Primary Aging: Thermoregulatory Sweating & Skin Blood Flow

by

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Honors Thesis

Appalachian State University

Submitted to
The Honors College
in partial fulfillment of the requirements for the degree of

Bachelor of Science

December, 2015

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ABSTRACT

Heat related morbidities and mortalities are disproportionately high in the aged population (≥60 yr). Aging without overt illness is associated with the attenuation of heat dissipation mechanisms including cutaneous vasodilation and eccrine sweating responses. These age-related decrements in thermoregulatory function are well represented in literature discussing heat dissipation, yet despite attempts to investigate a potential signaling mechanism between sweating and skin blood flow (SkBF), a functional link has not been demonstrated. Recent evidence supports a role of nitric oxide (NO), a potent signaling molecule in cutaneous vasodilation, as additionally signaling the eccrine sweating response. The aim of this study was to investigate the putative role of NO in eccrine sweat gland signaling in young and primary aged individuals. Prior to experimentation, pilot studies were conducted to develop experimental drug dilutions to achieve a successful flow matching protocol. In two subjects, three intradermal microdialysis (MD) probes were inserted into the left ventral forearm and perfused with 1) Lactated Ringer's solution, 2) Epoprostenol sodium (EPO) + N^G-Nitro-L-arginine (L-NNA), and 3) Sodium Nitroprusside (SNP) + L-NNA. Regional sweating rates (RSR) over each MD membrane were measured using ventilated capsules with a laser Doppler probe housed in each capsule for measurement of red cell flux (laser Doppler flux, LDF) and divided by mean arterial blood pressure (Cutaneous vascular conductance (CVC) = LDF/MAP) as an index of SkBF. Subjects completed a whole body heating protocol to a 1°C rise in sublingual temperature. Maximal CVC values were obtained pharmacologically at the end of each protocols using 25 mM SNP. During whole body heating in the second pilot, the L-NNA/EPO site displayed lower levels of CVC than the control site, implying the EPO had become inactive. A buffer solution was created to maintain EPO stability at physiological pH. The next steps are to complete pilot tests with EPO in buffer to ensure it remains active and to achieve a successful flow matching protocol before beginning the sweating study.

BACKGROUND AND SIGNIFICANCE

When exposed to thermal stress, the aged population (≥60 years of age) consistently exhibit the greatest vulnerability to heat related illnesses, with a reported 82-92% excess mortality occurring in this group.[1] Human primary aging is associated with attenuation of heat dissipation mechanisms, including the ability to increase skin blood flow (SkBf) and eccrine sweating responses.[2] Age-related thermoregulatory dysfunction has become of increasing relevance considering scientists predict a greater frequency and severity of heat waves which may exceed the physiological capacity of vulnerable groups.[1]

The physiological responses to heat stress include a significant increase in cutaneous vasodilation (VD) and stimulation of eccrine sweating, with the latter serving as the most effective avenue of heat loss.[2] Eccrine sweating becomes especially important when ambient air temperatures exceed skin temperature, due to convective heat loss via vasodilation being hindered.[3] Despite extensive literature concerning the physiological mechanisms involved in heat dissipation, the exact mechanisms governing heat dissipation processes and the relationship between sweating and SkBF remain unclear. It is speculated that a key active effector of cutaneous vasodilation, nitric oxide (NO) is also involved with signaling the eccrine sweat response. Demonstrating a functional link between cutaneous vasodilation and the sweat response would provide novel information on the regulation of eccrine sweating and potentially provide knowledge supporting the creation of treatment measures to improve heat dissipation of older adults.

Human Thermoregulation

Human thermoregulation is achieved via a balance of heat production and heat loss mechanisms. Humans are homeotherms, regulating core temperature (T_{core}) independently of the environment at a set point of approximately 37°C.[4] During temperate conditions, resting metabolic heat production is equalized predominately by dry heat exchange through means of conduction, convection, and radiation.[1] Elevations in T_{core} may occur during exercise or exposure to high ambient air temperatures, activating heat dissipation mechanisms including cutaneous vasodilation and eccrine sweating responses. In response to heat stress, initially vasoconstrictor tone is withdrawn; after a certain threshold is reached active cutaneous vasodilation and eccrine sweating are stimulated.[4]

Cutaneous vasodilation is achieved via increased cardiac output and redistribution of blood flow from renal and splanchnic vascular beds to vascular beds important in thermoregulation.[5] This redistribution of blood to the cutaneous circulation facilitates convective heat transfer from the core to the periphery. This thermoregulatory response provides dynamic results; modest changes in vasodilation produce profound increases in heat transfer. For example, SkBF may increase from a resting value of approximately 250 mL/min up to 6 to 8 L/min during hyperthermia, representing nearly 60% of cardiac output.[4]

Eccrine sweating serves as the most effective avenue of heat loss and is especially important for heat loss when ambient air temperature exceeds mean skin temperature (~34°C), rendering efforts of convective heat loss via vasodilation inefficient.[6] The exact mechanisms which govern the sweat response and cutaneous vasodilation remain elusive, although it is generally agreed that acetylcholine (ACh) is the primary signaling molecule for eccrine sweating while ACh and an unknown cotransmitter primarily induce cutaneous vasodilation. It has been observed that these thermoregulatory responses become

attenuated with primary aging and additionally that the aged become increasingly dependent on NO to induce vasodilation, yet this system is also blunted with age. Considering that eccrine sweating becomes especially important when ambient air temperatures exceed mean skin temperature and also that the attenuation of thermoregulation mechanisms is apparent with primary aging, investigation of age-related alterations in thermoregulation and the potential mechanistic relationship between skin blood flow and eccrine sweating has become an attractive area of study.

