The roles of nitric oxide, oxidative stress, and angiotensin II type 1 receptor in regulating cutaneous blood flow and sweating during prolonged exercise in the heat with and without fluid replacement

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B.Sc., McGill University 2014

THESIS PROPOSAL

Submitted to the
Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements
For the degree of Master's of Science – Human Kinetics

Faculty of Heath Science, School of Human Kinetics
University of Ottawa, Canada

Acknowledgements

I wish to thank the following individuals for their support and guidance in the development and completion of my thesis. Without your help and encouragement throughout this process, it would not have been possible.

To Dr. Glen Kenny –Thank you for giving me the opportunity to pursue my Master's of Science degree under your supervision. Your continuous guidance as well as your relentless encouragement and support have combined to make you an outstanding mentor for my development as a student, researcher, and colleague.

To my committee members Dr. Andrew Seely and Dr. Ronald Sigal - Thank you for your time and outstanding effort in providing me with timely and constructive feedback on my project.

To my fellow team members and colleagues at the Human and Environmental Physiology Research Unit - Thank you for your continuous help and encouragement throughout this project.

A special thanks to Dr. Naoto Fujii whose expertise and endless knowledge of mechanistic pathways was invaluable to me throughout my degree.

A special thanks to Mr. Robert Meade for his unending assistance throughout the project with data collection and all the long hours spent sweating in the thermal chamber.

Thank you to all of my participants who volunteered to take part in my gruelling project. Your time and commitment towards my thesis is greatly appreciated and I am indebted to you.

I would also like to thank my parents as well as my brothers Christopher and Jared. You have always encouraged me to purse my goals and have kept me grounded during times of struggle. You are a huge part of my ambition and passion to succeed in all areas of life.

Certainly not least, I would like to thank Katy who has been a consistent and unyielding force behind my drive and perseverance. You encourage me to become better every day and are an enormous part of my success in every endeavor.

Abstract

The current study evaluated whether NO synthase (NOS) contributes to cutaneous vasodilation and sweating during prolonged exercise in the heat. In addition, we determined if prolonged exercise-induced increases in reactive oxygen species (ROS) and activation of angiotensin II type 1 receptors (AT_1R) impair heat loss responses. On two separate days, eleven young men completed 90-min of continuous cycling at ~600W of metabolic heat production followed by 40-min of recovery in the heat (40ºC). To evaluate the role of excess fluid loss via sweating, participants completed a second session of the same protocol while receiving fluid replacement (FR) determined during the first session (No-FR). Cutaneous vascular conductance (CVC) and local sweat rate (LSR) were measured at four intradermal microdialysis forearm sites perfused with either: (1) lactated Ringer (Control); (2) 10 mM *N*G-nitro-L-arginine methyl ester (L-NAME, NOS inhibition); (3) 10 mM ascorbate (non-selective anti-oxidant); or (4) 4.34 nM Losartan (AT_1R inhibition). Ascorbate treatment increased CVC at 60- and 90-min of exercise versus Control during the FR ($P < 0.02$), but not the No-FR condition ($P > 0.31$). CVC was reduced at the L-NAME treated site ($P < 0.02$), but was not different relative to Control at the Losartan treated site ($P > 0.19$) irrespective of condition. LSR did not differ between sites or as a function of condition (all $P > 0.10$). We conclude that NO regulates cutaneous vasodilation but not sweating, irrespective of fluid replacement, and ascorbate sensitive ROS impair cutaneous vasodilation during prolonged exercise in the heat with FR.

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GLOSSARY

ANG II – angiotensin II

AT_1R – Angiotensin II Type 1 Receptor

CVC – cutaneous vascular conductance

CVC_{max} – maximal cutaneous vascular conductance

$_L$-NAME – N^G-nitro-$_L$-arginine methyl ester

NO – nitric oxide

NOS – nitric oxide synthase

PO/AH – pre-optic anterior hypothalamus

ROS – reactive oxygen species

SNP – sodium-nitroprusside

VO_{2peak} – peak rate of oxygen consumption

PART ONE: EMPIRICAL AND THEORETICAL CONSIDERATIONS

CHAPTER I

INTRODUCTION

1.1 Introduction

In humans the maintenance of a stable body core temperature of ~37˚C is essential for normal physiological function. However, during periods of physical activity and/or prolonged exposure to hot environments body core temperature will begin to rise beyond this homeostatic set point. In response to this increase in body core temperature, the human body is able to dissipate heat through cutaneous vasodilation (to increase blood flow to the skin) and sweating. However, if heat cannot be adequately dissipated, body core temperature will rise resulting in a greater risk for heat-related illnesses such as heat stroke, heat-related syncope, and ultimately death (Kenny & Jay, 2013). Despite the importance of cutaneous vasodilation and sweating to the maintenance of body core temperature within a physiologically safe range, the biochemical mechanisms that govern these heat loss responses have yet to be fully elucidated, particularly during prolonged dynamic exercise.

Many studies have indicated a role for nitric oxide (NO) in the mediation of human physiological heat loss responses during short-term exercise in the heat, wherein increases in NO bioavailability promote heat loss through full expression of cutaneous vasodilation (McGinn *et al.*, 2014b; Stapleton *et al.*, 2014; Fujii *et al.*, 2015b; Meade *et al.*, 2015; Fujii *et al.*, 2016; Louie *et al.*, 2016) and sweating (Fujii *et al.*, 2015b). However, the local concentration of NO in the skin can decrease through its interaction with other molecules in the body such as reactive oxygen species (ROS). ROS are byproducts generated from oxygen metabolism that readily react with other compounds in the body due to an unpaired valence electron such as superoxide and peroxides. Under normal resting conditions, ROS levels are regulated by the endogenous antioxidants found in the body but, particularly in prolonged or high-intensity exercise the increased oxidative metabolism produces a greater number of ROS such that these inherent

defenses can become overwhelmed (Lovlin *et al.*, 1987; Hartmann *et al.*, 1995; Poulsen *et al.*, 1996; Cooper *et al.*, 2002; Meade *et al.*, 2015). Consequently, excess ROS interact with and reduce NO bioavailability, thereby preventing maximum heat loss by preventing the full expression of cutaneous vasodilation (Stewart *et al.*, 2006; Stewart *et al.*, 2008a; Stewart *et al.*, 2008b; Fujii *et al.*, 2015b) and sweating (Fujii *et al.*, 2015b).

Recent research has suggested a role for angiotensin II (ANG II) in ROS-mediated reductions in NO-dependent heat loss during passive heat stress conditions (Stewart *et al.*, 2006; Stewart *et al.*, 2008a; Stewart *et al.*, 2008b). Specifically, activation of ANG II Type 1 Receptors (AT_1R) can induce the activation of NADPH oxidase ultimately resulting in the enhanced production of the ROS superoxide (Griendling & Ushio-Fukai, 2000; Hanna *et al.*, 2002; Harrison *et al.*, 2003). Moreover, intradermal infusion of exogenous ANG II has also produced reductions in cutaneous vasodilation and sweating prior to and following moderate exercise in the heat; thus clearly demonstrating a role for ANG II in modulating heat loss responses (Fujii *et al.*, 2015a). Although recent research has begun to elucidate the mechanisms that underlie cutaneous vasodilation and sweating during short-term and/or moderate intensity exercise in the heat, it has yet to be determined whether these mechanisms regulate cutaneous vasodilation and sweating similarly during exercise of prolonged duration.

In vivo, the circulating concentration of ANG II can become increased due to exercise (Staessen *et al.*, 1987; Bocqueraz *et al.*, 2004; Brothers *et al.*, 2006) and hypohydration (Francesconi *et al.*, 1985). With respect to heat loss responses, hypohydration is well known to cause decrements in cutaneous blood flow (Nishiyasu *et al.*, 1991; Montain & Coyle, 1992) and sweating (Senay, 1968; Cheuvront *et al.*, 2010) however, it remains to be determined if these reductions are mediated through ANG II-dependent mechanisms. Moreover, in addition to

oxidative stress, prolonged exercise can lead to hypohydration, thus further demonstrating a potential link between ROS- and ANG II-mediated pathways regulating heat loss responses (Sawka, 1992). In evaluating the role of hydration status on regulating the mechanisms of heat loss, this study will also provide a unique perspective relative to the previous research conducted by our group where natural fluid losses due to sweating occurred without intervention.

Altogether, it remains undetermined if NO, ROS, and ANG II regulate the heat loss mechanisms of cutaneous vasodilation and sweating during prolonged exercise in the heat and whether this regulation may differ as a function of hydration status.

1.2 Rationale and Statement of the problem

Prolonged exercise is known to increase the production of ROS and the concentration of circulating ANG II. Furthermore, recent research has shown that increased levels of ROS and ANG II in the skin can reduce cutaneous vasodilation (Stewart *et al.*, 2006; Stewart *et al.*, 2008a; Stewart *et al.*, 2008b; Fujii *et al.*, 2015a; b) and sweating (Fujii *et al.*, 2015a; b) through NO-mediated mechanisms. Prolonged exercise may also cause hypohydration, which can further reduce the physiological heat loss responses. However, it remains to be determined if this reduction in heat dissipation may be mediated through oxidative stress- and ANG II-mediated pathways. Moreover, there remains an important knowledge gap as to whether the observed roles NO, ROS and ANG II have in regulating cutaneous vasodilation and sweating during short-term and/or moderate intensity exercise in the heat translate similarly to prolonged exercise. Additionally, previous studies did not account for the natural fluid loss due to sweating during exercise in the heat; consequently, it remained to be determined whether hydration status can influence the role of these biochemical pathways on the mechanisms of heat loss. Therefore, we

employed intradermal microdialysis to administer pharmacological agents directly to the skin to inhibit the NO, ROS and ANG II pathways thereby assessing their relative contribution to the regulation of heat loss responses of cutaneous vasodilation and sweating during both prolonged exercise and recovery in the heat under both a hypohydrated and euhydrated state.

