulates heat transfer from the core to the periphery. At rest in a thermoneutral environment, skin blood flow is ~350 mL/min (i.e., ~5-10% of the cardiac output) (Rowell, 1977, 1983). Because there is a ~3^{o}C core-skin thermal gradient, metabolic heat is constantly delivered to the skin for removal. During cold exposure, reflex cutaneous vasoconstriction (VC) represents the first line of heat conservation by, 1) effectively minimizing skin blood flow and thus convective heat loss to the environment, and 2) reducing the thermal gradient between skin and the ambient temperature (Stocks *et al.*, 2004). The net result of fully expressed peripheral vasoconstriction is approximately a sixfold increase in tissue insulation, which is roughly equivalent to wearing a "light wool business suit" (Gisolfi & Mora, 2000).

Aging and Reflex Vasoconstriction

In older adults, thermoregulatory reflex VC is attenuated, thereby rendering them more susceptible to heat loss and potentially, hypothermia (Wagner *et al.*, 1974; Collins *et al.*, 1977; Khan *et al.*, 1992; Richardson *et al.*, 1992). Epidemiological evidence indicates that over 60% of hypothermia-related deaths occur in those over 65 years of age (CDC, 2002). Despite matching young and older subjects based on adiposity, fat-free mass, aerobic fitness, and polypharmacy, older subjects still exhibit reduced peripheral VC and a relative inability to defend against decreases in core temperature even during mild (22^{o}C) cold exposure (Kenney & Armstrong, 1996; Degroot & Kenney, 2007).

Thermoregulatory reflex VC is mediated by sympathetic adrenergic axonal release of norepinephrine (NE) and coreleased neurotransmitters, putatively neuropeptide Y (NPY) and ATP (Taddei *et al.*, 1990; Stephens *et al.*, 2004) (Figure 1.1). Cotransmitter-mediated VC is responsible for ~40% of the total reflex VC response to whole-body cooling (Stephens *et al.*, 2001); however, this component is functionally absent in aged skin (Thompson & Kenney, 2004). Thus, reflex VC in aged skin is

entirely dependent on a compromised noradrenergic-mediated mechanism. Moreover, this noradrenergic component may be impaired at multiple points along the efferent arm of the sympathetic reflex: 1) the neuronal signal, 2) NE biosynthesis and release, 3) end-organ adrenoreceptor (AR) responsiveness, and 4) the second messenger pathways involved in implementing vascular smooth muscle (VSM) contraction. Central to these impairments may be the age-associated increase in oxidative and nitrosative stress that affect key regulatory molecules mediating the reflex VC response, such as tetrahydrobiopterin (BH_4), L-tyrosine, and rho-kinase (ROCK) (Kohen, 1999; Lu *et al.*, 1999; Nishigori *et al.*, 2003; Hornig-Do *et al.*, 2007).

Aging and Prejunctional Noradrenergic Mechanisms

The neuronal signal required for reflex cutaneous VC may be dampened in older subjects. Sympathetic nerve traffic recordings from skin have demonstrated a ~60% decrease in cold-induced nerve activity (Grassi *et al.*, 2003). However, nerve traffic was expressed in bursts per minute and area under the curve, both of which may be inappropriate when comparing between groups because skin nerve recordings are, 1) highly asynchronous and 2) not reproducible due to the variability in the specific nerve fiber (i.e., sudomotor, cholinergic, and adrenergic) that is being assessed (Young *et al.*, 2009). Thus, it is unclear the extent to which skin sympathetic nerve activity or signal transduction mechanisms contribute to the age-related attenuation in the reflex VC response.

The synthesis and/or release of NE from axon terminals may, in part, account for the attenuated VC response in aged skin. Measuring NE release *per se* is complicated by concomitant changes in neuronal reuptake, receptor binding, and NE turnover (Esler *et al.*, 2002). However, indirect assessments have indicated that NE release in aged peripheral tissues is reduced in response to a variety of stressors (Cizza *et al.*, 1995; McCarty *et al.*, 1997; Donoso *et al.*, 2008). In response to a given absolute cold stimulus, older subjects exhibit diminished axonal release of NE (Frank *et al.*, 2000), which may be

linked to reduced NE synthesis and consequently bioavailability for vesicular packaging. **Specifically, elevated oxidative stress may deplete the substrate (L-tyrosine) and cofactor (BH_4) required by the rate-limiting enzyme, tyrosine hyroxylase (TH), to synthesize catecholamines (Figure 1.1).**

BH_4 is found throughout neural and vascular tissue and is an essential cofactor for both TH and nitric oxide synthase (NOS) (Kaufman, 1978; Kumer & Vrana, 1996; Moens & Kass, 2007). BH_4 may play a critical role in autonomic function because its intracellular concentration can regulate the synthesis of catecholamines (Iuvone *et al.*, 1985; Miwa *et al.*, 1985). Upon sympathetic activation, the affinity of TH for BH_4 markedly increases (Levine *et al.*, 1981; Dunkley *et al.*, 2004). BH_4 subsequently serves as a powerful reducing agent that maintains TH in the ferrous, active form, thereby enabling tyrosine hydroxylation and catecholamine production (Kaufman, 1978; Dunkley *et al.*, 2004; Urano *et al.*, 2006). However, BH_4 is vulnerable to oxidation; induction of oxidative stress in cultured sympathetic neurons decreases BH_4 concentration ~90% resulting in a ~75% reduction in catecholamine synthesis (Li *et al.*, 2003). Moreover, direct evidence of reduced BH_4 concentration has been detected in aged tissues (Williams *et al.*, 1980; Delp *et al.*, 2008). Oxidant-induced depletion of intraneuronal BH_4 in aged skin may decrease newly synthesized or stored pools of NE and contribute to the attenuated VC response. However, no studies to date have addressed the functional role of BH_4 in peripheral VC *in vivo*.

After sympathetic activation increases the affinity of TH for its cofactor, enzyme activity is ultimately dependent on its saturation with its amino acid substrate L-tyrosine (Weiner, 1978; Fluharty *et al.*, 1985; Kumer & Vrana, 1996). Because of its abundance in tissues, tyrosine is generally not thought to limit adrenergic function; however, in activated neurons, the immediate pool of tyrosine in the vicinity of TH may limit catecholamine production (Wurtman *et al.*, 1974; Iuvone *et al.*, 1978; Fernstrom, 1983; Fernstrom *et al.*, 1986). This tyrosine pool can be reduced by oxidative/nitrosative stress-induced conversion of tyrosine to the tyrosyl radical, which can further reduce free

tyrosine pools as well as nitrate other proteins thereby compromising their function (Ischiropoulos *et al.*, 1995; Reiter *et al.*, 2000; Kochman *et al.*, 2002). Studies in humans have indicated that tyrosine supplementation may enhance cognitive and psychomotor performance during cold stress (Banderet & Lieberman, 1989; O'Brien *et al.*, 2007). However, the functional role of tyrosine on thermoregulatory VC in aged human skin remains unknown.

Aging and Postjunctional Noradrenergic Mechanisms

Postjunctional noradrenergic mechanisms that may be affected with healthy aging include the responsivity of adrenoreceptors and/or the second messenger systems used to achieve VC (Figure 1.1). Noradrenergic VC in human skin depends on NE binding primarily to α_2-ARs on VSM (Borbujo *et al.*, 1989). In response to multiple physiological and supraphysiological perfusate concentrations of NE, VC responses were significantly blunted in aged skin (Thompson *et al.*, 2005b). Combined with the fact that resting plasma NE concentration increases ~10-15% per decade over the adult age range (Ziegler *et al.*, 1976; Goldstein *et al.*, 1983), these data suggest that adrenoreceptors in aged skin are desensitized (less responsive) to the reflex increase in NE released during cold exposure.

The second messenger systems downstream of adrenoreceptors rely in part on Ca^{2+}-independent pathways to elicit VC in aged skin. During localized cooling of skin, VC depends more on ROCK and less on adrenergic mechanisms (Thompson *et al.*, 2005a; Thompson-Torgerson *et al.*, 2007b). *In vitro* studies demonstrate that ROCK augments localized cold-induced VC through two distinct mechanisms, 1) inhibition of myosin light chain (MLC) phophatase thereby maintaining MLC phosphorylation without Ca^{2+} influx (i.e. Ca^{2+} sensitization) and 2) inducing the translocation of α_{2C} receptors from the Golgi apparatus to the cell membrane (Chotani *et al.*, 2000; Jeyaraj *et al.*, 2001; Bailey *et al.*, 2004). Interestingly, the stimulus for augmenting ROCK may be a cold-induced elevation in mitochondrial superoxide generation (Bailey *et al.*, 2005).

Additionally, upregulated ROCK in aged skin may also be a product of altered Ca^{2+} handling in VSM. Indirect evidence in aged rats demonstrates that contractile proteins in small vessels were less responsive to Ca^{2+}; however, in the absence of Ca^{2+}, the older animals exhibited a greater VC response to NE (Rubio *et al.*, 2002; Matz *et al.*, 2003). Thus, aged skin may rely on ROCK due to desensitization of contractile proteins to intracellular Ca^{2+}. The extent to which a Ca^{2+}-independent protein kinase such as ROCK is utilized during thermoregulatory reflex VC is unknown.

Upregulated ROCK favors a potentially pathological proconstrictor state due not only to its direct effects on VSM but also its mutually inhibitory influence on NOS (Ming *et al.*, 2002; Ming *et al.*, 2004; Noma *et al.*, 2006). *In vitro*, ROCK decreases NOS expression and activity and increases arginase activity thereby reducing NO bioavailability, whereas cyclic GMP-dependent protein kinase, a downstream product of NO metabolism, inhibits RhoA activation (Ming *et al.*, 2002; Ming *et al.*, 2004; Noma *et al.*, 2006). Furthermore, a greater dependence on ROCK may parallel that observed in other age-associated vascular pathologies such as atherosclerosis, hypertension, erectile dysfunction, and diabetes (Uehata *et al.*, 1997; Chitaley *et al.*, 2001; Mallat *et al.*, 2003; Bivalacqua *et al.*, 2004; Didion *et al.*, 2005; Noma *et al.*, 2006). Thus, ROCK inhibition may have a protective effect on the vasculature due in large part to its putative effects on NO bioavailability (Noma *et al.*, 2006).

Although BH_4 is required for optimal TH function prejunctionally, the extent with which BH_4 additionally affects postjunctional vascular adrenergic mechanisms remains unclear. Putative mechanisms through which BH_4 may affect the cutaneous vasculature include, 1) acting as a cofactor for NOS and 2) serving as an antioxidant. BH_4 prevents the uncoupling of the NOS dimer and subsequent superoxide production (Moens & Kass, 2007). Supplementation of BH_4 in older adults improves vasodilatory function by increasing NO bioavailability (Eskurza *et al.*, 2005), which may mask any potential effects that BH_4 would have on VC function. Additionally, BH_4 has the capacity to

effectively bind several oxidants, which may alter the redox state of VSM (Heales *et al.*, 1988; Gramsbergen *et al.*, 2002; Kuzkaya *et al.*, 2003).

Summary

Because reflex VC is mediated entirely by noradrenergic mechanisms in aged skin, four separate studies comprising this dissertation were performed to investigate how aging affects pre- and postjunctional noradrenergic reflex VC mechanisms. Two studies focused on the prejunctional role of BH_4 and tyrosine bioavailaibility as they relate to catecholamine biosynthesis during cutaneous VC in aged skin. Two studies were performed investigating the postjunctional role of ROCK as well as any potential effects of BH_4 on the aged cutaneous vasculature. For each study, cutaneous VC was evoked utilizing a reflex physiological (whole-body cooling) and/or pharmacological (tyramine or norepinephrine) stimulus. Specifically, tyramine elicits VC by causing the adrenergic release of neurotransmitters from axon terminals.

Specific Aims and Hypothesis

Specific Aim #1: The purpose of the study, "Local tetrahydrobiopterin administration augments cutaneous vasoconstriction in aged humans" was to determine the role of BH_4, a cofactor for TH, in the cutaneous VC response in aged skin.

Hypothesis 1: Acute localized BH_4 administration will enhance cutaneous VC in aged skin during 1) whole-body cooling and 2) during pharmacological depletion of adrenergic neurotransmitters via tyramine infusion.

Hypothesis 2: BH_4 affects the VC response through noradrenergic rather than cotransmitter-mediated mechanisms.

Specific Aim #2: The purpose of the study, "Localized tyrosine or tetrahydrobiopterin supplementation augments vasoconstriction in aged human skin" was to determine the role of tyrosine, the amino acid substrate for TH, in the cutaneous VC response in aged skin.

Hypothesis 1: Acute localized tyrosine administration will enhance cutaneous VC in aged skin during 1) whole-body cooling and 2) tyramine infusion.

Hypothesis 2: Combined BH_4 and tyrosine perfusion will increase the VC response in aged skin in an additive manner

Specific Aim #3: The purpose of the study, "Reflex vasoconstriction in aged human skin increasingly relies on rho-kinase dependent mechanisms during whole-body cooling" was to determine the role of ROCK in mediating the reflex VC response to whole-body cooling.

Hypothesis 1: Inhibiting ROCK by local fasudil administration will attenuate reflex VC to a greater extent in aged skin than in young skin.

Hypothesis 2: Fasudil will attenuate the cutaneous VC to exogenous NE to a greater extent in aged than in young skin.

Specific Aim #4: The purpose of the study, "Tetrahydrobiopterin does not affect end-organ responsiveness to norepinephrine-mediated vasoconstriction in aged skin" was to determine the extent to which the effects of BH_4 are localized to sympathetic nerve terminals as opposed to its potential non-specific effects BH_4 may have on cutaneous microvascular function.

Hypothesis 1: Local BH_4 administration does not affect the VC response to increasing doses of locally perfused exogenous NE in aged skin.

Figure **1.2**

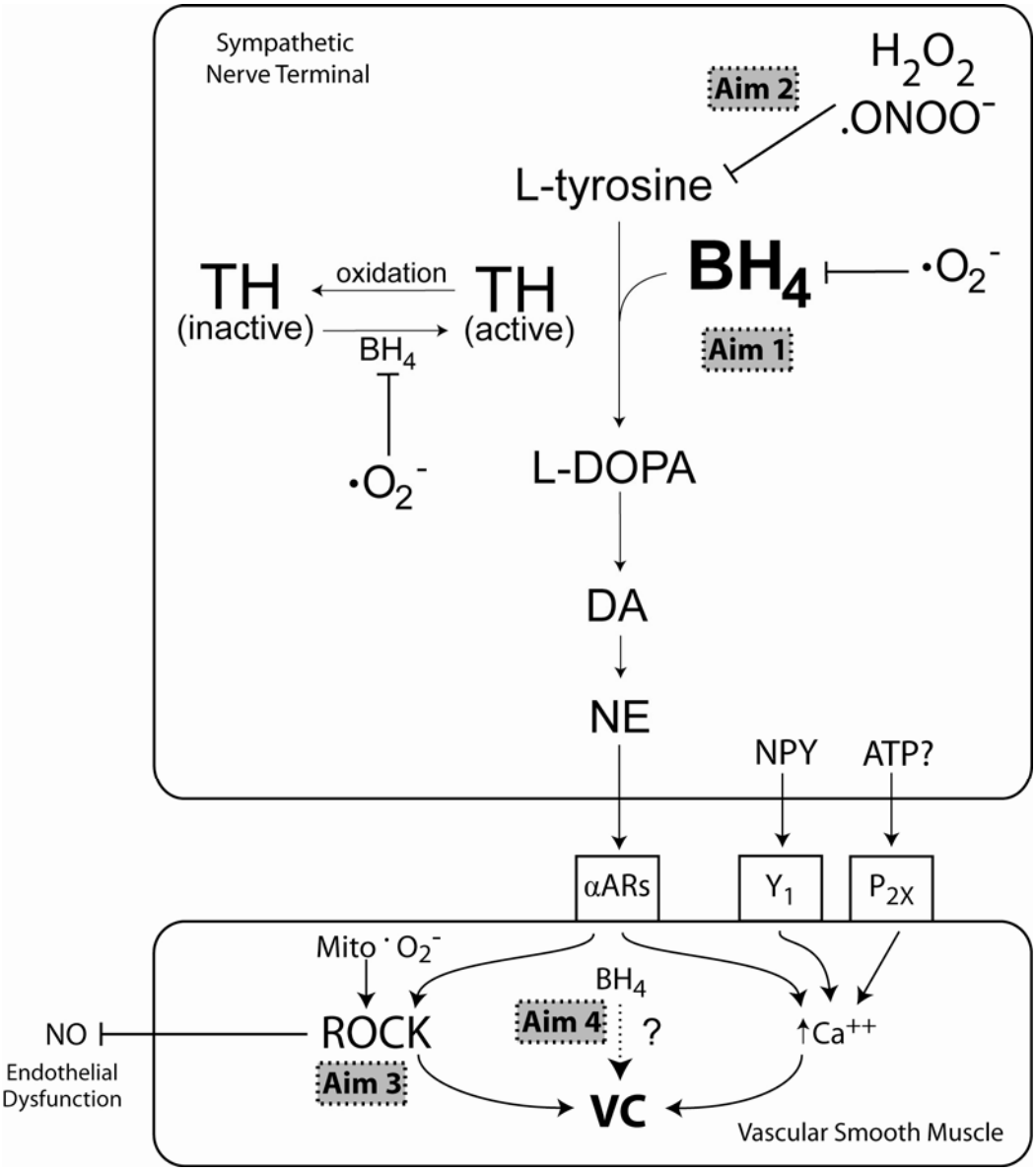

Figure **1.2**: Schematic representation of the prejunctional (Aims 1 and 2) and postjunctional (Aims 3 and 4) noradrenergic mechanisms. TH, tyrosine hydroxylase; BH_4, tetrahydrobiopterin; $ONOO^-$, peroxynitrite; $\bullet O_2^-$, superoxide; DA, dopamine; NE, norepinephrine; NPY, neuropeptide Y; αARs, α-adrenoreceptors; ROCK, rho kinase; NO, nitric oxide; Ca^{++}, calcium; VC, vasoconstriction

Chapter 2

REVIEW OF LITERATURE

Thermoregulation in humans is unique because of the role of skin as a primary effector organ for various homeostatic control mechanisms. No acceptable replicate can be found in other animals models. Human skin is dually innervated by the sympathetic nervous system, which consists of, 1) cholinergic fibers that stimulate sweating and vasodilation and 2) adrenergic fibers responsible for vasoconstrictor tone (Grant & Holling, 1938; Johnson & Proppe, 1996). The relative activity of these fiber subtypes modulates skin blood flow and convective heat loss during a thermal stimulus. Under thermoneutral conditions, skin blood flow is ~350 mL/min; however, during cold or hot ambient conditions, skin blood flow can range from nearly zero to as much as ~6-8 L/min, respectively (Rowell, 1977, 1983). The wide range of blood flows that skin accommodates as well as it being overperfused relative to its metabolic needs at rest suggests that the modulation of cutaneous vasomotor activity primarily serves a thermoregulatory purpose.

Activation of cholinergic nerves is essential to heat dissipation during heat stress or exercise. Even mild heating (i.e., less than the core temperature threshold for active vasodilation) is marked by increased vasodilator nerve activity; albeit insufficient to overcome vasoconstrictor tone (Kamijo *et al.*, 2005). However, under thermoneutral or cool conditions, body core temperature is maintained within narrow limits primarily through the modulation of the adrenergic vasoconstrictor system (Johnson & Proppe, 1996). Subtle changes in adrenergic nerve activity accompany the typical changes in physical activity or ambient temperature that is encountered throughout the day. These small changes in skin blood flow can alter convective heat transfer several fold (Johnson *et al.*, 1986).

Thermoregulatory reflex vasoconstriction

During cold exposure, distinct reflex and local VC mechanisms are implemented to minimize the return of cooled blood from the periphery to the core. Under these conditions, a reflex increase in sympathetic adrenergic activity evokes neurotransmitter release from peripheral nerve terminals. Localized intradermal administration of various antagonists of the adrenergic pathway using bretylium (presynaptic block of neurotransmitter release) or yohimbine and propranolol (postsynaptic block of adrenoreceptors) have revealed that reflex cutaneous VC relies entirely on adrenergic nerve fibers; however, only about ~60% of the VC response is mediated by norepinephrine (Kellogg *et al.*, 1989; Stephens *et al.*, 2001; Thompson & Kenney, 2004). Thus, the remaining ~40% is dependent on other coreleased sympathetic neurotransmitters.

There is evidence indentifying neuropeptide Y (NPY) and ATP as putative sympathetic cotransmitters. Both are coreleased with NE but in different vesicular populations and at different stimulation frequencies. Moreover, the relative role of these cotransmitters varies considerably depending on animal species and vascular bed (Burnstock, 2009). NPY is released from large dense core vesicles at high stimulation frequencies (e.g., severe and/or long duration cold exposure) and subsequently binds to postjunctional Y_1 receptors to directly elicit VC (Ekblad *et al.*, 1984; Fried *et al.*, 1985; De Potter *et al.*, 1988; Lundberg *et al.*, 1990; Racchi *et al.*, 1997; Han *et al.*, 1998). ATP is released from smaller synaptic vesicles at short, low stimulation frequencies and acts on postjunctional P_{2X} receptors to evoke VC (Taddei *et al.*, 1990; Ralevic & Burnstock, 1991; Burnstock, 2007). Both cotransmitters putatively function in modulating NE release and this may in fact be their primary function (Edvinsson *et al.*, 1984; Garcia-Villalon *et al.*, 2000; Li *et al.*, 2005; Ralevic, 2009). *In vivo* evidence in human skin demonstrates a direct role for NPY in reflex VC to whole-body cooling (Stephens *et al.*, 2004); however this result was not replicated in our lab using the same NPY antagonist, BIBP-3226 (Thompson, 2005). No similar studies have been performed in human skin

investigating the role of ATP. Thus, the identity of the sympathetic cotransmitter that functionally participates in the cutaneous VC response remains in question.

Prejunctional noradrenergic mechanisms

The efferent arm of the reflex arc of cutaneous VC begins with sympathetic nerve traffic originating from the brain stem and synapses upon the paravertebral ganglion. This houses the cell bodies and transcriptional machinery of peripheral neurons required to synthesize the enzymes needed for catecholamine biosynthesis. These enzymes must travel the length of the relatively long axons (rate of ~1-2 mm/hr) before they can be functionally relevant in the nerve terminal (Dahlstrom, 1973; Thoenen *et al.*, 1973). The distance between cell body and nerve terminal usually precludes any acute involvement of transcriptional regulation in catechoamine biosynthesis. For instance, increased expression of catecholamine synthesizing enzymes occurs ~3-6 days after prolonged cold exposure (Stachowiak *et al.*, 1986; Baruchin *et al.*, 1990; Andrews *et al.*, 1993).

Catecholamine synthesis almost exclusively occurs in the nerve terminal (Geffen & Rush, 1968). Although NE can be synthesized from phenylalanine, tyrosine is the primary substrate for catecholamine biosynthesis due to its abundance from dietary sources as well as that produced endogenously. As illustrated in Figure 1.1, the process of NE synthesis begins in the cytosol with 1) hydroxylation of tyrosine into L-DOPA by the rate-limiting enzyme tyrosine hydroxylase (TH), 2) the DOPA-decarboxylase catalyzed conversion of L-DOPA to dopamine, and then 3) dopamine is subsequently taken up by a storage vesicle where it is converted to NE by dopamine β-hydoxylase (Weiner, 1970; Dahlstrom, 1973; Langer & Hicks, 1984). Because dopamine β-hydoxylase is present in high concentration, negligible amounts of dopamine are produced in peripheral nerve terminals.

The rate-limiting and committed step of catecholamine synthesis is mediated by TH (Levitt *et al.*, 1965). The physiological importance of this enzyme is underscored by

the fact that disruption of the TH gene results in midgestational lethality (~97% of all embryos) in mice (Zhou *et al.*, 1995). In addition to L-tyrosine, the hydroxylation reaction requires BH_4, O_2, and Fe^{2+}. Activation of TH is mediated by the phosphorylation of a serine residue, primarily Ser-40, by a variety of protein kinases (Kumer & Vrana, 1996; Fujisawa & Okuno, 2005). Phosphorylation causes a conformational change in TH and increases its affinity for BH_4 (Levine *et al.*, 1981; Dunkley *et al.*, 2004). BH_4 is an essential cofactor because it acts as a natural reductant, thereby converting the iron moiety of TH from its inactive ferric (Fe^{3+}) form to its ferrous (Fe^{2+}) and catalytically active configuration (Dunkley *et al.*, 2004; Frantom *et al.*, 2006). Catecholamines can subsequently inactivate TH through two forms of feedback inhibition, 1) by competing with BH_4 for its binding site on TH thereby serving as a "sensor" for low catecholamine concentration and 2) by forming a stable, inactive complex with the ferric form of TH that is reversible only with phosphorylation of the enzyme (Ramsey & Fitzpatrick, 1998; Dunkley *et al.*, 2004).

The increase in sympathetic activity with acute cold exposure is the primary stimulus for TH activity (Rittenhouse & Zigmond, 1990). The factors that influence TH activity, apart from enzymatic phosphorylation, include the bioavailability of amino acid substrate or pterin cofactor (Levine *et al.*, 1981; Dunkley *et al.*, 2004). BH_4 is essential to catecholamine synthesis (Nagatsu *et al.*, 1964) and is primarily generated de novo from guanosine triphosphate (GTP) (Nagatsu & Ichinose, 1999). This process is catalyzed by the rate-limiting enzyme GTP cyclohydrolase (Viveros *et al.*, 1981). Additionally, BH_4 can be reconstituted from oxidized forms by utilizing a "salvage" pathway catalyzed by dihydrofolate reductase and dihydropteridine reductase (Thony *et al.*, 2000). Intracellular BH_4 concentration is ~10 μM (Zigmond *et al.*, 1989); however this can double with an elevation in sympathetic nerve activity (Baruchin *et al.*, 1990). After sympathetic activation and subsequent priming of TH with BH_4, catecholamine biosynthesis ultimately depends on the bioavailability of tyrosine. Although tyrosine is fairly abundant in tissues due to dietary intake as well as endogenous production, it may

become a limiting factor during neuronal activation (Wurtman *et al.*, 1974; Iuvone *et al.*, 1978; Fernstrom, 1983; Fernstrom *et al.*, 1986).

The final step of NE biosynthesis occurs within storage vesicles where DA is converted to NE. NE is colocalized with other neurotransmitter substances such as NPY in large dense core vesicles (80-100 nm) or ATP in small clear synaptic vesicles (40-50 nm) (Fried *et al.*, 1985; Lundberg *et al.*, 1990; Burnstock, 2009). These vesicle subtypes differ in function: 1) the latency for large dense core vesicles exocytosis is longer than with synaptic vesicles (De Camilli & Jahn, 1990) and 2) higher stimulation frequency favors the release from large dense core vesicles (Andersson *et al.*, 1982; Han *et al.*, 1998). In context of whole-body cooling, neuropeptides such as NPY may be putatively released during more severe or prolonged cooling whereas ATP may be released with minor perturbations in ambient temperature.

In addition to NE synthesis and storage, vesicular release also depends on neuronal reuptake. Approximately 85% of the NE that is released is taken back up into the nerve terminal; much of which (~70%) is repackaged into storage vesicles (Iversen, 1973). The remaining ~30% is metabolized by monoamine oxidase. Combined inhibition of monoamine oxidase and neuronal reuptake with amezinium increases the VC response to hand cooling; however, it is unknown if this response is altered with aging (Harada *et al.*, 1998). Alternatively, NE can be taken up by other tissues and metablized by catechol-O-methyltransferase (Eisenhofer, 2001). That which is not metabolized is available to bind adrenoreceptors and affect end-organ function.

Postjunctional noradrenergic mechanisms

NE released from sympathetic adrenergic nerves binds to both pre- and postjunctional ARs. In the cutaneous circulation, α_{2A}- and α_{2C}-ARs function as prejunctional autoreceptors that when stimulated, decreases NE release from axon terminals (Hein *et al.*, 1999; Philipp *et al.*, 2002). Postjunctionally, the receptor subtypes

that primarily elicit cutaneous VC are also α_{2A}- and α_{2C}-ARs; the former primarily mediates the VC to NE while the latter is translocated to the cell membrane after direct localized cooling of skin (Ekenvall *et al.*, 1988; Borbujo *et al.*, 1989; Nielsen *et al.*, 1990; Chotani *et al.*, 2000; Jeyaraj *et al.*, 2001). Additionally, α_1-ARs likely participate in the VC response but to a lesser extent (Guimaraes & Moura, 2001). Although β_2-ARs are sparsely found in skin and elicit VD when stimulated (Crandall *et al.*, 1997), their effect on reflex VC is unclear. Following AR binding, various second messenger pathways are initiated that affect contractile proteins in VSM.

NE induces VC in VSM through Ca^{2+}-dependent and Ca^{2+}-independent mechanisms (Figure 1.1). The former involves PIP_2 hydrolysis to diacylglycerol and IP_3, which mobilizes intracellular Ca^{2+} stores that subsequently activates MLC kinase. The latter can operate through a ROCK-dependent pathway that inhibits MLC phosphatase (i.e., Ca^{2+} sensitization), thereby maintaining MLC in a phosphorylated, pro-constrictor state (Somlyo & Somlyo, 2000; Sward *et al.*, 2003; Amobi *et al.*, 2006). In addition to MLC phosphatase inhibition, ROCK augments VC by the translocation of α_{2C} receptors from the Golgi apparatus to the plasma membrane thereby increasing the binding sites for NE approximately five fold (Chotani *et al.*, 2000; Jeyaraj *et al.*, 2001; Bailey *et al.*, 2004). *In vitro*, localized cooling of mouse tail arteries increases the generation of superoxide that can also directly stimulate ROCK (Bailey *et al.*, 2005). In humans, ROCK importantly contributes to resting vascular tone as well as the VC response to localized cooling (Bussemaker *et al.*, 2007; Thompson-Torgerson *et al.*, 2007a); however, the extent that ROCK participates in reflex thermoregulatory VC is unknown.

In addition to its effects on VSM, ROCK also reciprocally inhibits NOS (Ming *et al.*, 2002; Ming *et al.*, 2004; Noma *et al.*, 2006). ROCK can decrease NO bioavailability by inhibiting eNOS transcription and activity (Ming *et al.*, 2002) and by augmenting arginase activity (Ming *et al.*, 2004; Holowatz & Kenney, 2007). Conversely, cGMP-dependent protein kinase, a downstream product of NO production, inhibits ROCK (Noma *et al.*, 2006; Somlyo, 2007). Thus, an intricate balance exists between these

constrictor and dilator influences. How this reciprocal inhibition between these pathways factor into cold-induced VC is unclear.

From a thermoregulatory standpoint, upregulated ROCK may strengthen the effectiveness of cold-induced VC. However, this may occur at the expense of microvascular function by diminishing NO bioavailability (Ming *et al.*, 2002; Ming *et al.*, 2004; Noma *et al.*, 2006). In fact, increased ROCK activity precedes several vascular pathologies such as atherosclerosis (Mallat *et al.*, 2003), diabetes (Didion *et al.*, 2005), endothelial dysfunction (Chitaley *et al.*, 2001; Bivalacqua *et al.*, 2004), cerebral and coronary vasospasm (Sato *et al.*, 2000), and hypertension (Uehata *et al.*, 1997; Holowatz & Kenney, 2007). As such, inhibition of ROCK may be beneficial in mitigating these clinical pathologies (Noma *et al.*, 2006). Collectively, augmented ROCK activity appears to have a deleterious effect on vascular function; whether or not upregulated ROCK observed during local or whole-body cooling predates the onset of other vascular pathologies has yet to be determined.

Aging and themoregulatory reflex vasoconstriction

A series of studies were performed in elderly subjects by K.J. Collins and colleagues that ultimately identified accidental hypothermia as a "natural hazard of old age". This held true even in apparently fit older subjects. The first study they conducted was a longitudinal study measuring hand blood flow responses to cold in subjects >69 yrs of age. In the first test, 37 out of 43 subjects exhibited a VC response however, 4 years later, only 29 maintained VC function (Collins *et al.*, 1977). In a separate study, similarly clothed young and older subjects were free to adjust room temperature to a comfortable state. Older subjects made fewer temperature adjustments and allowed cooler and hotter air temperatures before responding (Collins *et al.*, 1981). Thus, both behavioral delays and physiological impairments, notably in the sensory and efferent VC response, may explain the overrepresentation of the elderly in hypothermia-related mortalities.

Subsequent studies in aged humans unanimously confirmed that cutaneous VC was attenuated in response to multiple modalities of cooling including intravenous cold saline infusion (Frank *et al.*, 2000), contralateral limb immersion in cold water (Khan *et al.*, 1992; Richardson *et al.*, 1992), cold air exposure (Wagner *et al.*, 1974; Budd *et al.*, 1991; Inoue *et al.*, 1992; Kenney & Armstrong, 1996; Degroot & Kenney, 2007), and water-perfusion suits (Thompson & Kenney, 2004). Only one study closely matched age groups in relation to body composition and aerobic fitness level (Kenney & Armstrong, 1996), both of which may be potentially confounding factors when identifying functional changes with primary aging (Smolander, 2002), and found that reflex VC was blunted in aged skin. Central to the age-related impairments in reflex VC may be elevated oxidative stress and nitrosative stress.

Human aging is associated with a systemic increase in oxidative and nitrosative stress (Harman, 1956; Beckman & Ames, 1998). This pro-oxidant environment results from both an increase in reactive oxygen/nitrogen species and a decrease in antioxidant scavenging mechanisms. Elevated intracellular oxidant concentration can cause structural damage to the cell and trigger the activation of redox-sensitive signaling pathways. Both of these effects can influence numerous cell processes and may precede the development of more serious age-related diseases (Finkel & Holbrook, 2000). In aged human skin, an increase in oxidant concentration has been observed and this may subsequently affect thermoregulatory reflex VC mechanisms both pre- and postjunctionally (Kohen, 1999; Lu *et al.*, 1999; Nishigori *et al.*, 2003; Hornig-Do *et al.*, 2007).