Age-Related Alterations in Thermoregulation

Aging in the absence of overt pathology is associated with attenuated reflex vasodilatory and sweating response during thermal stress.[5] These alterations appear to be peripheral in origin, due to decreased sensitivity of the active vasodilator system.[5] These age-related decrements in vasodilation occur in the cutaneous microcirculation, resulting in a delay in cutaneous microvascular responses and subsequent blunted thermoregulatory function.

Control of both reflex and local mechanisms of the cutaneous vasculature are altered with age.[2] Minson et al. [7] compared cardiac output and renal and splanchnic blood flow in young (~23 years) and older (~70 years) subjects during passive whole body heating and found that the increase in cardiac output and reductions in visceral blood flow observed in young subjects was reduced with age. The reduced cardiac output observed in aged individuals was largely attributed to a lower stroke volume and at the lower cardiac output, and less blood was redistricted towards the skin for thermoregulation.[7] Similarly, Johnson and colleagues [2] observed a 30% reduction in blood flow redistributed from visceral vascular beds to the cutaneous circulation in older individuals, reinforcing the significant decrements in their ability to dissipate heat. [2]

Holowatz and colleagues [8] have extensively studied the mechanisms involved in altered thermoregulatory control in aged skin. They have observed that when the enzyme involved in NO production, nitric oxide synthase (NOS), was inhibited, cutaneous vasodilation was reduced to a larger extent in aged skin compared to younger persons exposed to the same level of heat stress.[8] It was also found that the contribution of cholinergic active vasodilation co-transmitters to reflex vasodilation is functionally absent in the aged population.[5] Researchers have concluded that with advancing age, individuals become increasingly dependent on NO to vasodilate, yet the system in also blunted with age.[5]

Holowatz et al.[8] additionally concluded that with advancing age, arginase activity, which competes for the NOS substrate, L-arginine, limits L-arginine availability necessary for NO production (See Figure 1. for signaling pathways). The researchers additionally investigated whether cutaneous vasodilation alterations associated with age were related to increased oxidative stress.[8] They observed that the antioxidant, ascorbate, augmented cutaneous vasodilation responses in aged subjects compared to levels of younger subjects.[8] However, ascorbate did not alter a response in young subjects, implying attenuated active vasodilation functioning with advanced age is in part due to an age associated increase in oxidative stress.[8]

When considered alongside the right shift in the T_{core} threshold for vasodilation and sweating, older individuals are at considerable risk of heat-related illness and injury. Aged humans require a greater increase in T_{core} ($T_c \geq 0.9℃$) to stimulate non-NO dependent pathways in comparison to young subjects ($T_c \geq 0.6℃$) [5], causing a delay in thermoregulatory responses during heat exposure.

Reflex Cutaneous Vasodilation and Eccrine Sweating

Cutaneous vasodilation and eccrine sweating are achieved via sympathetic reflex (whole body) and locally mediated pathways. Central control of thermoregulation is attributed to the preoptic/anterior hypothalamus (PO/AH) of the brain.[4] Sensory input from skin and core thermosensors are integrated at the PO/AH which then sends an efferent signal to vascular endothelial cells and sweat glands. Once stimulated, cutaneous vasodilation and sweat responses increase proportionately to rising internal temperatures.[2] Appropriate responses from the PO/AH will continue until the internal temperature returns to normal, demonstrating a classic homeostatic negative feedback loop.[4]

It is generally accepted that acetylcholine (ACh) plays a role in mediation of cutaneous vasodilation and the eccrine sweat response. In support of this theory, Kellogg and colleagues (9) observed local presynaptic inhibition of cholinergic nerves with botulinum toxin completely inhibits both sweating and active cutaneous vasodilation.

Sweat glands are stimulated by cholinergic nerve terminals in addition to some adrenergic terminals. The predominance of ACh in eccrine sweating has been demonstrated via the administration of atropine, a muscarinic receptor antagonist which completely abolishes the sweat response. Further evidence suggesting a primary role of ACh in sweat production includes recent studies which have found that adrenergic receptors do not contribute to the sweat response.[6]

It is hypothesized that vasodilation is induced by the cotransmission ACh and an unknown cotransmitter.[9] This theory is supported by observations that muscarinic blockade with atropine slightly delays vasodilation responses during thermal stress.[10] The partial attenuation of SkBf indicates a role of ACh in mediating vasodilation.[10] As for identification of the unknown putative neurotransmitter, vasoactive neuropeptides including vasoactive

intestinal peptide (VIP), substance P, and calcitonin gene-related peptide are all purported possibilities.[5] ACh produces vasodilation through various mechanisms including NO-dependent, prostanoid-dependent, and non-NO-nonprostanoid-dependent pathways.[9]

NO is a highly reactive free radical that is involved with several physiological pathways, including cutaneous vasodilation. Endothelium-derived NO (ED-NO) is believed to be a major active effector of cutaneous vasodilation and generally thought to be required for full expression of vasodilation.[9] In support of this, the inhibition of NO synthase (NOS) reduces active vasodilation by approximately 30-40%.[8] In addition to its role as an effector of cutaneous vasodilation, ED-NO serves vascular health in several ways, including: inhibition of platelet aggregation, leukocyte adhesion to the endothelial surface and vascular smooth muscle proliferation.[11] ED-NO is synthesized in endothelial cells from the precursor L-arginine by the enzyme NOS and causes vasodilation by activation of soluble-guanylate cyclase-dependent mechanisms to increase cellular cGMP in vascular smooth muscle. [5] In addition to ACh, NO is produced via downstream activation of several putative neurotransmitters such as vasoactive intestinal peptide (VIP), substance P, and CGRP.[9]