1.3 Study objectives

The objective of the proposed study was to examine the underlying mechanisms that regulate cutaneous vasodilation and sweating during prolonged exercise in the heat. Specifically, this project aimed to:

1) Assess the separate roles of the nitric oxide synthase and AT_1R in the regulation of cutaneous vasodilation and sweating during prolonged exercise in the heat.
2) Evaluate whether local administration of the non-selective antioxidant ascorbate regulates cutaneous vasodilation and sweating during prolonged exercise in the heat.
3) Determine the effect, if any, of hydration status on NO, ROS and ANG II-mediated regulation of cutaneous vasodilation and sweating during prolonged exercise in the heat.

1.4 Hypotheses

This project evaluated the hypothesis that during prolonged exercise in the heat, cutaneous vasodilation and sweating will be augmented to a greater extent than control conditions with local antioxidant infusion and local AT_1R blockade. Conversely, it was hypothesized that local infusion of nitric oxide synthase blocker would reduce cutaneous

vasodilation and sweating relative to the control site. Furthermore, it was postulated that fluid replacement during prolonged exercise would reduce increases in body core temperature and diminish the ROS- and ANG II-mediated reductions in cutaneous vasoconstriction and sweating.

1.5 Relevance of the study

Cutaneous vasodilation and sweating are essential to the regulation of body temperature, especially during prolonged exercise and/or heat exposure. Although exercise induced oxidative stress and hypohydration may reduce heat loss responses in this situation, there remain significant knowledge gaps in our understanding of the mechanisms underpinning this response. This project addressed these important knowledge gaps by providing important mechanistic insight into the respective contributions of NO, ROS and ANG II in the regulation of the heat loss responses during prolonged exercise in the heat. Secondly, the results gained from this study has provided new data which serves new directions of research aimed at evaluating how hydration status may modulate the mechanisms regulating cutaneous vasodilation and sweating.

1.6 Delimitations and limitations

The cutaneous vasodilatory and sweating responses observed in the proposed study was confined to the forearm skin. Consequently, it is possible that the local mechanisms elucidated may not translate to various other parts of the body due to regional variability. Regardless, the insight gained from the forearm responses is furthering our understanding of the mechanisms underpinning the regulation of heat loss responses. Additionally, our study evaluated the mechanisms underlying the heat loss responses during prolonged exercise (≥ 90 mins) in the heat. For this reason, highly physically active (i.e.VO_{2peak} > 50 ml·min^{-1}·kg^{-1}) young adults were

recruited to ensure that they could complete the experimental protocols. Therefore, the findings may not be representative of young adults with lower aerobic capacity. Furthermore, individuals with chronic pathophysiologic conditions were excluded from the study. As a consequence, the findings of the proposed project may not apply to these individuals. Finally, this study was performed in young males only and thereby may not be representative of the older male or female populations.

CHAPTER II

REVIEW OF THE LITTERATURE

2.1 Human Heat Balance Equation

To best understand the physiological mechanisms that regulate human body core temperature, it is first important to understand the physical principles that underlie them. The human heat balance equation was developed to model the dynamic exchange of heat between the human body and its ambient environment (Gagge & Gonzalez, 1996; Kenny & Jay, 2013).

$$\mathbf{S = M - (\pm W) \pm (C+R) - E}$$

Where all terms are expressed in $W \cdot m^{-2}$ and,

S = rate of body heat storage

M = metabolic rate

W = rate of mechanical work

C = rate of convective and conductive heat loss from the skin

R = rate of radiative heat loss from the skin

E = rate of evaporative heat loss from the skin

This equation illustrates that for a human to maintain heat balance (no heat storage), the rate of metabolic heat production must be equal to the rate of heat lost to the environment. Metabolic heat load, or the heat that is generated within the body, is the difference in metabolic energy production and mechanical work, or M – (±W). Typically this is expressed as M – W given that work is being done by the body (e.g. exercise). Total heat loss is made up of dry (e.g. radiative, convective and conductive) and evaporative heat loss; however, dry heat loss is dependent upon the temperature gradient between the skin and the ambient environment. Consequently, environmental conditions that exceed skin temperature (~34˚C) result in dry heat loss becoming negative, or a dry heat gain (Gagge & Gonzalez, 1996; Kenny & Jay, 2013). In contrast, evaporative heat loss, which is dependent upon the vapour pressure gradient between the skin

and the surrounding air, can contribute to heat loss from the body at all elevated temperatures and represents the primary avenue for heat loss during exercise and/or exposure to hot ambient conditions (Gagge & Gonzalez, 1996; Gagnon *et al.*, 2013; Kenny & Jay, 2013). Taken together, the human heat balance equation provides a functional model to evaluate the many variables involved in human thermoregulation.

2.2 Basic Human Thermoregulation

Inherent to the principles of physiological homeostasis, it is essential that human body core temperature be maintained within functional limits (Taylor, 2006). This temperature regulation is coordinated at the brain level by the pre-optic anterior hypothalamus (POAH). In response to increases in metabolic heat production due to physical activity and/or exposure to hot environmental conditions, the POAH receives and integrates incoming sensory information from the core and skin thermoreceptors; from here the POAH is able to elicit the effector responses of cutaneous vasodilation and sweating (Boulant & Bignall, 1973; Hensel, 1981). It has been suggested that the POAH places a greater relative importance of the temperatures received from the core due to the physiological importance of internal temperature regulation versus skin temperature regulation on organ and systems function (Taylor, 2006). Ultimately, for temperature regulation the POAH functions via a negative feedback loop to balance the rate in which heat is gained by the body to the rate in which the body must dissipate heat to maintain body core temperature within a narrow range.

The effector responses of the POAH to heat stress can be defined by three distinct characteristics: the onset threshold, thermosensitivty and the plateau (Charkoudian, 2003). The onset threshold is described as the mean body temperature at which increases in skin blood flow and sweating occur. Following the onset threshold, the thermosensitivity can be defined as the

proportional increase in heat loss responses that is elicited in response to increases in mean body temperature. Lastly, the plateau phase may occur. The plateau refers to the point in which skin blood flow and sweating can no longer increase in response to further increases in mean body temperature. During this period the maximal capacity of the heat loss through skin blood flow and sweated has occurred (Charkoudian, 2003).

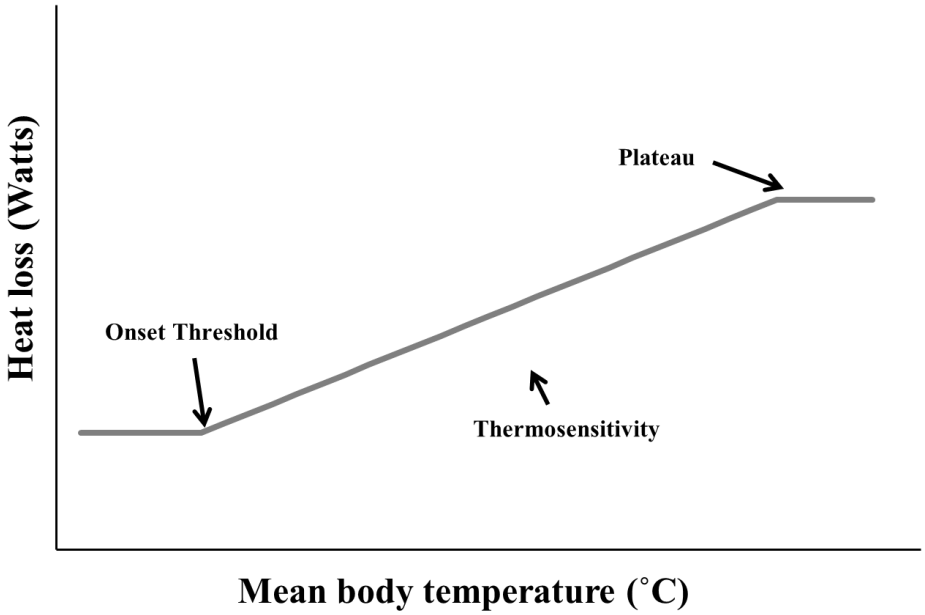

Figure 1. An illustration of the heat loss response to increases in mean body temperature during heat stress. The onset threshold indicates the mean body temperature threshold to activate increases in heat loss responses. Following the onset threshold, the thermosensitivity of the response refers to heat loss increasing proportionally to increases in mean body temperature. The plateau phase may follow; at this point increases in mean body temperature elicit no further increases in heat loss. This pattern of response has been demonstrated using local, whole-limb and whole-body measurements of heat loss. Adapted from (Gagnon & Kenny, 2012).

2.3 Mechanisms of Thermoregulation

In humans, heat stored within the body may be lost due to cutaneous vasodilation and sweating (Taylor, 2006). In response to increases in mean body temperature cutaneous blood vessels can widen, thereby distributing an increased amount of blood to the skin, where the heat it contains may be dissipated to the environment. In reference to the human heat balance equation, this increased skin blood flow also acts to elevate skin temperature, thus improving the

thermal gradient for dry heat loss to the environment. Cutaneous vasodilation, is an important means of temperature regulation during times of rest and mild heat stress; however, during situations that require substantial heat loss, sweating is the primary physiological mechanism of heat dissipation (Gagge & Gonzalez, 1996; Kenny & Jay, 2013). Notably, in instances of increased metabolic heat production and/or the prolonged exposure to high environmental temperatures there is a greater reliance on sweating as a form of heat loss, such that sweating contributes to ≥80% of total heat loss compared to ~25% at rest (Cain & McLellan, 1998; Gavin, 2003).

2.3.1 Cutaneous Blood Flow

Although it is not the primarily avenue of heat loss during a thermal challenge, cutaneous vasodilation plays an essential role in the maintenance of body core temperature (Charkoudian, 2003; Johnson & Kellogg, 2010; Johnson *et al.*, 2014). In fact, it has been discovered that skin blood flow can increase from ~250 $ml \cdot min^{-1}$ at rest to values as high as 8000 $ml \cdot min^{-1}$ during heat stress (Rowell, 1974; Crandall & Gonzalez-Alonso, 2010). These large changes in skin blood flow are a result of the relative contributions of the active vasodilator and the active vasoconstrictor sympathetic nerve systems that control its vasomotor activity (Johnson & Proppe, 1996). Sympathetic vasoconstriction is primarily mediated through presynaptic release of noradrenaline, and its withdrawal provides ~10-15% of the vasodilatory response to heat stress. In contrast, it is believed that the remaining 85-90% of the vascular tone during heat stress is controlled by the active vasodilator nerve system; however, relatively little is known about the regulation of this system and/or the substance(s) that control it (Charkoudian, 2003).