Aging and nonnoradrenergic function

There may be a link between the elevated oxidative stress observed with aging and the development of a pro-inflammatory state. Although which factor precedes the other is unclear, a proinflammatory NF-κB transcription factor complex is augmented in

endothelial cells in aged humans and this is positively associated with age-associated increases in 3-nitrotyrosine, a marker for oxidative stress (Donato *et al.*, 2007; Donato *et al.*, 2008). In the context of cotransmitter function, an inflammatory stimulus (lipopolysaccharide administration) in aged rats caused an increase in cytokine production as well as a concomitant reduction in serum NPY and ATP concentration; however, catecholamines were unaffected (Donoso *et al.*, 2008). Thus, the selective loss of cotransmitter function in aged humans may be due to oxidative stress secondary to chronic, low-grade inflammation.

The contribution of sympathetic cotransmitters in the reflex VC response is ~40% of the reflex VC response in young skin (Stephens *et al.*, 2001; Stephens *et al.*, 2004); however, in healthy aged skin, this cotransmitter component is functionally absent (Thompson & Kenney, 2004). Additionally, perivascular NPY innervation density gradually disappeared over the life span of the rat (Connat *et al.*, 2001). Moreover, end-organ responsivity to exogenously administered NPY or ATP is blunted in human dorsal hand veins and rat mesenteric vessels, respectively (Konishi *et al.*, 1999; Lambert *et al.*, 1999). Because cotransmitter-mediated VC is functionally nonexistent in aged skin, reflex VC is dependent on noradrenergic mechanisms.

Aging and prejunctional noradrenergic function

Little is known about how primary aging affects prejunctional noradrenergic mechanisms in skin, ranging from the neuronal signal to the synthesis and release of NE from axon terminals. One of the principal challenges of studying these mechanisms *in vivo* is verifying whether or not the observed experimental results are isolated to the neural compartment. Analyzing the neural signal driving reflex VC is also difficult based on its irregular pattern and the inability to discriminate between sympathetic fibers signals (i.e., cholinergic, sudomotor, and adrenergic) (Young *et al.*, 2009). Nevertheless, during cold air exposure, where the neural signal is primarily adrenergic in composition, the increase in skin sympathetic nerve activity was blunted in aged skin (Grassi *et al.*,

2003). Thus, the neuronal signal stimulating reflex VC appears to be dampened in older subjects. How this is coupled to age-associated changes in the catecholamine biosynthetic pathway is unclear.

Stimuli that increase NE release also accelerate the rate-limiting step in its biosynthesis. Any cold-induced increases in TH activity or content requires intact sympathetic input (Stachowiak *et al.*, 1986). However, the ability of sympathetic neurons to upregulate NE biosynthesis is impaired with age (Santer, 1979). Short-term increases in catecholamine synthesis and release are primarily mediated by covalent modification of TH resulting in a greater affinity for BH_4 (Kumer & Vrana, 1996). Additionally, a nearly twofold increase in BH_4 concentration can be observed during cold stress (Baruchin *et al.*, 1990). Because BH_4 has multiple functions (e.g., vasodilation and melanogenesis) in various cell types in skin (Moens & Kass, 2007; Schallreuter *et al.*, 2008), its direct assessment in axon terminals or endothelial cells in humans is challenging. Nevertheless, its bioavailability appears to be diminished in aged tissues and this may be due to an elevation in oxidative stress (Williams *et al.*, 1980; Delp *et al.*, 2008). An age-associated increase in oxidative stress in human skin has been well established (Kohen, 1999; Lu *et al.*, 1999; Nishigori *et al.*, 2003; Hornig-Do *et al.*, 2007). BH_4 is uniquely sensitive to oxidation such that induction of oxidative stress in cultured sympathetic neurons decreases BH_4 concentration ~90% resulting in a ~75% reduction in catecholamine synthesis (Heales *et al.*, 1988; Li *et al.*, 2003). Furthermore, the biosynthetic machinery for BH_4 may also be vulnerable to the effects of oxidative stress. The rate-limiting enzyme, GTP cyclohydrolase, and dihydrofolate reductase are dowregulated in the presence of oxidative stress (Moens & Kass, 2007). Collectively, this suggests that oxidant-induced depletion of BH_4 in aged skin may diminish the ability to synthesize catecholamines in response to a thermoregulatory stimulus.

In addition to the enzymatic cofactor, the pool of amino acid substrate available for hydroxylation may also be limited in older adults, particularly during neuronal activation. Animal studies have demonstrated that increasing tyrosine in activated

neurons augments the concentration of the NE precursor, L-DOPA, which suggests that tyrosine concentration in the vicinity of TH may be well below saturation (Wurtman *et al.*, 1974; Iuvone *et al.*, 1978; Fernstrom, 1983; Fernstrom *et al.*, 1986). In humans, tyrosine supplementation may enhance cognitive and psychomotor performance during cold stress (Banderet & Lieberman, 1989; O'Brien *et al.*, 2007). The bioavailability of tyrosine may be decreased by its oxidation to the tyrosyl radical, which can further reduce free tyrosine pools as well as nitrate other proteins (Ischiropoulos *et al.*, 1995; Reiter *et al.*, 2000; Kochman *et al.*, 2002). Additionally, peroxynitrite or its highly reactive degradation products may convert free tyrosine to a byproduct incapable of participating in NE biosynthesis (Kooy & Lewis, 1996). In human skin, one such byproduct, 3-nitrotyrosine, is elevated in photoaged skin and can acutely increase in response to oxidative stress generated from ultraviolet light exposure (Nishigori *et al.*, 2003). Thus, a subsaturable concentration of available tyrosine during sympathetic activation compounded with elevated oxidative and nitrosative stress may significantly limit catecholamine production in aged skin.

Reduced catecholamine synthesis may lower the amount of NE released during neuronal activation. In peripheral aged tissues, NE release is reduced in response to a variety of stressors (Cizza *et al.*, 1995; McCarty *et al.*, 1997; Donoso *et al.*, 2008). In response to a given absolute cold stimulus, older subjects exhibit diminished axonal release of NE (Frank *et al.*, 2000). However, the assessment of NE release may be obfuscated by age-related alterations in NE reuptake or metabolism. Because of these challenges, the concentration of NE in the synapse at rest or during neuronal activation is unknown. Neuronal reuptake in rat tissue preparations have yielded conflicting results ranging from reduced (Duckles, 1983; Borton & Docherty, 1989) or increased (Kreider *et al.*, 1984; Daly *et al.*, 1988) reuptake with aging. In humans, there is little conclusive evidence aside from one study that has demonstrated in cardiac tissue that the neuronal reuptake transporter density and activity diminishes with age (Leineweber *et al.*, 2002). Reduced neuronal reuptake would prolong the time that NE is in the synapse and may explain, in part, the desensitization of postjunctional ARs that occurs with aging.

Aging and postjunctional noradrenergic function

In human skin, NE released from peripheral axon terminals primarily binds with α_2-adrenoreceptors on VSM (Borbujo *et al.*, 1989). NE can also bind to prejunctional autoreceptors; however, conflicting evidence exists suggesting that their function is diminished (Bruck *et al.*, 2007) or unchanged (Dinenno *et al.*, 2002) with age. In contrast, the VC to multiple physiological and supraphysiological concentrations of NE in aged skin was reduced (Thompson *et al.*, 2005b). Moreover, when adding local cooling to multiple exogenous NE concentrations, older subjects exhibited a diminished capacity to respond (Thompson *et al.*, 2005a). Cumulatively, these results indicate that α_2-adrenoreceptors in aged skin are less responsive to a given adrenergic stimulus, thereby suggesting that the desensitization of these receptors may contribute to the age-related attenuation in reflex VC.

The second messenger responses coupling adrenoreceptor activation to reflex VC are altered in aged skin. NE-mediated VC may occur through Ca^{2+} dependent and Ca^{2+} independent mechanisms; the latter is stimulated by ROCK and sensitizes VSM to extant intracellular Ca^{2+} by deactivating MLC phosphatase. Aging is associated with greater reliance on Ca^{2+}-independent pathways such as ROCK to elicit VC (Thompson-Torgerson *et al.*, 2007b). This may be due in part to how Ca^{2+} is handled in VSM. Vessels from aged rats display an increase in basal intracellular Ca^{2+} and decreased sensitivity of contractile proteins to Ca^{2+} (Matz *et al.*, 2003). Furthermore, small vessels placed in a Ca^{2+} free medium display greater NE-evoked VC in older than in young rats (Rubio *et al.*, 2002; Amobi *et al.*, 2006; Naik *et al.*, 2006). Thus, it is plausible that compromised Ca^{2+}-induced signaling may explain the greater reliance on the ROCK component in aged vessels. However, the extent that ROCK contributes to reflex VC is unknown.

In addition to being stimulated by NE, ROCK can also be stimulated by an elevation in oxidative stress. In rat renal arteries, phenylephrine (α-AR agonist) infusion

can rapidly stimulate superoxide production (Just *et al.*, 2007). Superoxide can subsequently induce VC through ROCK-mediated pathways (Jin *et al.*, 2004). Perhaps the age-associated increase in reactive oxygen species tonically augments ROCK activity and increases the gain of the ROCK response under adrenoreceptor stimulation. Because of the inhibitory influence of ROCK on NOS, upregulated ROCK in aged vessels may diminish NO bioavailability and precede other age-associated vascular disease states.

Because BH_4 is prone to oxidation, its ability to react with oxidants confers antioxidative properties (Gramsbergen *et al.*, 2002; Foxton *et al.*, 2007; Katusic *et al.*, 2009). In fact, BH_4 has been demonstrated to be more effective than ascorbate in scavenging oxygen-derived radicals (Heales *et al.*, 1988; Kuzkaya *et al.*, 2003). Additionally, BH_4 minimizes NOS-derived ROS by preventing enzymatic uncoupling (Moens & Kass, 2007; Katusic *et al.*, 2009). Thus, BH_4 may affect end-organ responsiveness by altering the redox state of the vessel. The extent with which BH_4 affects VSM in aged skin or during thermoregulatory VC is unclear.

Clinical Aspects

Reduced BH_4 bioavailability is associated with various age-related disorders. BH_4-sensitive phenylketonuria is a form of hyperphenylalanemia that affects neonates and can be effectively treated with BH_4 supplementation prior to the development of any serious neurological deficits (Shintaku, 2002). In children ~6 years of age, mutations in the gene encoding GTP cyclohydrolase may dramatically reduce BH_4 production resulting in a L-DOPA responsive dystonia (Segawa disease). Dystonia can occur with as little as a 20% reduction in GTP cyclohydrolase; however, in Segawa's disease, enzyme activity is only ~10-20% of normal values (Segawa *et al.*, 2003). In older adults, a reduction in BH_4 secondary to elevated oxidative stress is also associated with neurodegenerative disorders such as Parkinson's and Alzheimer's disease. BH_4 supplementation has been attempted in these patients; however, very high doses are required to achieve any clinical efficacy (Foxton *et al.*, 2007).

An increase in ROCK activity is associated with multiple age-associated vascular disorders. ROCK inhibition may have a vasculoprotective effect due in large part to its effects on NO bioavailability. Part of the pleiotropic vascular protective effect of HMG-CoA reductase inhibitors (statins) is due to their inhibition of ROCK (Noma *et al.*, 2006). Furthermore, ROCK inhibition may be an effective treatment for Raynaud's phenomenon, which is characterized by a cold-induced amplification of ROCK-mediated VC in cutaneous vessels located in distal limbs (Flavahan, 2008).

Chapter 3

LOCAL TETRAHYDROBIOPTERIN ADMINISTRATION AUGMENTS CUTANEOUS VASOCONSTRICTION IN AGED HUMANS

Introduction

During whole-body cold exposure, a reflex increase in skin sympathetic nerve activity occurs resulting in constriction of peripheral cutaneous vessels. Reflex cutaneous vasoconstriction (VC) serves to increase effective tissue insulation and minimize convective heat loss in cool environments. This thermoregulatory response is attenuated in older adults rendering them more susceptible to heat loss and hypothermia (Khan *et al.*, 1992; Richardson *et al.*, 1992; Kenney & Armstrong, 1996). Even during mild cold stress (22^{o}C), aged humans may exhibit a relative inability to maintain body core temperature (Degroot & Kenney, 2007).

Reflex cutaneous VC is mediated by elevated efferent sympathetic nerve activity that stimulates the release of neurotransmitters and cotransmitters from perivascular axon nerve terminals. Studies using local intradermal administration of bretylium tosylate or combined adrenoreceptor antagonists such as yohimbine and propranolol have collectively revealed in young skin that ~60% of the VC response is dependent on noradrenaline (NA) and the remaining ~40% is mediated by nonadrenergic cotransmitters; putatively NPY and ATP (Stephens *et al.*, 2001; Stephens *et al.*, 2004; Thompson & Kenney, 2004). In contrast, aged skin exhibits 1) functionally absent cotransmitter-mediated VC (Thompson & Kenney, 2004), 2) reduced axonal release of NA for a given absolute cold stimulus (Frank *et al.*, 2000), and 3) diminished adrenoreceptor responsiveness for a given exogenous NA dose (Thompson *et al.*, 2005a) or tyramine dose (Dinenno *et al.*, 2002). Cumulatively, reflex VC in aged skin is entirely dependent on an already compromised noradrenergic-mediated mechanism.

Central to these impairments in aged skin may be the deleterious effects of elevated oxidative stress on key regulatory molecules in the VC response, such as tetrahydrobiopterin (BH_4). BH_4 is found throughout 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; Kumer & Vrana, 1996; Moens & Kass, 2007). BH_4 is uniquely sensitive to oxidation such that induction of oxidative stress in cultured sympathetic neurons decreases BH_4 concentration ~90% resulting in a ~75% reduction in catecholamine synthesis (Li *et al.*, 2003). BH_4 serves as a powerful reducing agent that maintains TH in its ferrous, active form and thus is necessary for catecholamine synthesis (Kaufman, 1978; Dunkley *et al.*, 2004; Urano *et al.*, 2006). Oxidant-induced depletion of intraneuronal BH_4 in aged skin may decrease newly synthesized or stored pools of NA, thereby functionally blunting cutaneous VC during sympathetic activation. Few, if any, in vivo studies in humans have addressed the functional role of BH_4 in reflex VC.

BH_4 has recently gained attention in its putative role in preventing NOS uncoupling and superoxide production. In the relative absence of BH_4, the active NOS dimer uncouples thereby disrupting the normal flow of electrons required to synthesize NO. In the uncoupled state, NOS produces superoxide, which further diminishes BH_4 bioavailability (Moens & Kass, 2007). Supplementation of BH_4 may increase NO production in aged individuals and thus, potentially mask any functional augmentation in VC function during whole-body cooling.

This study consisted of two protocols designed to examine the role of BH_4 in the attenuated VC response observed in aged skin during cold stress. For *protocol 1*, we hypothesized that supplementing BH_4 in aged skin would augment cutaneous VC in response to whole-body cold exposure and tyramine, which evokes NA release from storage vesicles in sympathetic nerve terminals. Also, we examined whether BH_4 mediates its effects through noradrenergic or sympathetic cotransmitter mechanisms by

pharmacologically blocking adrenoreceptors with yohimbine and propranolol. In *protocol 2*, we determined if the putative effects of BH_4 on NOS were masking any changes in VC responses to whole-body cooling.

Methods

Subjects

With the Pennsylvania State University Institutional Review Board approval and after verbal and written informed consent, eleven young (21 ± 1 yrs; 5 men, 6 women) and eleven older (72 ± 2 yrs; 5 men, 6 women) individuals participated in the study. Of the 22 total subjects, twelve (6y, 6o) participated in *protocol 1* and ten (5y, 5o) participated in *protocol 2*. All young women were tested in the early follicular phase (days 1-7) of the menstrual cycle, and all older women were post-menopausal. All subjects were healthy, non-obese, normotensive, normal cholesterolemic, non-smokers, and not taking any medications that would otherwise alter cardiovascular or thermoregulatory function. All procedures conformed to the standards set by the Declaration of Helsinki.

Instrumentation

On the morning of an experiment, between 0800-1000, subjects arrived at the laboratory and were instrumented with four microdialysis (MD) fibres (10mm, 30kDa cutoff membrane, MD 2000 Bioanalytical Systems, West Lafayette, IN, USA) placed intradermally in the ventral forearm using sterile technique. Prior to fibre placement, ice packs were applied to MD sites for 5 min to temporarily anesthetize the skin. For each fibre, a 25-gauge needle guide was inserted horizontally into the dermis such that entry and exit points were ~2.5 cm apart. MD fibres were threaded through the needle. The needle was then withdrawn leaving the membrane in place. After all fibres were taped in place, lactated Ringer solution was perfused at 2μL/min (Bee Hive controller and Baby Bee microinfusion pumps, Bioanalytical Systems) for 60-90min to allow for local hyperemia due to needle insertion trauma to subside.

To control skin temperature, subjects wore a water-perfused suit that covered the entire body excluding the face, feet, hands, and forearms. Copper-constantan thermocouples were placed on the surface of the skin at 6 different sites; calf, thigh, abdomen, chest, back, and upper arm. The unweighted mean of these sites provided an index of mean skin temperature (T_{sk}).

To obtain an index of skin blood flow, red blood cell flux was continuously measured with a laser Doppler flowmetry (LDF) probes (MoorLAB, Temperature Monitor SH02, Moor Instruments, Devon, UK). LDF probes were placed in local heaters and positioned directly over each MD fibre site. Local heaters were held at 33^{o}C to clamp local skin temperature throughout the experiment. Arterial blood pressure was taken every 5 min throughout the experiment via brachial auscultation. Mean arterial pressure (MAP) was calculated as the diastolic blood pressure plus one-third the pulse pressure. Cutaneous vascular conductance (CVC) was calculated as the ratio of LDF flux to MAP and expressed as percent change from baseline values (%ΔCVC_{base}).

Protocol

After instrumentation of MD fibres and resolution of local hyperemia, pharmacological agents were pefused for 45-60min. For *protocol 1* (n=12; 6y 6o), MD fibre sites were randomly assigned, 1) lactated Ringer's solution serving as control, 2) 5mM tetrahydrobiopterin (BH_4), 3) 5mM BH_4 + 10mM ascorbate (AA), and 4) BH_4 + α- and β-adrenoreceptor antagonists, yohimbine and propranolol (Y/P). Ascorbate was added to determine whether any additional increase in VC would be observed due to local antioxidant supplementation. Protocol 2 was similar to protocol 1 except for 2 MD fibres sites that controlled for the putative effects of BH_4 on NOS using the competitive NOS inhibitor, L-NAME. For *protocol 2* (n=10, 5y 5o), MD sites were randomly assigned, 1) Ringer's solution, 2) 5mM BH_4, 3) BH_4 + Y/P, 4) BH_4 + L-NAME, and 5) L-NAME. All drugs were purchased from Sigma-Aldrich, St. Louis, MO, USA; except for L-

NAME (Calbiochem, Gibbstown, NJ, USA). All drugs were mixed just prior to use, dissolved in lactated Ringer solution, and sterilized using syringe microfilters (Acrodisc, Pall, Ann Arbor, MI, USA). Pilot studies were performed to determine the optimal dose of BH_4, which was defined as a dose that would maximally affect VC function without altering baseline CVC. Doses exceeding 5mM did not further enhance the VC response to whole-body cooling. All other drug dosages were determined from previous studies in our laboratory. Throughout this baseline period, mean T_{sk} was held constant at 34°C by perfusing thermoneutral water though the suit.

After baseline measurements, cold water circulated through the suit to induce reflex VC. Mean T_{sk} decreased gradually from 34°C to 30.5°C over 30min and clamped an additional 10min at 30.5°C. Rewarming followed to bring T_{sk} back to 34°C. After which, a 10^{-6}M dose of NA was perfused at the Y/P site for 15min to test the efficacy of adrenoreceptor blockade. Then, all sites were perfused for 20min with 1mM tyramine to evoke endogenous NA release pharmacologically. NA (10^{-2}M) was then perfused at all sites for 15min to elicit further vasoconstriction. Full resolution of the robust VC responses to tyramine and NA prevented the randomization of these steps with whole-body cooling. Lastly, 28mM sodium nitroprusside (SNP) was perfused until a plateau in the vasodilatation response was achieved (~30min) to ensure that vascular responsivity was maintained at each MD site. Because baseline CVC was unchanged with addition of pharmacological agents, normalization to baseline ($\%\Delta CVC_{base}$) was used to standardize the data throughout the study.

Data Acquisition and Analysis

CVC data from the control, BH_4, and adrenoreceptor blockade sites were pooled across both protocols. Data were collected at 40Hz, digitized, recorded and stored in a personal computer until data analysis (Windaq, Dataq Instruments, Akron, OH). CVC data were averaged over 3 min intervals during baseline and at each 0.5°C drop in mean T_{sk} during the cooling period. A three-way repeated measures analysis of variance was

conducted to detect age and treatment differences over the decrease in mean T_{sk} (SAS, version 9.1.3, Cary, North Carolina, USA). Post hoc comparisons were performed with Bonferroni correction when appropriate to determine where significant differences occurred. The level of significance was set at α=0.05 for main effects and α=0.016 with Bonferroni correction. Data relating to subject characteristics and absolute baseline CVC values were assessed by paired student's t-test. Values are expressed as mean ± SE.

Results

Subject characteristics are presented in Table 1. Age groups were well matched with regard to height, weight, BMI, MAP, and cholesterol ratio (total cholesterol / HDL cholesterol).

The absolute baseline CVC, calculated as laser Doppler flux * $mmHg^{-1}$, for each MD fibre site is illustrated in Table 2. With the exception of BH_4 + Y/P site, there were no differences in baseline CVC between age groups or between experimental fibre sites and the control site (P<0.05). In the older subjects, the baseline CVC value at the BH_4 + Y/P site was significantly higher than control (P=0.04).

***Protocol 1*:** The effect of BH_4 on adrenergic and nonadrenergic VC function during whole-body cooling (T_{sk}=30.5^{o}C) is illustrated in Figure 1A. Compared to young subjects, older individuals exhibited a blunted VC response to cooling at control sites (Y: -34 ± 2, O: -17 ± 2% ΔCVC_{base}; P<0.0001). Local administration of BH_4 significantly enhanced VC in older subjects (-34 ± 2% ΔCVC_{base}; P<0.0001) but had no effect in young subjects (-34 ± 4 ΔCVC_{base}; P=0.93). Adding ascorbate to the BH_4 (Y: -33 ± 5, O: -34 ± 3% ΔCVC_{base}) did not increase the VC response more than BH_4 alone. Coadministration of BH_4 with Y/P abolished the VC response in older subjects (0 ± 4% ΔCVC_{base}); however, nearly half of the total VC response remained in young (Y: -16 ± 3 ΔCVC_{base}; P<0.0001 *versus* control and *versus* older).

Similar to whole-body cooling, older subjects exhibited an attenuated VC response to local tyramine perfusion at control sites (Y: -33 ± 4, O: -15 ± 3% ΔCVC_{base}; $P<0.0001$) (Figure 1B). Local BH_4 supplementation enhanced the VC response in older subjects (O: -35 ± 3% ΔCVC_{base}; $P<0.0001$) but had no significant effect in young subjects (Y: -38 ± 4% ΔCVC_{base}; $P=0.18$). VC responses at BH_4 sites were not different between age groups. Compared to BH_4, the BH_4 + AA site did not elicit greater VC responses in young or older subjects (Y: -44 ± 6; $P=0.26$, O: -34 ± 4 ΔCVC_{base}; $P=0.79$). In older subjects, the VC response was abolished in the adrenoreceptor blocked site (-3 ± 2% ΔCVC_{base}). In young, ~40% of the total VC response to cooling remained intact after adrenoreceptor blockade (Y: -14 ± 2 ΔCVC_{base}; $P<0.0001$ *versus* control and *versus* older).

In Figure 2, the CVC responses for every 0.5°C drop in T_{sk} during whole-body cooling is shown. Throughout cooling, the BH_4 treated site did not differ from the control site in the young subjects (Figure 2A); however, in older subjects (Figure 2B), BH_4 significantly increased VC at mean skin temperatures of 32.0°C and cooler ($P<0.017$). Compared to young, VC at control and BH_4+Y/P sites was significantly reduced in older subjects ($P<0.017$).

***Protocol 2*:** The effects of BH_4 on reflex vasoconstriction at NOS-intact and NOS-blocked sites are shown in Figure 3A. The cold-induced VC at the L-NAME site (Y: -36 ± 4; $P=0.45$, O: -15 ± 7% ΔCVC_{base}; $P=0.99$) did not differ from the VC response at the control site (from Figure 1A) in either young or older subjects. Combining L-NAME and BH_4 (Y: -33 ± 6; $P=0.83$, O: -23 ± 4% ΔCVC_{base}; $P=0.26$) did not alter the vasomotor response compared to perfusing BH_4 alone. The VC response at the BH_4 + L-NAME site was not significant between age groups ($P=0.09$); however, VC at the L-NAME site was different between age groups ($P<0.001$).

During tyramine perfusion (Figure 3B), there were no differences between BH_4 + L-NAME (Y: -38 ± 5, O: -40 ± 8% ΔCVC_{base}) and BH_4 alone in young or older subjects.

Compared to control (from Figure 1B), greater VC was observed in the L-NAME site in young but not older subjects (Y: -39 ± 2; P<0.017, O: -22 ± 8% ΔCVC_{base}; P=0.69). The VC response was significantly different between age groups at the L-NAME site (P<0.017).

During perfusion of a supraphysiological dose (10^{-2} M) of NA (Figure 4), there were no differences in NA-mediated VC between control (Y: -49 ± 3, O: -46 ± 5% ΔCVC_{base}) and BH_4 sites (Y: -54 ± 6, O: -54 ± 6% ΔCVC_{base}) in either age group. Adding ascorbate to BH_4 (Y: -69 ± 11% ΔCVC_{base}, O: -70 ± 3% ΔCVC_{base}; P<0.0001) increased the VC response to NA more than BH_4 alone, but this increase was significant only in older subjects. At the NOS-blocked sites, the VC response at the L-NAME site was greater than at the control site, however this was significant only in young subjects (Y: -67 ± 4, O: -61 ± 4% ΔCVC_{base}; P<0.001). No significant difference in CVC existed between young and older subjects at the L-NAME site. Combining L-NAME and BH_4 (Y: -72 ± 8; P<0.0001, O: -73 ± 6% ΔCVC_{base}; P=0.046) resulted in greater VC than perfusing BH_4 alone only in the young. No differences existed when comparing the BH_4 + L-NAME site or the L-NAME site between age groups.

Discussion

The primary finding from this study was that local BH_4 administration enhanced the VC response induced by either whole-body cooling or by tyramine infusion in aged but not young skin. In fact, BH_4 completely offset the age-associated decrement in cutaneous VC. Furthermore, BH_4 did not affect cotransmitter-mediated VC or VC in response to an exogenous supraphysiological dose of NA in aged skin, thereby suggesting that the effects of BH_4 were adrenergically-mediated and localized to peripheral nerve terminals, respectively. Lastly, the putative effects of BH_4 on NO bioavailability did not affect the physiological or pharmacological-induced VC response. In summary, these data suggest that reduced NA biosynthesis and release from sympathetic nerves likely contributes to attenuated cutaneous VC in older subjects.

Central to this impairment may be elevated oxidative stress which has been shown to deplete an essential cofactor required for NA biosynthesis, BH_4.

The data from the present study support the plausible mechanism by which the attenuated VC in aged skin may be related to oxidative stress and diminished NA release. In cultured sympathetic neurons, induction of oxidant stress decreased BH_4 concentration resulting in a ~75% reduction in catecholamine synthesis (Li *et al.*, 2003). Moreover, the elevation in catecholamines following depolarization of sympathetic nerves is much greater with optimal TH and BH_4 concentrations (Li et al., 2003). The putative oxidant-induced depletion of BH_4 with human aging results in suboptimal catecholamine release during whole-body cooling by 1) decreasing TH activity and 2) decreasing vesicular storage of NA. Furthermore, because NA release appears to be tonically higher in older individuals (Seals & Dinenno, 2004), there may be little reserve for increasing NA release during sympathetic stimulation. Cumulatively, these results may explain in part how attenuated NA biosynthesis and release contribute to attenuated VC and consequently, relatively greater skin blood flow and heat loss during cold exposure in older individuals.

BH_4 serves as an essential cofactor for pteridine-requiring monooxygenases including NOS and TH, thus BH_4 bioavailability is important to both cutaneous vasodilatation and VC (Kaufman, 1978; Moens & Kass, 2007). Oxidant stress affects BH_4 bioavailability by, 1) direct oxidation to inactive BH_2 and 2) decreased expression of GTP cyclohydrolase, the rate-limiting step in BH_4 de novo synthesis, and 3) downregulation of dihydrofolate reductase, involved in BH_4 regeneration from its oxidized form (Moens & Kass, 2007). Direct evidence of reduced BH_4 concentration in aged tissue has been demonstrated in other tissues (Williams *et al.*, 1980; Delp *et al.*, 2008). BH_4 is critical for cutaneous VC because of its central role in modulating TH activity and NA biosynthesis. BH_4 binding reduces the iron moiety of TH to its ferrous form ($TH\text{-}Fe^{2+}$) thereby priming TH for catalytic reaction (Kumer & Vrana, 1996; Dunkley et al., 2004; Thony et al., 2008). Thus, TH activity is highly dependent on BH_4

concentration in nerve terminals (Kaufman, 1978; Zigmond et al., 1989). In that regard, reduced BH_4 bioavailability in an aged population may result in suboptimal TH activity and reduced NA biosynthesis in aged skin.

Several impairments have been identified along the efferent arm of the sympathetic reflex in aged skin including, 1) a blunted increase in skin sympathetic nerve activity in response to cold exposure (Grassi et al., 2003), 2) depressed axonal release of NA for a given absolute cold stimulus (Frank et al., 2000), 3) diminished end-organ responsiveness to a given exogenous dose of NA (Thompson *et al.*, 2005b), 4) blunted postjunctional adrenergic responsiveness to increasing doses of tyramine in the human forearm (Dinenno *et al.*, 2002), and 5) abolished non-adrenergic (i.e. cotransmitter-mediated) VC (Thompson & Kenney, 2004). Cumulatively, these studies have demonstrated that VC in aged skin relies almost entirely on an albeit compromised adrenergic mechanism. The extent with which this is due to reduced NA release or diminished adrenoreceptor responsiveness remains in question. Further, it is unknown whether age-induced alterations in NA release precede reductions in end-organ responsiveness.

BH_4 enhances functional VC in aged skin presumably by augmenting NA biosynthesis and storage in axon nerve terminals; thereby allowing greater NA release during sympathetic adrenergic stimulation. This is supported by other studies where BH_4 supplementation 1) promoted in vivo TH activity (Nagatsu et al., 1994) and 2) augmented the kinetic stability of TH (Thony et al., 2008). Since NA biosynthesis mechanisms may be attenuated, this would suggest that NA storage in synaptic vesicles and release during sympathetic adrenergic stimulation are compromised in aged skin. Interestingly, the number of transporters for NA in synaptosomes decreases with age resulting in diminished ability to package NA for release (Snyder et al., 1998). In combination with the data from the present study, these results indicate that NA production and vesicular packaging mechanisms are compromised with advanced age in the skin, thereby

suggesting that diminished NA release may contribute significantly to the attenuated response to reflex- and pharmacologically-mediated VC in aged skin.

The apparent effects of BH_4 are isolated to TH in axon nerve terminals; however, additional effects on end-organ responsiveness cannot be ruled out. Aged skin exhibits blunted VC responsiveness as well as a diminished capacity to respond to exogenous NA (Thompson *et al.*, 2005a, b). We found that the magnitude of the VC response to an exogenous supraphysiological dose of NA is not different between control and BH_4 pretreated sites, thereby suggesting that BH_4 does not significantly influence end-organ responsivity. Further investigation is required to ascertain whether this is consistent with more physiological doses of NA.

We also investigated the role of BH_4 in cotransmitter-mediated VC. Putative neurotransmitters coreleased with NA during sympathetic stimulation include NPY and ATP (Ekblad et al., 1984; Racchi et al., 1999; Stephens et al., 2004; Burnstock, 2007). In young individuals, the total cotransmitter contribution to reflex VC is ~40%. However, cotransmitter-mediated VC is functionally absent in aged skin, thus the cutaneous VC response is entirely dependent on adrenergic mechanisms (Thompson & Kenney, 2004). In the present study, local supplementation of BH_4 in aged skin did not improve cotransmitter-mediated VC but alternatively increased the magnitude of the adrenergically-mediated VC response. We speculate that adrenergic VC may be compensating for absent cotransmitter function. Thus, the reason BH_4 completely normalized the VC response in older subjects may be due to augmented adrenergic function, thereby permitting a greater compensatory VC response. However, this point requires further investigation. Nevertheless, this reinforces the assertion that BH_4 is acting presynaptically on NA biosynthesis and release mechanisms to augment the VC response in aged skin.

In human skin, NO is capable of inhibiting sympathetic adrenergic VC; however, it is unclear whether NO acts pre- or post-synaptically in this regard (Durand *et al.*, 2005;

Shibasaki *et al.*, 2007; Shibasaki *et al.*, 2008). In isolated rat mesenteric arteries, incubation of NO donors reduced NA bioavailability yet did not affect NPY or ATP concentration (Kolo *et al.*, 2004). BH_4 increases NO-dependent vasodilatation through its putative effect of recoupling NOS and subsequent increase in NO bioavailability (Bevers et al., 2006; Moens & Kass, 2007; Cosentino et al., 2008) and thus, BH_4 could have the contrasting effect of augmenting the NO-mediated attenuation of sympathetic VC. To treat this mechanism, L-NAME and BH_4+L-NAME were administered in a small subset of subjects. The VC response at the L-NAME and BH_4+L-NAME sites were not different than the control and BH_4 sites, respectively. Interestingly, L-NAME treated sites increased the VC response to an exogenous supraphysiological dose of NA. Collectively, our data suggest that NO may prevent the full expression of cutaneous VC; however, the putative BH_4-mediated increase in NO did not appear to interfere with cold and tyramine-induced VC.

In summary, the present study suggests that reduced BH_4 bioavailability contributes to attenuated VC in aged skin. The global increase in ROS with aging may result in greater oxidation of BH_4 resulting in suboptimal tyrosine hydroxylase (TH) activity that is requisite for full expression of the VC response. Local supplementation of BH_4 restored the adrenergic VC response to both cooling and local tyramine perfusion. However, cotransmitter-mediated VC remained functionally absent in aged skin replete with BH_4. Finally, the VC to a supraphysiological dose of NA was not affected by BH_4 suggesting that receptor sensitivity was not affected at BH_4 pretreated sites.