In support of this NO-dependent theory, two studies in the late 1990s applied the blockage of NOS activity and tested cutaneous vasodilatory responses. Kellogg et al. (13) used intradermal MD to perfuse NOS inhibitor NG-nitro-L-arginine-methyl ester (L-NAME) into the skin while another study, Shastry et al.(12) utilized intra-arterial infusions of the NOS antagonist L-NNA. In both studies, cutaneous vasodilation was significantly attenuated, but not entirely inhibited, implying NO is a major signaling molecule for cutaneous VD, but there are other mechanisms involved.[12, 13] Wilkins and colleagues (14) examined the role of NO as an active effector, rather than a permissive effector via perfusion of SNP (NO donor) with L-NAME (NOS antagonist), ensuring a constant supply of NO. They observed an

attenuation in active vasodilation during heat stress, regardless of the exogenous NO addition. This indicates that NO mediates vasodilation actively, rather than permissively.[14]

In addition to NO, evidence suggests a role of prostanoids in active cutaneous vasodilation. Like NO, prostaglandins (PGI_2) are secreted by endothelial cells in blood vessels and serves as a vasodilator.[15] PGI_2 is a metabolic derivative of arachidonic acid (AA) which is produced by phospholipase A2 (PLA2) through a Ca^{+2}-dependent step and converted by cyclooxygenase (COX).[15] PGI_2 induces vasorelaxation by stimulation of adenylyl cyclase and the production of cyclic adenosine monophosphate (cAMP).[15]

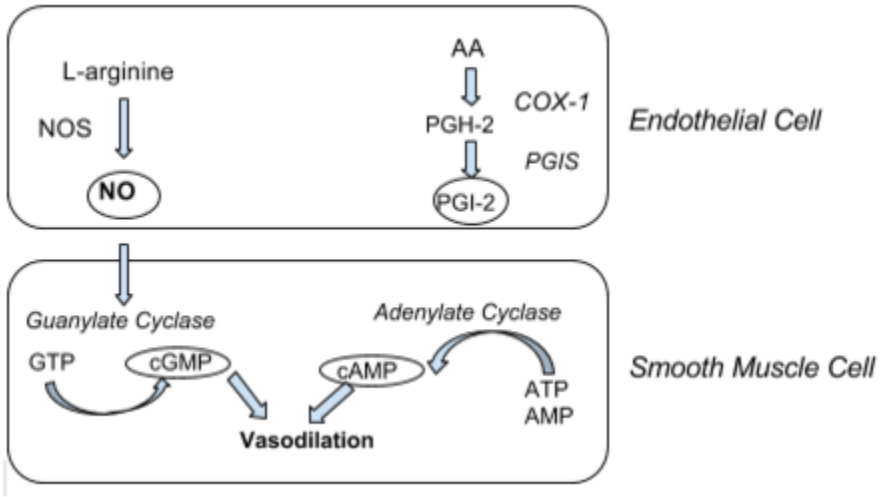

Figure 1. Schematic of NO and prostacyclin signaling pathways in regulation of vascular tone. Modified from reference [16].

NO is synthesized by NOS from the terminal nitrogen of L-arginine and further acts to stimulate vasodilation by soluble-guanylate cyclase-dependent mechanisms to increase cellular cGMP. PGH2 is an AA metabolite formed by cyclooxygenase (COX-1) and prostacyclin synthase (PGIS) located in vascular endothelium. Prostacyclin (PGI_2) further stimulates adenylate cyclase in vascular smooth muscle cells, increasing cellular cAMP which indirectly releases cytosolic calcium and resulting in vasorelaxation.[16]

The Relationship between Skin Blood Flow and Eccrine Sweating

It is logical to consider a functional link between sweating and SkBF since vasodilation provides the heat necessary for sweat evaporation as well as the precursor fluid to sweat production (blood plasma). It has additionally been observed that these two mechanisms of heat dissipation are often temporally correlated, thus it is suggested that cutaneous vasodilation is functionally related to the sweat response.[2] Cutaneous active vasodilation is mediated by sympathetic cholinergic active vasodilator nerves and eccrine sweating is controlled by sympathetic cholinergic sudomotor nerves. Whether these nerves comprise a single nerve or two separate nerves is still unclear.[2]

It is generally accepted that ACh is a key molecule in signaling both cutaneous vasodilation and the sweat response.[5] Sweat glands contain both adrenergic and cholinergic receptors, yet it appears that cholinergic receptors solely contribute to the sweat response.[6] It is therefore plausible to speculate that a major effector of cutaneous vasodilation and second-messenger pathway of ACh, NO is also involved in signaling the sweat response.[6] In addition, a study by Welch et al. [17] observed that sweating was lower in skin perfused with *N*G-nitro-L-arginine methyl ester [L-NAME, a non-selective NOS inhibitor] through intradermal MD, in comparison to during continuous cycling exercise (~47% of maximum oxygen uptake) performed in a hot environment. It has also been observed that NO augments sweat rate during both exogenous acetylcholine administration and active exercise heat stress.[10] These results have caused speculations over the potential role of NO in signaling sweating.