At a constant blood pressure, the rate of cutaneous blood volume delivery is primarily regulated by the diameter of connected arterioles and capillary beds. The supplying arterioles are lined with endothelial and vascular smooth muscle cells, whose diameter can be influenced by biochemical modulations originating from each of these cell types or in the skin (Edwards *et al.*, 2010). Produced by nitric oxide synthase (NOS) located in endothelial cells, nitic oxide (NO) is one such endothelium derived modulator that plays a major role in regulating vascular tone. By travelling across myoendothelial gap junctions to the vascular smooth muscle cells, NO ultimately causes relaxation of the smooth muscle, thereby widening the arterioles and permitting greater blood flow (Feletou & Vanhoutte, 2009).

2.3.2 Sweating

Sweat secreted onto the skin and the subsequent evaporation of this sweat is the primary avenue for humans to cope with elevated heat stress, particularly during exercise and/or in hot environmental conditions (Kenny and Jay, 2013). Importantly, if ambient temperature exceeds that of the skin, evaporative heat loss is the sole mechanism through which heat dissipation can occur. Particularly during exercise in hot environmental temperatures, maximal sweating can be elicited; during these times the ~2-4 million eccrine sweat glands of the body are able to produce up to 3.7 L/hr (Shibasaki & Crandall, 2010; Kenny & Jay, 2013). In humans, the millions of eccrine sweat glands that are responsible for heat loss can be found near the surface of most glabrous (hairless) skin. The eccrine sweat glands are under control from the cholinergic nervous system; as such, they are stimulated by acetylcholine (Sato, 1973). In parallel with cutaneous vasodilation, it has been demonstrated that NO is required for full expression of the sweat response during physical activity. Various studies reported diminished sweat rates for young

adults in areas treated with nitric oxide synthase blocker in response to local or whole body heat stress (Welch *et al.*, 2009; Fujii *et al.*, 2014; McGinn *et al.*, 2014b; Stapleton *et al.*, 2014; Fujii *et al.*, 2015b). However, the role of NO in sweating is thought only to be permissive such that NO itself cannot stimulate sweat production.

2.4 Prolonged exercise and mechanisms

Recent research has begun to elucidate the mechanisms that underlie cutaneous vasodilation and sweating during short-term and/or moderate intensity exercise in the heat; however, it has yet to be determined whether these mechanisms hold true for exercise of prolonged duration. Due to the strenuous nature of prolonged exercise, it can produce substantially different consequences to human physiology from short-term exercise. Namely, it is well documented that prolonged exercise in the heat can lead to oxidative stress (Lovlin *et al.*, 1987; Hartmann *et al.*, 1995; Poulsen *et al.*, 1996; Cooper *et al.*, 2002), hyponatremia (Montain *et al.*, 2001), and dehydration (Sawka, 1992). It remains unknown if these physiological consequences of prolonged exercise in the heat play a role in regulating cutaneous vasodilation and sweating.

Research has shown that increases in oxidative stress due to aging (Holowatz *et al.*, 2006) and high intensity exercise (Meade *et al.*, 2015), may act to reduce cutaneous vasodilation. Additionally, exercise-induced dehydration has been shown to impair cutaneous blood flow (Nishiyasu *et al.*, 1991; Montain & Coyle, 1992) and reduce sweat rate for a given increase in body core temperature (Senay, 1968; Cheuvront *et al.*, 2010). These decrements in heat loss responses may be partially mediated through angiotensin II (ANG II), whose circulating concentration is known to increase during exercise (Staessen *et al.*, 1987; Bocqueraz *et al.*, 2004;

Brothers *et al.*, 2006) and dehydration (Francesconi *et al.*, 1985). Moreover, ANG II has been shown to reduce cutaneous vasodilation during passive heat stress (Stewart *et al.*, 2006; Stewart *et al.*, 2008a; Stewart *et al.*, 2008b) as well as both cutaneous vasodilation and sweating following exercise in the heat (Fujii, 2015a). In addition, prolonged exercise in the heat can lead to substantial thermal stress due to the long-term cumulative metabolic heat production (Gagnon *et al.*, 2012). Consequently, these limitations on the mechanisms of heat dissipation in conjunction with prolonged thermal stress often result in an increase in core body temperature leading to reduced exercise performance and/or heat-related injuries (Sawka, 1992). Ultimately, although prolonged exercise in the heat poses many challenges to heat loss responses, whether oxidative stress and ANG II contribute to the regulation of cutaneous vasodilation and sweating remain undetermined.

2.4.1 Nitric Oxide

Although relatively little is known about the control of cutaneous vasodilation, a prominent role has been established for NO. NO is believed to be responsible for inducing ~30-45% of the vasodilatory response to whole-body passive heating (Kellogg *et al.*, 1998; Shastry *et al.*, 2000; McCord *et al.*, 2006) and has also been shown to be an important modulator of cutaneous blood flow during exercise (McCord *et al.*, 2006; Welch *et al.*, 2009; Fujii *et al.*, 2014; McGinn *et al.*, 2014a; McGinn *et al.*, 2014b; Fujii *et al.*, 2015b). Similarly, NO-dependent sweating has been observed in younger individuals during exercise (Welch *et al.*, 2009; Fujii *et al.*, 2014; McGinn *et al.*, 2014b; Stapleton *et al.*, 2014; Fujii *et al.*, 2015b). The degree to which NO may contribute to the control of cutaneous vasodilation and sweating during long-term exercise in the heat has yet to be determined. However, a potential role may exist for exercise

induced increases in oxidative stress and ANG II to decrease NO bioavailability in the skin and consequently, reduce cutaneous vasodilation and sweating (Figure 2).

2.4.2 Oxidative Stress

In situations such as high intensity or prolonged exercise, reactive oxygen species are produced in excess of the body's inherent anti-oxidant defense capacity (Lovlin *et al.*, 1987; Hartmann *et al.*, 1995; Poulsen *et al.*, 1996; Cooper *et al.*, 2002). Consequently, NO can react with the more readily available ROS, such as superoxide to form peroxynitrite, ultimately leading to diminished NO bioavailability (Mortensen & Lykkesfeldt, 2014). Additionally, elevated ROS levels can result in the uncoupling of NO synthase, creating a positive feedback loop producing more ROS and further reducing NO availability (Mortensen & Lykkesfeldt, 2014). As previously mentioned, without the full bioavailability of NO there may be subsequent reductions in cutaneous vasodilation and sweating.

In vivo, intradermal administration of ascorbate, a non-selective antioxidant, reduced cutaneous blood flow in response to local heating, albeit in older adults (Holowatz *et al.*, 2006). Similarly, intradermal administration of ascorbate in young adults exercising at high intensity in the heat has also been shown to increase cutaneous blood flow relative to the control condition (Meade *et al.*, 2015). The authors suggested the anti-oxidant likely functioned to stabilize the ROS thereby preventing the ROS from reducing NO bioavailability. Therefore, a clear role exists for oxidative stress mediating cutaneous blood flow during high intensity exercise in the heat; however, it remains to be determined if prolonged exercise is regulated similarly. With respect to the sweating response, it has been shown that increased oxidative stress levels are associated with attenuations in the sweating responses induced by local administration of the cholinergic

agonist, acetylcholine (Hoeldtke *et al.*, 2011). Although few studies have investigated the role of oxidative stress on exercise induced sweating, oxidative stress may modulate sweating through NO-mediated mechanisms as previous observed (Welch *et al.*, 2009; Fujii *et al.*, 2014; McGinn *et al.*, 2014b; Stapleton *et al.*, 2014; Fujii *et al.*, 2015b). Altogether, it is plausible that local cutaneous ROS produced during prolonged exercise in the heat may cause impairments in NO-dependent cutaneous vasodilation and sweating in young adults (Figure 2).

2.4.3 Angiotensin II

During elevated and sustained levels of sympathetic activity, such as occurs during prolonged exercise and dehydration, plasma renin activity increases, leading to increased production of the nonadrenergic signaling molecule ANG II (Kosunen & Pakarinen, 1976; Kosunen *et al.*, 1976; Tidgren *et al.*, 1991). ANG II is known to act directly on Angiotensin II Type 1 Receptors (AT_1R) of vascular smooth muscle cells to cause their contraction (Touyz & Schiffrin, 2000); however, this vasoconstriction may be mediated in-part through increased oxidative stress. Specifically in vascular smooth muscle cells, activated AT_1R's stimulates NAPDH oxidase resulting in the augmented production of superoxide and hydrogen peroxide (Greidling, 1998; Hanna, 1975). Superoxide may react with NO to form peroxynitrate and consequently reduce NO bioavailability (Mortensen & Lykkesfelt, 2014).

During conditions of heat stress, ANG II has also been shown to play a role in the regulation of cutaneous blood flow. Specifically, AT_1R blockade with Losartan improved NO-dependent cutaneous blood flow to passive heat stress (Stewart *et al.*, 2008a; Stewart *et al.*, 2008b). The mechanism of action for ANG II was shown to be isolated to AT_1R as no role in the regulation of cutaneous blood flow was found for the Angiotensin Type 2 Receptor (Stewart *et*

al., 2008b). Moreover, in young adults intradermal administration of ANG II reduced cutaneous blood flow, during passive resting conditions and recovery following exercise in the heat (Fujii *et al.*, 2015a). Similarly, this study also found that the intradermal administration of ANG II in young adults caused a ROS-mediated attenuation in local sweating during passive exposure and recovery from exercise in a hot environment (Figure 2) (Fujii *et al.*, 2015a). However, the amount of ANG II administered may not reflect endogenous concentrations of ANG II in circulation. Therefore to better determine the action of ANG II, AT_1R blockade is a more physiologically relevant technique.

Although ANG II has been shown to play a role in the regulation cutaneous blood flow and sweating during conditions of heat stress, it remains to be determined whether the vaso-constrictive and sudomotor effects of ANG II are present with physiological levels of circulating ANG II induced by prolonged exercise in the heat.