Table **3.1**

Table **3.1**: Subject characteristics. Values are means ± SE for young (n = 11) and older (n = 11) men and women. Data are pooled from both protocols 1 and 2. BMI, Body Mass Index; MAP, Mean Arterial Pressure; HDL, High-Density Lipoprotein; LDL, Low-Density Lipoprotein. **P*<0.05

Variable	**Young**	**Older**
Sex (M,F)	5,6	5,6
Age (yrs)	21 ± 1	72 ± 2*
Height (cm)	170 ± 4	170 ± 3
Weight (kg)	68 ± 4	71 ± 3
BMI (kg/m^2)	23 ± 1	24 ± 1
Resting MAP	84.9 ± 2.3	87.8 ± 1.4
Total Cholesterol (mg/dL)	161	204*
HDL (mg/dL)	55	65*
LDL (mg/dL)	90	121*
Cholesterol Ratio (total/HDL)	3.0	3.2

Table **3.2**

Table **3.2**: Baseline absolute cutaneous vascular conductance values: Baseline CVC values, calculated as laser Doppler flux * $mmHg^{-1}$, are means ± SE for each MD fibre site in young (Y) and older (O) subjects. AA, ascorbate; Y/P, yohimbine + propranolol. *P<0.05 versus control.

		Control	BH_4	BH_4 + AA	BH_4 + Y/P	BH_4 + L-NAME	L-NAME
Baseline	Y	0.22 ± 0.03	0.26 ± 0.05	0.34 ± 0.07	0.22 ± 0.02	0.17 ± 0.03	0.13 ± 0.02
	O	0.19 ± 0.03	0.24 ± 0.03	0.24 ± 0.04	0.37 ± 0.08*	0.25 ± 0.10	0.13 ± 0.02

Figure **3.1**

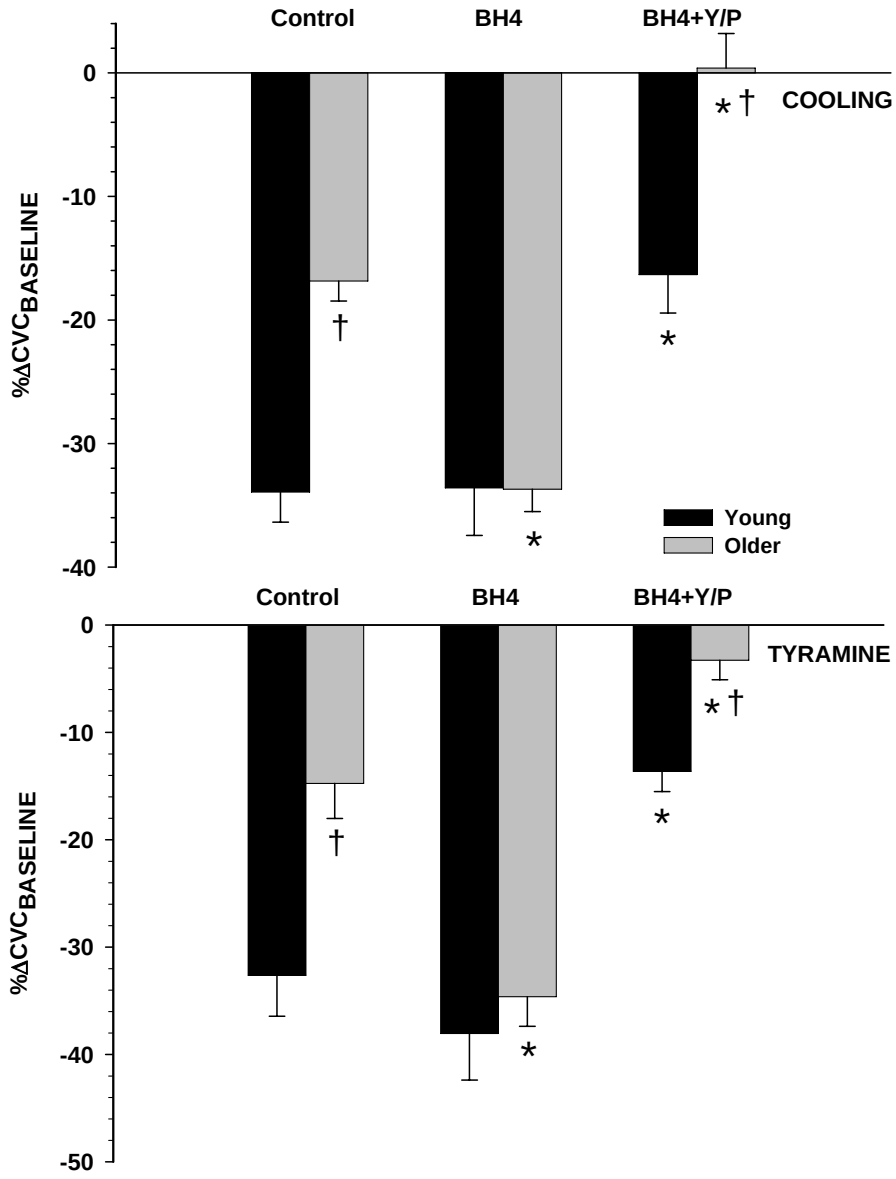

Figure **3.1**: Average change in maximal cutaneous vasoconstriction in response to whole-body cooling (T_{sk} = 30.5°C) and following 20 min perfusion of 1 mM tyramine at each microdialysis site in young and older subjects (protocol 1). The effects of whole-body cooling, A, and tyramine, B, at control, BH_4, and BH_4 + Y/P (yohimbine + propranolol) pretreated sites, n = 24 (12y, 12o) subjects. **P*<0.01 versus control; †*P*<0.01 versus young.

Figure **3.2**

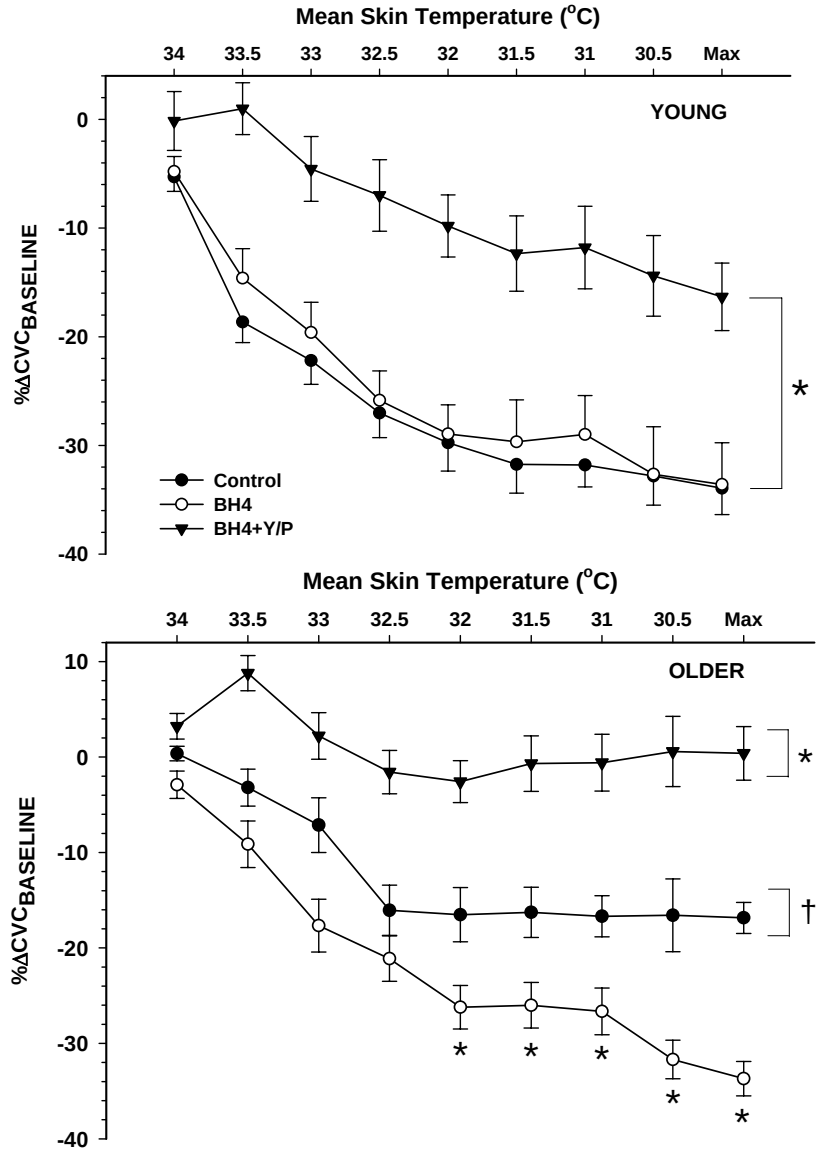

Figure **3.2**: CVC responses to 0.5°C incremental decreases in skin temperature during whole-body cooling in young and older subjects. A, young subjects (n = 12); B, older subjects (n = 12). Y/P, yohimbine + propranolol. **P*<0.0166 versus control; †*P*<0.0166 versus young.

Figure **3.3**

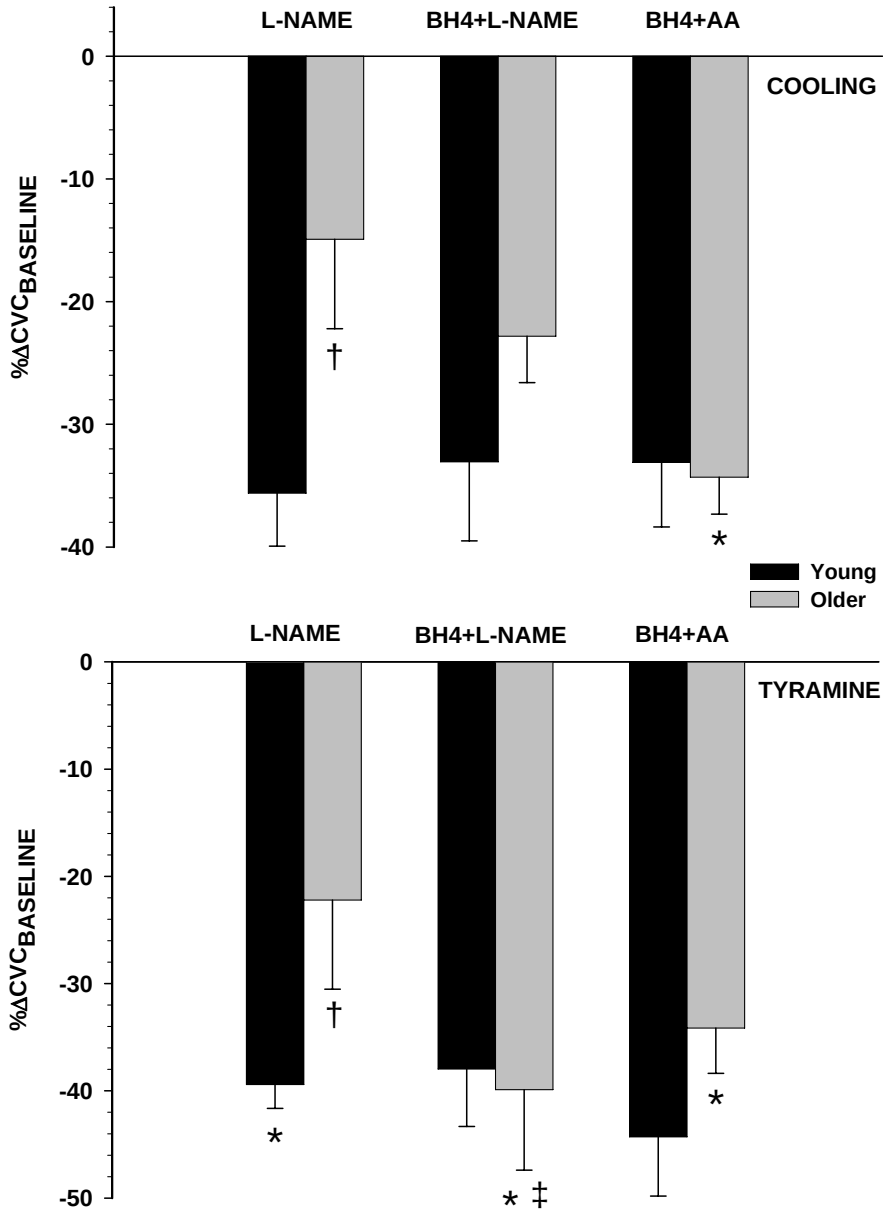

Figure **3.3**: Average change in maximal cutaneous vasoconstriction in response to whole-body cooling (T_{sk} = 30.5^{o}C) and following 20 min perfusion of 1 mM tyramine at each microdialysis site in young and older subjects (protocol 2). The effects of whole-body cooling, A, and tyramine, B, at L-NAME and BH_4 + L-NAME, n = 10 (5y, 5o), and BH_4 + AA (ascorbate), n = 12 (6y, 6o), pretreated sites. *P<0.0166 versus control; †P<0.0166 versus young; ‡P<0.0166 versus L-NAME

Figure **3.4**

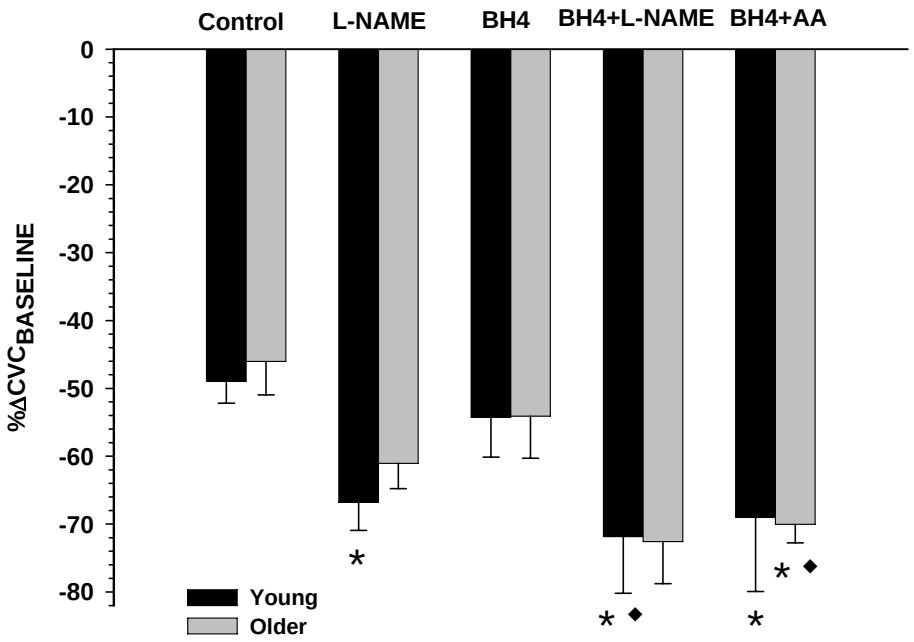

Figure **3.4**: Maximal vasoconstriction induced following a supraphysiological dose (10^{-2} M) of noradrenaline at each microdialysis site in young and older subjects. For control and BH_4, n = 24 (12y, 12o) subjects. For L-NAME and BH_4 + L-NAME, n = 10 (5y, 5o) and BH_4 + AA (ascorbate), n = 12 (6y, 6o). **P*<0.0166 versus control; ◆*P*<0.0166 versus BH_4.

Chapter 4

LOCALIZED TYROSINE OR TETRAHYDROBIOPTERIN SUPPLEMENTATION AUGMENTS VASOCONSTRICTION IN AGED HUMAN SKIN

Introduction

Reflex cutaneous vasoconstriction (VC) is an early and sustained response to whole-body cold exposure. This thermoregulatory response is attenuated in older adults rendering them more susceptible to greater convective heat loss to the environment (Wagner *et al.*, 1974; Kenney & Armstrong, 1996; Degroot & Kenney, 2007) and possibly hypothermia (CDC, 2002). Even when matched for body composition and fitness, older adults display reduced peripheral VC and a relative inability to maintain core temperature during even mild (22^{o}C) cold exposure (Degroot & Kenney, 2007).

Several studies have detailed functional deficits at various locations along the efferent arm of the sympathetic VC reflex in aged skin (Frank *et al.*, 2000; Thompson & Kenney, 2004; Thompson *et al.*, 2005b; Lang *et al.*, 2009a; Lang *et al.*, 2009b). Collectively, these data indicate that healthy aged skin relies entirely on functionally impaired adrenergic mechanisms to elicit VC. Central to this impairment may be elevated oxidative and nitrosative stress that affect key regulatory molecules in the biosynthetic pathway of noradrenaline (NA), putatively tyrosine and tetrahydrobiopterin (BH_4) (Lang *et al.*, 2009a). BH_4 is an essential cofactor for both nitric oxide synthase (NOS) and tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine biosynthesis (Kaufman, 1978; Kumer & Vrana, 1996; Moens & Kass, 2007). BH_4 serves as a powerful reducing agent that maintains TH in the ferrous, active form required for catecholamine production (Kaufman, 1978; Dunkley *et al.*, 2004; Urano *et al.*, 2006). Oxidative stress in cultured sympathetic neurons markedly reduces intracellular BH_4

concentration (~90%) resulting in a ~75% reduction in catecholamine synthesis (Li *et al.*, 2003). *In vivo*, localized intradermal administration of BH_4 offset the age-related attenuation in cold and tyramine-induced cutaneous VC; a result not related to effects of BH_4 on NOS (Lang *et al.*, 2009a). These studies suggest that suboptimal TH function contributes to the attenuated reflex VC response in aged skin.

Neuronal activation increases the affinity of TH for its cofactor BH_4, thereby making enzyme activity dependent on its saturation with its amino acid substrate L-tyrosine (Weiner, 1978; Fluharty *et al.*, 1985; Kumer & Vrana, 1996). Elevated oxidative and nitrosative stress converts tyrosine to the tyrosyl radical, which is unable to function in catecholamine biosynthesis and can further reduce free tyrosine pools as well as nitrate other proteins thereby compromising their function (Ischiropoulos *et al.*, 1995; Reiter *et al.*, 2000; Kochman *et al.*, 2002). Functionally, tyrosine bioavailability can affect catecholamine production in active nerve terminals (Wurtman *et al.*, 1974; Iuvone *et al.*, 1978; Fernstrom, 1983; Fernstrom *et al.*, 1986). *In vivo* human studies indicate that tyrosine supplementation may enhance cognitive and psychomotor performance during cold stress (Banderet & Lieberman, 1989; O'Brien *et al.*, 2007). However, few, if any, *in vivo* studies have addressed the functional role of tyrosine on thermoregulatory reflex VC in aged human skin.

The purpose of this study was to examine the relative roles of tyrosine and BH_4 during adrenergic cutaneous VC. We hypothesized that localized supplementation of tyrosine or BH_4 in aged skin would augment reflex and pharmacologically-induced VC elicited by whole-body cooling (physiological) and tyramine infusion (pharmacological stimulus), respectively. We further hypothesized that these compounds would not affect NA-mediated VC.

Methods

Subjects

With Pennsylvania State University Institutional Review Board approval and after verbal and written informed consent, ten young (23 ± 1 yrs; 5 men, 5 women) and ten older (73 ± 2 yrs; 5 men, 5 women) subjects participated in the study. Young women were tested in the early follicular phase (days 1-7) of the menstrual cycle, and older women were post-menopausal and not taking hormone replacement therapy. All subjects were healthy, non-obese, normotensive, normal cholesterolaemic, non-smokers, and not taking any medications or vitamin supplements that would otherwise alter cardiovascular or thermoregulatory function. All procedures conformed to the standards set by the Declaration of Helsinki.

Instrumentation

On the morning of an experiment, between 07:00-10:00, subjects arrived at the laboratory and were instrumented with four microdialysis (MD) fibres (10 mm, 20 kDa cutoff membrane, MD 2000 Bioanalytical Systems, West Lafayette, IN, USA) placed intradermally in the left ventral forearm using aseptic technique. MD sites were at least 4.0 cm apart to prevent cross-reactivity of pharmacological agents between sites. Throughout the protocol, subjects were in a semisupine position with the experimental forearm at heart level. Prior to fibre placement, ice packs were applied to MD sites for 5 min to temporarily anaesthetize the skin (Hodges *et al.*, 2009). For each fibre, a 25-gauge needle was inserted horizontally into the dermis such that entry and exit points were ~2.5 cm apart. After MD fibres were threaded through the needle, the needle was withdrawn leaving the membrane in place. All fibres were taped in place and lactated Ringer's solution was initially perfused to test the integrity of the fibre and during the resolution period following needle insertion trauma. After which, MD sites were

randomly assigned with respect to position on the forearm and were perfused with, 1) lactated Ringer's solution serving as control, 2) 0.5 mM L-tyrosine, 3) 5 mM tetrahydrobiopterin (BH_4), and 4) L-tyrosine + BH_4. Pilot studies were performed to determine the optimal concentration of tyrosine, which was defined as a dose that would maximally affect VC function without altering baseline CVC. Concentrations exceeding 0.5 mM did not further enhance the VC response. All other drug dosages were determined from previous studies in our laboratory (Lang *et al.*, 2009a).

To control skin temperature, subjects wore a water-perfused suit that covered the entire body except for the face, feet, hands, and forearms. Copper-constantan thermocouples were placed on the surface of the skin at 6 sites; calf, thigh, abdomen, chest, back, and upper arm. The unweighted mean of these sites provided an index of mean skin temperature (T_{sk}).

To obtain an index of skin blood flow, red blood cell flux was continuously measured with a laser Doppler flowmetry (LDF) probes (MoorLAB, Temperature Monitor SH02, Moor Instruments, Devon, UK). LDF probes were placed in the center of local heaters and positioned directly over each MD fibre site. To specifically isolate reflex mechanisms, local skin temperature was clamped at 34^{o}C throughout the experiment. Arterial blood pressure was measured every 5 min throughout the experiment via brachial auscultation. Mean arterial pressure (MAP) was calculated as the diastolic blood pressure plus one-third the pulse pressure. Cutaneous vascular conductance (CVC) was calculated as the ratio of LDF flux to MAP and expressed as per cent change from baseline values ($\%\Delta CVC_{baseline}$).

Protocol

After instrumentation with MD fibres, local hyperemia was allowed to resolve for 60-90 min while prefusing sites with their assigned pharamacological agent. All drugs were mixed just prior to use, dissolved in lactated Ringer's solution, sterilized using

syringe microfilters (Acrodisc, Pall, Ann Arbor, MI, USA), and perfused at 2 μL/min (Bee Hive controller and Baby Bee microinfusion pumps, Bioanalytical Systems). Throughout the baseline period, mean T_{sk} was held constant at 34°C by perfusing thermoneutral water through the suit.

After baseline measurements, cold water was circulated through the suit to induce reflex VC. Mean T_{sk} decreased gradually from 34°C to 30.5°C over 30 min and was then clamped for an additional 10 min at 30.5°C. Rewarming for approximately 30 min followed to return T_{sk} back to 34°C. After which, all sites were perfused for 20 min with 1 mM tyramine to evoke endogenous NA release pharmacologically. Exogenous NA (1 x 10^{-2}M) was then perfused at all sites for 10 min to elicit further vasoconstriction. The CVC established after rewarming was utilized as a baseline to assess tyramine and NA-mediated VC. Full resolution of the robust VC responses to tyramine and NA prevented the randomization of these steps with whole-body cooling. Lastly, 28 mM sodium nitroprusside (SNP) was perfused through all sites at a rate of 4 μL/min in combination with local heating of the skin to 43°C until a plateau in the vasodilatation response was achieved (~30 min) at each MD site to ensure that vascular responsivity remained intact post-cooling.

Data Acquisition and Analysis

Data were collected at 40 Hz, digitized, recorded and stored in a personal computer until data analysis (Windaq, Dataq Instruments, Akron, OH). CVC data were averaged over 3 min intervals during baseline and at each 0.5°C drop in mean T_{sk} during the cooling period. A three-way mixed model repeated measures analysis of variance was conducted to detect age and treatment differences during whole-body cooling, tyramine, and NA administration (SAS, version 9.1.3, Cary, NC, USA). Tukey post hoc tests were performed when appropriate to determine where age and drug treatment differences occurred. Data relating to subject characteristics and absolute baseline CVC

values were assessed by Student's paired t tests. Statistical significance for all analyses were set at α=0.05. Values are expressed as mean ± S.E.M.

Results

Subject characteristics are presented in Table 1. Age groups were well matched with regard to height, weight, BMI, MAP, and cholesterol ratio (total cholesterol / HDL cholesterol).

The absolute baseline CVC, calculated as laser Doppler flux * $mmHg^{-1}$, for each MD fibre site is illustrated in Table 2. There were no significant differences in baseline CVC between age groups or between drug treated fibre sites and the control site (P<0.05).

The effect of tyrosine and BH_4 on adrenergic VC function during whole-body cooling (T_{sk}=30.5°C) is illustrated in Figure 1A. Compared to young subjects, older subjects exhibited a blunted VC response to cooling at control sites (Y: -39 ± 3, O: -17 ± 3 $\%\Delta CVC_{base}$; P<0.01). Localized administration of BH_4 significantly augmented VC in older subjects (-36 ± 3 $\%\Delta CVC_{base}$; P<0.01) but had no effect in young subjects (-37 ± 3 $\%\Delta CVC_{base}$; P=0.82). Localized tyrosine also augmented VC in older (-35 ± 3 $\%\Delta CVC_{base}$; P<0.01) but not in young subjects (-38 ± 4 $\%\Delta CVC_{base}$; P=0.87). Similar to the other experimental sites, coadministration of BH_4 with tyrosine enhanced VC in older (-34 ± 3 $\%\Delta CVC_{base}$; P<0.01) but not young subjects (-39 ± 4 $\%\Delta CVC_{base}$; P=0.96). However, the BH_4 + tyrosine site did not further augment VC more than either BH_4 (Y: P=0.78, O: P=0.69) or tyrosine (Y: P=0.83, O: P=0.86) alone.

Similar to whole-body cooling, older subjects exhibited an attenuated VC response to local tyramine infusion at control sites (Y: -41 ± 4, O: -21 ± 4 $\%\Delta CVC_{base}$; P<0.01) (Figure 1B). Localized BH_4 supplementation enhanced VC in older subjects (-35 ± 3 $\%\Delta CVC_{base}$; P<0.02) but not in young subjects (-41 ± 2 $\%\Delta CVC_{base}$; P=0.94).

Localized tyrosine also augmented VC in older (-44 ± 4 $\%\Delta CVC_{base}$; $P<0.01$) but not young subjects (-40 ± 4% ΔCVC_{base}; $P=0.95$). The increase in VC with tyrosine was not greater than the increase in VC observed with BH_4 in older subjects ($P=0.13$). Compared to the control site, coinfusion of BH_4 with tyrosine enhanced VC in older (-37 ± 6 $\%\Delta CVC_{base}$; $P<0.01$) but not young subjects (-37 ± 3 $\%\Delta CVC_{base}$; $P=0.57$). However, the BH_4 + tyrosine site did not further augment VC more than BH_4 (Y: $P=0.52$, O: $P=0.75$) or tyrosine (Y: $P=0.62$, O: $P=0.23$) alone.

In Figure 2, the CVC response at every 0.5°C decrease in mean T_{sk} during whole-body cooling is illustrated. Throughout cooling, the drug treated sites did not differ from the control site in young subjects (Figure 2A). Compared to young subjects, older subjects exhibited a blunted VC response in the control site (mean $T_{sk} < 32.5$°C; $P<0.05$). In older subjects (Figure 2B), the VC response was augmented at the drug treated sites, which was significant at mean $T_{sk} \leq 32.0$°C (BH_4, BH_4 + tyrosine) and mean $T_{sk} \leq 33.0$°C (tyrosine; $P<0.05$). No differences were observed between the drug-treated sites in older subjects.

During infusion of a supraphysiological concentrations (1×10^{-2} M) of NA (Figure 3), there were no differences in NA-mediated VC between control (Y: -70 ± 5, O: -67 ± 4 $\%\Delta CVC_{base}$; $P=0.52$), BH_4 (Y: -74 ± 3, O: -66 ± 5 $\%\Delta CVC_{base}$; $P=0.14$), tyrosine (Y: -64 ± 5, O: -73 ± 4 $\%\Delta CVC_{base}$; $P=0.12$), and BH_4 + tyrosine (Y: -71 ± 6, O: -74 ± 4 $\%\Delta CVC_{base}$; $P=0.57$) sites.

Discussion

The primary finding from this study was that both local tyrosine and BH_4 administration augmented the VC response induced by either whole-body cooling or by tyramine infusion in aged, but not in young, skin. Similar to BH_4, localized tyrosine supplementation offset the age-associated decrement in cutaneous VC. Furthermore, perfusing tyrosine and BH_4 concomitantly did not have an additive effect on the

cutaneous VC response in older subjects. Lastly, neither tyrosine nor BH_4 affected the VC response to an exogenous supraphysiological dose of NA, which suggests that the putative effects of these compounds were localized to peripheral nerve terminals. In summary, these data suggest that the synthesis of NA in response to a physiological or pharmacological stimulus is compromised in older subjects and this may be occurring at the tyrosine hydroxylase (TH), or rate-limiting, step in the biosynthetic pathway. Consequently, blunted NA synthesis and axonal release from sympathetic nerves likely contributes to the attenuated cutaneous VC observed in older subjects.

These data corroborate previous findings in our laboratory indicating that BH_4 augments reflex- and tyramine-induced VC in aged skin even after controlling for the putative effects of BH_4 on nitric oxide synthase (Lang *et al.*, 2009a). Additionally, BH_4 did not affect cotransmitter- or NA (1×10^{-2} M)-mediated VC suggesting that its effects were adrenergically mediated and localized to peripheral nerve terminals. Using a similar experimental design, the current study demonstrates that tyrosine also selectively augments cutaneous VC in aged skin. Reduced NA biosynthesis due to diminished tyrosine and BH_4 bioavailability likely contribute to the attenuated VC response in aged skin.

Catecholamine synthesis and storage requires functionally active TH. To this end, both BH_4 and tyrosine must be present in sufficient concentrations to maintain optimal TH function (Kumer & Vrana, 1996; Dunkley *et al.*, 2004). BH_4 is a critical cofactor in this process because it catalytically activates the enzyme and enables hydroxylation of the amino acid substrate tyrosine (Kumer & Vrana, 1996; Dunkley *et al.*, 2004; Thony *et al.*, 2008). In response to cold exposure, the affinity of TH for BH_4 is considerably enhanced, thereby augmenting NA biosynthesis (Weiner, 1978; Fluharty *et al.*, 1985; Kumer & Vrana, 1996). However, in aged sympathetic ganglia, markedly reduced NA fluorescence has been reported (Hervonen *et al.*, 1978; Santer, 1979). Furthermore, the number of transporters for NA in synaptomsomes declines with age (Snyder *et al.*, 1998). Thus, the present data support the hypothesis that the apparent age-

associated deficits in NA synthesis and storage may be secondary to reduced precursor substrates and cofactors.

It is plausible that reduced BH_4 and tyrosine bioavailability may be secondary to the globalized elevation in oxidative and nitrosative stress associated with aging (Finkel & Holbrook, 2000). Although BH_4 has not been directly measured in human skin, its concentration is diminished in other aged tissues and this has been linked to oxidative stress at various points along the BH_4 biosynthetic and salvage pathways (Williams *et al.*, 1980; Moens & Kass, 2007; Delp *et al.*, 2008). Tyrosine bioavailability may be decreased by its oxidation to tyrosyl radical. Peroxynitrite or its highly reactive degradation products may convert free tyrosine to an inactive NA substrate (Ischiropoulos *et al.*, 1995; Reiter *et al.*, 2000; Kochman *et al.*, 2002). For example, in human skin 3-nitrotyrosine is elevated in photoaged skin and can increase acutely in response to oxidative stress generated from ultraviolet light exposure (Nishigori *et al.*, 2003). Thus, elevated peroxynitrite in aged skin may reduce the tyrosine pool available for catecholamine production.

In addition to oxidative stress, tyrosine bioavailability may be diminished due to increased tonic NA release in aged skin (Seals & Dinenno, 2004). At rest, tyrosine is not thought to be limiting because its concentration is well above the substrate K_m of TH (Fernstrom, 1983). Moreover, plasma tyrosine concentrations appear to be unaltered with age (Caballero *et al.*, 1991). However, in animal studies tyrosine supplementation augments the concentration of the NA precursor, L-DOPA, in activated neurons (Wurtman *et al.*, 1974; Iuvone *et al.*, 1978; Fernstrom, 1983; Fernstrom *et al.*, 1986). This suggests that upon neuronal activation tyrosine concentration in the vicinity of TH may be well below saturation, and in the case of aged skin, this may be compounded by elevated oxidative stress. The cumulative effects of oxidative stress and elevated tonic NA synthesis may deplete tyrosine in aged skin, thereby limiting NA biosynthesis and consequently the ability to sustain a significant VC response during cold exposure.

In addition to prejunctional noradrenergic mechanisms, attenuated cutaneous VC function in aged skin may be explained by multiple deficits along the efferent arm of the sympathetic reflex including, 1) absent functional contribution of coreleased sympathetic neurotransmitters, putatively ATP and NPY (Thompson & Kenney, 2004), 2) reduced adrenoreceptor responsivity (Thompson *et al.*, 2005b), and 3) altered downstream vascular signaling mechanisms (Lang *et al.*, 2009b). Furthermore, although the apparent effects of BH_4 and tyrosine are likely isolated to TH in sympathetic nerve terminals, additional effects on end-organ responsiveness cannot be ruled out. In the present study, VC induced by a supraphysiological dose of NA was not different between control and drug treatment sites suggesting that neither BH_4 nor tyrosine significantly influenced end-organ responsivity; however, further investigation is required to assess whether this is consistent with more physiological concentrations of NA.

We also investigated the combined role of BH_4 and tyrosine on VC function. Contrary to our hypothesis, BH_4 did not additionally enhance VC function when administered with tyrosine. Thus, administering either BH_4 or tyrosine appears sufficient to augment VC function in older adults during sympathetic activation. This presumably occurs through two different mechanisms acting on TH, 1) greater cofactor increases the amount of catalytically active enzyme and 2) greater substrate increases the saturation of enzyme that is already active. Alternatively, it is possible that cooling of greater duration or intensity is required to observe additive effects of BH_4 and tyrosine. Moreover, a basement effect may have occurred where the signal gain is minimized to a point where no detectable changes in the combination site could be observed. However, further reduction of the LDF signal occurred with exogenous NA infusion.