SPECIFIC AIMS

1.) Develop drug dilutions to elicit cutaneous vasodilation with EPO sodium L-NNA.
2.) Complete pilot testing to achieve flow matching of cutaneous vasodilatation during a whole body heating protocol via
    a. Lactated Ringers
    b. EPO sodium (PGI-II agonist) and L-NNA (NOS inhibitor).
    c. SNP (NO donor) and L-NNA
3.) To investigate the putative role of NO in eccrine sweat gland signaling in young individuals and in primary aging.

The rationale for the drugs perfused at each of the MD sites is as follows: Lactated Ringers is perfused at site A as a control site. L-NNA is perfused at sites B and C to block NOS and therefore endothelial NO production, whilst simultaneously perfusing a drug which stimulates cutaneous vasodilation by a specific pathways. This controls the level of vasodilation at each of the drug sites and the mechanism causing these responses. EPO is a PGI-2 agonist and serves to mediate cutaneous vasodilation through prostaglandin-dependent mechanisms while SNP, an NO donor, serves to induce cutaneous vasodilation through NO-dependent mechanisms. By blocking the physiological vasodilation response to heat stress (NOS-inhibition) and supplying a drug which elicits vasodilation, this allows for comparison of sweating responses associated with each specific pathway. To successfully compare sweating responses associated with prostaglandin-dependent mechanisms and sweating responses associated with NO-dependent mechanisms, blood flow as a measure of cutaneous vascular conductance (CVC) at each site must be matched to the control site, requiring experimentation with differing concentrations of L-NNA, SNP, and EPO. Once this step is completed sweating responses between each site may be compared. It is expected that the SNP+L-NNA site and control site will show higher levels of eccrine sweating

compared to the EPO+L-NNA site, indicating a role of NO in eccrine sweat gland signaling. During flow matching protocol development it was found that EPO does not remain active in physiological pH, therefore a buffer was created to maintain drug stability. Buffer development is further explained in Appendix I.

HYPOTHESIS

Based on current literature and a putative relation between sweating and SkBF, we hypothesize that sweating will be attenuated at the EPO and L-NNA site compared with the SNP and L-NNA, and control sites indicating a role for NO in eccrine sweating.

EXPERIMENTAL METHODS

A series of pilot studies were conducted to develop experimental drug dilutions to achieve a successful flow matching protocol. This required identifying appropriate concentrations of vasodilatory drugs combined with a non-specific NO inhibitor to match vasodilation at a control site during a whole body warming procedure.

Subjects

All experimental procedures were approved by the Appalachian State University Institutional Review Board and met the ethical guidelines set forth by the declaration of Helinski. Both procedures and pharmacological dilutions were approved by the Food and Drug Administration (FDA). Voluntary verbal and written consent were both obtained from subjects prior to participation screening. Two normotensive young women aged 22 and 23 years of age participated. All subjects completed a health screen questionnaire prior to participation. Upon arrival at the laboratory, the subjects' heart rate, blood pressure and sublingual temperature were recorded. Participants provided a urine sample for assessment of hydration status via urine specific gravity, and body mass was recorded. All

participants were untrained (neither sedentary nor highly exercise trained, were nonsmokers, nondiabetic and were not taking medications including antihypertensives or other drugs that may affect the cardiovascular system, including antioxidants, hormone replacement therapy, or oral contraceptives.) Both women were tested in the low hormone phase of their menstrual cycle.

Calibration of Laser Doppler Flowmetry

Laser Doppler probes (model HMT330; Vaisala, Helsinki, Finland) were sequentially placed in the calibration fluid, ensuring it was not touching the sides or bottom of the container and submerged in the fluid at a 90 degree angle to the bottom of the calibration bottle). During the calibration it was ensured that no excessive movement or vibration was within the laboratory. Values were measured with a flux between 200-220 and DC of 80-90. If values were not in the above ranges probes were recalibrated and calibrated prior to every experiment.

Calibration of Capacitance Hygrometers

Prior to experimentation, all equipment provided with the calibration system was were washed with alcohol, rinsed with water and then triple rinsed with deionized water (conductivity < 0.25μS/cm). Salts and equipment were handled carefully with gloves to ensure salts were not contaminated.

Capacitance hygrometers were calibrated using Lithium chloride (LiCl) (dry end, offset) and Sodium chloride (NaCl) (wet end, gain) as reference points (model HMT330; Vaisala, Helsinki, Finland). Each probe was calibrated using LiCl as a reference of 11.3% relative humidity (RH) and NaCl as a reference of 75.5% RH.

To mix each salt solution, the transit cover of each chamber was opened and pressed on the holder. Ion exchanged water was then poured into the chamber; 12 mL of water for LiCl and 10 mL of water for NaCl. The contents of each prepared salt package were then poured into each chamber and stirred constantly. It was ensured that each saturated salt solution had the ratio of 60-90% undissolved salt to 10-40% liquid. It was ensured no more than 1 cm of undissolved salt and liquid was at the bottom of each salt chamber. Each chamber was sealed and then left undisturbed for at least 48 hours prior to probe calibration to ensure the appropriate humidity was formed inside the chamber above the salt solutions in the air.

For calibration, each T2/RH probe was inserted into a salt chamber containing Lithium chloride which creates an 11% RH inside the chamber. The probe remained in the salt chamber for 30 minutes, allowing the sensor to stabilize and the value was recorded. This process was repeated for the NaCl salt solution. It was ensured that the difference between the humidity references was at least 50%.

Additional Calibration

Prior to each experiment a two-point calibration was performed by inserting , thermocouples and thermistors into 25°C and 45°C circulating water baths, respectively and recording the voltage in the data acquisition system.