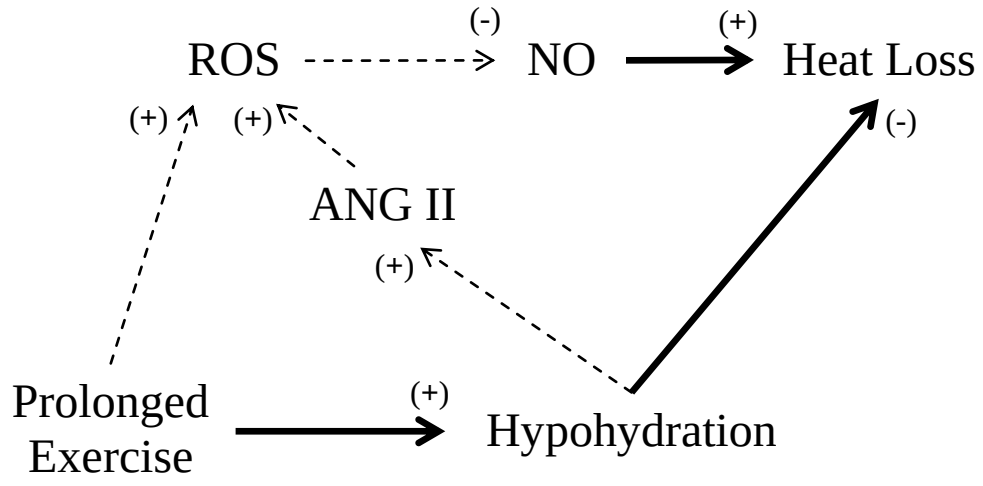

Figure 2. Summary of the potential mechanisms contributing to the impairments in NO-dependent heat loss (i.e., cutaneous vasodilation and sweating) during prolonged exercise in the heat. In this model, prolonged exercise induced increases in cutaneous levels of reactive oxygen species (ROS) reduce nitric oxide (NO) bioavailability, thereby limiting heat loss. Furthermore, prolonged exercise may cause hypohydration, which in turn increases circulating levels of angiotensin II (ANG II). Hypohydration is known to cause impairments in heat loss responses; however, the increased concentration of ANG II due to hypohydration may serve to increase ROS levels in the skin, thereby further reducing NO bioavailability and consequently reducing heat loss as well.

PART TWO: METHODS AND RESULTS OF THE THESIS

Investigating the mechanisms regulating cutaneous blood flow and sweating during prolonged exercise in the heat

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Running Head: Oxidative stress and heat exchange during prolonged exercise

[Paper submitted to American Journal of Physiology – Regulatory, Integrative, and Comparative Physiology (R-00284-2017)]

Word Count: 5,387

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ABSTRACT:

The roles of nitric oxide synthase (NOS), reactive oxygen species (ROS) and angiotensin II type 1 receptor (AT_1R) activation in regulating cutaneous vasodilation and sweating during prolonged (≥60 min) exercise are currently unclear. Moreover, it remains to be determined whether fluid replacement (FR) modulates the contribution of these mechanisms to heat loss. To investigate, eleven young men completed 90-min of continuous moderate intensity (46% of VO_{2peak}) cycling performed at a fixed rate of 600 W (No-FR condition). On a separate day, participants completed a second session of the same protocol while receiving FR to offset sweat losses (FR condition). Cutaneous vascular conductance (CVC) and local sweat rate (LSR) were measured at four intradermal microdialysis forearm sites perfused with: (1) lactated Ringer (Control); (2) 10 mM *N*G-nitro-L-arginine methyl ester (LNAME, NOS inhibition); (3) 10 mM ascorbate (non-selective anti-oxidant); or (4) 4.34 nM Losartan (AT_1R inhibition). Relative to Control (71% CVC_{max} at both time points), CVC with Ascorbate (80% and 83% CVC_{max}) was elevated at 60- and 90-min of exercise during FR (both $P<0.02$) but not at any time during No-FR (all $P>0.31$). In both conditions, CVC was reduced at end exercise with LNAME (60% CVC_{max}; both $P<0.02$), but was not different relative to Control at the Losartan site (76% CVC_{max}; both $P>0.19$). LSR did not differ between sites in either condition (all $P>0.10$). We conclude that NOS regulates cutaneous vasodilation but not sweating, irrespective of FR, and that ROS influence cutaneous vasodilation during prolonged exercise with FR.

Key words: Angiotensin II, heat loss, nitric oxide, prolonged exercise, reactive oxygen species

Abbreviations: ANG II, angiotensin II; AT_1R, Angiotensin II Type 1 Receptor; CVC, cutaneous vascular conductance; NO, nitric oxide; NOS, nitric oxide synthase; ROS ,reactive oxygen species

INTRODUCTION

Despite the importance of cutaneous vasodilation and sweating to the maintenance of a stable body core temperature during exercise and/or exposure to a hot environment, the physiological mechanisms governing these heat loss responses at the level of the end-organ (i.e., the cutaneous vasculature and eccrine sweat gland) have yet to be fully elucidated. Numerous studies have shown nitric oxide (NO) to be a key modulator of cutaneous vasodilation and sweating during dynamic exercise lasting 30-45 min (7, 27, 30-33, 55) or passive heat stress (18, 20, 22, 53). However, exercise of longer duration (i.e., ≥60 min) may reduce NO bioavailability. Specifically, prolonged exercise is associated with the increased production of reactive oxygen species (ROS), such as superoxide, which may gradually overwhelm the scavenging capacity of endogenous anti-oxidants (15, 28, 40). The accumulation of superoxide can subsequently reduce NO bioavailability by directly interacting with NO to form peroxynitrate (37, 41, 44, 47), a potent ROS itself, and/or by uncoupling NO synthase (NOS). Indeed, a role for exercise-induced ROS in limiting NO-dependent cutaneous vasodilation was confirmed during 30-min bouts of high intensity (70% VO_{2peak}) exercise in the heat (35°C) (33). However, it remains to be determined if oxidative stress produced during prolonged exercise limits cutaneous vasodilation and sweating.

During prolonged exercise in the heat, there can be an increase in the circulating concentration of angiotensin II (ANG II) (1, 48). Intradermal administration of ANG II has been shown to reduce cutaneous vasodilation and sweating during passive exposure to a hot environment (35°C) both prior to and following moderate-intensity exercise (9). ANG II mediated reductions in cutaneous blood flow are thought to be due to ROS-dependent mechanisms (50-52). Specifically, the activation of ANG II Type 1 Receptors (AT_1R) elevates ROS production through NADPH oxidase activation (11, 12, 14). Thus, the AT_1R mediated reductions in heat loss could be a result of increased oxidative stress.

Prolonged exercise in the heat may cause substantial disruptions to the regulation of cutaneous vasodilation and sweating due to excess fluid losses. Hypohydration is well known to directly impair thermoregulatory function (42). Notably, passive heat stress induced hypohydration has also been shown to increase markers of oxidative stress (39). Thus, prolonged exercise without fluid replacement could further exacerbate oxidative stress inherent to exercise of long duration. Further, hypohydration resulting from prolonged exercise without fluid replacement also increases circulating ANG II (29, 36), ultimately potentiating further ROS

production and the consequent reductions in cutaneous vasodilation and sweating as mentioned above.

Prolonged exercise without fluid replacement can cause significant disruptions to the physiological mechanisms of heat loss; therefore, gaining a better understanding of the ROS- and AT_1R-mediated regulation of cutaneous blood flow and sweating could have profound implications for body core temperature regulation. Thus, the present study aimed to evaluate the influence of NO, ROS and AT_1R on cutaneous vasodilation and sweating during prolonged exercise (90 min) in the heat (40°C) with and without fluid replacement. Given that previous work showed ROS-mediated reductions in cutaneous vasodilation through NO- (33) and ANG II-dependent mechanisms (51, 52), we hypothesized that cutaneous vasodilation and sweating would be augmented by local ascorbate (a non-selective antioxidant) administration and AT_1R blockade during prolonged moderate-intensity exercise in the heat. Secondly, we hypothesized that progressive fluid replacement would reverse the ROS- and ANG II-mediated reductions, if any, in the heat loss responses.

METHODS

Ethical Approval

The current study was approved by the University of Ottawa Health Sciences and Science Research Ethics Board and is in accordance with the guidelines set forth by the *Declaration of Helsinki*. Verbal and written informed consent was obtained from all volunteers prior to their participation in the study.

Participant Information

Eleven young males participated in this study. All participants were healthy (i.e., no known history of cardiovascular, respiratory, and/or metabolic diseases), highly active, non-smoking, and not taking prescription medications at the time of the study. The physical characteristics of the participants (mean ± standard deviation) were: age, 26 ± 5 years; height, 1.81 ± 0.04 m; body mass, 78.4 ± 8.1 kg; body surface area, 2.0 ± 0.1 m^2; body fat percentage, 11 ± 4%; and peak rate of oxygen consumption (VO_{2peak}), 60.0 ± 8.2 ml $O_2 \cdot kg^{-1} \cdot min^{-1}$.

Experimental Procedures

Each participant completed one screening and two experimental sessions. All participants were instructed to refrain from over-the-counter medications (including non-steroidal anti-inflammatory drugs and supplements) for a minimum of 48 hours, as well as alcohol, caffeine, and heavy exercise at least 24 hours prior to each session. Further, on the day of each session participants were instructed not to consume food for 2 hours prior to arriving to the laboratory. During the screening session, body height, mass, surface area, density, fat percentage, and VO_{2peak} were determined. Body height was measured using an eye-level physician stadiometer (Model 2391; Detecto, Webb City, MO, USA) while body mass was measured using a digital weight scale platform (Model CBU150X, Mettler Toledo Inc., OH, USA) with a weighing terminal (Model IND560, Mettler Toledo, Inc.). Subsequently, body surface area was calculated from the measurements of body height and mass (4). Body density was evaluated by means of hydrostatic weighing and used to calculate body fat percentage (46). VO_{2peak} was assessed using an automated indirect calorimetry system (MCD Medgraphics Ultima Series, Sun Tech Medical, Morrisville, NC, USA) during a progressive incremental cycling protocol on a semi-recumbent cycle ergometer (Corival; Lode BV, Groningen, Netherlands). During this protocol, participants were instructed to maintain a pedaling cadence between 60-90 revolutions·min^{-1} at a starting workload of 120 W for 1 min. The resistance was increased incrementally by 20 W·min^{-1} thereafter until volitional failure and/or until a pedaling cadence above 50 revolutions·min^{-1} - could no longer be maintained. VO_{2peak} was then evaluated as the highest average rate of oxygen uptake measured over 30s.