In summary, local supplementation of either tyrosine or BH_4 resolved the compromised adrenergic VC response to both cooling and local tyramine infusion in aged skin. However, combined tyrosine and BH_4 infusion did not further enhance cutaneous VC. Additionally, VC to a supraphysiological dose of NA was not affected by either tyrosine or BH_4 indicating that adrenoreceptor sensitivity was unaltered by these

compounds. These results suggest that optimal TH function is required to fully express the cutaneous VC response, and that reduced functional substrate and cofactor for TH contributes to the attenuated VC in aged skin.

Table **4.1**

Table **4.1**: Subject characteristics. Values are means ± S.E.M. for young (n = 10) and older (n = 10) men and women. BMI, Body Mass Index; MAP, Mean Arterial Pressure; HDL, High-Density Lipoprotein; LDL, Low-Density Lipoprotein. **P*<0.05.

Variable	**Young**	**Older**
Sex (M,F)	5,5	5,5
Age (yrs)	23 ± 1	73 ± 2*
Height (cm)	174 ± 3	166 ± 2
Weight (kg)	72 ± 5	68 ± 3
BMI (kg/m^2)	24 ± 1	25 ± 1
Resting MAP (mmHg)	85.4 ± 2.4	88.9 ± 1.5
Glucose (mg/dL)	88 ± 2	90 ± 2
Total Cholesterol (mg/dL)	153 ± 6	190 ± 7*
HDL (mg/dL)	53 ± 3	68 ± 4*
LDL (mg/dL)	83 ± 7	106 ± 6*
Cholesterol Ratio (total/HDL)	3.0 ± 0.2	2.9 ± 0.2

Table **4.2**

Table **4.2**: Baseline absolute cutaneous vascular conductance: Baseline CVC values, calculated as laser Doppler flux * $mmHg^{-1}$, are means ± S.E.M. for each MD fiber site in young (Y) and older (O) subjects.

		Control	BH_4	Tyrosine	BH_4 + Tyrosine
Baseline	Y	0.23 ± 0.03	0.30 ± 0.06	0.19 ± 0.03	0.36 ± 0.06
	O	0.22 ± 0.03	0.28 ± 0.04	0.25 ± 0.02	0.36 ± 0.07

Figure **4.1**

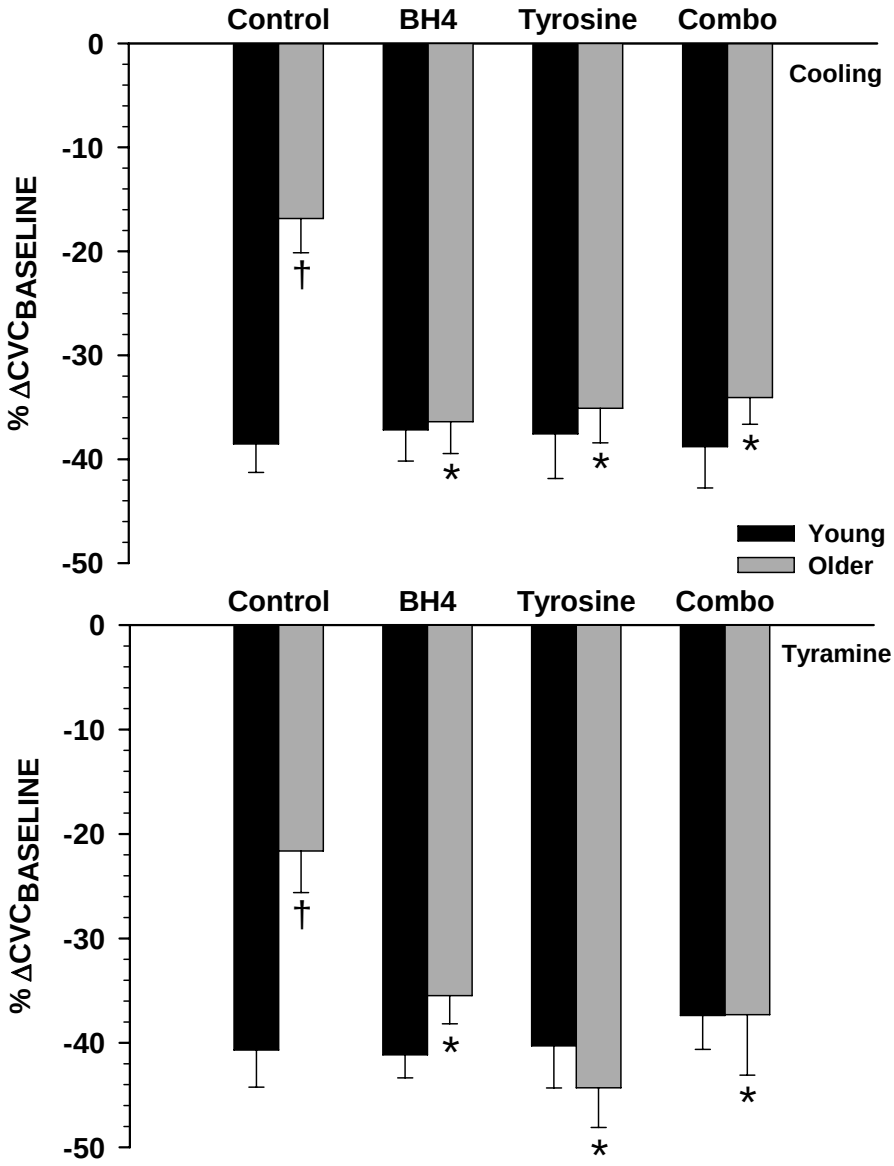

Figure **4.1**: Average change in maximal cutaneous vasoconstriction in response to whole-body cooling (T_{sk} = 30.5°C) and following 20 min infusion of 1 mM tyramine at each microdialysis site in young and older subjects. The effects of whole-body cooling, A, and tyramine, B, at control, BH_4, tyrosine, and BH_4 + tyrosine (combo) pretreated sites, n = 20 (10 young, 10 older) subjects. **P*<0.05 versus control; †*P*<0.05 versus young.

Figure **4.2**

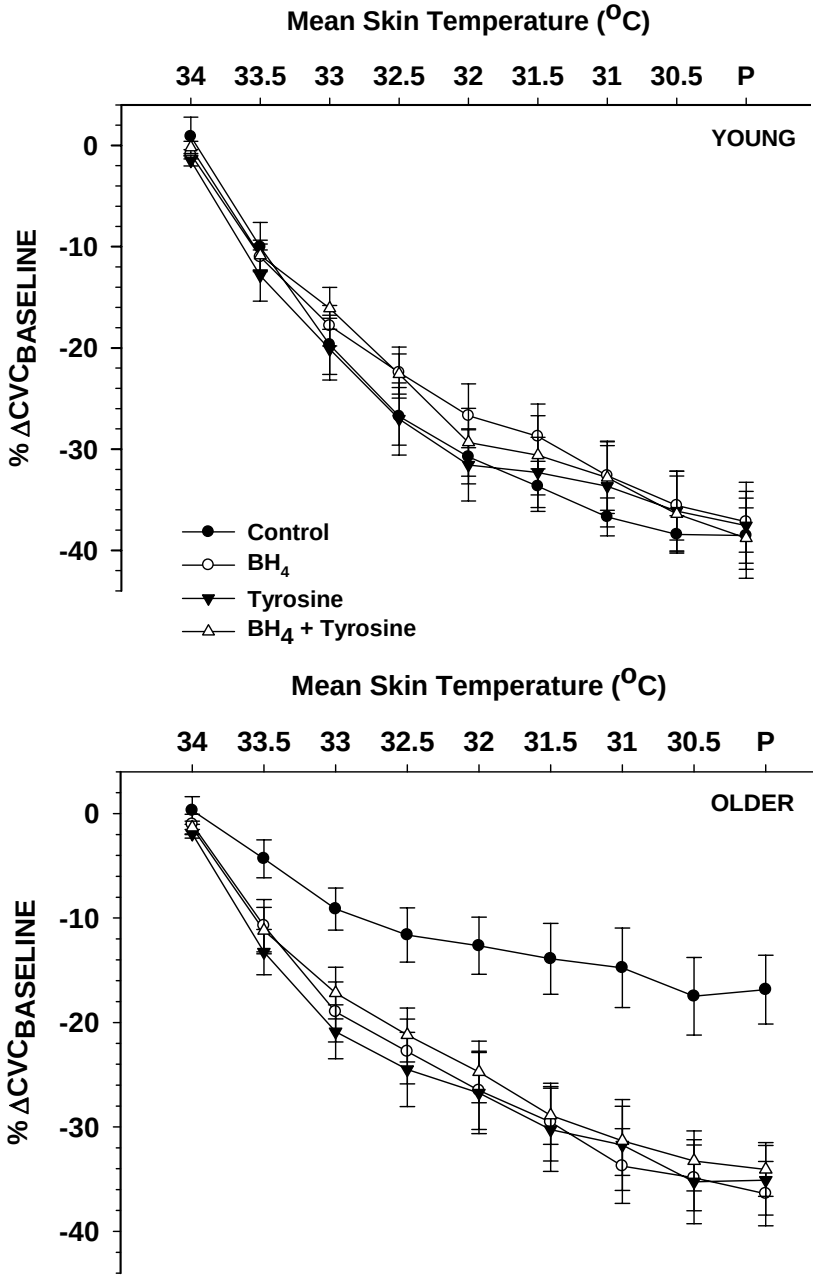

Figure **4.2**: CVC responses to 0.5°C incremental decreases in skin temperature during whole-body cooling in young and older subjects. A, young subjects (n = 10); B, older subjects (n = 10). **P*<0.05 versus control; †*P*<0.05 versus young.

Figure **4.3**

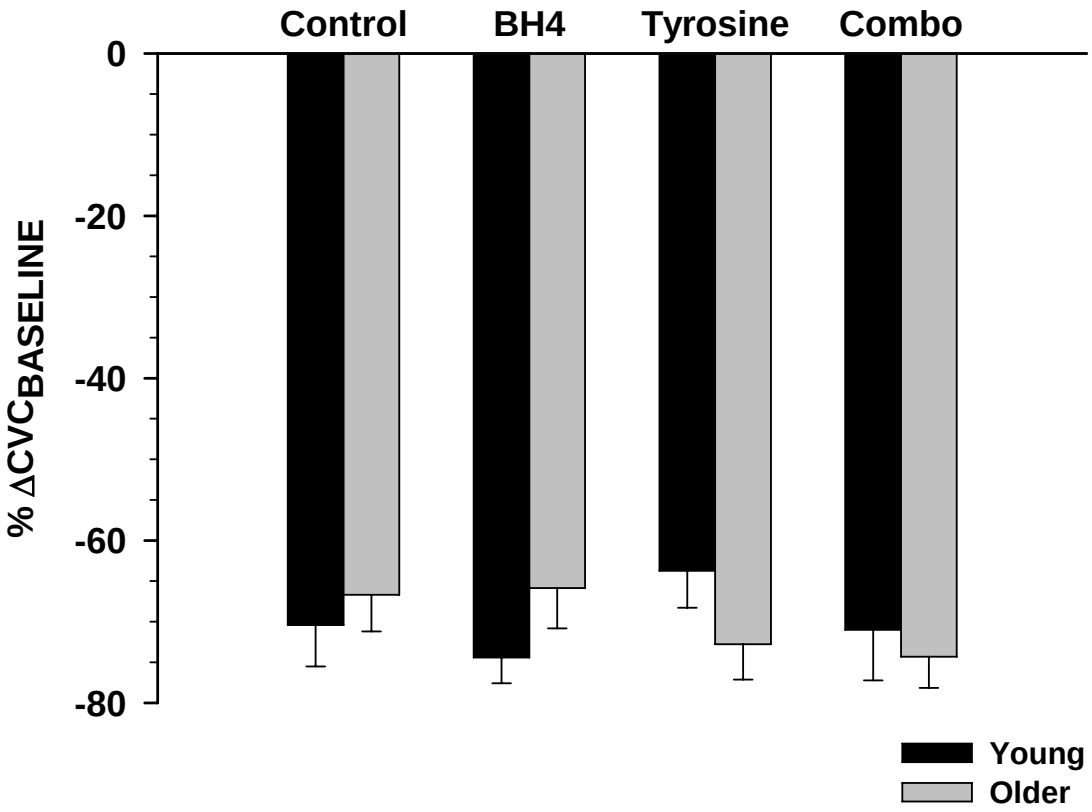

Figure **4.3**: Maximal vasoconstriction induced following a supraphysiological dose (1 x 10^{-2} M) of noradrenaline at each microdialysis site in young and older subjects. The effects of noradrenaline at control, BH_4, tyrosine, and BH_4 + tyrosine (combo) pretreated sites, n = 20 (10 young, 10 older) subjects. **P*<0.05 versus control; †*P*<0.05 versus young.

Chapter 5

REFLEX VASOCONSTRICTION IN AGED HUMAN SKIN INCREASINGLY RELIES ON RHO-KINASE DEPENDENT MECHANISMS DURING WHOLE-BODY COOLING

Introduction

Peripheral cutaneous vessels constrict in response to cold exposure through distinct reflex and locally regulated mechanisms. Localized cooling of skin induces vasoconstriction (VC) that is partly mediated by both stimulation of α_2-adrenoreceptors by norepinephrine (NE) (Ekenvall *et al.*, 1988; Freedman *et al.*, 1992) and upregulation of the downstream intracellular messenger, Rho-kinase (ROCK) (Thompson-Torgerson *et al.*, 2007a, b), whereas reflex sympathetically mediated VC relies on norepinephrine (NE) (~60% of the VC response) and other coreleased sympathetic neurotransmitters (~40%) (Stephens *et al.*, 2001; Thompson & Kenney, 2004).

In aged skin (>60 yrs), the reflex VC response is not only blunted but relies entirely on an already compromised adrenergic mechanism (Kenney & Armstrong, 1996; Thompson & Kenney, 2004; Thompson *et al.*, 2005a, b). Furthermore, the vascular signaling pathways that couple adrenoreceptor activation to VC may be altered (Matz *et al.*, 2003; Thompson-Torgerson *et al.*, 2007b). During localized cooling in aged skin, the magnitude of the VC response is not diminished but relies more on ROCK and less on other adrenergically-stimulated protein kinase signaling cascades (Thompson *et al.*, 2005a, b; Thompson-Torgerson *et al.*, 2007b). ROCK can be stimulated by NE or mitochondrial superoxide generated in response to localized cooling (Somlyo & Somlyo, 2000; Bailey *et al.*, 2005). Activated ROCK elicits VC through two distinct mechanisms, 1) inhibition of myosin light chain (MLC) phosphatase, thereby maintaining MLC phosphorylation without Ca^{2+} influx (i.e. Ca^{2+} sensitization) and 2) inducing the

translocation of α_{2C} receptors from the Golgi apparatus to the cell membrane (Chotani *et al.*, 2000; Jeyaraj *et al.*, 2001; Bailey *et al.*, 2004). Although ROCK has a clear role in VC during localized cooling, the extent with which ROCK mediates reflex VC remains unclear.

A greater dependence on ROCK in primary aging may parallel that observed in other age-associated vascular pathologies such as atherosclerosis, hypertension, erectile dysfunction, and diabetes (Uehata *et al.*, 1997; Chitaley *et al.*, 2001; Mallat *et al.*, 2003; Bivalacqua *et al.*, 2004; Didion *et al.*, 2005; Noma *et al.*, 2006; Holowatz & Kenney, 2007). From a thermoregulatory standpoint, ROCK may serve as an important mechanism in aged skin to sustain cutaneous VC and prevent excessive heat loss during cold exposure; however, this may occur at the expense of microvascular function since ROCK has a mutually inhibitory influence on endothelial nitric oxide synthase (eNOS) (Ming *et al.*, 2002; Ming *et al.*, 2004; Noma *et al.*, 2006). *In vitro*, ROCK decreases eNOS expression and activity and increases arginase activity thereby reducing NO bioavailability, whereas cyclic GMP-dependent protein kinase, a product of NO metabolism, inhibits Rho activation (Ming *et al.*, 2002; Ming *et al.*, 2004; Noma *et al.*, 2006). Thus, ROCK inhibition may have a vasoprotective effect due in large part to its putative effects on NO bioavailability (Noma *et al.*, 2006). Collectively, augmented ROCK activity appears to have a deleterious effect on vascular function; whether or not upregulated ROCK during whole-body cooling predates the onset of other vascular pathologies has yet to be determined.

The purpose of this study was to determine the extent with which ROCK participates in reflex VC during whole-body cooling. We hypothesized that ROCK-dependent mechanisms would contribute to the reflex VC response to a greater extent in aged skin. We further hypothesized that ROCK inhibition with local fasudil supplementation would attenuate the VC response to 1) gradual whole-body cooling and 2) localized NE perfusion, and that this reduction would be larger in aged than in young skin, which would suggest that ROCK is upregulated or unmasked due to reduced

activity of other adrenergically-stimulated protein kinase signaling mechanisms. Finally, we sought to determine whether nitric oxide synthase (NOS) inhibition, which would putatively function to disinhibit ROCK, would have a differential effect on the cutaneous VC response between age groups.

Methods

Subjects

With Pennsylvania State University Institutional Review Board approval and after verbal and written informed consent, eight young (20 ± 1 yrs; 3 men, 5 women) and eight older (73 ± 2 yrs; 5 men, 3 women) subjects participated in the study (Table 1). Young women were tested in the early follicular phase (days 1-7) of the menstrual cycle, and older women were post-menopausal and not taking hormone replacement therapy. All subjects were healthy, non-obese, normotensive, normal cholesterolemic, non-smokers, and not taking any medications that would otherwise alter cardiovascular or thermoregulatory function. All procedures conformed to the standards set by the Declaration of Helsinki.

Instrumentation

On the morning of an experiment, between 0800-1000, subjects arrived at the laboratory and were instrumented with four microdialysis (MD) fibers (10 mm, 20 kDa cutoff membrane, MD 2000 Bioanalytical Systems, West Lafayette, IN, USA) placed intradermally in the ventral forearm using aseptic technique. Prior to fiber placement, ice packs were applied to MD sites for 5 min to temporarily anesthetize the skin. For each fiber, a 25-gauge needle guide was inserted horizontally into the dermis such that entry and exit points were ~2.5 cm apart. MD fibers were threaded through the needle. The needle was then withdrawn leaving the membrane in place. After all fibers were taped in

place, lactated Ringer solution was perfused at 2 μL/min (Bee Hive controller and Baby Bee microinfusion pumps, Bioanalytical Systems) for 60-90 min to allow for local hyperemia due to needle insertion trauma to subside.

To control skin temperature, subjects wore a water-perfused suit that covered the entire body except for the face, feet, hands, and forearms. Copper-constantan thermocouples were placed on the surface of the skin at 6 sites; calf, thigh, abdomen, chest, back, and upper arm. The unweighted mean of these sites provided an index of mean skin temperature (T_{sk}).

To obtain an index of skin blood flow, red blood cell flux was continuously measured with a laser Doppler flowmetry (LDF) probes (MoorLAB, Temperature Monitor SH02, Moor Instruments, Devon, UK). LDF probes were placed in the center of local heaters and positioned directly over each MD fiber site. To specifically isolate reflex mechanisms, local skin temperature was clamped at 33^{o}C throughout the experiment. Arterial blood pressure was measured every 5 min throughout the experiment via brachial auscultation. Mean arterial pressure (MAP) was calculated as the diastolic blood pressure plus one-third pulse pressure. Cutaneous vascular conductance (CVC) was calculated as the ratio of LDF flux to MAP and expressed as percent change from baseline ($\%\Delta CVC_{baseline}$).

Protocol

After instrumentation of MD fibers and resolution of local hyperemia, pharmacological agents were perfused for 45-60 min. MD fiber sites were randomly assigned with respect to position on the forearm and were perfused with, 1) lactated Ringer's solution serving as control, 2) 3 mM fasudil (ROCK inhibitor), 3) 20 mM L-NAME, and 4) fasudil + L-NAME. All drugs were mixed just prior to use, dissolved in lactated Ringer solution, and sterilized using syringe microfilters (Acrodisc, Pall, Ann Arbor, MI, USA). Drug dosages were determined from previous studies where the

concentrations of fasudil and L-NAME used maximally inhibited ROCK and NOS, respectively (Hodges *et al.*, 2006; Thompson-Torgerson *et al.*, 2007b, a). Throughout the baseline period, mean T_{sk} was held constant at 34°C by perfusing thermoneutral water through the suit.

After baseline measurements, cold water was circulated through the suit to induce reflex VC. Mean T_{sk} decreased gradually from 34°C to 30.5°C over 30 min and was then clamped for an additional 10 min at 30.5°C (i.e., above the threshold for shivering). Rewarming for approximately 30 min followed to return T_{sk} back to 34°C, after which a $1x10^{-6}$M dose of NE was perfused at all sites for 15 min to verify that VC responsivity was preserved post cooling. The CVC established after rewarming was utilized as a baseline to assess NE-mediated VC. Lastly, 28 mM sodium nitroprusside (SNP) was perfused until a plateau in the vasodilatation response was achieved (~30 min) at each MD site to ensure that vascular function remained intact post-cooling. Due to the VC stimuli used in the protocol, the SNP-induced vasodilation was likely submaximal (Table 1).

Data Acquisition and Analysis

Data were collected at 40Hz, digitized, recorded and stored in a personal computer until data analysis (Windaq, Dataq Instruments, Akron, OH). CVC data were averaged over 3 min intervals during baseline and at each 0.5°C drop in mean T_{sk} during the cooling period. A three-way mixed model repeated measures analysis of variance was conducted to detect age and treatment differences during both whole-body cooling and NE administration. Post hoc comparisons were performed when appropriate to determine where age and treatment differences occurred. The false discovery rate procedure was utilized to control for multiple comparisons (Curran-Everett, 2000). Data relating to subject characteristics were assessed by paired Student's t-test. Statistical significance for all analyses were set at α=0.05. Values are expressed as mean ± SEM.

Results

Age groups were well matched with regard to height (Y: 170 ± 3, O: 171 ± 3 cm), weight (Y: 67 ± 3, O: 71 ± 3 kg), BMI (Y: 23.3 ± 1.0, O: 24.5 ± 0.5 kg/m^2), resting MAP (Y: 85.6 ± 2.4, O: 85.7 ± 3.0 mmHg), and cholesterol ratio (total cholesterol / HDL cholesterol) (Y: 3.1 ± 0.2, O: 3.1 ± 0.3).

The absolute CVC values, calculated as laser Doppler flux * $mmHg^{-1}$, for each MD fiber site are illustrated in Table 1. At the fasudil treated sites, CVC values differed from the control site at baseline, during cooling, and during NE perfusion (P<0.01). CVC values at the fasudil site during both cooling and NE perfusion were higher in aged skin (P<0.01), as was the CVC during SNP perfusion at the control site (P<0.01). Compared to the control site, CVC during SNP was greater in both fasudil (P=0.01) and L-NAME (P<0.01) treated sites in young skin, whereas in aged skin CVC was lower in the fasudil (P<0.01) and fasudil + L-NAME (P<0.01) treated sites.

In Figure 1, the CVC response at every 0.5°C drop in mean T_{sk} during whole-body cooling is illustrated. In young subjects (Figure 1A), fasudil attenuated the VC response to mild cooling (mean $T_{sk} \geq 31.0$°C; P<0.05) but VC was unaffected at lower mean skin temperatures. In contrast, older subjects exhibited blunted VC at the fasudil site as cooling became more severe (mean Tsk ≤ 33.0°C; P<0.05) (Figure 1B).

The effect of ROCK inhibition with fasudil on VC function during whole-body cooling (T_{sk}=30.5°C) is illustrated in Figure 2A. Compared to young subjects, older subjects exhibited a blunted VC response at the control site (Y: -34 ± 3, O: -18 ± 3% $\Delta CVC_{baseline}$; P<0.01). Local administration of fasudil significantly attenuated VC in older subjects (-7 ± 1% $\Delta CVC_{baseline}$; P=0.02) but had no effect in young subjects (-30 ± 5% $\Delta CVC_{baseline}$; P=0.33). Similarly, compared to L-NAME alone (Y: -38 ± 4, O: -23 ± 4% $\Delta CVC_{baseline}$), combined administration of fasudil and L-NAME (Y: -29 ± 4, O: -11 ±

4% $\Delta CVC_{baseline}$) blunted the VC response in older (P=0.01) and young subjects (P=0.03).

During NE ($1x10^{-6}$ M) perfusion (Figure 2B), VC at the control site was significantly lower in aged skin (Y: -53 ± 4, O: -41± 9% $\Delta CVC_{baseline}$; P<0.01). Compared to control, fasudil attenuated the VC response in both young and older subjects (Y: -23 ± 4, O: -7 ± 3% $\Delta CVC_{baseline}$; P<0.01). Moreover, NE-mediated VC at L-NAME (Y: -34 ± 3, O: -24 ± 4% $\Delta CVC_{baseline}$; P<0.01) and fasudil + L-NAME (Y: -21 ± 4, O: -19 ± 5% $\Delta CVC_{baseline}$; P<0.01) sites were also reduced relative to the VC at the control site. Compared to L-NAME alone, adding fasudil to L-NAME blunted the VC response in young (P<0.01) but not older subjects (P=0.35).

Figure 3 illustrates the ROCK contribution to the VC response, expressed as a percentage of the total VC elicited by moderate whole-body cooling (mean T_{sk} = 30.5°C) or by NE ($1x10^{-6}$M), and calculated as, % of VC mediated by ROCK = [(%$\Delta CVC_{baseline}$ at control site - %$\Delta CVC_{baseline}$ at fasudil site) / %$\Delta CVC_{baseline}$] at control site]. During whole-body cooling, ROCK contributed to reflex VC to a greater extent in aged (52 ± 9%; P<0.01) than in young skin (13 ± 13%). However, the ROCK contribution to NE-mediated VC was not different between age groups (Y: 56 ± 7, O: 77 ± 17%).

Discussion

The primary finding from this study was that ROCK inhibition diminished the VC response to mild cooling in both age groups, and this reduction remained during more severe cooling in aged but not young skin. In fact, fasudil attenuated ~50% of the VC response to more severe whole-body cooling (mean T_{sk} = 30.5°C) in aged skin. Similar reductions in the VC response due to ROCK inhibition were observed at NOS-inhibited sites. In contrast, the VC response to an exogenous physiological dose of NE was blunted by fasudil in both age groups. Cumulatively, these data suggest that ROCK

mediates approximately half of the reflex VC response to whole-body cooling in aged skin.

The data from the present investigation are consistent with previous studies demonstrating that reflex VC to whole-body cooling is not only attenuated in aged skin but relies almost entirely on a compromised adrenergic mechanism (Kenney & Armstrong, 1996; Frank *et al.*, 2000; Thompson & Kenney, 2004; Thompson *et al.*, 2005a, b; Degroot & Kenney, 2007). In addition, the second messenger responses coupling adrenoreceptor activation to reflex VC are altered in aged skin such that VC relies more on ROCK (Thompson & Kenney, 2004; Thompson *et al.*, 2005a, b; Thompson-Torgerson *et al.*, 2007b). This may be due in part to how Ca^{2+} is handled in vascular smooth muscle. NE induces VC through Ca^{2+} dependent and Ca^{2+} independent mechanisms; the latter is stimulated by ROCK and sensitizes vascular smooth muscle to extant intracellular Ca^{2+} by deactivating MLC phosphatase. In aged rats, small vessels demonstrate reduced sensitivity of contractile proteins to Ca^{2+} as well as greater NE-evoked VC when placed in a Ca^{2+} free medium. Thus, it is plausible that compromised Ca^{2+}-induced signaling explains the augmented ROCK component of cutaneous VC in older humans.

In addition to altered Ca^{2+} handling in vascular smooth muscle, ROCK may be unmasked during reflex VC in light of absent cotransmitter function in aged skin. At milder skin temperatures (>31.0^{o}C), ROCK inhibition attenuated reflex VC in both young and older skin, which suggests that ROCK not only contributes to this response but that the ROCK-mediated component of reflex VC is not different between age groups at these temperatures. However, with more severe cooling (<31.0^{o}C), ROCK had no appreciable affect on the VC response in young but remained a considerable component in older skin. Interestingly, it is in this skin temperature range that cotransmitter-mediated VC significantly contributes to the reflex VC response in younger skin (Stephens *et al.*, 2001; Thompson & Kenney, 2004). Because cotransmitter and noradrenergic-mediated VC are absent or reduced, respectively, in aged skin, it is plausible that the ROCK component is

what functionally remains to contribute to reflex VC. In contrast, sympathetic cotransmitters may be inhibiting the ROCK pathway in young skin; thus, the VC differences with fasudil may actually be a consequence of absent cotransmitter function, or disinhibited ROCK, in aged skin rather than differences in the ROCK-mediated component. However, the interaction between ROCK and cotransmitter function during whole-body cooling requires further investigation.

Much of what is known about how ROCK implements cold-induced VC comes from *in vivo* localized cooling studies in humans (Thompson-Torgerson *et al.*, 2007b, a) and *in vitro* work using mouse tail arteries. The *in vitro* studies revealed that localized cooling of vessels increases the generation of mitochondrial superoxide which directly stimulates ROCK (Bailey *et al.*, 2005). Elevated ROCK activity augments VC through two distinct mechanisms, 1) calcium sensitization by inhibiting myosin light chain phophatase and maintaining myosin light chain phosphorylation in the absence of Ca^{2+} influx and 2) translocation of α_{2C} receptors from the Golgi apparatus to the plasma membrane thereby augmenting adrenoreceptor binding sites for NE by as much as five fold (Chotani *et al.*, 2000; Jeyaraj *et al.*, 2001; Bailey *et al.*, 2004). The *in vivo* studies have examined the functional role of ROCK in the VC response to a locally applied cold stimulus, demonstrating that ~60% of the VC response is ROCK-mediated in young, and the VC becomes more dependent on ROCK with age (Thompson-Torgerson *et al.*, 2007b, a). In contrast to local cooling, our data demonstrate that during moderate whole-body cooling (T_{sk} = 30.5^{o}C), young subjects exhibited little ROCK-mediated VC (~10%) whereas over half of the VC response in older skin was ROCK dependent. The collective results from both localized and whole-body cooling in humans suggest that cutaneous VC is more reliant on ROCK with primary human aging.

The mechanism of how ROCK participates in localized versus reflex VC may differ. In contrast to whole-body cooling, the VC to local cooling in aged skin 1) does not differ in magnitude from the VC response observed in young skin (Thompson *et al.*, 2005b; Thompson-Torgerson *et al.*, 2007b), 2) is independent of efferent sympathetic

reflex activity (Ekenvall *et al.*, 1988; Pergola *et al.*, 1993), and 3) is only modestly attenuated by adrenoreceptor blockade during more prolonged (i.e. "late phase") cooling (Johnson *et al.*, 2005; Thompson-Torgerson *et al.*, 2007b). However, the VC to whole-body cooling in aged skin is completely abolished in response to bretylium tosylate and to adrenoreceptor blockade indicating that reflex VC is entirely dependent on axonal release of NE from sympathetic adrenergic nerves (Pergola *et al.*, 1993; Thompson & Kenney, 2004). Moreover, we found that fasudil substantially reduced the VC in response to exogenous NE (10^{-6} M) in older subjects (i.e., ROCK contributed to ~80% of the NE-mediated VC response). Cumulatively, these data suggest that NE relies primarily on ROCK to elicit vasoconstriction in aged skin.

Oxidative stress may also play a role in NE-induced stimulation of ROCK. In rat renal arteries, phenylephrine (α-adrenoreceptor agonist) infusion can rapidly stimulate superoxide production (Just *et al.*, 2007). Subsequently, superoxide induces VC through ROCK-mediated pathways (Jin *et al.*, 2004). Perhaps the globalized increase in reactive oxygen species that occurs with primary aging tonically augments ROCK activity and increases the gain of the ROCK response under adrenoreceptor stimulation. However, this mechanism requires further study.

In addition to its effects on vascular smooth muscle, ROCK also reciprocally inhibits NOS (Ming *et al.*, 2002; Ming *et al.*, 2004; Noma *et al.*, 2006). ROCK can decrease NO bioavailability by inhibiting eNOS transcription and activity (Ming *et al.*, 2002) and by augmenting arginase activity (Ming *et al.*, 2004; Holowatz & Kenney, 2007). As a result, increased ROCK activity may precede more serious age-related clinical pathologies such as atherosclerosis (Mallat *et al.*, 2003), diabetes (Didion *et al.*, 2005), endothelial dysfunction (Chitaley *et al.*, 2001; Bivalacqua *et al.*, 2004), cerebral and coronary vasospasm (Sato *et al.*, 2000), and hypertension (Uehata *et al.*, 1997; Holowatz & Kenney, 2007). As such, inhibition of ROCK may be beneficial in mitigating these vascular pathologies (Noma *et al.*, 2006). Much of the underlying protective effects of ROCK inhibition are mediated by the upregulation of eNOS

(Chitaley *et al.*, 2001; Ming *et al.*, 2002; Bivalacqua *et al.*, 2004; Ming *et al.*, 2004; Noma *et al.*, 2006).

We examined the interactive role of NOS and ROCK during whole-body cooling and found that the reduction in VC associated with fasudil was similar at NOS-inhibited and NOS-intact sites. Thus, NOS inhibition does not appear to affect the ROCK contribution to VC during whole-body cooling in aged skin. However, in young skin, the VC response was reduced when comparing the affect of fasudil across the NOS-blocked sites but was unaffected when comparing fasudil with control. This would suggest that NOS inhibition is required to fully express the ROCK-mediated component of reflex VC. In contrast, L-NAME blunted the VC response to NE in both young and older subjects. This was counter to what we hypothesized based on previous evidence that NOS inhibition augments cutaneous VC (Shibasaki *et al.*, 2007; Shibasaki *et al.*, 2008). Possible explanations for the discrepancy with L-NAME may be that it is dose related or that there is an order effect of perfusing NE following cooling. Additionally, baseline CVC at the L-NAME site tended to be lower than other sites (Table 1), which may limit the signal gain during a VC stimulus resulting in an artificially blunted response. Nevertheless, ROCK inhibition clearly attenuated NE-mediated VC in both young and older subjects suggesting that ROCK has an important role in NE-mediated VC.