Instrumentation

Experiments were conducted in a thermoneutral environment. Following subject check-in, MD fibers were inserted into the ventral forearm. Entry and exit points for MD fiber insertion were marked on the skin for three MD sites, avoiding visible blood vessels. The skin was cleaned using sterile technique with betadine and alcohol. A sterile bag of ice was then

placed over the sites for 5 minutes to numb the skin. For each site a 25-gauge needle was guided horizontally through the dermis and exited the skin near the exit mark. Three intradermal MD fibers (25 gauge, MD 2000 Bioanalytical Systems, West Lafayette, IN) were inserted into the left ventral forearm and were spaced 4.0 cm apart to prevent cross-reactivity of pharmacological agents.[9] The MD probes, consisting a membrane (320 μm outer diameter) and connective tubing (650 μm outer diameter), were threaded through the lumen of the 25-gauge needle. The needle was then withdrawn leaving the probe membrane in the dermal layer of the skin to deliver pharmacological agents directly to the cutaneous vasculature and eccrine sweat glands.

After MD insertion, lactated Ringer's solution was perfused at a rate of 2μL/min via microinfusion pumps for 60- 90 min to ensure attenuation of the local hyperemic response from needle insertion trauma. Following hyperemia, the subject was instrumented in a water perfused suit that covered the entire body except the head, hands, and feet to facilitate whole body heating. Copper-constantan thermocouples were attached at six sites on the skin surface to continuously measure skin temperature and an unweighted mean skin temperature ($T_{sk\ mean}$) was calculated. A thermistor was placed in the sublingual sulcus throughout baseline and whole body heating to measure oral temperature (T_{or}) as an index of body T_{core}. Appropriate placement was ensured by temperature readings and the thermistor was taped in place. Subjects were instructed to maintain a closed mouth throughout baseline and whole body heating to prevent evaporation of moisture from the mouth and an inaccurate T_{or}. Mean body temperature (T_{body}) was calculated as $T_{body} = (0.9 \times T_{or}) + (0.1 \times \text{mean } T_{sk})$.[3] Laser-Doppler flowmetry probes (MoorLAB; Moor Instruments) were placed over each MD site to measure cutaneous red blood cell flux as an index of SkBF. Measurements were continuously recorded during the experiment. Arterial blood pressure was measured via brachial auscultation every 5 minutes following resolution of

hyperemia induced by needle insertion trauma. Mean arterial pressure (MAP) was calculated as 1/3 systolic blood pressure + 2/3 diastolic blood pressure.[9] SkBF was expressed as CVC (red blood cell flux/MAP) and normalized as percent of maximal CVC values (%CVC_{max}).[9]

Pharmacological agents were mounted on the microinfusion pumps throughout the experimental protocol. The delivery of drugs was accomplished through a mixture of lactated Ringer's solution mounted on microinfusion pumps (Bioanalytical Systems Bee hive and Baby Bee, West Lafayette, IN) and the desired drugs that reached an isolated area of the skin (~2.5 cm^2). A summary table of pharmacological agents and function can be found below in Table 1 for further clarification. Drugs were perfused as follows throughout the whole body heating protocol as follows:

Site 1. Lactated Ringers Solution
Site 2. EPO & L-NNA
Site 3. SNP & L-NNA

Drug dilution ranges were 10/50/100/200 ng/mL of EPO mixed with 10mM L-NNA. The SNP ranged from 50 μM 100/200/500 μM mixed with 10mM L-NNA. A summary of the actions of each drug are shown in table 1:

Table 1. Summary of experimental pharmacological agents and their functions. CVC; cutaneous vascular conductance.

Pharmacological Agent	**Abbreviation**	**Function**	**Expected effect on CVC**
N^G-Nitro-L-arginine	L-NNA	Non-specific NO inhibitor	Blocks NO-dependent vasodilation
Sodium nitroprusside	SNP	NO donor	Induces vasodilation
Epoprostenol Sodium	EPO	Prostacyclin (PGI-2) agonist	Induces vasodilation

Local sweating rates were measured using ventilated capsules perfused with compressed medical-grade dry nitrogen with a relative humidity of 1-2%.[3] Sweat capsules (0.5 cm^2) were positioned over the center of each MD fiber. The temperature and humidity of the air flowing out of the capsules were measured using capacitance hygrometers that were calibrated using reference solutions of known temperature and humidity (model HMT330; Vaisala, Helsinki, Finland).[3] Sweating rate (SR) was calculated based on the change in relative humidity of the air as it passed through the capsule (Δrh) at a determined air flow (AF), the density of saturated steam at the given temperature (D), and the capsules surface area (SA), using the following equation:[3]

$$SR=[AF\times(\Delta rh/100)\times D]/SA.$$

Experimental Protocol

Whole body heating

Following resolution of insertion trauma, baseline data were collected for 20 min ensuring mean T_{sk} was maintained at a thermoneutral temperature using a water-perfused suit (34°C). A thermal comfort scale was verbally asked (1= cold, 5=neutral, 10= hot) at baseline and throughout heating. After the collection of baseline, T_{sk}, T_{or}, SkBf and SR data, 50°C water was perfused through the water-perfused suit to elevate T_{or} by 1°C. T_{or} was then clamped for 5 min by reducing the temperature of the water perfusion. After 5 min of steady-state laser-Doppler flux values, water perfusing the suit was returned to 34°C and 25 mM SNP (Nitropress; Abott Laboratories, Chicago, IL) was perfused for 20 min through each MD site at a rate of 4 µl/min to obtain maximum SkBF for normalization of CVC values to a percentage of maximal SkBF (%CVCmax).