Participants performed two experimental trials on separate days, with a minimum of 48 h between trials. Upon arrival to the laboratory, participants provided a urine sample for the determination of urine specific gravity and voided their bladders prior to a nude body mass measurement. Next, participants rested passively in an upright seated position for a 30 min instrumentation period at ambient room temperature (~23˚C). During this time, four microdialysis membranes fibers (30 kDa cutoff; MD 2000, Bioanalytical Systems Inc., West Lafayette, IN, USA) were placed in the dermal layer of the left dorsal forearm of the participants. All microdialysis fibers were separated by a minimum of 4 cm and inserted under aseptic conditions using a 25 gauge needle. The needle entry and exit sites were 20-25 mm apart thereby ensuring that the full membrane rested below the surface of the skin. The microdialysis fiber was

then threaded through the lumen of the needle, which when subsequently withdrawn, left the 10 mm dialysis membrane of the fiber beneath the skin. All fibers were secured in place for the remainder of the trial with surgical tape.

Following fiber insertion, participants were moved to a thermally controlled chamber (Can-Trol Environmental Systems Limited, Markham, ON, Canada) regulated to 40ºC and 20% relative humidity. In the chamber, participants rested quietly while seated on a semi-recumbent cycle ergometer (Corival; Lode BV) while the microdialysis fibers were perfused in a counter-balanced manner with either: (1) lactated Ringer's solution (Baxter, Deerfield, IL, US) (Control); (2) 10 mM *N*G-nitro-L-arginine methyl ester (LNAME, Sigma-Aldrich, St. Louis, MO, USA), to non-selectively inhibit NOS; (3) 10 mM ascorbate (Sigma-Aldrich), to non-selectively reduce reactive oxygen species; or (4) 4.34 nM Losartan (Sigma-Aldrich), to inhibit the action of AT_1R at a rate of 4 μl·min^{-1} via a perfusion pump (model 400; CMA Microdialysis, Solna, Sweden). Each site was instrumented for the measurement of local sweat rate and cutaneous blood flow (see below). The concentration of LNAME was determined based on previous literature of microdialysis on human skin (7, 8, 18, 20, 33, 49) as were the concentrations for ascorbate (19, 20, 33, 52) and Losartan (26, 51, 52).

All fibers were perfused with their respective physiological agents throughout a habituation period to ensure complete pharmacological blockade and for needle trauma to subside (~90 min) (17). Next, a 10 min baseline resting data collection period ensued. After baseline, participants performed 90 min of semi-recumbent cycling exercise at 600 W of metabolic heat production followed by a 40 min recovery period. A fixed rate of heat production of 600 W was determined from pilot work that evaluated the metabolic heat load that was achieved during an established prolonged exercise protocol where participants exercised against a constant load of 120 W (10). This workload was equivalent to an exercise intensity of 46 ± 3% (standard deviation) VO_{2peak} for the study participants. In controlling for the rate of the metabolic heat production between trials, rather than a given percentage of maximum oxygen consumption, we aimed to reduce potential differences in fluid loss between experimental sessions and between participants (10). At the end of the recovery period, each microdialysis fiber was perfused with 50 mM of sodium nitroprusside (SNP, Sigma-Aldrich), to determine the maximum cutaneous blood flow at each skin site. SNP administration continued at a rate of 6 μl·min^{-1} until a stable plateau cutaneous blood flow was achieved for a minimum of 2 min. Thereafter, blood

pressure was measured to evaluate a maximum cutaneous vascular conductance (CVC_{max}). Finally, all instrumentation was removed from the participants' forearms and a post-trial nude body mass was measured. The difference between pre-trial nude body mass and post-trial nude body mass was used to evaluate fluid loss via sweating. Participants also provided a post-trial urine sample to evaluate any changes in hydration status.

In the first experimental session, participants performed the above-mentioned experimental protocol with no fluid replacement (No-FR). The change in body weight exhibited in the No-FR trial was measured and used to determine the amount of water required to maintain the individual's body weight during the second or fluid replacement trial (FR). Fluid replacement consisted of tap water, which was kept within the environmental chamber to stabilize its temperature to that of the room (40ºC). The water was administered in boluses of 500–700 ml prior to the start of exercise and every 30 min of exercise thereafter. This range of fluid replacement is in line with a previous study using a similar experimental protocol (10). The No-FR condition allowed for the observation of the influence of progressive exercise-induced fluid loss via sweating, while the FR condition allowed for the observation of responses due to prolonged exercise without progressive dehydration. Aside from the fluid replacement, the experimental protocol for the two sessions was identical.

Measurements

Cutaneous red blood cell flux, an index of cutaneous blood flow, was measured at a sampling rate of 32 Hz with laser Doppler flowmetry (PeriFlux System 5000, Perimed, Stockholm, Sweden). Four integrated 7-laser array Doppler flowmetry probes were each housed in a specialized sweat capsule positioned directly over the centre of each of the four microdialysis membrane to allow for the simultaneous measurement of cutaneous red blood cell flux and local sweat rate (34). Cutaneous vascular conductance (CVC) was calculated as the red blood cell flux divided by mean arterial pressure and presented as a percentage of CVC_{max} obtained during the SNP-induced maximum vasodilation protocol. Mean arterial pressure was calculated as diastolic pressure plus one-third of the difference between systolic and diastolic pressures (i.e., pulse pressure), which were measured using manual auscultation with a validated mercury column sphygmomanometer (Baumanometer Standby Model, WA Baum Co., Copiague, NY, USA) at 5 min intervals through the experimental protocol.

Local forearm sweat rate was simultaneously evaluated at each skin site by a ventilated capsule (1.1 cm^2) specifically designed to encompass the area of skin perfused by the intradermal microdialysis fiber (34). The sweat capsules were secured directly over the center of the microdialysis membrane using adhesive rings and topical skin glue (Collodion HV, Mavidon Medical Products, Lake Worth, FL, USA). Anhydrous air was passed through each sweat capsule at a rate of 0.4 $l \cdot min^{-1}$, while the water content of the effluent air was measured using capacitance hygrometers (Model HMT333, Vaisala, Helsinki, Finland). To ensure that the internal gas tanks were equilibrated to near room temperature, the gas tanks were located in the thermal chamber and connected to the sweat capsules and hygrometers via vinyl tubing. Local sweat rate was calculated every 5 s using the water content of the effluent air multiplied by flow rate and normalized for skin surface area beneath the capsule (expressed in $mg \cdot min^{-1} \cdot cm^{-2}$).

Esophageal temperature was measured using a pediatric thermocouple probe of ~2 mm diameter (Mon-a-therm; Mallinckrodt Medical, St Louis, MO, USA) inserted ~40 cm past the entrance of the nostril and confirmed every 5 min with aural canal temperature measurement (Welch Allyn Braun ThermoScan Pro 6000, Braun GmbH, Kronberg, Germany). Mean skin temperature was calculated from the temperature measured at four skin sites using T-type copper thermocouples (Concept Engineering, Old Saybrook, CT, USA), weighted to the following regional proportions: chest, 30%; biceps, 30%; quadriceps, 20%; and calf, 20% (13). All temperature data were collected using a data acquisition module (Model 34970A; Agilent Technologies Canada, Mississauga, ON, Canada), at a sampling rate of 15 s and simultaneously displayed and recorded using LabVIEW software, version 7.0 (National Instruments, Austin, TX, USA). Mean body temperature was calculated as (0.9 × esophageal temperature) + (0.1 × mean skin temperature). Heart rate was measured using a Polar coded WearLink and transmitter, Polar RS400 interface, and Polar Trainer 5 software (Polar Electro, Kempele, Finland).

A total solids refractometer (Model TS400, Reichter Inc., Depew, NY, USA) was used to evaluate urine specific gravity, from the urine samples obtained prior to and following the experimental protocol.

Metabolic energy expenditure was measured using indirect calorimetry. Electrochemical gas analyzers (AMETEK model S-3A/1 and CD3A, Applied Electrochemistry, Pittsburgh, PA, USA) measured expired air for concentrations of O_2 and CO_2. The gas analyzers were calibrated ~20 min before baseline using a gas mixture of known concentration. The turbine ventilometer

was calibrated using a 3-litre syringe. Participants wore a partial face mask (Model 7600 V2, Hans-Rudolph, Kansas City, MO, USA) attached to a two-way T-shape non-rebreathing valve (Model 2700, Hans-Rudolph). Metabolic rate was calculated using oxygen uptake and respiratory exchange ratio values averaged over 30s. The rate of metabolic heat production was taken as the difference between metabolic rate and external work.

Data Analysis

Baseline resting values were determined by averaging the data collected over the 5 min prior to the start of the 90 min exercise bout. CVC and local sweat rate at each skin treatment site, all temperature measurements and heart rate, were evaluated by averaging data collected during the final 10 min of each 30 min interval during exercise and the final 10 min of each 20 min interval during recovery. Furthermore, blood pressure data were presented as the average of the two measurements taken during the final 10 min of each interval mentioned above. CVC_{max} was obtained from data averaged over a 2 min period once a stabilized plateau occurred during the maximal vasodilation protocol.