Limitations. The baseline vasodilation observed in the fasudil sites may be a source of concern. Although fasudil is a selective inhibitor of ROCK, it may be acting on other signaling pathways in addition to ROCK. It is apparent that at least a portion of the dilatory response to fasudil was NO-mediated. However, more selective inhibitors could not be substituted as they are not available for in vivo administration in humans. As a result, it is difficult to determine the influence of ROCK on resting tone. Because normalization occurred at higher baseline CVCs, it is possible that the prior dilation to fasudil may have blunted the subsequent VC to cooling or NE. This is refuted in part by the observation that the VC to whole-body cooling at the fasudil site did not differ from the control site in young skin. Furthermore, the magnitude of the VC reduction with

fasudil was similar when comparing the reduction among NOS-intact sites (control vs. fasudil) with the reduction among NOS-blocked sites (L-NAME vs. fasudil + L-NAME).

In summary, the present study suggests that VC is more dependent on ROCK during more severe whole-body cooling in aged but not young skin, which was largely unaffected by NOS inhibition. Furthermore, much of the VC to exogenous NE relies on ROCK, particularly in aged skin. Thus, reflex VC in aged skin relies entirely on noradrenergic function and approximately half of this response is ROCK-mediated. Greater dependence on ROCK to elicit VC in primary aging may predate more serious clinical pathologies due primarily to its inhibitory effects on NOS.

Table **5.1**

Table **5.1**: Group mean ± SEM for absolute cutaneous vascular conductance. Absolute CVC values (laser Doppler flux * $mmHg^{-1}$) at baseline, maximal cooling

		Control	Fasudil	Fasudil + L-NAME	L-NAME
Baseline	Y	0.29 ± 0.06	1.60 ± 0.24*	1.28 ± 0.24*	0.22 ± 0.04
	O	0.30 ± 0.09	1.71 ± 0.41*	1.16 ± 0.29*	0.19 ± 0.02
Cooling	Y	0.19 ± 0.05	1.16 ± 0.20*	0.89 ± 0.16*	0.14 ± 0.02
		(-34 ± 3)	(-30 ± 5)	(-28 ± 4)	(-38 ± 4)
	O	0.23 ± 0.06	1.59 ± 0.38*†	1.01 ± 0.25*	0.15 ± 0.02
		(-18 ± 3)	(-7 ± 1)	(-11 ± 4)	(-23 ± 4)
Rewarm	Y	0.30 ± 0.06	1.31 ± 0.27*	0.95 ± 0.17*	0.20 ± 0.04
	O	0.35 ± 0.07	1.64 ± 0.37*†	1.07 ± 0.32*	0.20 ± 0.02
NE	Y	0.14 ± 0.03	1.06 ± 0.24*	0.76 ± 0.15*	0.12 ± 0.02
		(-53 ± 4)	(-23 ± 4)	(-21 ± 4)	(-34 ± 3)
	O	0.18 ± 0.03	1.52 ± 0.36*†	0.87 ± 0.29*	0.15 ± 0.01
		(-41 ± 9)	(-7 ± 3)	(-19 ± 5)	(-24 ± 4)
SNP	Y	2.17 ± 0.22	2.51 ± 0.33*	2.03 ± 0.23	2.53 ± 039*
	O	2.86 ± 0.43†	2.49 ± 0.47*	2.31 ± 0.44*	2.65 ± 036

Figure **5.1**

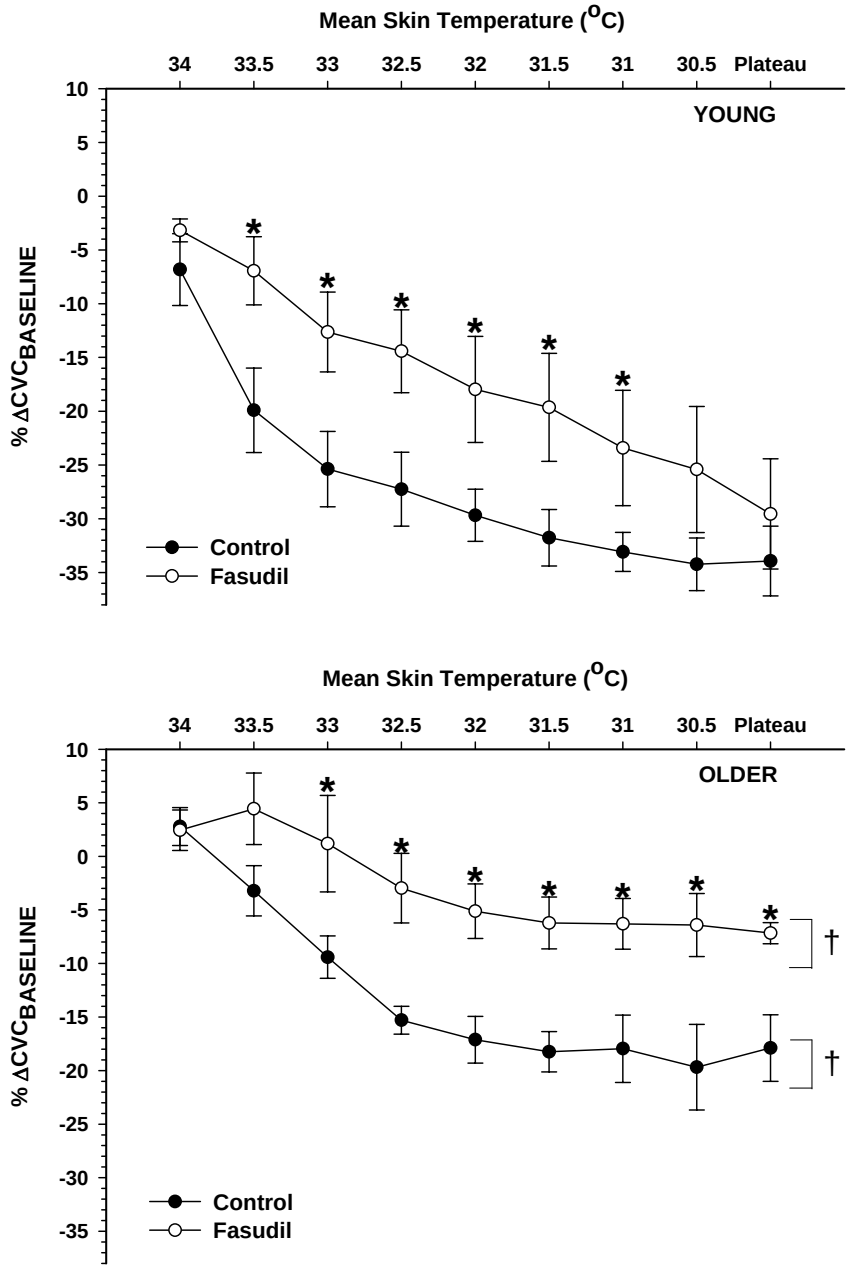

Figure **5.1**: CVC responses to 0.5°C incremental decreases in mean skin temperature during whole-body cooling in control and Rho-kinase inhibited sites. A, young subjects (n = 8); B, older subjects (n = 8). **P*<0.05 versus control; †*P*<0.05 versus young.

Figure **5.2**

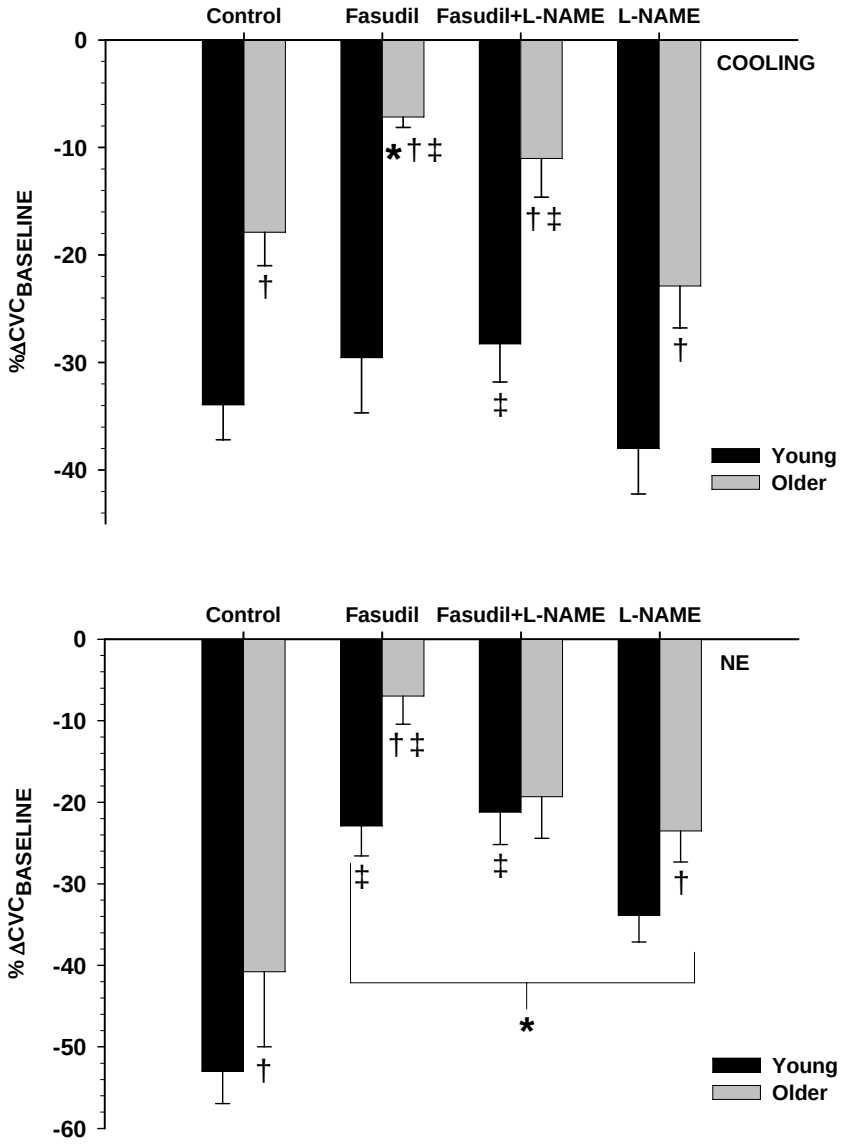

Figure **5.2**: Average cutaneous vasoconstriction in response to moderate whole-body cooling (T_{sk} = 30.5°C) or NE (1 x 10^{-6} M) at each microdialysis site in young and older subjects. A, whole-body cooling; B, NE perfusion. The mean percent change from baseline CVC at control, fasudil (Rho-kinase inhibited), L-NAME, and fasudil + L-NAME pretreated sites, n = 16 (8y, 8o) subjects. **P*<0.05 versus control; †*P*<0.05 versus young; ‡*P*<0.05 versus L-NAME.

Figure **5.3**

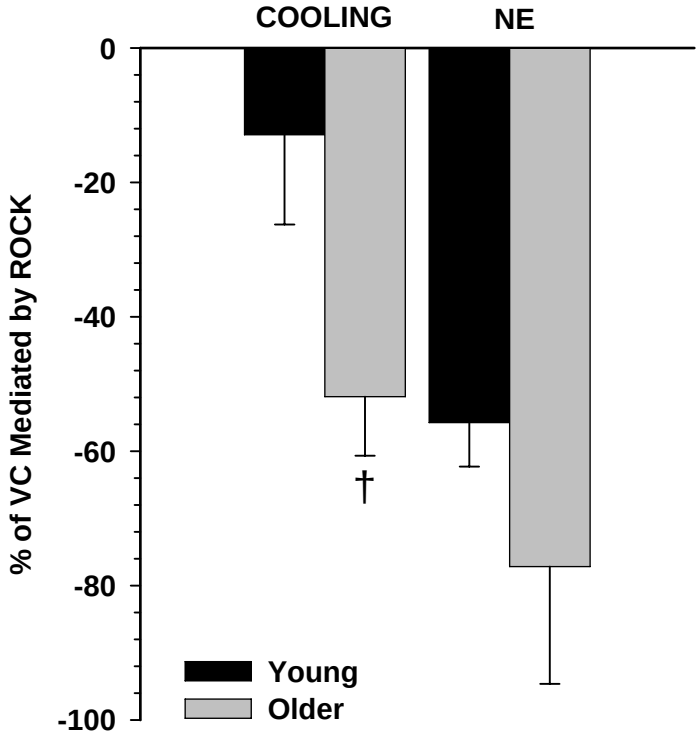

Figure **5.3**: The proportion of the VC response that is Rho-kinase mediated; expressed as percentage of the total VC response during moderate whole-body cooling (T_{sk} = 30.5°C) or norepinephrine (1 x 10^{-6} M). The percent of VC mediated by ROCK is calculated as [(%ΔCVC$_{baseline}$ at control site - %ΔCVC$_{baseline}$ at fasudil site) / %ΔCVC$_{baseline}$]. †P<0.05 versus young.

Chapter 6

TETRAHYDROBIOPTERIN DOES NOT AFFECT END-ORGAN RESPONSIVENESS TO NOREPINEPHRINE-MEDIATED VASOCONSTRICTION IN AGED SKIN

Introduction

Reflex-mediated cutaneous vasoconstriction (VC) is an immediate and sustained thermoregulatory response to cold exposure that effectively minimizes convective heat loss to the environment. In older subjects this response is impaired thereby rendering them more susceptible to excessive heat loss and possibly hypothermia (Collins *et al.*, 1977; Kenney & Armstrong, 1996). Even when matched for body composition and aerobic fitness, older adults exhibit reduced peripheral VC and a relative inability to defend against decreases in core temperature even during mild (22^{o}C) cold exposure (Kenney & Armstrong, 1996; Degroot & Kenney, 2007).

We have recently demonstrated that localized administration of tetrahydrobiopterin (BH_4) offsets the attenuated VC response in aged skin (Lang *et al.*, 2009a). BH_4 is found throughout neural and vascular tissue and serves as an essential cofactor for nitric oxide synthase (NOS) and tyrosine hydroxylase (TH), the rate-limiting step in catecholamine biosynthesis (Kaufman, 1978; Kumer & Vrana, 1996; Dunkley *et al.*, 2004; Urano *et al.*, 2006; Moens & Kass, 2007). Because BH_4 is a powerful reducing agent and antioxidant, it is also vulnerable to reactive oxygen species (ROS). In cultured sympathetic neurons induction of oxidative stress reduced BH_4 ~90% resulting in a ~75% reduction in catecholamine biosynthesis (Li *et al.*, 2003). Elevated oxidative stress in aged skin may deplete BH4, and compromise enzymatic function.. Reduced BH_4 bioavailability may result in suboptimal TH function and contribute to the attenuated

cutaneous VC response in aged skin; however, the extent with which BH_4 additionally affects vascular mechanisms is unclear.

BH_4 may affect the postjunctional component of cutaneous VC by, 1) acting as a potent antioxidant, 2) preventing NOS uncoupling and increasing NO bioavailability. BH_4 readily scavenges several oxidants including superoxide and peroxynitrite (Heales *et al.*, 1988; Gramsbergen *et al.*, 2002; Katusic *et al.*, 2009). In fact, BH_4 is 6-10 times more effective than ascorbate or reduced thiols in binding peroxynitrite (Kuzkaya *et al.*, 2003). This may be particularly relevant to aged skin considering recent evidence indicating that rho-kinase, which is directly stimulated by ROS, mediates ~50% of the reflex VC response in older adults (Lang *et al.*, 2009b). Additionally, BH_4 minimizes NOS-derived ROS by preventing enzymatic uncoupling (Moens & Kass, 2007; Katusic *et al.*, 2009). Cumulative, BH_4 may affect end-organ responsiveness to NE by altering the redox state in the cutaneous vasculature.

In conjunction with what we have previously demonstrated (Lang *et al.*, 2009a), the purpose of this study was to determine the extent with which the effects of BH_4 in improving the VC response in aged skin are mechanistically isolated to the nerve terminal as opposed to the cutaneous vasculature. We hypothesized that localized BH_4 supplementation would minimally affect VC responsiveness to exogenous NE at sites where sympathetic nerves were blocked, which underscores the role of BH_4 on TH during cutaneous VC.

Methods

Subjects

With Pennsylvania State University Institutional Review Board approval and after verbal and written informed consent, ten young (22 ± 1 yrs; 5 men, 5 women) and ten

older (73 ± 3 yrs; 4 men, 6 women) subjects participated in the study. Young women were tested in the early follicular phase (days 1-7) of the menstrual cycle, and older women were post-menopausal and not taking hormone replacement therapy. All subjects were healthy, non-obese, normotensive, normal cholesterolemic, non-smokers, and not taking any medications or vitamin supplements that would otherwise alter cardiovascular or thermoregulatory function. All procedures conformed to the standards set by the Declaration of Helsinki.

Instrumentation

Subjects arrived at the laboratory between 07:00-09:00 and remained in a semisupine position with the experimental forearm at heart level throughout the protocol. Two sites were marked with ink on the left ventral forearm and spaced at least 4.0 cm apart to prevent cross-reactivity of pharmacological agents between sites. At each marked site, 10 mM bretylium was iontophoresed (USP Pharmacopeia, Rockville, MD) at 200 μA for 20 min over a 1 cm^2 area of skin. After iontophoresis at the final site, ~45 min was allowed for resolution of hyperemia. After which, the efficacy of the bretylium block was tested with 3 min of vigorous whole-body cooling (water perfusion temperature = 19^{o}C) to verify that reflex VC was abolished. An additional laser Doppler was placed on a nontreated site during cooling to ensure that the reflex VC response remained intact. Full resolution of the VC response to the supraphysiological concentrations of NE prevented the ability to test the integrity of the bretylium block post-experiment. However, pilot studies from our laboratory performed in 3 subjects demonstrated that the block remains intact for ~7 hrs after iontophoresis.

After the whole-body cooling test, two microdialysis (MD) fibers (10 mm, 20 kDa cutoff membrane, MD 2000 Bioanalytical Systems, West Lafayette, IN, USA) were placed at each bretylium pretreated site using aseptic technique. Prior to fiber placement, ice packs were applied to MD sites for 5 min to temporarily anesthetize the skin (Hodges *et al.*, 2009). For each fiber, a 25-gauge needle was inserted horizontally into the dermis

such that entry and exit points were ~2.5 cm apart. After MD fibers were threaded through the needle, the needle was withdrawn leaving the membrane in place. All fibers were taped in place and lactated Ringer's solution was initially perfused to test the integrity of the fiber and during the resolution period following needle insertion trauma. After which, MD sites were randomly assigned with respect to position on the forearm and were perfused with, 1) lactated Ringer's solution serving as control and 2) 5 mM tetrahydrobiopterin (BH_4). The BH_4 dosage was determined from a previous study in our laboratory (Lang *et al.*, 2009a).

To obtain an index of skin blood flow, red blood cell flux was continuously measured with a laser Doppler flowmetry (LDF) probes (MoorLAB, Temperature Monitor SH02, Moor Instruments, Devon, UK). LDF probes were placed in the center of local heaters and positioned directly over each MD fiber site. Local skin temperature was clamped at a thermoneutral temperature (34^{o}C) throughout the experiment. Arterial blood pressure was measured every 5 min throughout the experiment via brachial auscultation. Mean arterial pressure (MAP) was calculated as the diastolic blood pressure plus one-third the pulse pressure. Cutaneous vascular conductance (CVC) was calculated as the ratio of LDF flux to MAP and expressed as percent change from baseline values ($\%\Delta CVC_{baseline}$).

Protocol

After instrumentation with MD fibers at bretylium pretreated sites, local hyperemia was allowed to resolve for ~90 min while perfusing sites with their assigned pharamacological agent. All drugs (IND#: 103180) were mixed just prior to use, dissolved in lactated Ringer's solution, sterilized using syringe microfilters (Acrodisc, Pall, Ann Arbor, MI, USA), and perfused at 2 μL/min (Bee Hive controller and Baby Bee microinfusion pumps, Bioanalytical Systems).

After baseline measurements, NE (preserved with 1 mg/mL of ascorbic acid) was perfused for 5 min at all sites followed by immediate washout with its designated pharmacologic (i.e., lactated Ringer's solution or BH_4) for ~20-30 min to allow skin blood flow to recover to initial baseline values (CMA 110 Liquid Switch, CMA Microdialysis Inc). This protocol was repeated for each NE dose (10^{-12}, 10^{-10}, 10^{-8}, 10^{-6}, 10^{-4}, 10^{-2} M). The initial three concentrations (10^{-12}, 10^{-10}, 10^{-8} M) were performed in random order followed by sequential perfusion of the final three more concentrated doses (10^{-6}, 10^{-4}, 10^{-2} M). Full resolution of the VC to the more potent concentrations of NE prevented the randomization of these final doses. Lastly, 28 mM sodium nitroprusside was perfused through all sites at a rate of 4 μL/min in combination with local heating of the skin to 43^{o}C for ~30 min at each MD site to ensure that vascular responsivity remained intact.

Data Acquisition and Analysis

Data were collected at 40 Hz, digitized, recorded and stored in a personal computer until data analysis (Windaq, Dataq Instruments, Akron, OH). CVC data were averaged over 3 min intervals during baseline. During NE perfusion, the VC response was defined as the lowest CVC 1-min average observed during the washout period. Typically, the maximal VC response for a given NE dose was achieved ~2-3 min into its washout. Data were analyzed using three-way mixed model repeated measures analysis of variance (i.e., NE dose x age x drug treatment) and Student's paired t test for subject characteristics (SAS, version 9.1.3, Cary, NC, USA). Tukey post hoc tests were performed when appropriate to determine where age and drug treatment differences occurred. Statistical significance for all analyses were set at α=0.05. Values are expressed as mean ± S.E.M.

Results

Subject characteristics are presented in Table 1. Age groups were well matched with regard to height, weight, BMI, MAP, blood glucose, and cholesterol ratio (total cholesterol / HDL cholesterol). There were no significant differences in absolute baseline CVC, calculated as laser Doppler flux * $mmHg^{-1}$, between age groups or between the control (Y: 0.18 ± 0.03, O: 0.22 ± 0.05; P=0.50) and BH_4 site (Y: 0.24 ± 0.05 P=0.38 vs control, O: 0.22 ± 0.04 P=0.97 vs control).

The efficacy of the presynaptic adrenergic blockade is displayed in Figure 1, which compares the reflex VC response to whole-body cooling observed at the bretylium-treated and untreated sites in young and older subjects. Compared to non-treated sites, no VC was observed at bretylium treated sites during whole-body cold stress (P<0.01).

Figure 2 illustrates VC in response to 6 different concentrations of NE (10^{-12}, 10^{-10}, 10^{-8}, 10^{-6}, 10^{-4}, 10^{-2} M) in young and older subjects. The VC response was attenuated at all doses in aged skin (P<0.01).

The VC response to NE in BH_4 pretreated sites is displayed in Figure 3. Panel A demonstrates that BH_4 had no effect on the VC response in young subjects (10^{-12}: P=0.68; 10^{-10}: P=0.49; 10^{-8}: P=0.81; 10^{-6}: P=0.40; 10^{-4}: P=0.81; 10^{-2}: P= 0.25). Similarly, BH_4 did not affect NE-mediated VC in older subjects (panel B) (10^{-12}: P=0.10; 10^{-10}: P=0.14; 10^{-8}: P=0.15; 10^{-6}: P=0.12; 10^{-4}: P=0.12; 10^{-2}: P= 0.05).

Discussion

The primary finding from this study was that localized BH_4 supplementation did not affect the VC response to NE in bretylium pretreated skin of young or older subjects. In conjunction with our previous findings (Lang *et al.*, 2009a), this suggests that the primary site of action of BH_4 during cutaneous VC is isolated to the axon terminal of adrenergic nerves. Moreover, BH_4 has a negligible role on vascular adrenergic VC mechanisms. Additionally, we verified that VC in aged skin is blunted at various physiological and supraphysiological concentrations of NE (Thompson *et al.*, 2005b). This result occurred even when controlling for endogenous NE release with bretylium.

The present study was an important follow-up to previous findings in our laboratory in which localized BH_4 infusion in aged skin offset the attenuated VC response to both physiological (whole-body cooling) and pharmacologically (tyramine)-mediated VC (Lang *et al.*, 2009a). Additionally, BH4 did not alter the cotransmitter-mediated component of reflex VC. Moreover, the BH4-mediated augmentation of the VC response in aged skin was not affected by NOS inhibition. Thus, increase in NO bioavailability secondary to the coupling effect of BH4 on NOS did not affect the VC response. However, it is possible that we observed a basement effect in the signal gain, thereby making any additional VC due to NOS inhibition undetectable. Furthermore, exogenous and endogenous sources of NO attenuated cutaneous VC (Durand *et al.*, 2005; Shibasaki *et al.*, 2007; Shibasaki *et al.*, 2008) and presumably, NOS inhibition would have the contrasting effect of augmenting the VC response. Collectively, BH4 augmented the VC response in aged skin; however this study (Lang *et al.*, 2009a) was unable to determine whether the underlying mechanism for BH4 is localized to sympathetic adrenergic nerve terminals or the cutaneous vasculature.

In addition to acting as a cofactor for NOS, the putative postjunctional effects of BH_4 may be related to its capacity to act as an antioxidant. BH4 readily neutralizes ROS

and in some cases, demonstrating greater reactivity than ascorbate (Heales *et al.*, 1988; Gramsbergen *et al.*, 2002; Kuzkaya *et al.*, 2003; Katusic *et al.*, 2009). Additionally, BH4 minimizes NOS-derived ROS by preventing enzymatic uncoupling (Moens & Kass, 2007; Katusic *et al.*, 2009). However, the reaction of BH4 with various oxidants may limit its ability to act as an enzymatic cofactor.

In sympathetic adrenergic nerve terminals, BH_4 is important to NE biosynthesis because it acts as a cofactor for the rate-limiting enzyme, tyrosine hydroxylase (TH). During neuronal activation, the affinity of TH for its cofactor markedly increases (Kaufman, 1978; Zigmond *et al.*, 1989). BH_4 subsequently reduces the iron moiety of TH, thereby catalytically activating the enzyme for hydroxylation of tyrosine (Kumer & Vrana, 1996; Dunkley *et al.*, 2004; Urano *et al.*, 2006). However, in the relative absence of BH_4, NE biosynthesis and storage may be compromised resulting in blunted adrenergic VC. Reduced BH_4 concentration is apparent in aged tissues and this may be secondary to elevated oxidative stress (Williams *et al.*, 1980; Delp *et al.*, 2008). Additionally, the number of transporters for NE in synaptomsomes decreases with age (Snyder *et al.*, 1998). Thus, reduced BH_4 bioavailability may result in suboptimal TH function, thereby compromising the available pool of NE required to fully express the VC response in aged skin.

The importance of noradrenergic function is particularly evident in aged skin since this is the only mechanism responsible for eliciting thermoregulatory reflex VC (i.e., cotransmitter-mediated VC is functionally absent) (Thompson & Kenney, 2004; Lang *et al.*, 2009a). However, deficits in noradrenergic function have also been identified in adrenoreceptor and second messenger systems of vascular smooth muscle (Hogikyan & Supiano, 1994; Dinenno *et al.*, 2002; Thompson *et al.*, 2005b; Thompson-Torgerson *et al.*, 2007b; Lang *et al.*, 2009b). Although conflicting accounts exist regarding the effect of age on the cutaneous VC response to NE (Wilson *et al.*, 2004; Thompson *et al.*, 2005b),of whether autoreceptors are affected with aging (Dinenno *et al.*, 2002; Bruck *et al.*, 2007), we verified that the VC response to multiple doses of NE

was attenuated in aged skin even after controlling for presynaptic adrenergic function with bretylium tosylate. The cutaneous VC response primarily operates through postjunctional α_2 receptors (Ekenvall *et al.*, 1988; Borbujo *et al.*, 1989); however, this receptor subtype also exists presynaptically as an autoreceptor that functionally inhibits NE release (Hein *et al.*, 1999). Conflicting evidence exists indicating that presynaptic adrenergic function is diminished (Bruck *et al.*, 2007) or unchanged (Dinenno *et al.*, 2002) with age. Nevertheless, these data collectively suggest that in response to a given physiological dose of NE, postjunctional adrenoreceptor sensitivity is functionally blunted in aged skin.

Because BH_4 normalizes adrenergic VC and couples NOS to increase NO bioavailability (Moens & Kass, 2007; Lang *et al.*, 2009a), which putatively improves vasodilator function and minimizes superoxide generation, future studies may be warranted in assessing the effects of acute and chronic oral supplementation of this cofactor on vascular and thermoregulatory function. Acute oral BH_4 improves flow-mediated dilation in older sedentary men by ~45% (Eskurza *et al.*, 2005). Four week administration of BH_4 (800 mg/day) reversed endothelial dysfunction and oxidative stress in hypercholesterolemic humans (Cosentino *et al.*, 2008). Whether or not the effects of oral BH_4 enhance thermoregulatory function in healthy older subjects remains unknown.

Limitations. Bretylium tosylate inhibits adrenergic function presynaptically only after an initial release in neurotransmitter substance. This may result in acute desensitization of adrenoreceptors. However, bretylium administered at concentrations that causes presynaptic blockade does not affect exogenous NE-mediated VC (Blair *et al.*, 1960). Furthermore, iontophoresis may induce a current-related vasodilation (Durand *et al.*, 2002). However, baseline absolute CVC was not different from those observed from previous protocols where iontophoresis was not used. Also, these limitations were minimized by the ~3 hr interim between iontophoresis of the final site and infusion of the first NE dose.

In summary, the present study indicates that the effects of localized BH_4 supplementation on cutaneous VC are primarily localized to adrenergic nerve terminals. This corroborates our previous study that demonstrated an augmentation in the physiological- (whole-body cooling) and pharmacological- (tyramine) induced VC in aged skin. Thus, reduced NE biosynthesis due to depletion of BH_4 in aged skin contributes to the attenuated VC in aged skin. Lastly, we verified that NE-mediated VC was attenuated in older subjects even when blocking presynaptic function.

Table **6.1**

Table **6.1**: Subject characteristics. Values are means ± S.E.M. for young (n = 10) and older (n = 10) men and women. BMI, Body Mass Index; MAP, Mean Arterial Pressure; HDL, High-Density Lipoprotein; LDL, Low-Density Lipoprotein. **P*<0.05.

Variable	**Young**	**Older**
Sex (M,F)	5,5	4,6
Age (yrs)	22 ± 1	73 ± 3*
Height (cm)	172 ± 3	166 ± 2
Weight (kg)	73 ± 5	68 ± 3
BMI (kg/m^2)	24 ± 1	24 ± 1
Resting MAP (mmHg)	82.1 ± 2.2	86.7 ± 1.7
Glucose (mg/dL)	87 ± 2	89 ± 2
Total Cholesterol (mg/dL)	151 ± 6	191 ± 7*
HDL (mg/dL)	51 ± 2	67 ± 4*
LDL (mg/dL)	82 ± 7	107 ± 6*
Cholesterol Ratio (total/HDL)	3.0 ± 0.3	3.0 ± 0.2

Figure **6.1**

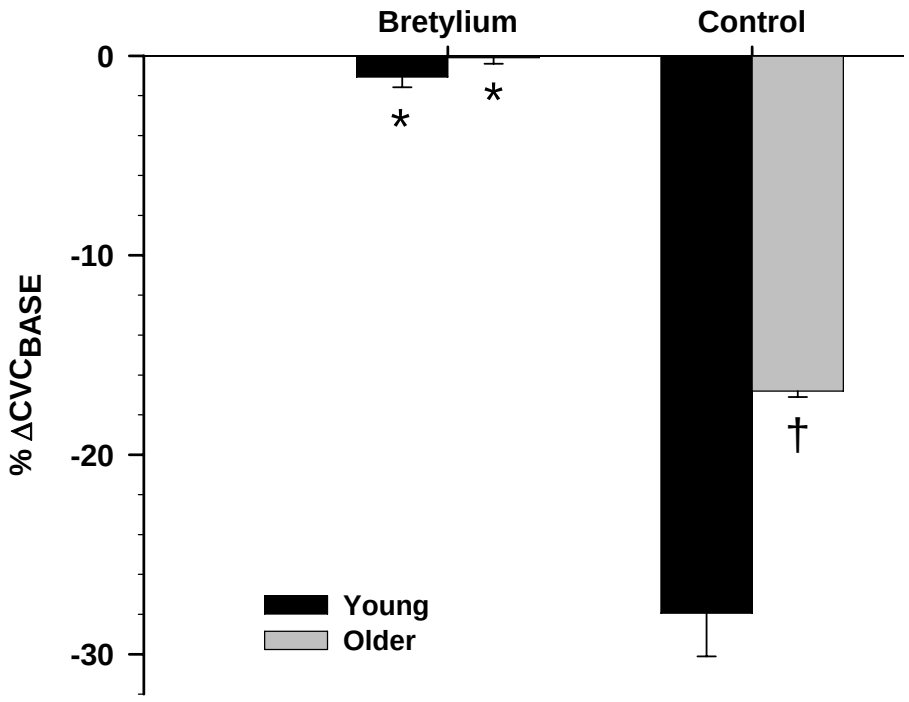

Figure **6.1**: Average cutaneous vasoconstriction in response to whole-body cold stress at bretylium treated and nontreated (control) sites. The mean percent change from baseline CVC at bretylium treated and control sites, n = 20 (10 young, 10 older) subjects. Whole-body cooling was utilized to test the efficacy of bretylium blockade. **P*<0.05 versus control; †*P*<0.05 versus young.