MD Drug Perfusion

Baseline data (BL 1) were collected for 20 min following hyperemia. After BL 1 measurements were collected, T_{sk} was clamped at 34°C (water-perfused suit) during perfusion. Ringer's solution was perfused as a control site and two sites were perfused with a NOS inhibitor, L-NNA for 30 minutes before a second baseline was recorded. Following baseline 2 (BL 2), whole body warming was initiated by perfusing 50 °C water through the water-perfused suit. During heating, SNP and EPO sodium were perfused in combination with L-NNA at two separate sites:

Site 1: Control
Site 2: SNP + 10 mM L-NNA
Site 3: EPO sodium + 10 mM L-NNA

The dilutions of SNP and EPO sodium were progressively increased on their respective sites to match the vasodilation achieved at the control site. After completion of the perfusion, 25 mM SNP was perfused through all sites at a rate of 4 µl/min to induce maximal cutaneous vasodilation for normalization of data.

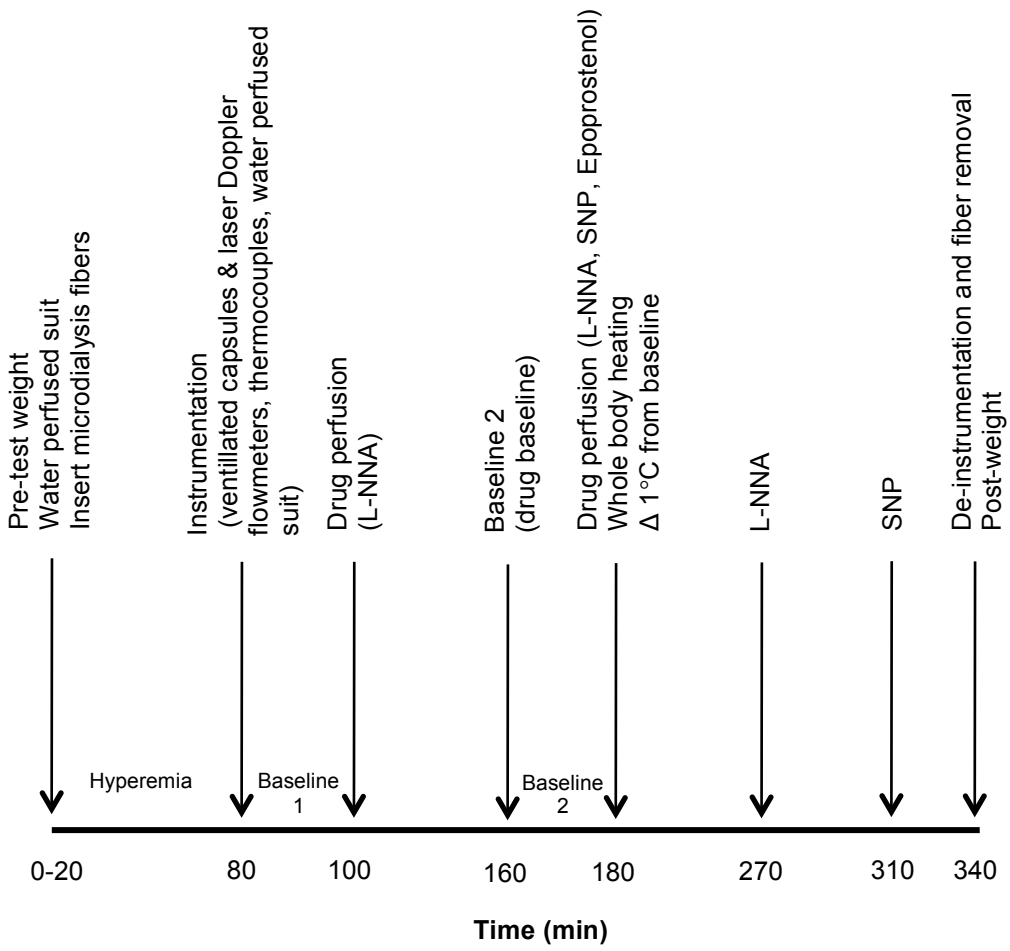

Figure 1. A schematic representative of the experimental protocol.

*Unexpectedly, it was found that EPO is inactive in physiological pH and therefore a buffer was created to maintain drug stability throughout the experiment. Further details may be found in Appendix I.

RESULTS

Two pilot studies were conducted to test the drug dilutions of EPO+L-NNA and SNP+L-NNA to produce sufficient vasodilation (CVC%max) to match the control site during a whole body heating protocol (Figures 1 and 2). CVC % max values were calculated for each 0.1°C rise in T_{core} during whole body heating and values were compared across sites. Figure 1 illustrates the SkBF responses at the control site versus the EPO+L-NNA and SNP+L-NNA sites.