Statistical Analysis

For the purpose of statistical comparison, the exercise and recovery periods of the experimental protocol were defined based on the following time periods: -5 to 0 min: Baseline, 0 to 90 min: Exercise, 90 to 130 min: Recovery. To assess the influence of each treatment, a two way repeated measures ANOVA was performed with the factors of treatment site (4 levels: Control, LNAME, ascorbate, and Losartan) and time (6 levels: baseline, 30, 60, 90, 110, and 130 min) for both CVC and local sweat rate. Furthermore, to assess the influence of fluid replacement on the observed responses, an additional two-way mixed model ANOVA was performed with the factors of time (6 levels: baseline, 30, 60, 90, 110, and 130 min) and condition (2 levels: No-FR and FR) to compare the cutaneous vasodilatory and sweating responses at the Control site between groups as well as the body temperature (i.e., esophageal temperature, mean skin and mean body temperature) and cardiovascular (i.e., heart rate and blood pressure) responses. To compare CVC_{max} (expressed as perfusion units $mmHg^{-1}$) between each treatment site in both trials obtained during SNP administration, we performed a two-way repeated measures ANOVA with the factors of treatment site (4 levels: Control, LNAME, ascorbate, and Losartan) and

condition (2 levels: No-FR and FR). A Student's paired samples t-test was used to compare pre-trial body mass and the percent change in body mass during the experimental sessions between No-FR and FR trials. *Post hoc* comparisons were conducted using Student's paired two-tailed t-tests, in which *P* values were adjusted using a modified Bonferroni correction (Holm-Bonferroni's method) when a significant interaction or main effect was detected. For all analyses, $P \leq 0.05$ was considered statistically significant. All values were presented as mean ± 95% confidence intervals (calculated as 1.96 × standard error of the mean) unless otherwise indicated. Statistical analyses were performed using software package SPSS 24.0 for Windows (IBM, Armonk, NY).

RESULTS

Local forearm cutaneous vascular conductance response

No Fluid Replacement Condition. During No-FR, CVC (Figure 1A; main effect of time, $P \leq 0.01$) was elevated throughout exercise and recovery at all sites relative to their respective baseline values (all $P \leq 0.01$). Throughout baseline, exercise and recovery, CVC (Figure 1A; main effect of condition $P \leq 0.01$) at the LNAME treated site was reduced relative to Control (all $P \leq 0.04$); whereas, ascorbate or Losartan sites were similar to Control (all $P \geq 0.14$).

Fluid Replacement Condition. In parallel to the No-FR condition, CVC (Figure 1B; interaction of time and treatment site, $P \leq 0.01$) in the FR condition was elevated during exercise and recovery at all sites relative to their respective baseline values (all $P \leq 0.01$). CVC was reduced at the LNAME treated site compared to Control during baseline resting ($P < 0.01$) and throughout exercise and recovery (all $P < 0.01$). In contrast, CVC was similar at the ascorbate or Losartan treated sites during baseline resting (both $P > 0.29$). While no differences were observed between the Losartan treated site and Control throughout the protocol (all $P > 0.19$), CVC at the ascorbate treated site was increased relative to Control for the final 30 min of exercise (both $P < 0.03$). CVC at the ascorbate treated site returned to values similar to the Control site throughout the 40 min recovery period (both $P > 0.38$).

Between Fluid Replacement Conditions. No effect of condition was observed on CVC at the Control site ($P = 0.56$), indicating that CVC was similar with or without fluid replacement.

Maximal CVC Response. Maximum CVC during administration of 50 mM of SNP did not differ between treatment sites within each condition (Table 1; main effect of treatment site, $P = 0.35$). Further, there was a main effect of condition on Maximum CVC (Table 1; $P = 0.04$); however, no statistical differences were observed after correcting for multiple comparisons (all $P \geq 0.09$).

Local forearm sweating response

Local sweat rate (Figure 2; main effect of time, both $P \leq 0.01$) was elevated relative to baseline values throughout exercise and recovery at all treatment sites during both No-FR and FR (all $P \leq 0.01$). Local sweat rate (main effect of condition, $P = 0.20$) did not differ between treatment sites in either condition. Similarly, no differences at the Control site (main effect of time, $P = 0.16$) were observed between trial conditions.

Hydration status and fluid balance

No differences in pre-trial urine specific gravity (No-FR, 1.008 ± 0.002; FR, 1.009 ± 0.003; $P = 0.78$) were observed, while post-trial urine specific gravity was greater following No-FR (1.019 ± 0.004) trial in comparison to FR (1.007 ± 0.001; $P < 0.01$). Pre-trial body weight (No-FR, 79 ± 5 kg; FR, 79 ± 9 kg; $P = 0.08$) was not different between conditions; however, the percent change in body weight was greater during No-FR (Table 2; -3.4 ± 0.5%) compared to FR (-0.1 ± 0.0%; $P < 0.01$). In FR, participants received an average of 2.6 ± 0.5 L of water, accounting for 89% of total fluid loss in No-FR.

Body temperature and cardiovascular responses

Esophageal, mean skin and mean body temperatures (Table 3; main effect of time, all $P < 0.01$) were elevated from baseline values throughout exercise and recovery (all $P \leq 0.01$). Moreover, esophageal and mean body temperatures (main effect of condition, all $P \leq 0.01$) were reduced during the FR condition relative to No-FR from the 60-min time point of exercise until the end of recovery (all $P \leq 0.02$). By contrast, mean skin temperature was reduced in FR relative to No-FR at 20 min of recovery only (Table 3; $P = 0.05$).

In both conditions, mean arterial pressure (Table 3; main effect of time, $P = 0.03$) was increased relative to baseline, during the first 30 min of exercise but, similar thereafter until the

end of exercise (all $P \geq 0.15$). Mean arterial pressure (main effect of condition, $P = 0.04$) was elevated during the first 30 min of exercise in the No-FR condition relative to the FR condition ($P = 0.01$) but not different at any other time point (all $P > 0.07$). Mean arterial pressure was reduced relative to baseline throughout the recovery period in the No-FR (>7 mm Hg decrease, both $P < 0.01$) but not the FR condition (both $P \geq 0.07$). Heart rate (Table 3; main effect of time, $P < 0.01$) was increased relative to baseline throughout exercise and recovery in both conditions (all $P < 0.01$) and was increased in the No-FR condition relative to the FR condition from 30 min of exercise until the end of the protocol (main effect of condition, all $P \leq 0.02$).

DISCUSSION

The primary finding of the present study is that intradermal administration of ascorbate augmented cutaneous vasodilation but not sweating during moderate intensity (46% VO_{2peak}) prolonged exercise in the heat after 60 min of exercise when fluid was provided to offset losses via sweat. This observation indicates that long duration exercise even at moderate intensity can cause a substantial physiological burden limiting heat loss, likely through increased ROS accumulation. Additionally, NOS was shown to be a key modulator of cutaneous vasodilation but not sweating in response to prolonged exercise in the heat, irrespective of fluid replacement. In contrast, we did not see an effect of local AT_1R inhibition on cutaneous vasodilation or sweating during or following exercise in the heat with or without fluid replacement. Altogether, these data demonstrate that during prolonged exercise in the heat, the role of NO on cutaneous vasodilation is not dependent upon fluid replacement albeit we showed that local ascorbate treatment augments cutaneous vasodilation with fluid replacement.

Cutaneous Vascular Response

Consistent with previous reports we show NOS inhibition (7, 8, 18, 20, 22, 33, 55) but not ascorbate (33) nor Losartan (26) administration, to be an essential modulator of cutaneous vascular tone during passive exposure to a hot environment (40ºC). At the initiation of exercise in both conditions, CVC at the NOS inhibited site remained reduced relative to Control throughout the experimental protocol, thereby demonstrating the importance of NO to cutaneous vasodilation during and following prolonged exercise induced heat stress. Importantly, this role

for NOS inhibition was conserved irrespective of condition, suggesting NOS mediated cutaneous vasodilation is not dependent upon fluid replacement.

In the current study, we show that intradermal administration of ascorbate increased cutaneous vasodilation during prolonged exercise in the heat when fluid was provided to offset fluid losses via sweat. Recently, local ascorbate administration has been shown to augment cutaneous vasodilation in young adults performing 30 min of high intensity (70% VO_{2peak}) exercise in the heat in a NO-dependent manner (33). It was suggested that ascorbate administration served to reduce an increase in ROS resulting from high intensity exercise and thereby promoted NO bioavailability and cutaneous vasodilation (33). However, while there is also a progressive accumulation of ROS during prolonged exercise (3, 15, 28, 40), it remained to be determined if it was sufficient to modulate the body's heat loss responses. Importantly, no effect of ascorbate supplementation was observed until 60 min of exercise. While speculative, it may be that the later stages (≥ 60 min) of prolonged exercise in the heat resulted in the progressive accumulation of oxidative stress such that the accumulation of ROS limited the ability to increase cutaneous vasodilation.

Contrary to our hypothesis, the augmented cutaneous vasodilation at the ascorbate treated site was only present during the FR but not the No-FR condition. It is possible that the progressive dehydration during the No-FR condition could have caused a further increase in oxidative stress (i.e. increased superoxide levels) relative to the FR condition (16, 25). In the presence of superoxide dismutase, highly elevated levels of superoxide facilitate the conversion of superoxide to H_2O_2, which can act as a vasodilator and thereby increase cutaneous blood flow (35), counteracting the superoxide-mediated reduction in cutaneous vasodilation. Therefore, under these conditions ascorbate would have a minimal effect on CVC as it non-selectively reduces levels of both superoxide and H_2O_2, each pathway yielding the opposite action on cutaneous vascular regulation.

During recovery from exercise in the FR condition, CVC at the ascorbate treated site returned to levels similar to Control. This observation may indicate that once exercise was halted ROS production declined and the body's endogenous anti-oxidants were able to scavenge the ROS, restoring NO bioavailability and thereby NO-dependent cutaneous vasodilation. In fact, it has been shown that endogenous serum anti-oxidant capacity is raised following prolonged (~87 min) running (2). Moreover, it is believed that post-exercise suppression of cutaneous

vasodilation is largely mediated centrally through non-thermal factors (e.g. baroreceptors etc.) (24) and is in part due to cutaneous vasoconstriction associated with adenosine and α-adrenergic mechanisms (30, 31). Therefore, perhaps vasoconstriction induced by such mechanisms may have an overriding influence on the ascorbate-mediated vasodilation during recovery from exercise in the heat.