Figure **6.2**

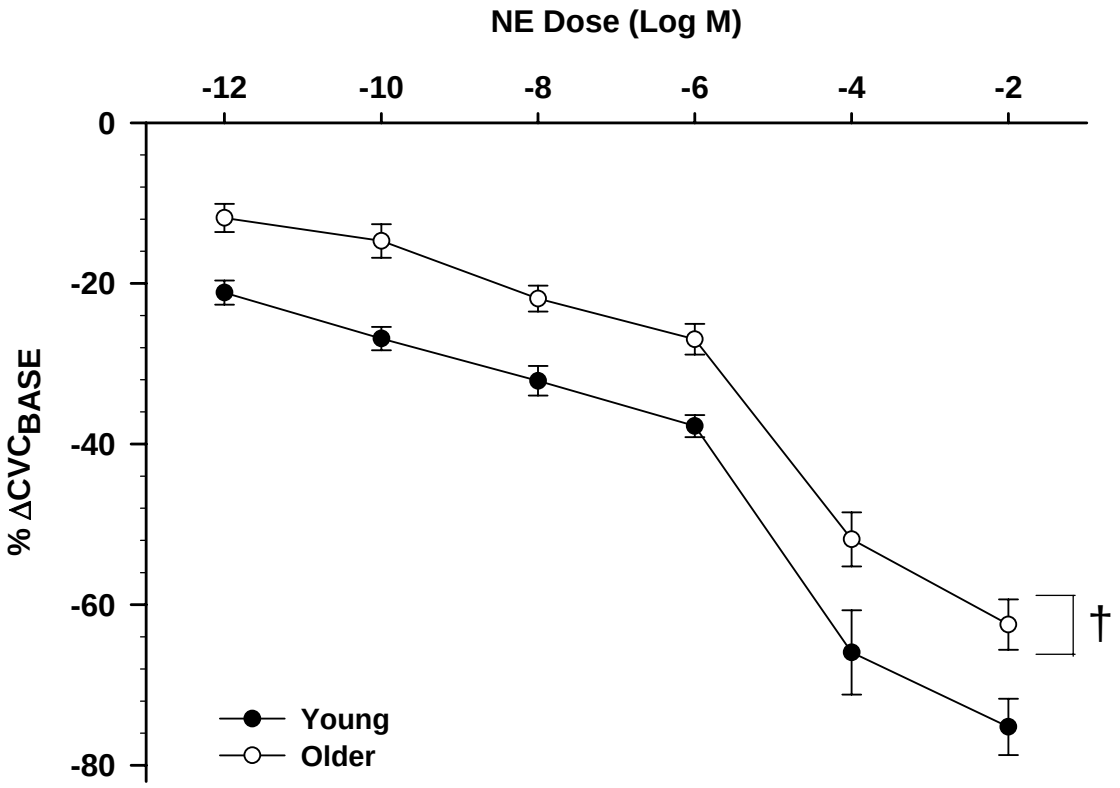

Figure **6.2**: Average cutaneous vasoconstriction in response to sequential 5 min infusions of various concentrations of norepinephrine. The mean percent change from baseline CVC at the bretylium pretreated control site of young (n = 10) and older (n = 10) subjects. The VC response was attenuated at each NE concentration in older subjects. †P<0.05 versus young.

Figure **6.3**

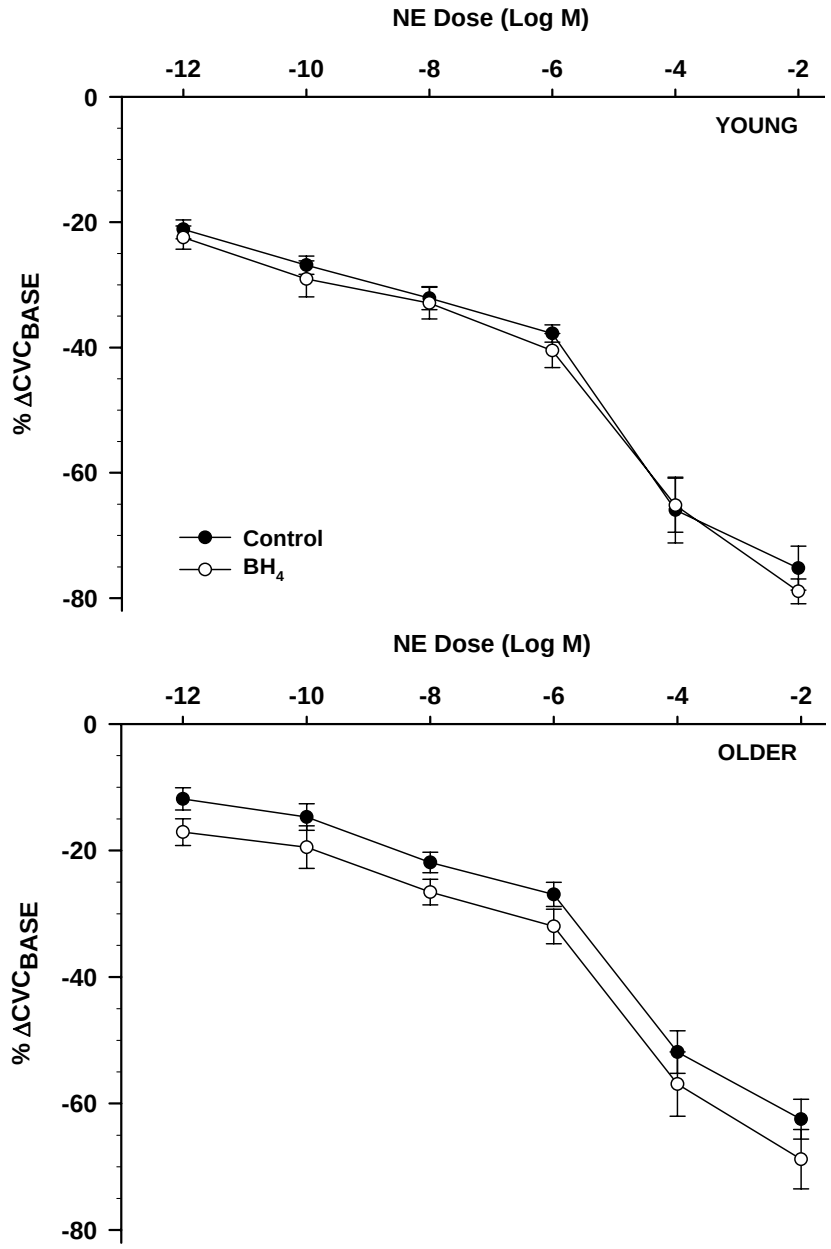

Figure **6.3**: Effects of tetrahydrobiopterin (BH_4) on the average cutaneous vasoconstrictor response to sequential 5 min infusions of various concentrations of norepinephrine. The mean percent change from baseline CVC at the control and BH_4 pretreated sites of young (n = 10) (panel A) and older (n = 10) (panel B) subjects. Both sites received pretreatment of bretylium tosylate to block presynaptic adrenergic function.

Chapter 7

CONCLUSIONS

The four studies comprising this thesis were designed to examine the age-related impairments in the prejunctional (neural) and postjunctional (vascular) noradrenergic mechanisms controlling reflex VC in response to whole-body cooling. This chapter is intended to summarize the primary findings, integrate them into a broader physiological context, and provide future directions of research to further clarify the effects of healthy aging on thermoregulatory VC.

Tetrahydrobiopterin and Reflex Vasoconstriction

The primary finding from this study was that localized BH_4 supplementation in aged skin selectively augmented the VC response induced by either whole-body cooling or by tyramine infusion. In fact, BH_4 completely offset the age-associated decrement in cutaneous VC. This result was not a function of the cofactor role of BH_4 on NOS. Furthermore, BH_4 did not affect cotransmitter-mediated VC, which suggests that the effects of BH_4 were isolated to the noradrenergic component of VC. In summary, these data suggest that reduced BH_4 bioavailability contributes to attenuated VC in aged skin. Because BH_4 is an essential cofactor for TH, these data also suggest that reduced NE biosynthesis and release from sympathetic nerves likely contributes to the attenuated reflex VC response in older subjects.

Tyrosine and Reflex Vasoconstriction

The primary finding from this study was that local tyrosine administration augmented the VC response induced by either whole-body cooling or by tyramine

infusion in aged but not in young skin. Similar to BH_4, adding tyrosine completely offset the age-associated decrement in cutaneous VC. Moreover, perfusing tyrosine and BH_4 concomitantly did not have an additive effect on the cutaneous VC response in older subjects. In summary, these data suggest that reduced bioavailability of the pterin cofactor and amino acid substrate required by TH to synthesize NE is reduced in aged skin, thereby contributing to the attenuated noradrenergic component of reflex VC in older subjects.

Rho-kinase and Reflex Vasoconstriction

The primary finding from this study was that ROCK inhibition diminished the VC response to mild cooling in both age groups, and this reduction remained during more severe cooling in aged but not young skin. In fact, fasudil attenuated ~50% of the VC response to more severe whole-body cooling (mean T_{sk} = 30.5^{o}C) in aged skin. This result was largely unaffected by NOS inhibition. In contrast, the VC response to an exogenous physiological dose of NE was blunted by fasudil in both age groups. Cumulatively, these data suggest that the reflex VC response to whole-body cooling in aged skin relies more on a ROCK-mediated (Ca^{2+}-independent) component.

Tetrahydrobiopterin and Norepinephrine Dose Response

The primary finding from this study was that localized BH_4 supplementation did not affect the VC response to NE in bretylium pretreated skin of young or older subjects. This suggests that BH_4 has a negligible role on vascular VC mechanisms, and that its primary site of action during peripheral VC is localized to the axon terminal of adrenergic nerves. Additionally, we verified that VC in aged skin is blunted at various physiological and supraphysiological concentrations of NE. This result occurred even when controlling for endogenous NE release with bretylium.

Implications

Healthy aging is associated with impairments in peripheral autonomic and thermoregulatory function. Specifically, this series of studies addresses how primary aging affects the sympathetic VC response to whole-body cooling. Previous work in aged skin has demonstrated that reflex VC relies exclusively on a blunted noradrenergic component. Furthermore, we and others have provided evidence that noradrenergic function may be compromised or altered at several levels during neuronal activation in aged skin: 1) blunted neural drive, 2) relative inability to upregulate TH secondary to reduced amino acid and cofactor bioavailability, 3) desensitization of adrenoreceptors, 4) greater reliance on Ca^{2+}-independent pathways such as ROCK to elicit VC in VSM.

These impairments may be related to the elevated oxidative and nitrosative stress observed in aged skin (Kohen, 1999; Lu *et al.*, 1999; Nishigori *et al.*, 2003; Hornig-Do *et al.*, 2007). Prejunctionally, oxidative stress may convert tyrosine and BH_4 to byproducts unable to participate in catecholamine biosynthesis. Postjunctionally, an increase in reactive oxygen species may upregulate ROCK, thereby placing greater dependence on ROCK to elicit VC. Although ROCK-mediated VC may be beneficial from a thermoregulatory standpoint, its activity may precede other age-related vascular pathologies due in large part to its mutual inhibition of NOS. In summary, elevated oxidative stress may serve as a primary contributor to the attenuated reflex VC response observed in aged skin. However, reflex VC represents only one thermoregulatory mechanism that is utilized during cold stress. Other mechanisms such as shivering thermogenesis and tissue insulation (both adipose and skeletal muscle properties) may also play a role.

Future Research Directions

1. The results presented in the BH_4 study (chapter 3) confirmed previous findings indicating that cotransmitter-mediated VC mediates ~40% of the reflex VC

response. However, the identity of these coreleased substances remains in question. NPY has been identified as a putative cotransmitter that contributes to the VC response to whole-body cooling (Stephens *et al.*, 2004). However, this finding has not been replicated in our laboratory using the same NPY receptor antagonist, BIBP-3226 (Thompson, 2005). As a result, this raises the following concerns, 1) whether BIBP-3226 is indeed an effective NPY antagonist, 2) whether the 10.5 μM dose utilized in the aforementioned studies is efficacious in consistently blocking NPY, and 3) whether NPY has any contribution to reflex VC at all.

2. In addition to NPY, ATP may also importantly contribute to the reflex cutaneous VC response. ATP may elicit VC directly by binding to P_{2X} receptors on VSM and indirectly by binding to prejunctional purinergic receptors to augment NE release (Li *et al.*, 2005; Burnstock, 2009). In combination, these putative effects may substantially affect the VC response particularly at the onset of cooling. The few *in vivo* human studies that have addressed the effects of ATP on vascular function have demonstrated that ATP has a vasodilatory influence (Kirby *et al.*, 2008). However, these studies used brachial artery infusions of ATP, thereby influencing primarily endothelial P_{2Y} receptors rather than the P_{2X} receptors situated on VSM. Thus, the question remains as to the importance of ATP in the cotransmitter-mediated component of reflex VC in human skin.

3. Apart from identifying the sympathetic cotransmitter substance, the reason why this component is functionally absent in aged skin requires further investigation. One potential explanation may be how these neurotransmitters are packaged. Whereas NE is loaded primarily within synaptic vesicles, larger neuropeptide cotransmitters are packaged in large dense core vesicles. No studies to date have addressed the underlying mechanism for the age-related elimination of cotransmitter-mediated VC.

4. Because BH_4 acts as a cofactor for both NOS and TH, it likely augments both NO and NE bioavailability in aged skin during VD and VC, respectively. Whether or not these results can be replicated with systemic oral administration of BH_4 has yet to be investigated. Thus, oral BH_4 may expand the range of blood flow perfusing the cutaneous vasculature and improve thermoregulatory function in older adults.

5. In addition to its role in improving cognitive and psychomotor performance during cold stress (Banderet & Lieberman, 1989; O'Brien *et al.*, 2007), systemic tyrosine and/or BH_4 may more effectively guard against decreases in core temperature during prolonged cold stress in older adults. In that sense, these TH precursors may serve as a useful tool in assessing the relative importance of VC to the overall impairment in the thermoregulatory response to cold exposure in older adults.

6. Because the bioavailability of BH_4 is important to both microcirculatory and thermoregulatory function, the regulation of its biosynthetic pathway may be an important area of study. In fact, the expression of GTP cyclohydrolase, the rate-limiting enzymatic step, may be altered with physical exercise, depression, or aging (Hashimoto *et al.*, 2004). Furthermore, the role of folate in reconstituting BH_4 from its oxidized form may significantly affect its bioavailability (Moens *et al.*, 2008).

7. Rho-kinase and NO mutually inhibit one another at several points along their respective vasomotor pathways (Thompson-Torgerson *et al.*, 2008); however, the functional extent that ROCK blunts NO-mediated reflex VD during heating or NO decreases ROCK-mediated VC remains unclear. In addition to thermoregulatory function, this interaction may play an important role in the etiology of various vascular disease states. Thus, the functional interaction between ROCK and NO requires further study.

8. Interestingly, one of the pleiotropic effects of HMG-CoA reductace inhibitors (statins) is an inhibition of ROCK. Although this may improve vascular function and reflex cutaneous VD, its affect on reflex VC has yet to be investigated. The results from the ROCK study (chapter 5) demonstrate that over half of the reflex VC response in aged skin is ROCK-mediated. Effectively blocking this component in older adults may have an additive effect in impairing thermoregulatory function in response to cold stress.

Figure **7.1**

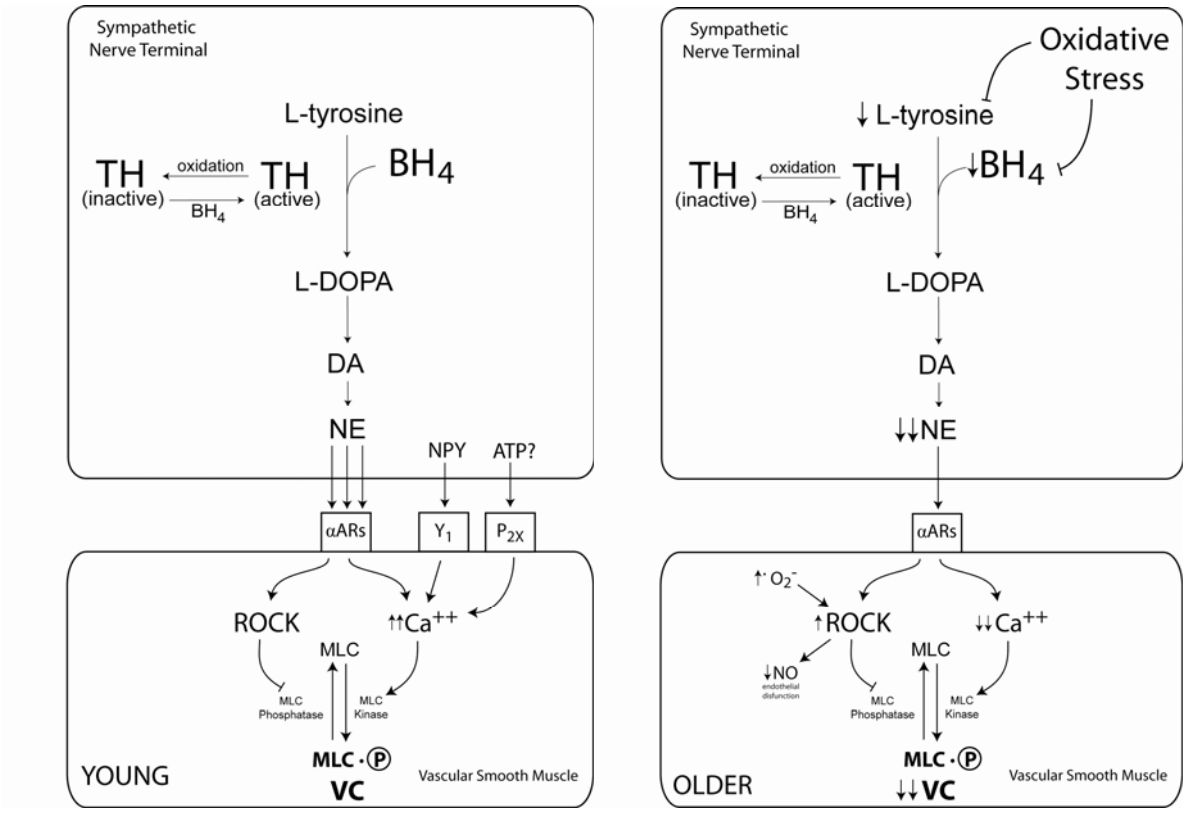

Figure **7.1**: Schematic comparison of the mechanism of reflex vasoconstriction in young and older humans. TH, tyrosine hydroxylase; BH_4, tetrahydrobiopterin; O_2^-, superoxide; DA, dopamine; NE, norepinephrine; NPY, neuropeptide Y; αARs, α-adrenoreceptors; MLC, myosin light chain; ROCK, rho kinase; NO, nitric oxide; Ca^{++}, calcium; VC, vasoconstriction

Figure **7.2**

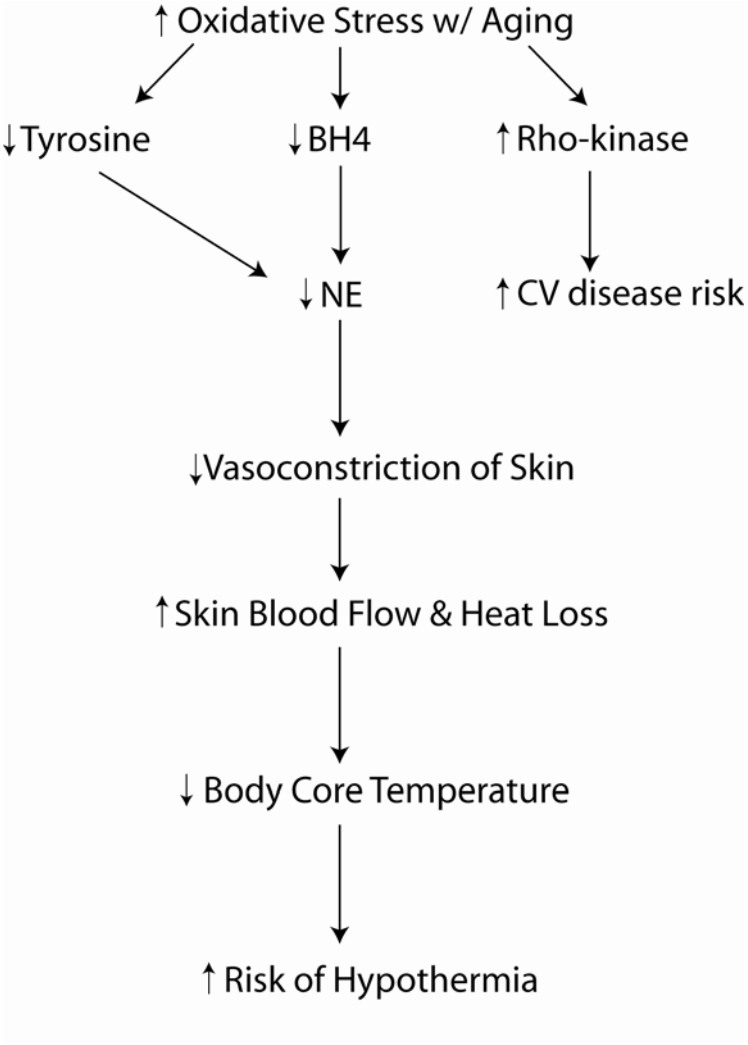

Figure **7.2**: Conceptual summary illustrating the contribution of the studies comprising this thesis to the understanding of how oxidative stress may be altering vasoconstrictor function in human skin.

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Appendix A

ADDITIONAL DATA FROM CH. 6: NOS INHIBITION DURING NE-MEDIATED VC IN AGED SKIN

Introduction

Cutaneous VC responsiveness may be impaired by substances that elicit vasodilation such as nitric oxide (NO). In human skin, both exogenous and endogenous NO has been demonstrated to attenuate the VC response to cold stress, NE, or an orthostatic stress (Durand *et al.*, 2005; Shibasaki *et al.*, 2007; Shibasaki *et al.*, 2008). These investigators also found an enhanced VC when blocking NOS with L-arginine analogues; however, an elevation in the signal gain with adenosine, heating, or isoproterenol was necessary to observe this effect. Without this artificial rise in baseline, NOS inhibition had no effect on the VC response to cold stress (Shibasaki *et al.*, 2007). In contrast, NOS blockade enhanced cutaneous VC only in response to low doses of NE (i.e., 10^{-12}, 10^{-10} M) (Bruck *et al.*, 2001). Thus, NO clearly has an effect on cutaneous vessels during VC; however, whether NOS blockade has the contrasting effect of enhancing VC is unclear. Moreover, whether on not this response is affected by healthy aging is unknown.

In the study, "Tetrahydrobiopterin does not Affect End-Organ Responsiveness to Norepinephrine Mediated Vasoconstriction in Aged Skin" (chapter 6), additional data was collected and analyzed at a third and fourth microdialysis site but not included in chapter 6 because of the questionable efficaciousness of a batch of L-NAME ("old" L-NAME) used in the study. The purpose for collecting these data was to test the hypothesis that localized NOS blockade with L-NAME would not significantly affect end-organ responsiveness to exogenous NE in young and aged skin.

Methods

Because the appended data was collected during the full study that is reported in chapter 6, all methodological procedures (subjects, inclusion criteria, microdialysis instrumentation, techniques of assessing blood flow, NE dose-response protocol, and statistical analyses) were the same as chapter 6. The only variation from this methodology was the addition of a third and fourth microdialysis fiber that were inserted into the ventral forearm during the insertion of the other two fibers. These additional two fibers were perfused with 1) 10 mM N^G-nitro-L-arginine methyl ester (L-NAME) and 2) 5 mM BH4 + 10 mM L-NAME at a rate of 2 μL min^{-1}. Following hyperemia associated with fiber placement, the NE dose-response protocol commenced. Details for which can be referenced in chapter 6.

Results

These data were collected prior to discovering that some of the L-NAME ("old" L-NAME) that was used in the study proved ineffective in predictably attenuating the local heating response. However, Figure A1 illustrates in young (panel A) and older (panel B) subjects that there were no observable differences when comparing the NE-mediated VC response between the "old" L-NAME and a different or "new" batch. The reason for this discrepancy in efficaciousness between the local heating and our protocol may be that L-NAME was perfused over a long enough time to reach an effective dose or perhaps the optimal concentration is much lower than that required for local heating.

Figure A2 illustrates the VC response to 6 different concentrations of NE (10^{-12}, 10^{-10}, 10^{-8}, 10^{-6}, 10^{-4}, 10^{-2} M) in L-NAME pretreated sites ("old" and "new" batches were pooled for analysis). Panel A demonstrates that L-NAME augmented the VC response at most NE doses in aged skin (10^{-12}: $P<0.01$; 10^{-10}: $P<0.01$; 10^{-8}: $P=0.01$; 10^{-6}: $P=0.01$; 10^{-4}: $P=0.01$; 10^{-2}: $P= 0.06$). In contrast (panel B), L-NAME did not affect the VC response

in young skin (10^{-12}: P=0.91; 10^{-10}: P=0.77; 10^{-8}: P=0.84; 10^{-6}: P=0.48; 10^{-4}: P=0.69; 10^{-2}: P= 0.66).

The VC response at the BH_4 + L-NAME site was augmented compared to the control site in older (P<0.01) but not young subjects (P>0.05). Combining BH_4 with L-NAME did not further augment the VC response in young or older individuals more than L-NAME alone (P>0.05).

Discussion

The primary result from these additional sites was that NOS inhibition selectively augmented the VC response to NE in aged but not young skin. We have previously demonstrated (chapter 3) that L-NAME did not affect the VC response to cold stress in young or older subjects. Conflicting evidence exists indicating that L-NAME enhances the cold-induced VC response; however, adenosine was required to increase the signal gain to observe this effect (Shibasaki *et al.*, 2007). This result may be obfuscated by the fact that adenosine promotes the release of NO in peripheral vascular beds (Clifford & Hellsten, 2004). However, the relatively lower baseline used in the present study may have attenuated any subsequent response to NE (Hodges *et al.*, 2007); although this is likely mimimized particularly at more dilute concentrations of NE.

Our data suggests that NOS inhibition selectively augments the VC response in aged but not young skin. It is improbable that this is due to inhibition of a greater NO component in aged skin considering that the production of NO in aged skin is limited by decreased L-arginine bioavailability, upregulated arginase, and elevated oxidative stress (Holowatz *et al.*, 2006a, b). A more plausible explanation may be that NOS inhibition is disinhibiting a greater ROCK component in aged skin. This is supported by the observation that ROCK inhibition attenuates the VC response to a greater extent in aged skin in response to local (Thompson-Torgerson *et al.*, 2007b) or whole-body cooling (chapter 5). Thus, aged skin relies more heavily on ROCK to elicit cutaneous VC.

Constriction through the ROCK pathway occurs by 1) α_{2C} translocation to the plasma membrane of VSM, 2) inhibition of MLC phosphatase (i.e., Ca^{2+} sensitization), and 3) the mutual inhibition of NOS. In combination with what we have found previously (chapter 5), the latter mechanism may explain the selective augmentation of the VC response with L-NAME in aged skin. The balance between ROCK and NOS is tilted toward ROCK in aged skin. ROCK decreases NO bioavailability by decreasing NOS expression and activity as well as stimulating arginase (Ming *et al.*, 2002; Ming *et al.*, 2004). Also, the age-associated elevation is oxidative stress may also quench NO before it can bind with soluble guanylate cyclase. Thus, further inhibition of NOS with L-NAME may disinhibit an already upregulated ROCK pathway in aged skin.

The effects of NOS inhibition on cutaneous VC may primarily affect postjunctional noradrenergic mechanisms. In isolated rat mesenteric arteries, incubation of NO donors reduced NE bioavailability yet did not affect NPY or ATP concentration (Kolo *et al.*, 2004). Cutaneous vessels from rabbits demonstrated that NO had a negligible influence on nerve-induced VC compared with NE-mediated VC (Smith *et al.*, 1999). Cumulatively, these data suggest that NO has negligible effects on cotransmitter function or presynpatic VC mechanisms. Thus, the effects of NO are primarily limited to postjunctional noradrenergic mechanisms, which supports a ROCK disinhibition component in eliciting an augmented constrictor response to NE.

In summary, these data indicate that the augmentation in NE-mediated VC at NOS-blocked sites is specific to aged skin, which contrasts with what has been demonstrated in young subjects infused with L-NAME (Shibasaki *et al.*, 2007). Because ROCK has been previously demonstrated to be upregulated in aged skin (chapter 5) (Thompson-Torgerson *et al.*, 2007b), it is plausible that NOS blockade is disinhibiting this upregulated ROCK component in aged skin.

Figure **A.1**

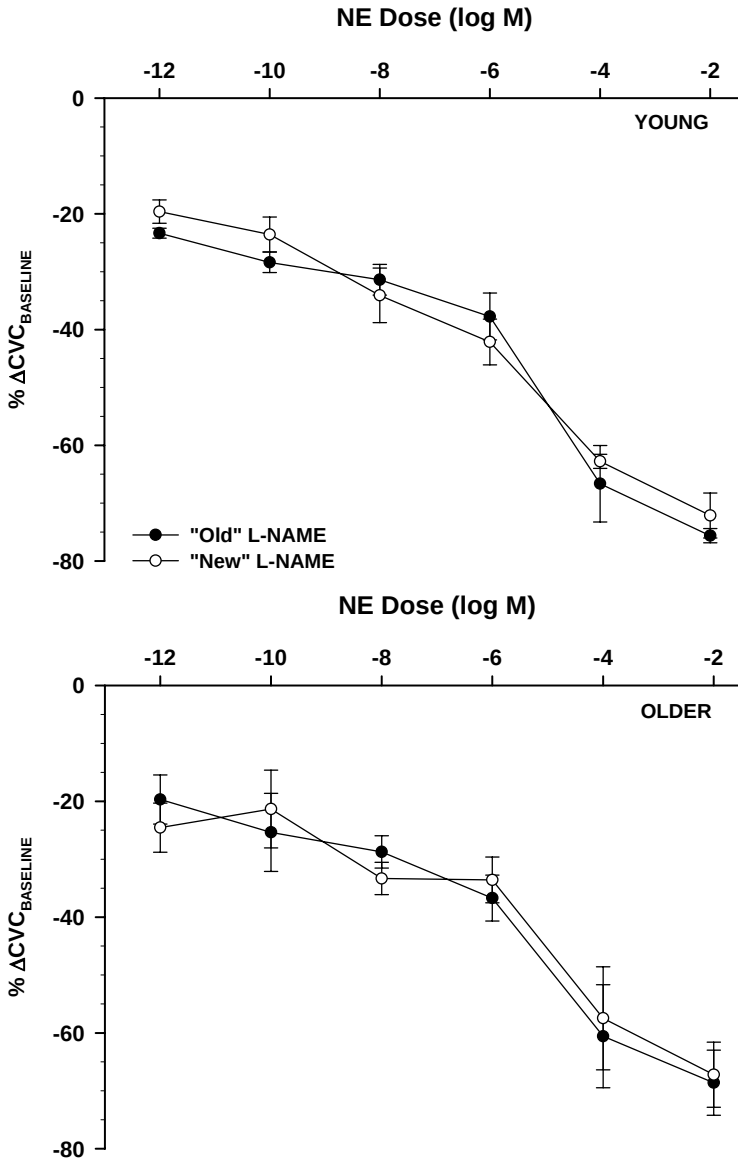

Figure **A.1**: Effect of different batches of L-NAME on the average cutaneous vasoconstrictor response to sequential 5 min infusions of various concentrations of norepinephrine. The mean percent change from baseline CVC at the control and L-NAME pretreated sites of young (panel A) (n = 10; 5 received the "old" batch; 5 received the "new" batch) and older (panel B) (n = 10; 7 received the "old" batch while 3 received "new" batch) subjects. Prior to NE infusion, both sites received pretreatment of bretylium tosylate to block presynaptic adrenergic function.

Figure **A.2**

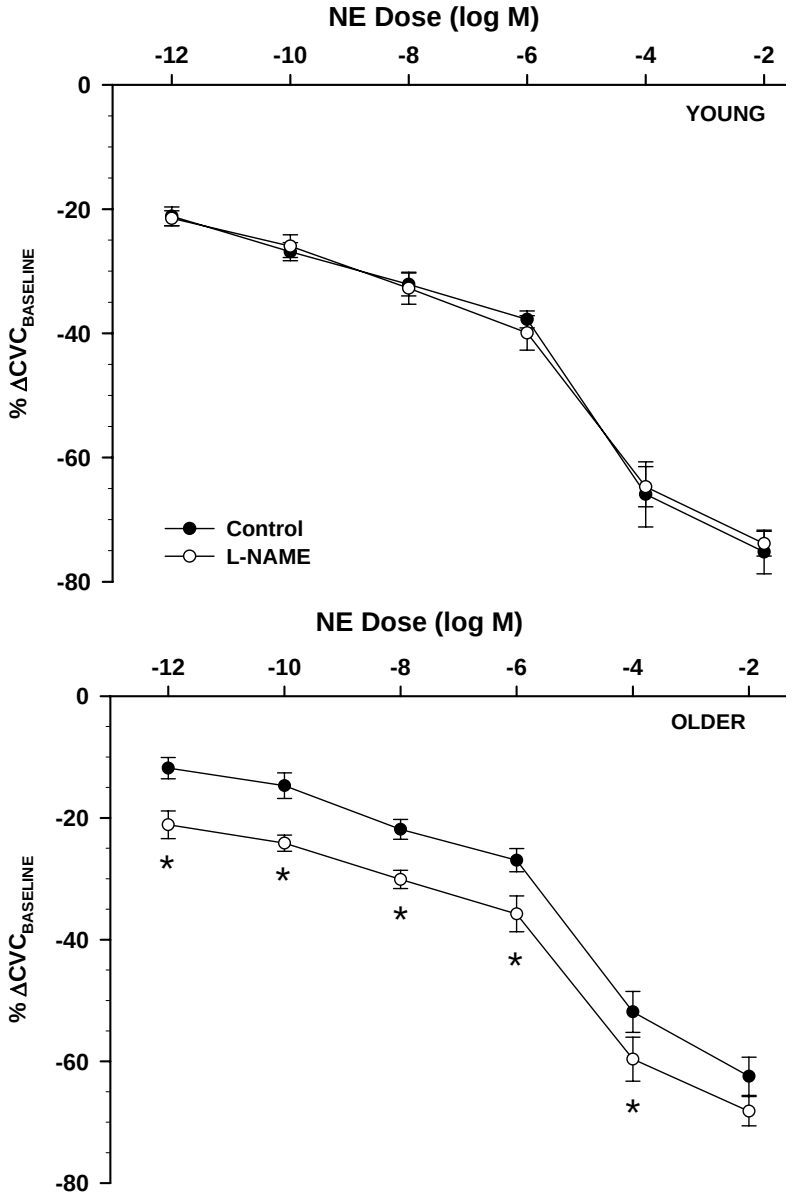

Figure **A.2**: Effect of L-NAME (pooled from "old" and "new" batches) on the average cutaneous vasoconstriction to sequential 5 min infusions of various concentrations of norepinephrine. The mean percent change from baseline CVC at the control and L-NAME pretreated sites of young (n = 10) (panel A) and older (n = 10) (panel B) subjects. Both sites received pretreatment of bretylium tosylate to block presynaptic adrenergic function. **P*<0.05 versus control.