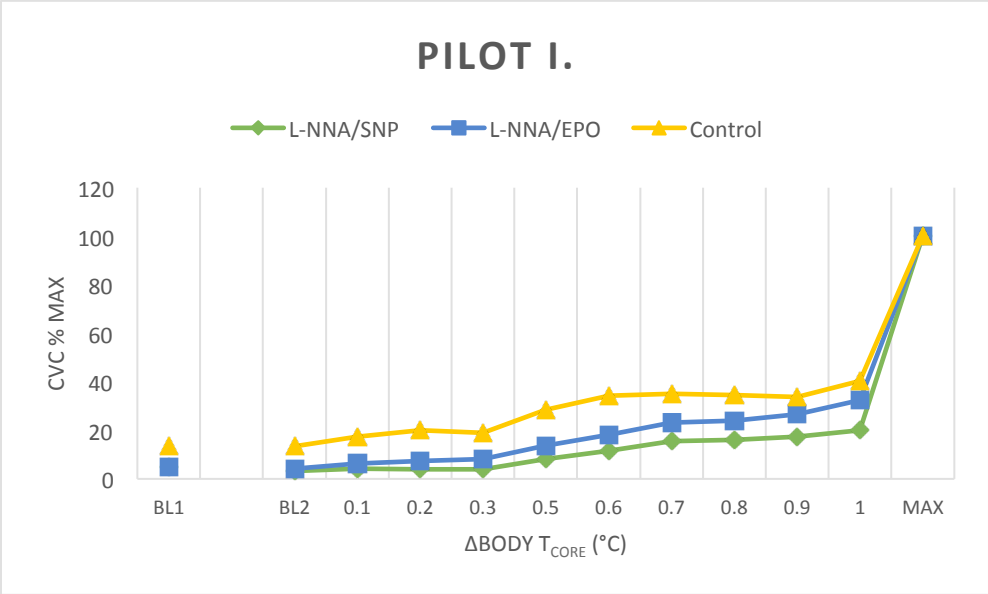

Figure 1. Cutaneous blood flow responses during pilot study 1 expressed as a percentage of CVC%max during a whole body heating protocol to a 1°C rise in core temperature (ΔT_{core}) at three sites.

No differences were observed between the maximal values at each site when perfused with 25mM SNP, which was required for data normalization. Throughout the whole body heating procedure for pilot I, all sites showed a similar pattern of vasodilation, however both the L-NNA+SNP and L-NNA+EPO sites displayed lower rates of vasodilation than at the control site, with L-NNA+SNP demonstrating the lowest values of CVC (%max). At 0.9°C and 1.0°C rise in T_{core} the EPO+L-NNA site and control site indicated similar levels of vasodilation.

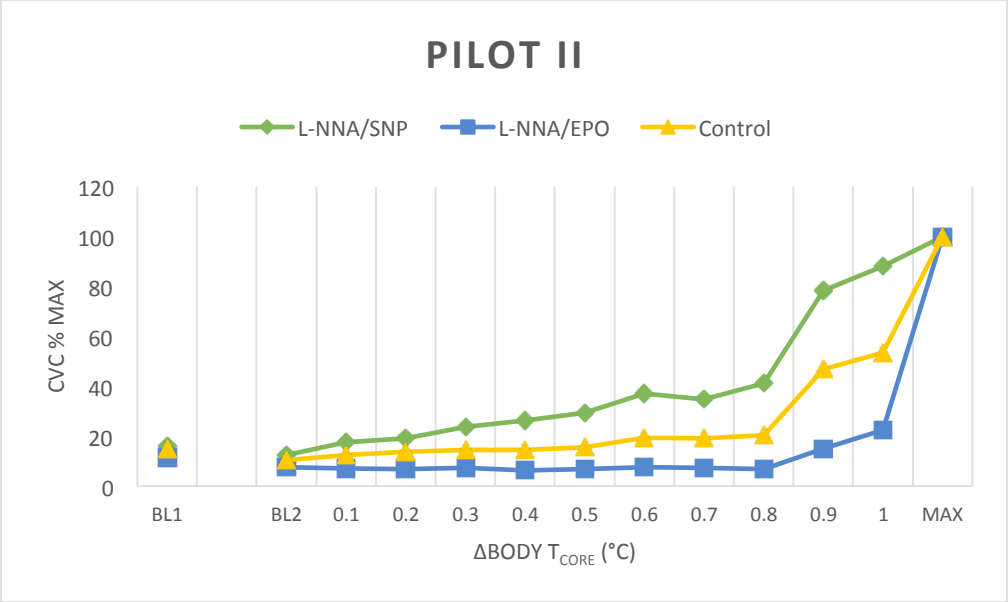

Figure 2. Cutaneous blood flow responses during pilot study 2, expressed as a percentage of CVC%max during a whole body heating protocol to a 1°C rise in core temperature (ΔT_{core}) at three sites.

Similar to pilot study 1, no differences were observed between sites for maximal cutaneous vasodilation in response to 25mM SNP. Throughout the whole body heating protocol the SNP/L-NNA site showed greater vasodilation in compared to the other sites. At the 1°C rise in T_{core}, the SNP/L-NNA site showed a value of 88.2 CVC%max versus 53.6 and 22.4 CVC%max at the Control and EPO+L-NNA sites respectively. The control site demonstrated higher SkBF values compared to the EPO+ L-NNA site throughout the protocol. This indicated that EPO was not stimulating PGI-mediated vasodilation as was expected, yet the L-NNA was likely blocking NO production.