Losartan did not influence cutaneous vasodilation during either the No-FR or FR conditions. This observation parallels the findings by Fujii *et al.*, (2015) wherein no role for ANG II administration was found during exercise in the heat. The authors postulate exercise-induced increases in NO bioavailability may be an overriding influence of ANG II-mediated vasoconstriction. Our observations contrasted with findings from Stewart and colleagues where local AT_1R inhibition diminished an exogenous ANG II-induced reduction in CVC during a local heating protocol (50-52). Importantly, AT_1R inhibition was evaluated in response to the administration of exogenous ANG II which does not necessarily mimic physiological concentrations of circulating ANG II. Moreover, whole-body heat stress, such as exercise in the heat, has been shown to affect NO mediated cutaneous vascular regulation differently than local heating (23, 56).

Following prolonged exercise in the heat, we show that AT_1R inhibition did not influence CVC. Previously, ANG II administration was shown to cause a reduction in cutaneous vasodilation following exercise in the heat (9); however, the administration of exogenous ANG II may not be an exact representation of true physiological concentrations as mentioned above. In addition, body core temperatures achieved in our study surpassed (~1.5˚C greater) those in the study by Fujii et al. (9) and, as such, the greater requirement to dissipate heat (and therefore muscarinic receptor activation) in our study may have overridden ANG II mediated vasoconstriction through increased NO bioavailability as mentioned above. Future research is warranted to determine at what elevation in body core and skin temperatures ANG II-mediated pathways regulate cutaneous vasodilation during post-exercise recovery periods.

Sweating

In contrast to the comparatively well-established modulators of NO-dependent cutaneous vasodilation during whole-body heat stress, the mechanisms underpinning the involvement of NO in the sweating response to exercise are less understood. Contrary to our hypothesis, we did

not observe a role for LNAME in the regulation of local forearm sweating during or following prolonged exercise in the heat. Although increased NO bioavailability has been shown to be required for the full expression of local forearm sweating, its involvement has also been shown to be exercise intensity-dependent (7, 33). Specifically, this NOS inhibition has been shown to diminish local sweating during low exercise intensity (400 W metabolic heat load) but not high exercise intensity (700 W metabolic heat load) (6). Therefore, given that our participants exercised at a fixed rate of metabolic heat load of 600 W metabolic heat load and in conjunction with an increased ambient temperature relative to the study conducted by Fujii *et al.* (9), (i.e., room temperature in their study was 35˚C relative to 40˚C in the present study), the cumulative (metabolic + environmental) heat stress experienced in the current study may have caused sufficient thermal drive such that the skin treatment specific changes in sweating could not be observed.

Sweat production is induced via muscarinic stimulation of the sweat gland by acetylcholine released from cholinergic nerves (45). While NO alone can induce cutaneous vasodilation (7, 18, 20-22, 30-32), the current view is that NO plays a synergistic role to muscarinic sweating such that it acts to augment cholinergic sweating but cannot activate sweat production directly. In support of this conclusion, we have previously observed no contribution of NO to intradermal administration of relatively high doses of methacholine, an acetylcholine mimetic (1-2000 mM) (6). In the context of whole-body heat stress, these findings suggest that NO may not contribute to the sweating response at high levels of thermal drive and therefore cholinergic stimulation. Similarly, we did not observe a role for ascorbate administration during or following prolonged exercise in the heat. Given that ROS are produced during situations of high thermal drive, this would support the conclusion that ROS do not mediate NO-dependent sweating during prolonged exercise in the heat.

During rest in the heat (40°C) as well as during and following prolonged exercise in the heat we found no role for AT_1R inhibition irrespective of fluid replacement (Figure 2). While ANG II administration reduced sweating during resting in a hot environment (35°C) both prior to and following moderate intensity exercise (9), exogenous administration of an agent does not necessarily reflect true physiological conditions as previously mentioned. Moreover, levels of hyperthermia at the end of exercise were substantially greater in our study relative to Fujii *et al.* (39.0˚C vs 37.5˚C body core temperature, respectively). Therefore the increased thermal drive,

(and therefore muscarinic receptor activation), to dissipate heat via sweating may have overridden ANG II mediated attenuations in sweating.

Perspectives

Much of the previous research investigating the mechanisms underlying the regulation of heat loss responses did not incorporate compensation for the natural fluid loss due to sweating during exercise in the heat. In fact, previous work has demonstrated that vascular regulation is in part dependent on hydration status (5, 38, 43). As the role of ascorbate differed between trial conditions, our results could further indicate that fluid replacement may be necessary to delineate the mechanisms regulating cutaneous blood flow and sweating during prolonged exercise in the heat. Importantly, participants in our study lost 3.6% body weight during the No-FR condition thereby indicating hypohydration levels sufficient to impair heat loss responses (42). In addition, post-trial urine specific gravity was significantly greater in the No-FR trials (1.019) relative to the FR trials (1.006; $P < 0.01$). Recent work has shown that even moderate hypohydration (~2% body mass loss) requires greater postsynaptic (cholinergic) output acting through the endothelium to elicit the same degree of cutaneous vasodilation as the euhydrated condition (54). Altogether, our results suggest that both hydration status and fluid replacement are important factors to consider when interpreting and comparing the results between different protocols.

The results of the current study have important physiological and clinical implications as augmented levels of oxidative stress occur during prolonged or high intensity exercise but are also inherent to advanced aging. Given that older adults have been shown to have reduced heat loss responses relative to their young counterparts (24), hypohydration may further exacerbate these deficits, ultimately putting this population at a greater risk of heat-related injury during thermal challenges. As such future research is warranted to determine how exercise-induced fluid loss via sweating and subsequent fluid replacement may modulate heat loss responses in older adults.

CONCLUSION

We showed that NOS modulates cutaneous vasodilation during prolonged moderate intensity exercise in the heat irrespective of progressive fluid replacement while ascorbate-sensitive ROS impair cutaneous vasodilation (exercise duration of >60 min) only with fluid replacement. In

contrast, no role for AT_1R inhibition was found for the regulation of heat loss responses with or without fluid replacement.

ACKNOWLEDGEMENTS

We sincerely thank all volunteers for using their time and energy to participate in this study.

GRANTS

This study was supported by the Natural Sciences and Engineering Research Council of Canada (Discover grant, RGPIN-06313-2014 and Discovery Grants Program - Accelerator Supplement, RGPAS-462252-2014; funds held by Dr. Glen P. Kenny). B. D. McNeely is supported by the Queen Elizabeth II Graduate Scholarship in Science and Technology. G. P. Kenny is supported by a University of Ottawa Research Chair Award. R.D. Meade is supported by a Natural Sciences and Engineering Research Council of Canada Alexander Graham Bell graduate scholarships (CGS-D). N. Fujii was supported by the Human and Environmental Physiology Research Unit. The current affiliation of N. Fujii is the University of Tsukuba, Institute of Health and Sport Sciences, Tsukuba City, Japan

DISCLOSURES

None.

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Table 1. Absolute maximal cutaneous vascular conductance at the four skin sites.

	Control	**LNAME**	**Ascorbate**	**Losartan**
CVC_{max} (PU mm Hg^{-1})				
No-FR	2.3 ± 0.3	2.3 ± 0.5	2.1 ± 0.3	2.4 ± 0.6
FR	2.6 ± 0.3	2.7 ± 0.4	2.5 ± 0.5	3.0 ± 0.9

Presented values are mean ± 95% confidence interval. Eleven young adults performed a continuous 90-min exercise protocol followed by a 40-min recovery period. Exercise was performed at a fixed rate of heat production of 600 W (46% VO_{2peak}). CVC_{max} in perfusion units (PU $mmHg^{-1}$) measured at the four skin sites previously perfused with either: (1) lactated Ringer's solution (Control); (2) 10 mM LNAME, to non-selectively inhibit NOS; (3) 10 mM ascorbate, an antioxidant; or (4) 4.34 nM Losartan, to inhibit the action of AT_1R. No statistically significant differences were detected.

Table 2. Body weight changes prior to and following exercise in the heat.

	Pre-Weight (kg)	Post-Weight (kg)	% change in Body weight	Volume of water given (L)
Trial Condition				
No-FR	78.8 ± 4.7	76.2 ± 4.5	-3.57 ± 0.44	0.4 ± 0.1
FR	79.4 ± 5.0	78.8 ± 4.8†	-0.01 ± 0.00†	2.6± 0.3†

Presented values are mean ± 95% confidence interval. Eleven young adults performed a continuous 90-min exercise protocol followed by a 40-min recovery period. Exercise was performed at a fixed rate of heat production of 600 W (46% VO_{2peak}). †, significant difference vs. No-FR; $P \leq 0.05$.

Table 3. Body temperatures and cardiovascular responses at rest, during and following prolonged exercise.

	Baseline	Exercise			Recovery	
	Resting	**30 min**	**60 min**	**90 min**	**20 min**	**40 min**
Esophageal Temperature (°C)						
No-FR	37.1 ± 0.1	37.9 ± 0.2*	38.5 ± 0.2*	39.0 ± 0.3*	38.3 ± 0.3*	37.9 ± 0.2*
FR	37.0 ± 0.1	37.7 ± 0.2*	38.3 ± 0.3*†	38.7 ± 0.3*†	37.9 ± 0.3*†	37.6 ± 0.3*†
Mean Skin Temperature (°C)						
No-FR	35.6 ± 0.2	36.6 ± 0.2*	37.1 ± 0.3*	37.5 ± 0.3*	37.1 ± 0.5*	36.5 ± 0.5*
FR	35.7 ± 0.2	36.6 ± 0.2*	37.0 ± 0.2*	37.4 ± 0.3*	36.8 ± 0.3*†	36.2 ± 0.3*
Mean Body Temperature (°C)						
No-FR	37.0 ± 0.1	37.7 ± 0.2*	38.3 ± 0.2*	38.9 ± 0.3*	38.2 ± 0.3*	37.7 ± 0.3*
FR	36.9 ± 0.1	37.6 ± 0.2*	38.1 ± 0.3*†	38.6 ± 0.3*†	37.8 ± 0.3*†	37.4 ± 0.3*†
Mean Arterial Pressure (mmHg)						
No-FR	93 ± 3	99 ± 4*	97 ± 4	97 ± 4	85 ± 4*	86 ± 3*
FR	91 ± 3	95 ± 3*†	94 ± 4	94 ± 4	86 ± 5	89 ± 4
Heart Rate (beats·min^{-1})						
No-FR	70 ± 3	131 ± 6*	146 ± 8*	157 ± 8*	114 ± 8*	103 ± 7*
FR	69 ± 5	124 ± 8*†	132 ± 8*†	141 ± 9*†	97 ± 7*†	86 ± 7*†

Presented values are mean ± 95% confidence interval. Eleven young adults performed a continuous 90-min exercise protocol followed by a 40-min recovery period. Exercise was performed at a fixed rate of heat production of 600 W (46% VO_{2peak}). Esophageal and mean skin temperatures as well as heart rate responses represent an average of the final 10 min of the corresponding time period. Mean arterial pressure values represent an average of two measurements taken over the final 10 min of the corresponding time period. *, significant difference vs. Baseline; †, significant difference vs. No-FR; $P \leq 0.05$.