Appendix B

INFORMED CONSENTS

Informed Consent form for chapter 3:

Informed Consent Form for Biomedical Research

The Pennsylvania State University

Title of Project:	***Age and Sympathetic Cotransmitter Function in Human Skin- Part 1***
Principal Investigator:	**James A. Lang, M.S.** **229 Noll Lab** **jal493@psu.edu; 814-863-2948**
Advisor:	**W. Larry Kenney, Ph.D.** **102 Noll Lab** **w7k@psu.edu; 814-863-1672**
Other Investigator(s):	**Lacy A. Holowatz, Ph.D.** **204 Noll Lab** **lma191@psu.edu; 814-867-1781** **Jane Pierzga, M.S.** **228 Noll Lab** **jmp141@psu.edu; 814-865-1236**

This is to certify that I, _____ have been given the following information with respect to my participation as a volunteer in a program of investigation under the supervision of Dr. W. Larry Kenney.

Note: This study has 3 parts. Part 1 has a main experiment and optional pilot experiment.

Part 2 has one experiment. Part 3 has one experiment. You do not have to participate with all parts. If you were to complete all parts, you could have as many a 4 experiments total. You must read and sign separate informed consent forms to participate with Parts 1, 2, and 3.

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

When you are exposed to the cold, your body wants to save heat. It saves heat by making the blood vessels in your skin get smaller (constriction). This reduces the amount of warm blood flowing through your cold skin. As you grow older, your skin's blood vessels are less able to constrict. This causes older people to have a higher skin blood flow in the cold and makes them more prone to heat loss. Our project looks at how cooling causes blood vessels to constrict and how the ability to constrict changes when getting older. To do this, we measure the flow in the blood vessels by shining a weak laser light onto your skin. Also, we use a special technique called "microdialysis" (MD). MD involves placing very thin plastic tubing between the layers of your skin. The largest part of the tubing is about 6x the diameter of a human hair. We pump fluid like that found in your body's tissues (Lactated Ringer's solution) through the thin tubing. The thin tubing acts like the very small blood vessels in your skin by allowing the exchange of substances between the fluid in the tubing and the body fluid in the surrounding layers of your skin. During the experiment, we will add substances to the fluid in the tubing. The substances can only reach a 2.5 cm^2 (0.4 $inch^2$), nickel-sized area of skin. Most of these substances keep your blood vessels from constricting. They do this by blocking some of the natural substances that your body makes that cause your vessels to constrict. We have the ability to manipulate any of these substances in your skin in order to look at the effects of your skin's blood vessel response to cooling; however, we will manipulate only a few of these substances in any one visit/experiment. These substances will help us determine how the skin responds to whole body cooling in healthy volunteers of different ages.

2. <u>Procedures</u>: *You will participate on the circled days. Please read the descriptions of the circled days. Then write your initials by the circled days.*

_____ initial <u>Day 1: Screening</u>

You will report to the nurses' station at the General Clinical Research Center for your appointment. The GCRC staff measures your blood pressure, height and weight, reviews your medical history, conducts a physical exam, and measures the electrical activity of the heart using ECG (12 leads will be taped to the chest to obtain the measurement). GCRC staff will also draw 15 mL (1 Tbsp) of blood from a vein in your arm to measure the concentrations of various substances including cholesterol and glucose. If you take a thyroid drug, you need to supply the results of a thyroid test taken within the past 6 months, because unusual thyroid levels can affect how blood vessels in your skin respond to cooling. If you do not have thyroid test results, a blood sample will be sent to a lab that tests it for thyroid levels. The lab destroys the

sample after testing. If you are a woman of childbearing age, you will submit a urine sample for a pregnancy test. A positive result will result will exclude you from the study. We may measure the thickness of folds of skin at several places on your body to determine your percent body fat.

_____**initial** **Day 2: Experiment** **(If you are participating in experimental protocol)**

_____initial a) When you arrive at the laboratory, the GCRC checks your heart rate and blood pressure. Then you wash your forearm and pat it dry.

_____initial b) We will escort you to the Microdialysis Lab (room 205 Noll Lab) where the remainder of the experiment will be completed. You will put on a whole-body suit that has tubing lining the inside. Men wear shorts under the suit. Women wear shorts and a sports bra.

_____initial c) You will have wires taped to 6 places on your body to measure skin temperature.

_____initial d) You rest on a bed for the experiment, and we place a tight band around your upper arm so your veins are easily seen. We mark places on your arm 2.5 cm (1 inch) apart where the tubing will enter and exit your skin. You will have from 5 sets of marks on your arm. After the tight band is removed, we will clean your arm with an orange fluid called Betadine and alcohol. We will 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 will travel between the layers of skin for 2.5 cm (1 inch) and leave your skin at the matching exit mark. The tubing is threaded through the needle. Next, we withdraw the needle, leaving the tubing in your skin. Any redness of your skin will subside in 30-45 minutes.

_____initial e) While we are waiting for the redness on your arm to go away, we will begin to run lukewarm water through the whole-body suit to keep your skin at a normal temperature (34^{o}C or 93^{o}F). Also, during this time we will start clear fluid (resembling body fluid) flowing through all the sets of tubing. The clear fluid flows through the tubing in your skin during the whole experiment. We will add the following substances to the clear fluid flowing through the sites on your arm:

- site 1 = clear fluid only
- site 2 = tetrahydrobiopterin
- site 3 = tetrahydrobiopterin + vitamin C + yohimbine + propranolol
- site 4 = L-NAME
- site 5 = tetrahydrobiopterin + L-NAME

After these fluids have flowed through the tubing for about 60 minutes, the redness in your skin should be gone, and we will continue with the rest of the experiment. During the experiment, we also measure your blood pressure by inflating a cuff on your upper arm and listening to your pulse with a stethoscope at the inside of your elbow.

_____initial f) After the redness in your skin has gone away, we tape a pencil-sized probe over each test site to measure the blood flow in the skin. The probes use a weak laser light to measure this

blood flow through the whole experiment. The laser light probes will be held in place with special metal holders that control the temperature of your skin.

_____initial g) We will set the temperature controllers to keep your skin at a comfortable temperature (34°C or 93°F)

_____initial h) After 40 min to allow your blood vessels to return to normal, cool water will run through the suit for 30 minutes so that your skin reaches 30.5°C (86.9°F). Your skin temperature will be held an additional 10 minutes at 30.5°C. During the entire cooling part of the experiment (40 min), you will have a small probe placed under the tongue to measure your body core temperature. After cooling, re-warming to a more comfortable temperature will follow.

_____initial i) At site 3, norepinephrine will be added to the fluid flowing through the tubing in your skin for 10 minutes to test whether the constriction of the small vessels at that site was blocked by the yohimbine and propranolol that was infused there previously

_____initial j) Then tyramine will be added to all sites for an additional 20 minutes. This will cause your small vessels in the skin around each site to constrict.

_____initial l) Norepinephrine will then be infused at all sites for 20 minutes to maximally constrict the small vessels in the skin.

_____initial m) We will warm the temperature controllers to 42°C (108°F) and switch to fluid with only sodium nitroprusside flowing through the tubing at all sites for about 40 minutes. Heating and adding sodium nitroprusside to the fluid helps the blood vessels in your skin to get as big as they can (dilation).

_____initial n) We will remove the MD tubing from your skin and place sterile bandages over the sites. If you want, we can also place a bag of ice on your arm for 10 minutes to reduce any bruising that may occur. Finally, a member of the GCRC medical staff measures your blood pressure and heart rate before you leave.

_____ initial <u>OPTIONAL: Pilot experiment</u> (If you are participating in pilot work)

_____initial a) When you arrive at the laboratory, the GCRC checks your heart rate and blood pressure. Then you wash your forearm and pat it dry.

_____initial b) We will escort you to the Microdialysis Lab (room 205 Noll Lab) where the remainder of the experiment will be completed. You will put on a whole-body suit that has tubing lining the inside. Men wear shorts under the suit. Women wear shorts and a sports bra.

_____initial c) You will have wires taped to 6 places on my body to measure skin temperature.

_____initial d) You rest on a bed for the experiment, and we place a tight band around your upper arm so your veins are easily seen. We mark places on your arm 2.5 cm (1 inch) apart where the tubing will enter and exit your skin. You will have from 2 to 6 sets of marks on your arm. After the tight band is removed, we will clean your arm with an orange fluid called Betadine and alcohol. We will 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 will travel between the layers of skin for 2.5 cm (1 inch) and leave your skin at the matching exit mark. The tubing is threaded through the needle. Next, we withdraw the needle, leaving the tubing in your skin. Any redness of your skin will subside in 30-45 minutes.

_____initial e) While we are waiting for the redness on your arm to go away, we will begin to run lukewarm water through the whole-body suit to keep your skin at a normal temperature (34^{o}C or 93^{o}F). Also, during this time we will start clear fluid (resembling body fluid) flowing through all the sets of tubing. The clear fluid flows through the tubing in your skin during the whole experiment. We will add substances to the clear fluid flowing through the sites on your arm, so that site 1 only has clear fluid flowing through it and the remaining sites will have up to 5 different types of substances flowing through the tubing in your arm (one type of substance at each site on your arm):

_____ (initial) If you are participating in a pilot protocol designed to look at the role of **ATP** in the skin, you may have up to 5 of the following different types of substances selected for each site:

_____(initial) One site has fluid + yohimbine + propranolol (Y/P)
_____(initial) One site has fluid + L-NAME
_____(initial) One site has fluid + theophylline
_____(initial) One site has fluid + L-NAME + theophylline (L/T)
_____(initial) One site has fluid + suramin
_____(initial) One site has fluid + suramin + Y/P + BIBP-3226
_____(initial) One site has fluid + suramin + BIBP-3226
_____(initial) One site has fluid + suramin + Y/P
_____(initial) One site has fluid + Y/P + BIBP-3226

_____ (initial) If you are participating in a pilot protocol designed to look at the role of **NPY** in the skin, you may have up to 5 of the following different types of substances selected for each site:

_____(initial) One site has fluid + yohimbine + propranolol (Y/P)

_____(initial) One site has fluid + BIBP-3226

_____(initial) One site has fluid + suramin

_____(initial) One site has fluid + NPY

_____(initial) One site has fluid + NPY + BIBP-3226

_____ (initial) If you are participating in a pilot protocol designed to look at the role of the **interaction of ATP and NPY** in the skin, you may have up to 5 of the following different types of substances selected for each site:

_____(initial) One site has fluid + reserpine

_____(initial) One site has fluid + reserpine + Y/P

_____(initial) One site has fluid + reserpine + BIBP-3226

_____(initial) One site has fluid + reserpine + suramin

_____(initial) One site has fluid + reserpine + theophylline

_____(initial) One site has fluid + reserpine + L-NAME

_____(initial) One site has fluid + reserpine + L-NAME + theophylline

_____(initial) One site has fluid + tyramine

_____initial f) After these fluid have flowed through the tubing for about 60 minutes, the redness in your skin should be gone, and we will continue with the rest of the experiment. During the experiment, we also measure your blood pressure by inflating a cuff on your upper arm and listening to your pulse with a stethoscope at the inside of your elbow.

_____initial g) After the redness in your skin has gone away, we tape a pencil-sized probe over each test site to measure the blood flow in the skin. The probes use a weak laser light to measure this blood flow through the whole experiment. The laser light probes will be held in place on your skin with either plain metal holders or special metal holders that also control the temperature of your skin.

_____(initial) The laser light probes are held in place in plain holders

_____(initial) The laser light probes are held in place with special metal holders that can also control the temperature of your skin

_____initial h) We will set the temperature controllers to keep your skin at a comfortable temperature (34°C or 93°F)

_____initial i) At all sites, the following substances will be added to the fluid flowing through the tubing in your skin for 10-40 minutes:

_____(initial) Tyramine
_____(initial) Suramin
_____(initial) ATP
_____(initial) NPY
_____(initial) Norepinephrine
_____(initial) Y/P

_____initial j) After 40 min to allow your blood vessels to return to normal, cool water will run through the suit for 30 minutes so that your skin reaches 30.5°C (86.9°F). Your skin temperature will be held an additional 10 minutes at 30.5°C. During the entire cooling part of the experiment (40 min), you will have a small probe placed under the tongue to measure your body core temperature. After cooling, rewarming to a more comfortable temperature will follow.

_____initial k) We will warm the temperature controllers to 42°C (108°F) and switch to fluid with only sodium nitroprusside flowing through the tubing at all sites for about 40 mintues. Heating and adding sodium nitroprusside to the fluid helps the blood vessels in your skin to get as big as they can (dilation).

_____initial l) We will remove the MD tubing from your skin and place sterile bandages over the sites. If you want, we can also place a bag of ice on your arm for 10 minutes to reduce any bruising that may occur. Finally, a member of the GCRC medical staff measures your blood pressure and heart rate before you leave.

3. <u>Discomforts and risks</u>: ***You will experience the circled procedures. Please read the descriptions of the circled procedures. Then write your initials by the circled procedures.***

_____initial a) <u>Microdialysis:</u> 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. But you may feel a little pain when the tubing is removed from your skin. You may become lightheaded or may faint. Although rare, it is possible for the tubing to break during removal from your skin. In that case, we remove the tubing by pulling on the other end of it. This produces no additional risk for you. Even rarer, the tubing could break so that a piece of the tubing is left under

your skin. In this case, any tubing remaining in your skin would be treated like a splinter. If slight bleeding occurs, applying mild pressure with sterile gauze will stop it. Infection is possible, but sterile techniques and supplies like those used in hospitals will be used to keep the risk minimal. We apply a sterile bandage to the site after the experiment. We will tell you how to take care of the site.

Note: In the approximately 1500 to 2000 fibers that have been placed in our lab, we have never had a subject report an infection at the microdialysis sites.

_____initial b) Fluid flowing through the tubing: The substances flowing through the tubing only go to a 2.5 cm^2 (0.4 $inch^2$) area of skin at each tubing site. The amount that enters the skin is very small. However, there is a minimal chance of having a bad reaction to the substances. This reaction could produce redness, itching, rash, and/or swelling limited to the small area of skin around the microdialysis sites. A worse reaction could also cause more diffuse swelling/rash, fever, breathing problems, changes in pulse, convulsions, and/or collapse. Other researchers have used these substances with microdialysis in skin. The researchers have not reported that these substances caused bad reactions. If a bad reaction should occur, medical help will be summoned. After the experiment, you will receive an instruction sheet on how to care for the microdialysis sites.

_____(initial) *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 your body's natural fluids. A bad reaction to this fluid is highly unlikely.

_____(initial) *Yohimbine, Propranolol, BIBP-3226, Suramin, Theophylline, L-NAME, Reserpine, Tyramine*: These substances stop the action of your body's natural chemicals upon the blood vessels in the skin. A small amount of these substances will enter the skin around the tubing. This only affects the blood flow in the vessels in that small area of skin. The effect of these substances is gone within an hour after the experiment. These substances have been used in microdialysis and/or in clinical medical treatment before. The researchers and doctors using these substances have not reported that these substances caused bad reactions in microdialysis.

_____(initial) *Norepinephrine, Neuropeptide Y, ATP, Tetrahydrobiopterin, Vitamin C*: Norepinephrine and neuropeptide Y are natural chemicals made by the nerves in the skin. Your body constantly makes ATP to provide your cells with energy. Tetrahydrobiopterin is produced naturally in the body and acts to preserve norepinephrine. Vitamin C occurs naturally in foods and preserves norepinephrine. Only a small amount of these substances will enter the skin around the tubing. This only affects the blood flow in the vessels in that small area of skin. The effect of these substances is gone within an hour after the experiment. These substances have been used in microdialysis and/or in clinical medical treatment before. The researchers and doctors using these substances have not reported that these substances caused bad reactions in microdialysis.

_____(initial) *Sodium Nitroprusside (SNP)*: Only a small amount of SNP will enter your skin around the tubing. SNP increases the blood flow in the vessels and reddens that small area of skin. This effect is gone within an hour after the experiment. Other researchers have used SNP with microdialysis in skin. They have reported no bad reactions with SNP.

_____initial c) 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.

_____initial d) Blood Pressure: We measure your blood pressure using the method common in a doctor's office. A cuff will be inflated on your upper arm. As the cuff slowly deflates, we listen with a stethoscope at the bend in your elbow. During the short time the cuff is inflated, your arm may feel numb.

_____initial e) Betadine: Hospitals and researchers use this orange-colored fluid to clean and sterilize the skin. You could have a bad reaction to Betadine if you are allergic to iodine or shellfish. You will inform us if you have these allergies so that we will use alcohol instead. A bad reaction could cause redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or collapse.

_____initial f) Latex: Some gloves and medical materials are made of latex rubber. You will inform us if you are allergic to latex so that we will use non-latex gloves and research materials.

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

_____initial h) Whole body cooling: Cooling may cause goose bumps, shivering and chilly sensations. The 10-minute period where skin temperature is kept at 30.5°C (86.9°F) is the coldest part of the study. The rest of the cooling is milder. If your body feels too cold, you will tell us, and we will reduce or stop the cooling.

_____initial i) Blood draw: Blood draws may cause anxiety, mild pain, swelling, nausea, lightheadedness, fainting, or bleeding. There is a slight chance of infection. Competent GCRC or GCRC-approved staff using standard precautions with aseptic techniques will perform the blood draw. You will be reclining for the procedure. You may decline the blood draw if you feel uncomfortable at any time during or before the procedure.

_____initial j) ECG: This machine measures the electrical activity of my heart. There have been no adverse effects from this measure. The tape may irritate your skin.

_____initial k) Body core temperature: Body core temperature is measured during the cooling part of the experiment with a small probe placed under the tongue. If this causes discomfort, you will tell us and we can either reposition or remove the probe.

_____initial j) 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.

4. a. Benefits to me: You will receive a medical screening that could inform you about your health. You will know what your blood cholesterol levels, fasting blood glucose levels, and blood pressure are. Also, you could gain knowledge about how your body works.

b. Potential benefits to society: We will find out why older people are less able to withstand a cold stress. These results could suggest ways to prevent or treat the changes that make older people prone to greater loss of body heat in the cold. This could help prevent illness and death related to cold in this age group.

5. Duration/time of the procedures and study: ***The circled statements apply to you. Please read the circled statements. Then write your initials by the circled statements.***

_____ initial a) Day 1 (Screening): typically about ½ hour, no longer than 1 hour

_____ initial b) Day 2 (Experiment): typically about 5 ½ hours, no longer than 6 hours

_____ initial c) Day 2 (Optional Pilot Work): The time spent in the pilot experiment can vary depending on the parts of the study in which you are involved. You should not expect the time on any given day to exceed 6 hours.

Note: You are not obligated to participate in both pilot and experimental protocols. For example, participating in the experimental protocol does not obligate you to participate in the pilot work and vice versa. However, if you wish to participate in both experimental and pilot protocols, you will perform these experiments on two separate occasions on the same forearm provided that enough time (approximately 5 days) has elapsed between experiments. If you come in for a second experiment before the 5-day period, the opposite forearm will be used.

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

7. Statement of confidentiality: Volunteers will be 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 and The Penn State University Biomedical Institutional Review Board may review records related to this project.

8. Right to ask questions: Please contact James Lang at work (814) 863-2948 or at home (814-466-6961) with questions, complaints or concerns about this research. You may also contact Jane Pierzga at work (814-865-1236) or at home (814-692-4720). You can also call these numbers if you feel this study has harmed you. If you have any questions, concerns, problems about your rights as a research participant or would like to offer input, please contact 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 principal investigator.

9. Payment for participation: The circled statements apply to you. Please read the circled statements. Then write your initials by the circled statements.

_____initial a) You will receive $10.00 for each microdialysis probe inserted into your skin (maximum $50.00). You will receive another $20.00 for completing the study. In the event that you do not complete the full experiment, you are paid an amount of money equal to the part of the experiment that you do complete. For example, if you complete only half of the experiment but have all 5 microdialysis probes in place, you will receive $50.00 ($10.00 x 5) + $10.00 ($20.00 x ½ of the total experiment) = $60.00 (Total maximum payment: $70.00). You may be asked if you would like to repeat the experiment on another day. If you agree to repeat the experiment, you will be paid for the repeated trial as stated above. You will also receive a lab t-shirt.

Note: 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. Voluntary participation: Your decision to be in this research is voluntary. You can stop at any time. You do not have to answer any questions you do not want to answer. Refusal to take part in or withdrawing from this study will involve no penalty or loss of benefits you would receive otherwise. Your participation in the study may be ended without your consent if the researcher deems that your health or behavior adversely affects the study or increases risks to you beyond those approved by The Penn State University Biomedical Institutional Review Board and agreed upon by you in this document.

11. Injury Clause: In the unlikely event you become injured as a result of your participation in this study, medical care is available. 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. In the event of a research related injury, you may call James Lang (office: 814-863-2948; home: 814-466-6961) or Jane Pierzga (office: 814-865-1236; home: 814-692-4720).

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

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

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

_____ _____

Participant Signature Date

_____ _____

Person Obtaining Consent Date

Addendum informed consent form for chapter 3:

This is an addendum informed consent form **Part 1**. All information in the first informed consent **Part 1** that you signed still applies except as described below:

Reason for the Addendum: The purpose of this project remains the same. We will look at how cooling causes blood vessels to constrict and how the ability to constrict changes when you get older. We conducted pilot studies at the start of this project. The results of the pilot studies suggested minor changes that would allow us to better fulfill the purpose of this project. Therefore, we made minor changes to the procedure described for "Day 2" on the first consent form. We describe the new procedure for "Day 2" below. The researcher will discuss the changes with you. You may choose whether you wish to help with this new procedure.

_____**initial Day 2: Experiment (If you are participating in experimental protocol)**

_____initial a) When you arrive at the laboratory, the GCRC checks your heart rate and blood pressure. Then you wash your forearm and pat it dry.

_____initial b) We will escort you to the Microdialysis Lab (room 205 Noll Lab) where the remainder of the experiment will be completed. You will put on a whole-body suit that has tubing lining the inside. Men wear shorts under the suit. Women wear shorts and a sports bra.

_____initial c) You will have wires taped to 6 places on your body to measure skin temperature.

_____initial d) You rest on a bed for the experiment, and we place a tight band around your upper arm so your veins are easily seen. We mark places on your arm 2.5 cm (1 inch) apart where the tubing will enter and exit your skin. You will have from 5 sets of marks on your arm. After the tight band is removed, we will clean your arm with an orange fluid called Betadine and alcohol. We will 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 will travel between the layers of skin for 2.5 cm (1 inch) and leave your skin at the matching exit mark. The tubing is threaded through the needle. Next, we withdraw the needle, leaving the tubing in your skin. Any redness of your skin will subside in 30-45 minutes.

_____initial e) While we are waiting for the redness on your arm to go away, we will begin to run lukewarm water through the whole-body suit to keep your skin at a normal temperature (34^{o}C or 93^{o}F). Also, during this time we will start clear fluid (resembling body fluid) flowing through all the sets of tubing. The clear fluid flows through the tubing in your skin during the whole experiment. We will add the following substances to the clear fluid flowing through the sites on your arm:

- site 1 = clear fluid only
- site 2 = tetrahydrobiopterin
- site 3 = tetrahydrobiopterin + vitamin C + yohimbine + propranolol
- site 4 = L-NAME
- site 5 = tetrahydrobiopterin + L-NAME

After these fluids have flowed through the tubing for about 60 minutes, the redness in your skin should be gone, and we will continue with the rest of the experiment. During the experiment, we also measure your blood pressure by inflating a cuff on your upper arm and listening to your pulse with a stethoscope at the inside of your elbow.

_____initial f) After the redness in your skin has gone away, we tape a pencil-sized probe over each test site to measure the blood flow in the skin. The probes use a weak laser light to measure this blood flow through the whole experiment. The laser light probes will be held in place with special metal holders that control the temperature of your skin.

_____initial g) We will set the temperature controllers to keep your skin at a comfortable temperature (34°C or 93°F)

_____initial h) After 40 min to allow your blood vessels to return to normal, cool water will run through the suit for 30 minutes so that your skin reaches 30.5°C (86.9°F). Your skin temperature will be held an additional 10 minutes at 30.5°C. During the entire cooling part of the experiment (40 min),

you will have a small probe placed under the tongue to measure your body core temperature. After cooling, re-warming to a more comfortable temperature will follow.

_____initial i) At site 3, norepinephrine will be added to the fluid flowing through the tubing in your skin for 10 minutes to test whether the constriction of the small vessels at that site was blocked by the yohimbine and propranolol that was infused there previously

_____initial j) Then tyramine will be added to all sites for an additional 20 minutes. This will cause your small vessels in the skin around each site to constrict.

_____initial k) Norepinephrine will then be infused at all sites for 20 minutes to maximally constrict the small vessels in the skin.

_____initial l) We will warm the temperature controllers to 42°C (108°F) and switch to fluid with only sodium nitroprusside flowing through the tubing at all sites for about 40 minutes. Heating and adding sodium nitroprusside to the fluid help the blood vessels in your skin to get as big as they can (dilation).

_____initial m) We will remove the MD tubing from your skin and place sterile bandages over the sites. If you want, we can also place a bag of ice on your arm for 10 minutes to reduce any bruising that may occur. Finally, a member of the GCRC medical staff measures your blood pressure and heart rate before you leave.

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

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

_____ _____

Participant Signature Date

_____ _____

Person Obtaining Consent Date

Informed Consent form for chapter 4:

Informed Consent Form for Biomedical Research

The Pennsylvania State University

Title of Project: ***Age and Sympathetic Cotransmitter Function in Human Skin – Part 2***

Principal Investigator: **James A. Lang, M.S.**
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Advisor: **W. Larry Kenney, Ph.D.**
102 Noll Lab
w7k@psu.edu; 814-863-1672

Other Investigator(s): **Lacy A. Holowatz, Ph.D.**
204 Noll Lab
lma191@psu.edu; 814-867-1781

Jane Pierzga, M.S.
228 Noll Lab
jmp141@psu.edu; 814-865-1236

This is to certify that I, _____ have been given the following information with respect to my participation as a volunteer in a program of investigation under the supervision of Dr. W. Larry Kenney.

Note: This study has 3 parts. Part 1 has a main experiment and optional pilot experiment. Part 2 has one experiment. Part 3 has one experiment. You do not have to participate with all parts. If you were to complete all parts, you could have as many a 4 experiments total. You must read and sign separate informed consent forms to participate with Parts 1, 2, and 3.

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

When you are exposed to the cold, your body wants to save heat. It saves heat by making the blood vessels in your skin get smaller (constriction). This reduces the amount of warm blood flowing through your cold skin. As you grow older, your skin's blood vessels are less able to constrict. This causes older people to have a higher skin blood flow in the cold and makes them more prone to heat loss. Our project looks at how cooling causes blood vessels to constrict and how the ability to constrict changes when getting older. To do this, we measure the flow in the blood vessels by shining a weak laser light onto your skin. Also, we use a special technique called "microdialysis" (MD). MD involves placing very thin plastic tubing between the layers of your skin. The largest part of the tubing is about 6x the diameter of a human hair. We pump fluid like that found in your body's tissues (Lactated Ringer's solution) through the thin tubing. The thin tubing acts like the very small blood vessels in your skin by allowing the exchange of substances between the fluid in the tubing and the body fluid in the surrounding layers of your skin. During the experiment, we will add substances to the fluid in the tubing. The substances can only reach a 2.5 cm^2 (0.4 $inch^2$), nickel-sized area of skin. Most of these substances keep your blood vessels from constricting. They do this by blocking some of the natural substances that your body makes that cause your vessels to constrict. We have the ability to manipulate any of these substances in your skin in order to look at the effects of your skin's blood vessel response to cooling; however, we will manipulate only a few of these substances in any one visit/experiment. These substances will help us determine how the skin responds to whole body cooling in healthy volunteers of different ages.

2. <u>Procedures</u>: ***You will participate on the circled days. Please read the descriptions of the circled days. Then write your initials by the circled days.***

_____ initial <u>Day 1: Screening</u>

You will report to the nurses' station at the General Clinical Research Center for your appointment. The GCRC staff measures your blood pressure, height and weight, reviews your medical history, conducts a physical exam, and measures the electrical activity of the heart using ECG (12 leads will be taped to the chest to obtain the measurement). GCRC staff will also draw 15 mL (1 Tbsp) of blood from a vein in your arm to measure the concentrations of various substances including cholesterol and glucose. If you take a thyroid drug, you need to supply the results of a thyroid test taken within the past 6 months, because unusual thyroid levels can affect how blood vessels in your skin respond to cooling. If you do not have thyroid test results, a blood sample will be sent to a lab that tests it for thyroid levels. The lab destroys the sample after testing. If you are a woman of childbearing age, you will submit a urine sample for a pregnancy test. A positive result will result will exclude you from the study. We may measure the thickness of folds of skin at several places on your body to determine your percent body fat.

_____**initial** **Day 2: Experiment - "Role of Tyrosine Availability in Vasoconstriction"**

_____initial a) When you arrive at the laboratory, the GCRC checks your heart rate and blood pressure. Then you wash your forearm and pat it dry.

_____initial b) We will escort you to the Microdialysis Lab (room 205 Noll Lab) where the remainder of the experiment will be completed. You will put on a whole-body suit that has tubing lining the inside. Men wear shorts under the suit. Women wear shorts and a sports bra.

_____initial c) You will have wires taped to 6 places on your body to measure skin temperature.

_____initial d) You will rest on a bed for the experiment, and we place a tight band around your upper arm so your veins are easily seen. We mark places on your arm 2.5 cm (1 inch) apart where the tubing will enter and exit your skin. You will have from 5 sets of marks on your arm. After the tight band is removed, we will clean your arm with an orange fluid called Betadine and alcohol. We will 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 will travel between the layers of skin for 2.5 cm (1 inch) and leave your skin at the matching exit mark. The tubing is threaded through the needle. Next, we withdraw the needle, leaving the tubing in your skin. Any redness of your skin will subside in 30-45 minutes.

_____initial e) While we are waiting for the redness on your arm to go away, we will begin to run lukewarm water through the whole-body suit to keep your skin at a normal temperature (34^{o}C or 93^{o}F). Also, during this time we will start clear fluid (resembling body fluid) flowing through all the sets of tubing. The clear fluid flows through the tubing in your skin during the whole experiment. We will add the following substances to the clear fluid flowing through the sites on your arm:

- site 1 = clear fluid only
- site 2 = tetrahydrobiopterin
- site 3 = tetrahydrobiopterin + metyrosine
- site 4 = tetrahydrobiopterin + L-tyrosine
- site 5 = L-tyrosine

After these fluids have flowed through the tubing for about 60 minutes, the redness in your skin should be gone, and we will continue with the rest of the experiment. During the experiment, we also measure your blood pressure by inflating a cuff on your upper arm and listening to your pulse with a stethoscope at the inside of your elbow.

_____initial f) After the redness in your skin has gone away, we tape a pencil-sized probe over each test site to measure the blood flow in the skin. The probes use a weak laser light to measure this blood flow through the whole experiment. The laser light probes will be held in place with special metal holders that control the temperature of your skin.

_____initial g) We will set the temperature controllers to keep your skin at a comfortable temperature (34°C or 93°F).

_____initial h) We will collect dialysate (the fluid dripping from the end of the microdialysis fiber) for 20 minutes. Samples will be analyzed later and provide us an index of neural activity at each test site.

_____initial i) After 40 min to allow your blood vessels to return to normal, cool water will run through the suit for 30 minutes so that your skin reaches 30.5°C (86.9°F). At this time dialysate will be collected while your skin temperature is held an additional 20 minutes at 30.5°C. During the entire cooling part of the experiment (50 min), you will have a small probe placed under the tongue to measure your body core temperature. After cooling, re-warming to a more comfortable temperature will follow.

_____initial j) Tyramine will be added to all sites for an additional 20 minutes. This will cause your small vessels in the skin around each site to constrict. Dialysate will be collected throughout during this 20 minute period.

_____initial k) Norepinephrine will then be infused at all sites for 20 minutes to maximally constrict the small vessels in the skin.

_____initial l) We will warm the temperature controllers to 42°C (108°F) and switch to fluid with only sodium nitroprusside flowing through the tubing at all sites for about 40 minutes. Heating and adding sodium nitroprusside to the fluid helps the blood vessels in your skin to get as big as they can (dilation).

_____initial m) We will remove the MD tubing from your skin and place sterile bandages over the sites. If you want, we can also place a bag of ice on your arm for 10 minutes to reduce any bruising that may occur. Finally, a member of the GCRC medical staff measures your blood pressure and heart rate before you leave.

3. <u>Discomforts and risks</u>: ***You will experience the circled procedures. Please read the descriptions of the circled procedures. Then write your initials by the circled procedures.***

_____initial a) 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. But you may feel a little pain when the tubing is removed from your skin. You may become lightheaded or may faint. Although rare, it is possible for the tubing to break during removal from your skin. In that case, we remove the tubing by pulling on the other end of it. This produces no additional risk for you. Even rarer, the tubing could break so that a piece of the tubing is left under your skin. In this case, any tubing remaining in your skin would be treated like a splinter. If slight bleeding occurs, applying mild pressure with sterile gauze will stop it. Infection is possible, but sterile techniques and supplies like those used in hospitals will be used to keep the risk minimal. We apply a sterile bandage to the site after the experiment. We will tell you how to take care of the site.

Note: In the approximately 1500 to 2000 fibers that have been placed in our lab, we have never had a subject report an infection at the microdialysis sites.

_____initial b) Fluid flowing through the tubing: The substances flowing through the tubing only go to a 2.5 cm^2 (0.4 $inch^2$) area of skin at each tubing site. The amount that enters the skin is very small. However, there is a minimal chance of having a bad reaction to the substances. This reaction could produce redness, itching, rash, and/or swelling limited to the small area of skin around the microdialysis sites. A worse reaction could also cause more diffuse swelling/rash, fever, breathing problems, changes in pulse, convulsions, and/or collapse. Other researchers have used these substances with microdialysis in skin. The researchers have not reported that these substances caused bad reactions. If a bad reaction should occur, medical help will be summoned. After the experiment, you will receive an instruction sheet on how to care for the microdialysis sites.

_____(initial) *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 your body's natural fluids. A bad reaction to this fluid is highly unlikely.