DISCUSSION

Throughout both pilot studies a whole body heating protocol was successfully utilized to elicit cutaneous vasodilation. The magnitude of cutaneous vasodilation at the control sites was 53.6% CVC max, similar to that observed in young healthy individuals in other studies using a similar protocol.[8] During pilot study 1, the SNP+ L-NNA and EPO+ L-NNA sites both demonstrated lower values of CVC%max compared to the control site, indicating that higher concentrations of both drugs were required to flow match the vasodilation with the control site. During the subsequent pilot study (pilot 2), the SNP L-NNA site demonstrated a significantly higher rate of CVC%max than the control site, suggesting higher vasodilation occurred compared to the control and therefore a greater concentration of SNP was administered than necessary. Unexpectedly, the EPO+ L-NNA site displayed values that were lower than the control site, indicating the EPO+ L-NNA solution was ineffective. After analyzing the cutaneous vasodilation data it was evident that the EPO was not effectively stimulating PGI-2-mediated vasodilation as had been anticipated. Considering the SkBF was lower than the control site during pilot study 2, this indicates that the L-NNA was effectively blocking NO-dependent vasodilation, but PGI-2 mediated vasodilation not was occurring, thus resulting in low blood flow. After further investigation, despite the EPO being soluble in Lactated Ringer's (physiological pH), the pharmacological company supplying the drug could not confirm it would remain stable. The half-life of EPO is reported to be short in vivo and in vitro reported to range from 30 seconds to a few minutes at a physiological pH, making it difficult to mix and administer successfully in Lactated Ringer's. To remain active EPO sodium requires a pH of 10.4 and therefore must be dissolved in a buffer. A suitable buffer was developed involving a mixture of sodium bicarbonate and sodium carbonate to achieve a pH of 10.48. The solution was then mixed with (HCl) to return the buffer to physiological pH OF 6.74.

The next step in this study includes ensuring that the EPO does not precipitate out of the buffer as it is brought back to a physiological pH with HCl. Additional pilot studies would then be completed to ensure EPO remains active when dissolved in the buffer, brought back to physiological pH with HCl and administered via MD (dose response pilot.) Pilot studies for whole body heating to match blood flow at each site would then be conducted, including a control site, an EPO+ L-NNA site and an SNP+ L-NNA site. Once flow matching has been achieved between sites, the sweating study in which the whole body heating protocol is utilized with the addition of the sweat system to measure regional sweat rates over each MD site may begin. It is expected that sweating responses will be lower at the EPO+L-NNA site versus the SNP+L-NNA site, indicating a role for NO in signaling the eccrine sweat response. If sweat rates do not significantly differ evidence will not support a role for neither prostaglandin-dependent nor NO-dependent vasodilation mechanisms in additionally signaling the sweat response.

It has been consistently observed that the aged population experience attenuated cutaneous vasodilation and sweat responses as well as increasingly rely on blunted NO-dependent mechanisms to achieve cutaneous vasodilation. Considering these age-related decrements associated with NO, it is likely that sweat rate will be observed as lower with aging resulting in a reduction in NO-signaling for the sweat response.

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APPENDIX I

Buffer Development

Following analysis of data from pilot study 2, it was observed that the EPO sodium was inactive when administered via MD (see discussion for further details). It was established that EPO sodium becomes inactive in solutions with a pH lower than 10.4. Considering physiological pH is approximately 7.4, EPO sodium will not remain active when dissolved and administered under these conditions. To ensure maintenance of an active form of EPO during experimentation, a buffer was created at room temperature to provide a stock solution. The following procedures were utilized to develop an appropriate buffer:

A two-point calibration was performed before each use of the pH probe with two solutions of a known pH (pH 7, pH 10). The probe was thoroughly rinsed with ultrapure water prior to and between calibrations. Following calibration, separate formulas of either sodium bicarbonate or sodium carbonate dissolved in ultrapure water were mixed to determine their pH. Table 1 shows the pH achieved utilizing these separate mixtures, neither of which were of a suitable pH for use with EPO sodium.

Table 1. Details of initial buffer development utilizing sodium bicarbonate and sodium carbonate.

Chemical Compound	**g/100 mL ultrapure water**	**pH**
Sodium bicarbonate ($NaHCO_3$)	0.851	8.27
Sodium carbonate (Na_2CO_3)	2.831	11.48

To achieve the desired pH of ~10.4, a combination of sodium bicarbonate and sodium carbonate was required.

Table 2. Details of buffer development utilizing a combined mixture of sodium bicarbonate and sodium carbonate.

Solution	**Volume of Sodium bicarbonate ($NaHCO_3$) solution of 8.27 pH (ml)**	**Volume of Sodium carbonate (Na_2CO_3) solution of 11.48 pH (ml)**	**pH**
1	15	35	10.70
2	20	35	10.57
3	25	35	10.48
4	30	35	10.41

The next step required returning the buffer to a physiological pH. Lactated Ringer's was added to the sodium bicarbonate/sodium carbonate buffer mixture (pH 10.41). As shown in Table 3, Lactated Ringer's was unable to bring the buffer to a pH lower than 10.19.

Table 3. Dilutions of the sodium bicarbonate/sodium carbonate buffer with Lactated Ringer's.

Solution	Volume of Sodium bicarbonate/sodium carbonate buffer solution of 10.41 pH (ml)	Volume of Lactated Ringer's (ml)	**Final pH**
1	3	3	10.45
2	1	9	10.36
3	0.1	9.9	10.19

Due to the inability of Lactated Ringer's to sufficiently reduce the pH of the buffer solution, 1 molar (1M) hydrochloric acid (HCl) was utilized. A suitable pH for administration of the EPO

sodium via MD was achieved, as show in from the dilutions of the buffer solution to the HCl(1M) in Table 4.

Table 4. Dilutions of the sodium bicarbonate/sodium carbonate buffer with (HCl).

Solution	**Volume of Sodium bicarbonate/sodium carbonate buffer solution of 10.41 pH (ml)**	**Volume of 1M HCl (ml)**	**Final pH**
1	9	0.5	9.38
2	9	1.0	6.74