_____(initial) *Tyramine*: Tyramine stops the action of your body's natural chemicals upon the blood vessels in the skin. A small amount of tyramine will enter the skin around the tubing. This only affects the blood flow in the vessels in that small area of skin. The effect of tyramine is gone within an hour after the experiment. Tyramine has been used in microdialysis and/or in clinical medical treatment before. The researchers and doctors using tyramine with microdialysis have not reported any bad reactions.

_____(initial) *Norepinephrine, Tetrahydrobiopterin*: Norepinephrine is a natural chemical made by the nerves in the skin. Tetrahydrobiopterin is produced naturally in the body and acts to preserve norepinephrine. Only a small amount of these substances will enter the skin around the tubing. This only affects the blood flow in the vessels in that small area of skin. The effect of these substances is gone within an hour after the experiment. These substances have been used in microdialysis and/or in clinical medical treatment before. The researchers and doctors using these substances have not reported that these substances caused bad reactions in microdialysis.

_____(initial) *Sodium Nitroprusside (SNP)*: Only a small amount of SNP will enter your skin around the tubing. SNP increases the blood flow in the vessels and reddens that small area of skin. This effect is gone within an hour after the experiment. Other researchers have used SNP with microdialysis in skin. They have reported no bad reactions with SNP.

_____(initial) *Metyrosine, L-Tyrosine*: L-tyrosine is a natural chemical that is found in your body. Your nerves use L-tyrosine to make norepinephrine. Metyrosine can reduce the amount of norepinephrine made by your nerves. Doctors have used Metyrosine in patients whose nerves make too much norepinephrine and related chemicals. In this study, a small amount of metyrosine and L-tyrosine will enter and affect an area of your skin about the size of a dime.

_____initial c) 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.

_____initial d) Blood Pressure: We measure your blood pressure using the method common in a doctor's office. A cuff will be inflated on your upper arm. As the cuff slowly deflates, we listen with a stethoscope at the bend in your elbow. During the short time the cuff is inflated, your arm may feel numb.

_____initial e) Betadine: Hospitals and researchers use this orange-colored fluid to clean and sterilize the skin. You could have a bad reaction to Betadine if you are allergic to iodine or shellfish. You will inform us if you have these allergies so that we will use alcohol instead. A bad reaction could cause redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or collapse.

_____initial f) Latex: Some gloves and medical materials are made of latex rubber. You will inform us if you are allergic to latex so that we will use non-latex gloves and research materials.

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

_____initial h) Whole body cooling: Cooling may cause goose bumps, shivering and chilly sensations. The 20-minute period where skin temperature is kept at 30.5°C (86.9°F) is the coldest part of the study. The rest of the cooling is milder. If your body feels too cold, you will tell us, and we will reduce or stop the cooling.

_____initial i) Blood draw: Blood draws may cause anxiety, mild pain, swelling, nausea, lightheadedness, fainting, or bleeding. There is a slight chance of infection. Competent GCRC or GCRC-approved staff using standard precautions with aseptic techniques will perform the blood draw. You will be reclining for the procedure. You may decline the blood draw if you feel uncomfortable at any time during or before the procedure.

_____initial j) ECG: This machine measures the electrical activity of my heart. There have been no adverse effects from this measure. The tape may irritate your skin.

_____initial k) Body core temperature: Body core temperature is measured during the cooling part of the experiment with a small probe placed under the tongue. If this causes discomfort, you will tell us and we can either reposition or remove the probe.

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.

4. a. Benefits to me: You will receive a medical screening that could inform you about your health. You will know what your blood cholesterol levels, fasting blood glucose levels, and blood pressure are. Also, you could gain knowledge about how your body works.

b. Potential benefits to society: We will find out why older people are less able to withstand a cold stress. These results could suggest ways to prevent or treat the changes that make older people prone to greater loss of body heat in the cold. This could help prevent illness and death related to cold in this age group.

5. Duration/time of the procedures and study: ***The circled statements apply to you. Please read the circled statements. Then write your initials by the circled statements.***

_____ initial a) Day 1 (Screening): typically about ½ hour, no longer than 1 hour

_____ initial b) Day 2 (Experiment): typically about 5 ½ hours, no longer than 6 hours

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

7. Statement of confidentiality*:* Volunteers will be 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 and The Penn State University Biomedical Institutional Review Board may review records related to this project.

8. Right to ask questions: Please contact James Lang at work (814) 863-2948 or at home (814-466-6961) with questions, complaints or concerns about this research. You may also contact Jane Pierzga at work (814-865-1236) or at home (814-692-4720). You can also call these numbers if you feel this study has harmed you. If you have any questions, concerns, problems about your rights as a research participant or would like to offer input, please contact 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 principal investigator.

9. Payment for participation: The circled statements apply to you. Please read the circled statements. Then write your initials by the circled statements.

_____initial a) You will receive $10.00 for each microdialysis probe inserted into your skin (maximum $50.00). You will receive another $20.00 for completing the study. In the event that you do not complete the full experiment, you are paid an amount of money equal to the part of the experiment that you do complete. For example, if you complete only half of the experiment but have all 5 microdialysis probes in place, you will receive $50.00 ($10.00 x 5) + $10.00 ($20.00 x ½ of the total experiment) = $60.00 (Total maximum payment: $70.00). You may be asked if you would like to repeat the experiment on another day. If you agree to repeat the experiment, you will be paid for the repeated trial as stated above. You will also receive a lab t-shirt.

Note: 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. Voluntary participation: Your decision to be in this research is voluntary. You can stop at any time. You do not have to answer any questions you do not want to answer. Refusal to take part in or withdrawing from this study will involve no penalty or loss of benefits you would receive otherwise. Your participation in the study may be ended without your consent if the researcher deems that your health or behavior adversely affects the study or increases risks to you beyond those approved by The Penn State University Biomedical Institutional Review Board and agreed upon by you in this document.

11. Injury Clause: In the unlikely event you become injured as a result of your participation in this study, medical care is available. 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. In the event of a research related injury, you may call James Lang (office: 814-863-2948; home: 814-466-6961), or Jane Pierzga (office: 814-865-1236; home: 814-692-4720).

12. <u>Abnormal Test Results</u>: In the event that abnormal test results are obtained, you will be made aware of the results immediately and recommended to contact your private medical provider for follow-up.

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

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

_____ _____

Participant Signature Date

_____ _____

Person Obtaining Consent Date

Informed Consent form for chapter 5:

Informed Consent Form for Biomedical Research

The Pennsylvania State University

Title of Project: ***Age and Sympathetic Cotransmitter Function in Human Skin- Part 3***

Principal Investigator: **James A. Lang, M.S.**
229 Noll Lab
jal493@psu.edu; 814-863-2948

Advisor: **W. Larry Kenney, Ph.D.**
102 Noll Lab
w7k@psu.edu; 814-863-1672

Other Investigator(s): **Lacy A. Holowatz, Ph.D.**
204 Noll Lab
lma191@psu.edu; 814-867-1781

Jane Pierzga, M.S.
228 Noll Lab
jmp141@psu.edu; 814-865-1236

This is to certify that I, _____ have been given the following information with respect to my participation as a volunteer in a program of investigation under the supervision of Dr. W. Larry Kenney.

Note: This study has 3 parts. Part 1 has a main experiment and optional pilot experiment. Part 2 has one experiment. Part 3 has one experiment. You do not have to participate with all parts. If you were to complete all parts, you could have as many a 4 experiments total. You must read and sign separate informed consent forms to participate with Parts 1, 2, and 3.

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

When you are exposed to the cold, your body wants to save heat. It saves heat by making the blood vessels in your skin get smaller (constriction). This reduces the amount of warm blood flowing through your cold skin. As you grow older, your skin's blood vessels are less able to constrict. This causes older people to have a higher skin blood flow in the cold and makes them more prone to heat loss. Our project looks at how cooling causes blood vessels to constrict and how the ability to constrict changes when getting older. To do this, we measure the flow in the blood vessels by shining a weak laser light onto your skin. Also, we use a special technique called "microdialysis" (MD). MD involves placing very thin plastic tubing between the layers of your skin. The largest part of the tubing is about 6x the diameter of a human hair. We pump fluid like that found in your body's tissues (Lactated Ringer's solution) through the thin tubing. The thin tubing acts like the very small blood vessels in your skin by allowing the exchange of substances between the fluid in the tubing and the body fluid in the surrounding layers of your skin. During the experiment, we will add substances to the fluid in the tubing. The substances can only reach a 2.5 cm^2 (0.4 $inch^2$), nickel-sized area of skin. Most of these substances keep your blood vessels from constricting. They do this by blocking some of the natural substances that your body makes that cause your vessels to constrict. We have the ability to manipulate any of these substances in your skin in order to look at the effects of your skin's blood vessel response to cooling; however, we will manipulate only a few of these substances in any one visit/experiment. These substances will help us determine how the skin responds to whole body cooling in healthy volunteers of different ages.

2. <u>Procedures</u>: ***You will participate on the circled days. Please read the descriptions of the circled days. Then write your initials by the circled days.***

_____ initial <u>Day 1: Screening</u>

You will report to the nurses' station at the General Clinical Research Center for your appointment. The GCRC staff measures your blood pressure, height and weight, reviews your medical history, conducts a physical exam, and measures the electrical activity of the heart using ECG (12 leads will be taped to the chest to obtain the measurement). GCRC staff will also draw 15 mL (1 Tbsp) of blood from a vein in your arm to measure the concentrations of various substances including cholesterol and glucose. If you take a thyroid drug, you need to supply the results of a thyroid test taken within the past 6 months, because unusual thyroid levels can affect how blood vessels in your skin respond to cooling. If you do not have thyroid test results, a blood sample will be sent to a lab that tests it for thyroid levels. The lab destroys the sample after testing. If you are a woman of childbearing age, you will submit a urine sample for a pregnancy test. A positive result will result will exclude you from the study. We may measure the thickness of folds of skin at several places on your body to determine your percent body fat.

_____**initial** **Day 2: Experiment** **(If you are participating in experimental protocol)**

_____initial a) When you arrive at the laboratory, the GCRC checks your heart rate and blood pressure. Then you wash your forearm and pat it dry.

_____initial b) We will escort you to the Microdialysis Lab (room 205 Noll Lab) where the remainder of the experiment will be completed. You will put on a whole-body suit that has tubing lining the inside. Men wear shorts under the suit. Women wear shorts and a sports bra.

_____initial c) You will have wires taped to 6 places on your body to measure skin temperature.

_____initial d) You rest on a bed for the experiment, and we place a tight band around your upper arm so your veins are easily seen. We mark places on your arm 2.5 cm (1 inch) apart where the tubing will enter and exit your skin. You will have from **4** sets of marks on your arm. After the tight band is removed, we will clean your arm with an orange fluid called Betadine and alcohol. We will 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 will travel between the layers of skin for 2.5 cm (1 inch) and leave your skin at the matching exit mark. The tubing is threaded through the needle. Next, we withdraw the needle, leaving the tubing in your skin. Any redness of your skin will subside in 30-45 minutes.

_____initial e) While we are waiting for the redness on your arm to go away, we will begin to run lukewarm water through the whole-body suit to keep your skin at a normal temperature (34^{o}C or 93^{o}F). Also, during this time we will start clear fluid (resembling body fluid) flowing through all the sets of tubing. The clear fluid flows through the tubing in your skin during the whole experiment. We will add the following substances to the clear fluid flowing through the sites on your arm:

- site 1 = clear fluid only
- site 2 = **Fasudil**
- site 3 = L-NAME
- site 4 = **Fasudil +** L-NAME

After these fluids have flowed through the tubing for about 60 minutes, the redness in your skin should be gone, and we will continue with the rest of the experiment. During the experiment, we also measure your blood pressure by inflating a cuff on your upper arm and listening to your pulse with a stethoscope at the inside of your elbow.

_____initial f) After the redness in your skin has gone away, we tape a pencil-sized probe over each test site to measure the blood flow in the skin. The probes use a weak laser light to measure this blood flow through the whole experiment. The laser light probes will be held in place with special metal holders that control the temperature of your skin.

_____initial g) We will set the temperature controllers to keep your skin at a comfortable temperature (34°C or 93°F)

_____initial h) After 40 min to allow your blood vessels to return to normal, cool water will run through the suit for 30 minutes so that your skin reaches 30.5°C (86.9°F). Your skin temperature will be held an additional 10 minutes at 30.5°C. During the entire cooling part of the experiment (40 min), you will have a small probe placed under the tongue to measure your body core temperature. After cooling, re-warming to a more comfortable temperature will follow.

_____initial i) At **all sites**, **a very small amount of** norepinephrine will be added to the fluid flowing through the tubing in your skin for about 10 minutes. **The blood flow at the sites will reach a new, stable level.**

_____initial j) Then, **more** norepinephrine will then be infused at all sites for 20 minutes to maximally constrict the small vessels in the skin.

_____initial k) We will warm the temperature controllers to 42°C (108°F) and switch to fluid with only sodium nitroprusside flowing through the tubing at all sites for about 40 minutes. Heating and adding sodium nitroprusside to the fluid helps the blood vessels in your skin to get as big as they can (dilation).

_____initial l) We will remove the MD tubing from your skin and place sterile bandages over the sites. If you want, we can also place a bag of ice on your arm for 10 minutes to reduce any bruising that may occur. Finally, a member of the GCRC medical staff measures your blood pressure and heart rate before you leave.

3. <u>Discomforts and risks</u>: ***You will experience the circled procedures. Please read the descriptions of the circled procedures. Then write your initials by the circled procedures.***

_____initial a) <u>Microdialysis:</u> 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. But you may feel a little pain when the tubing is removed from your skin. You may become lightheaded or may faint. Although rare, it is possible for the tubing to break during removal from your skin. In that case, we remove the tubing by pulling on the other end of it. This produces no additional risk for you. Even rarer, the tubing could break so that a piece of the tubing is left under your skin. In this case, any tubing remaining in your skin would be treated like a splinter. If slight bleeding occurs, applying mild pressure with sterile gauze will stop it. Infection is possible, but sterile

techniques and supplies like those used in hospitals will be used to keep the risk minimal. We apply a sterile bandage to the site after the experiment. We will tell you how to take care of the site.

Note: In the approximately 1500 to 2000 fibers that have been placed in our lab, we have never had a subject report an infection at the microdialysis sites.

_____initial b) Fluid flowing through the tubing: The substances flowing through the tubing only go to a 2.5 cm^2 (0.4 $inch^2$) area of skin at each tubing site. The amount that enters the skin is very small. However, there is a minimal chance of having a bad reaction to the substances. This reaction could produce redness, itching, rash, and/or swelling limited to the small area of skin around the microdialysis sites. A worse reaction could also cause more diffuse swelling/rash, fever, breathing problems, changes in pulse, convulsions, and/or collapse. Other researchers have used these substances with microdialysis in skin. The researchers have not reported that these substances caused bad reactions. If a bad reaction should occur, medical help will be summoned. After the experiment, you will receive an instruction sheet on how to care for the microdialysis sites.

_____(initial) *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 your body's natural fluids. A bad reaction to this fluid is highly unlikely.

_____(initial) *L-NAME, **Fasudil***: These substances stop the action of your body's natural chemicals upon the blood vessels in the skin. A small amount of these substances will enter the skin around the tubing. This only affects the blood flow in the vessels in that small area of skin. The effect of these substances is gone within an hour after the experiment. These substances have been used in microdialysis and/or in clinical medical treatment before. The researchers and doctors using these substances have not reported that these substances caused bad reactions in microdialysis.

_____(initial) *Norepinephrine, Vitamin C*: Norepinephrine is a natural chemical made by the nerves in the skin. Vitamin C occurs naturally in foods and preserves norepinephrine. **A small amount is in the fluid that contains norepinephrine.** Only a small amount of these substances will enter the skin around the tubing. This only affects the blood flow in the vessels in that small area of skin. The effect of these substances is gone within an hour after the experiment. These substances have been used in microdialysis and/or in clinical medical treatment before. The researchers and doctors using these substances have not reported that these substances caused bad reactions in microdialysis.

_____(initial) *Sodium Nitroprusside (SNP)*: Only a small amount of SNP will enter your skin around the tubing. SNP increases the blood flow in the vessels and reddens that small area of skin. This effect is gone within an hour after the experiment. Other researchers have used SNP with microdialysis in skin. They have reported no bad reactions with SNP.

_____initial c) 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.

_____initial d) Blood Pressure: We measure your blood pressure using the method common in a doctor's office. A cuff will be inflated on your upper arm. As the cuff slowly deflates, we listen with a stethoscope at the bend in your elbow. During the short time the cuff is inflated, your arm may feel numb.

_____initial e) Betadine: Hospitals and researchers use this orange-colored fluid to clean and sterilize the skin. You could have a bad reaction to Betadine if you are allergic to iodine or shellfish.

You will inform us if you have these allergies so that we will use alcohol instead. A bad reaction could cause redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or collapse.

_____initial f) Latex: Some gloves and medical materials are made of latex rubber. You will inform us if you are allergic to latex so that we will use non-latex gloves and research materials.

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

_____initial h) Whole body cooling: Cooling may cause goose bumps, shivering and chilly sensations. The 10-minute period where skin temperature is kept at 30.5°C (86.9°F) is the coldest part of the study. The rest of the cooling is milder. If your body feels too cold, you will tell us, and we will reduce or stop the cooling.

_____initial i) Blood draw: Blood draws may cause anxiety, mild pain, swelling, nausea, lightheadedness, fainting, or bleeding. There is a slight chance of infection. Competent GCRC or GCRC-approved staff using standard precautions with aseptic techniques will perform the blood draw. You will be reclining for the procedure. You may decline the blood draw if you feel uncomfortable at any time during or before the procedure.

_____initial j) ECG: This machine measures the electrical activity of my heart. There have been no adverse effects from this measure. The tape may irritate your skin.

_____initial k) Body core temperature: Body core temperature is measured during the cooling part of the experiment with a small probe placed under the tongue. If this causes discomfort, you will tell us and we can either reposition or remove the probe.

_____initial j) 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.

4. a. Benefits to me: You will receive a medical screening that could inform you about your health. You will know what your blood cholesterol levels, fasting blood glucose levels, and blood pressure are. Also, you could gain knowledge about how your body works.

b. Potential benefits to society: We will find out why older people are less able to withstand a cold stress. These results could suggest ways to prevent or treat the changes that make older people prone to greater loss of body heat in the cold. This could help prevent illness and death related to cold in this age group.

5. Duration/time of the procedures and study: ***The circled statements apply to you. Please read the circled statements. Then write your initials by the circled statements.***

_____ initial a) Day 1 (Screening): typically about ½ hour, no longer than 1 hour

_____ initial b) Day 2 (Experiment): typically about 5 ½ hours, no longer than 6 hours

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

7. Statement of confidentiality*:* Volunteers will be 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 and The Penn State University Biomedical Institutional Review Board may review records related to this project.

8. Right to ask questions: Please contact James Lang at work (814) 863-2948 or at home (814-466-6961) with questions, complaints or concerns about this research. You may also contact Jane Pierzga at work (814-865-1236) or at home (814-692-4720). You can also call these numbers if you feel this study has harmed you. If you have any questions, concerns, problems about your rights as a research participant or would like to offer input, please contact 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 principal investigator.

9. Payment for participation: The circled statements apply to you. Please read the circled statements. Then write your initials by the circled statements.

_____initial a) You will receive $10.00 for each microdialysis probe inserted into your skin (maximum $**4**0.00). You will receive another $20.00 for completing the study. In the event that you do not complete the full experiment, you are paid an amount of money equal to the part of the experiment that you do complete. For example, if you complete only half of the experiment but have all **4** microdialysis probes in place, you will receive $**4**0.00 ($10.00 x **4**) + $10.00 ($20.00 x

½ of the total experiment) = $**5**0.00 (Total maximum payment: $**6**0.00). You may be asked if you would like to repeat the experiment on another day. If you agree to repeat the experiment, you will be paid for the repeated trial as stated above. You will also receive a lab t-shirt.

Note: 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. Voluntary participation: Your decision to be in this research is voluntary. You can stop at any time. You do not have to answer any questions you do not want to answer. Refusal to take part in or withdrawing from this study will involve no penalty or loss of benefits you would receive otherwise. Your participation in the study may be ended without your consent if the researcher deems that your health or behavior adversely affects the study or increases risks to you beyond those approved by The Penn State University Biomedical Institutional Review Board and agreed upon by you in this document.

11. Injury Clause: In the unlikely event you become injured as a result of your participation in this study, medical care is available. 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. In the event of a research related injury, you may call James Lang (office: 814-863-2948; home: 814-466-6961) or Jane Pierzga (office: 814-865-1236; home: 814-692-4720).

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

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

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

_____ _____

Participant Signature Date

_____ _____

Person Obtaining Consent Date

Informed Consent form for chapter 6:

Informed Consent Form for Biomedical Research

The Pennsylvania State University

Title of Project: ***Age and Sympathetic Cotransmitter Function in Human Skin- Part 4***

Principal Investigator: **James A. Lang, M.S.**
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Advisor: **W. Larry Kenney, Ph.D.**
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Other Investigator(s): **Lacy A. Holowatz, Ph.D.**
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Jane Pierzga, M.S.
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This is to certify that I, _____ have been given the following information with respect to my participation as a volunteer in a program of investigation under the supervision of Dr. W. Larry Kenney.

Note: This study has 4 parts. Part 1 has a main experiment and optional pilot experiment. Parts 2, 3, and 4 each have one experiment. You do not have to participate with all parts. If you were to complete all parts, you could have as many a 5 experiments total. You must read and sign separate informed consent forms to participate in Parts 1, 2, 3, and 4.

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

When you are exposed to the cold, your body wants to save heat. It saves heat by making the blood vessels in your skin get smaller (constriction). This reduces the amount of warm blood flowing through your cold skin. As you grow older, your skin's blood vessels are less able to constrict. This causes older people to have a higher skin blood flow in the cold and makes them more prone to heat loss. Our project looks at how cooling causes blood vessels to constrict and how the ability to constrict changes when getting older. To do this, we measure the flow in the blood vessels by shining a weak laser light onto your skin. Also, we use a special technique called "microdialysis" (MD). MD involves placing very thin plastic tubing between the layers of your skin. The largest part of the tubing is about 6x the diameter of a human hair. We pump fluid like that found in your body's tissues (Lactated Ringer's solution) through the thin tubing. The thin tubing acts like the very small blood vessels in your skin by allowing the exchange of substances between the fluid in the tubing and the body fluid in the surrounding layers of your skin. During the experiment, we will add substances to the fluid in the tubing. The substances can only reach a 2.5 cm^2 (0.4 $inch^2$), nickel-sized area of skin. Most of these substances keep your blood vessels from constricting. They do this by blocking some of the natural substances that your body makes that cause your vessels to constrict. We have the ability to manipulate any of these substances in your skin in order to look at the effects of your skin's blood vessel response to cooling; however, we will manipulate only a few of these substances in any one visit/experiment. These substances will help us determine how the skin responds to whole body cooling in healthy volunteers of different ages.

2. <u>Procedures</u>: ***You will participate on the circled days. Please read the descriptions of the circled days. Then write your initials by the circled days.***

_____ initial <u>Day 1: Screening</u>

You will report to the nurses' station at the General Clinical Research Center for your appointment. The GCRC staff measures your blood pressure, height and weight, reviews your medical history, conducts a physical exam, and measures the electrical activity of the heart using ECG (12 leads will be taped to the chest to obtain the measurement). GCRC staff will also draw 15 mL (1 Tbsp) of blood from a vein in your arm to measure the concentrations of various substances including cholesterol and glucose. If you take a thyroid drug, you need to supply the results of a thyroid test taken within the past 6 months, because unusual thyroid levels can affect how blood vessels in your skin respond to cooling. If you do not have thyroid test results, a blood sample will be sent to a lab that tests it for thyroid levels. The lab destroys the sample after testing. If you are a woman of childbearing age, you will submit a urine sample for a pregnancy test. A positive result will result will exclude you from the study. We may measure the thickness of folds of skin at several places on your body to determine your percent body fat.

_____initial <u>Day 2: Experiment</u> (If you are participating in experimental protocol)

_____initial a) When you arrive at the laboratory, the GCRC checks your heart rate and blood pressure. Then you wash your forearm and pat it dry.

_____initial b) We will escort you to the Microdialysis Lab (room 205 Noll Lab) where the remainder of the experiment will be completed. You will put on a whole-body suit that has tubing lining the inside. Men wear shorts under the suit. Women wear shorts and a sports bra.

_____initial c) You rest on a bed for the experiment, and bretylium tosylate, a drug that prevents blood vessels from getting smaller, will be introduced into four 4-5 cm^2 (1.57-1.97 $inch^2$) sites on the skin of the forearm using AC electric current generated by a small battery-powered device. Any redness of your skin will subside in 30-45 minutes.

_____initial d) After approximately 60-80 minutes (to ensure any redness is gone), cold water will run through the whole-body suit for 3 minutes to test bretylium. If bretylium fails to prevent the blood vessels from getting smaller, the experiment will be postponed, and you will be asked to return on another day.

_____initial d) After testing bretylium, we place a tight band around your upper arm so your veins are easily seen. We mark places on your arm 2.5 cm (1 inch) apart where the tubing will enter and exit your skin. You will have 4 sets of marks on your arm. After the tight band is removed, we will clean your arm with an orange fluid called Betadine and alcohol. We will 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 will travel between the layers of skin for 2.5 cm (1 inch) and leave your skin at the matching exit mark. The tubing is threaded through the needle. Next, we withdraw the needle, leaving the tubing in your skin. Any redness of your skin will subside in 30-45 minutes.

_____initial e) While we are waiting for the redness on your arm to go away, we will start clear fluid (resembling body fluid) flowing through all the sets of tubing. The clear fluid flows through the tubing in your skin during the whole experiment. We will add the following substances to the clear fluid flowing through the sites on your arm:

_____initial

- site 1 = clear fluid only
- site 2 = L-NAME
- site 3 = Tetrahydrobiopterin (BH4)
- site 4 = L-NAME + BH4

OR

_____initial

- site 1 = clear fluid only
- site 2 = Tyrosine

- site 3 = Tetrahydrobiopterin (BH4)
- site 4 = Tyrosine+ BH4

After these fluids have flowed through the tubing for about 60 minutes, the redness in your skin should be gone, and we will continue with the rest of the experiment. During the experiment, we also measure your blood pressure by inflating a cuff on your upper arm and listening to your pulse with a stethoscope at the inside of your elbow.

_____initial f) After the redness in your skin has gone away, we tape a pencil-sized probe over each test site to measure the blood flow in the skin. The probes use a weak laser light to measure this blood flow through the whole experiment. The laser light probes will be held in place with special metal holders that control the temperature of your skin.

_____initial g) We will set the temperature controllers to keep your skin at a comfortable temperature (34°C or 93°F).

_____initial i) At all sites, norepinephrine will be added at five separate times to the fluid flowing through the tubing in your skin. Specifically, there will 6 timed perfusions (each perfusion lasting 5 minutes) of different concentrations of norepinephrine. Between each 5-minute perfusion, there will be a 30 minute wash-out period where Ringers will be perfused. The maximal dose of norepinephrine that will be used will maximally constrict the small vessels in the skin.

_____initial j) We will warm the temperature controllers to 42°C (108°F) and switch to fluid with only sodium nitroprusside flowing through the tubing at all sites for about 40 minutes. Heating and adding sodium nitroprusside to the fluid helps the blood vessels in your skin to get as big as they can (dilation).

_____initial k) We will remove the MD tubing from your skin and place sterile bandages over the sites. If you want, we can also place a bag of ice on your arm for 10 minutes to reduce any bruising that may occur. Finally, a member of the GCRC medical staff measures your blood pressure and heart rate before you leave.

3. <u>Discomforts and risks</u>: ***You will experience the circled procedures. Please read the descriptions of the circled procedures. Then write your initials by the circled procedures.***

_____initial a) <u>Microdialysis:</u> 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. But you may feel a little pain when the tubing is removed from your skin. You may become lightheaded or may faint. Although rare, it is possible for the tubing to break during removal from your skin. In that case, we remove the tubing by pulling on the other end of it. This produces no additional risk for you. Even rarer, the tubing could break so that a piece of the tubing is left under your skin. In this case, any tubing remaining in your skin would be treated like a splinter. If slight bleeding occurs, applying mild pressure with sterile gauze will stop it. Infection is possible, but sterile techniques and supplies like those used in hospitals will be used to keep the risk minimal. We apply a sterile bandage to the site after the experiment. We will tell you how to take care of the site.

Note: In the approximately 1500 to 2000 fibers that have been placed in our lab, we have never had a subject report an infection at the microdialysis sites.

_____initial b) Fluid flowing through the tubing: The substances flowing through the tubing only go to a 2.5 cm^2 (0.4 $inch^2$) area of skin at each tubing site. The amount that enters the skin is very small. However, there is a minimal chance of having a bad reaction to the substances. This reaction could produce redness, itching, rash, and/or swelling limited to the small area of skin around the microdialysis sites. A worse reaction could also cause more diffuse swelling/rash, fever, breathing problems, changes in pulse, convulsions, and/or collapse. Other researchers have used these substances with microdialysis in skin. The researchers have not reported that these substances caused bad reactions. If a bad reaction should occur, medical help will be summoned. After the experiment, you will receive an instruction sheet on how to care for the microdialysis sites.

_____(initial) *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 your body's natural fluids. A bad reaction to this fluid is highly unlikely.

_____(initial) *Tyrosine, Norepinephrine, Vitamin C, Tetrahydrobiopterin (BH4), L-NAME*:: Tyrosine is a natural chemical that is found naturally within nearly all cells in the body. Norepinephrine is a natural chemical made by the nerves in the skin. Vitamin C occurs naturally in foods and preserves norepinephrine. BH4 is produced naturally in the body and also acts to preserve norepinephrine. L-NAME stops the action of your body's natural chemicals to dilate blood vessels in the skin. Only a small amount of these substances will enter the skin around the tubing. This only affects the blood flow in the vessels in that small area of skin (approximately the size of a dime). The effect of these substances is gone within an hour after the experiment. These substances have been used in microdialysis and/or in clinical medical treatment before. The researchers and doctors using these substances have not reported that these substances caused bad reactions in microdialysis.

_____(initial) *Sodium Nitroprusside (SNP)*: Only a small amount of SNP will enter your skin around the tubing. SNP increases the blood flow in the vessels and reddens that small area of skin. This effect is gone within an hour after the experiment. Other researchers have used SNP with microdialysis in skin. They have reported no bad reactions with SNP.

_____initial c) 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.

_____initial d) Bretylium Iontophoresis: Bretylium's effects, limited to only these 4 small areas of skin, last for at least 6 hours and are completely gone in 24 hours. The iontophoresis may cause a slight tingling sensation. If this becomes uncomfortable, you will tell us, and we will reduce the current or stop iontophoresis. Although no bad effects to this amount of bretylium administered in this fashion have been recorded, there remains a remote change of an allergic reaction that could include rash, swelling, and itching.

_____initial e) Blood Pressure: We measure your blood pressure using the method common in a doctor's office. A cuff will be inflated on your upper arm. As the cuff slowly deflates, we listen with a stethoscope at the bend in your elbow. During the short time the cuff is inflated, your arm may feel numb.

_____initial f) Betadine: Hospitals and researchers use this orange-colored fluid to clean and sterilize the skin. You could have a bad reaction to Betadine if you are allergic to iodine or shellfish. You will inform us if you have these allergies so that we will use alcohol instead. A bad reaction could cause redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or collapse.

_____initial g) Latex: Some gloves and medical materials are made of latex rubber. You will inform us if you are allergic to latex so that we will use non-latex gloves and research materials.

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

_____ initial i) Whole body cooling: Cooling may cause goose bumps, shivering and chilly sensations. If your body feels too cold, you will tell us, and we will reduce or stop the cooling.

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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.

4. a. Benefits to me: You will receive a medical screening that could inform you about your health. You will know what your blood cholesterol levels, fasting blood glucose levels, and blood pressure are. Also, you could gain knowledge about how your body works.

b. Potential benefits to society: We will find out why older people are less able to withstand a cold stress. These results could suggest ways to prevent or treat the changes that make older people prone to greater loss of body heat in the cold since norepinephrine is the principle substance responsible for eliciting cold-induced responses in older individuals. This could help prevent illness and death related to cold in this age group.

5. Duration/time of the procedures and study: ***The circled statements apply to you. Please read the circled statements. Then write your initials by the circled statements.***

_____ initial a) Day 1 (Screening): typically about ½ hour, no longer than 1 hour

_____ initial b) Day 2 (Experiment): typically about 8 hours, no longer than 9 hours

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

7. Statement of confidentiality*:* Volunteers will be 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 and The Penn State University Institutional Review Board may review records related to this project.

8. Right to ask questions: Please contact James Lang at work (814) 863-2948 or at home (814-466-6961) with questions, complaints or concerns about this research. You may also contact Jane Pierzga at work (814-865-1236) or at home (814-692-4720). You can also call these numbers if you feel this study has harmed you. If you have any questions, concerns, or problems about your rights as a research participant or would like to offer input, please contact Penn State University's Office for Research Protections (ORP) at (814) 865-1775. The ORP cannot answer questions about research procedures. All questions about research procedures can only be answered by the principal investigator.

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_____initial a) You will receive $10.00 for each microdialysis probe inserted into your skin (maximum $40.00). You will receive another $40.00 for completing the study. In the event that you do not complete the full experiment, you are paid an amount of money equal to the part of the experiment that you do complete. For example, if you complete only half of the experiment but

have all 4 microdialysis probes in place, you will receive $40.00 ($10.00 x 4) + $20.00 ($40.00 x ½ of the total experiment) = $60.00 (Total maximum payment: $80.00). You may be asked if you would like to repeat the experiment on another day. If you agree to repeat the experiment, you will be paid for the repeated trial as stated above. You will also receive a lab t-shirt.

Note: 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.

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

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

_____ _____

Participant Signature Date

_____ _____

Person Obtaining Consent Date

Curriculum Vitae: James A. Lang, M.S.

Ph.D.Cand.	Kinesiology, expected graduation date May 2010, Penn State University
M.S.	Department of Integrative Physiology, August 2002, University of Iowa
B.S.	Department of Integrative Physiology, July 1999, University of Iowa

HONORS AND AWARDS

2010 Predoctoral Recognition Award, EEP Section, APS

2009 Environmental Graduate Student Research Award, American College of Sports Medicine

2009 Caroline tum Suden /Francis Hellebrandt Professional Opportunity Award, APS
2008 Carl V. Gisolfi Memorial Research Fund, American College of Sports Medicine

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