FIGURE LEGENDS

Figure 1: Time-course changes in cutaneous vascular conductance (CVC) for the No-FR condition (panel A) and FR condition (panel B) at baseline resting (BL), during a 90 min prolonged exercise bout performed at a fixed rate of metabolic heat production (600 W), and during the post-exercise recovery period. Four skin sites were continuously administered with: 1) lactated Ringer's solution (CON, open circles); (2) 10 mM *N*G-nitro-L-arginine methyl ester (LNAME, light gray triangles); (3) 10 mM ascorbate (ASC, dark gray diamonds); or (4) 4.34 nM Losartan (LOS, black squares). *, Control significantly different from LNAME ($P < 0.05$); †, Control significantly different from ASC ($P < 0.05$).

Figure 2: Time-course changes in local sweat rate (LSR) for the without fluid replacement condition (No-FR, panel A) and with fluid replacement condition (FR, panel B) at baseline resting (BL), during a 90 min prolonged exercise bout performed at a fixed rate of metabolic heat production (600 W), and during the post-exercise recovery period. Four skin sites were continuously administered with: 1) lactated Ringer's solution (CON, open circles); (2) 10 mM *N*G-nitro-L-arginine methyl ester (LNAME, light gray triangles); (3) 10 mM ascorbate (ASC, dark gray diamonds); or (4) 4.34 nM Losartan (LOS, black squares). No statistical differences between sites were detected.

Figure 1

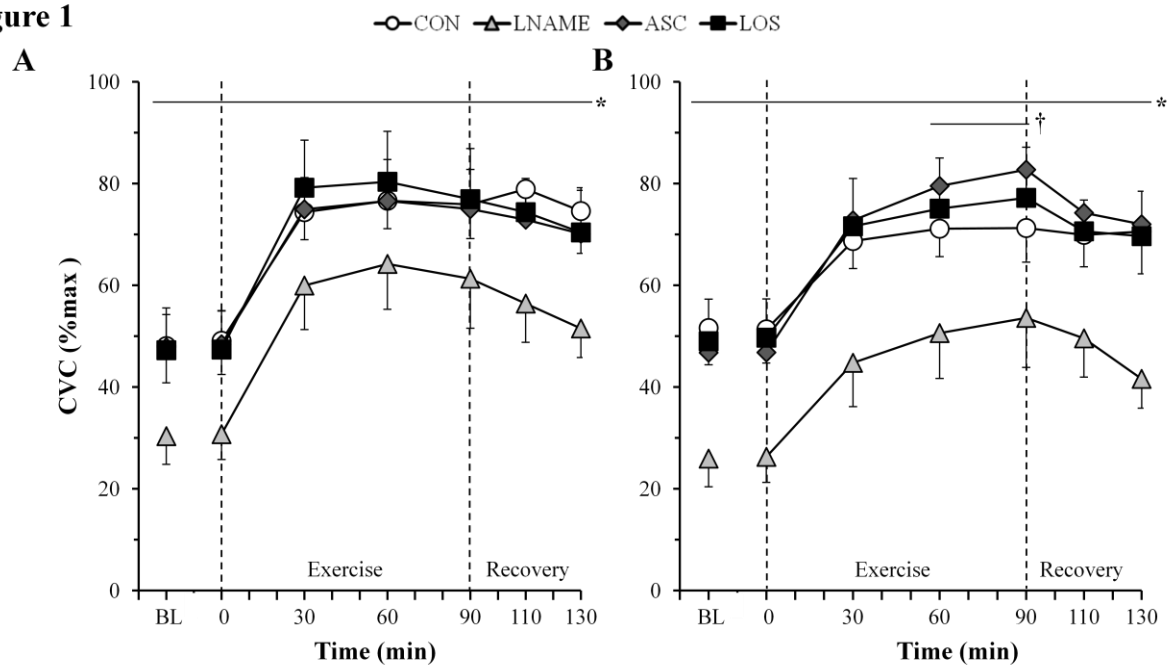
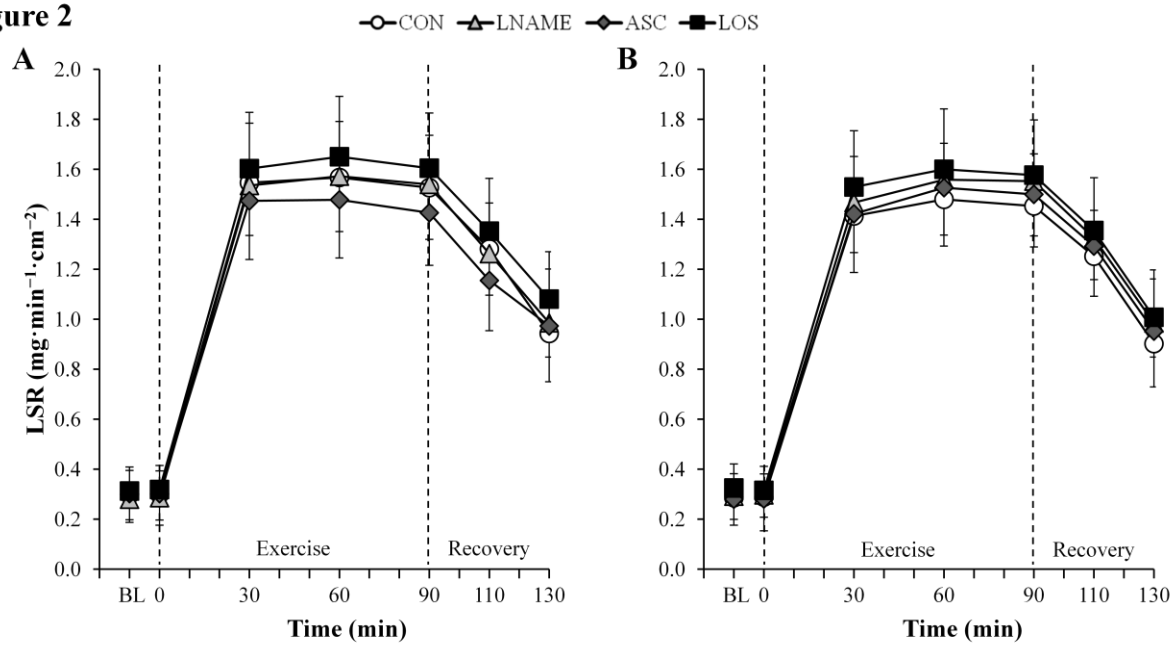

CON
LNAME
ASC
LOS
A
B
CVC (%max)
100
80
60
40
20
0
*
†
Exercise
Recovery
BL
0
30
60
90
110
130
Time (min)

Figure 2


CON
LNAME
ASC
LOS
A
B
LSR (mg·min⁻¹·cm⁻²)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
BL
0
30
60
90
110
130
Exercise
Recovery
Time (min)

PART THREE: GENERAL CONCLUSIONS OF THE THESIS

The current thesis examined the separate effects of nitric oxide synthase (NOS), ascorbate and Losartan on cutaneous vasodilation and sweating during prolonged exercise in the heat. In addition, the current thesis evaluated whether excess fluid loss via sweating and subsequent fluid replacement modulated the role of these pathways to regulate heat loss responses. The primary findings of the present thesis implicate an important role for ascorbate-sensitive reactive oxygen species (ROS) and NOS in the regulation of cutaneous vasodilation during prolonged exercise in the heat. Moreover, the results show that the NOS-mediated reductions in cutaneous vasodilation are conserved irrespective of fluid replacement while the ascorbate-mediated cutaneous vasodilation is not. Specifically, intradermal administration of ascorbate augmented cutaneous vasodilation during prolonged exercise in the heat relative to Control after 60 min of exercise with fluid replacement only. Importantly, no effect of ascorbate administration was observed until 60 minutes of exercise likely indicating a progressive accumulation of ROS during prolonged exercise as anticipated. In contrast, there was no effect of Losartan administration on cutaneous vasodilation during or following prolonged exercise in the heat with or without fluid replacement, suggesting that angiotensin II Type 1 Receptor (AT_1R) inhitibition does not participate in cutaneous regulation under these conditions. Lastly, the manipulation of NOS, ascorbate-sensitive ROS, or AT_1R pathways did not amount to any measurable effect on local forearm sweating during prolonged exercise in the heat irrespective of fluid replacement. The results of this thesis also confirmed previous reports of an increase in body core and mean body temperature during progressive dehydration exercise relative to exercise with fluid replacement. Altogether, at the level of the end-organs (i.e., skin vessels and sweat glands), these data suggest that during prolonged exercise in the heat the role of nitric oxide on cutaneous vasodilation is not dependent upon fluid replacement but, there is a progressive accumulation of ascorbate sensitive ROS that impairs cutaneous vasodilation with fluid replacement.

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APPENDIX

File Number: H10-04-04B

Date (mm/dd/yyyy): 12/01/2016

Université d'Ottawa
Bureau d'éthique et d'intégrité de la recherche

University of Ottawa
Office of Research Ethics and Integrity

Ethics Approval Notice

Health Sciences and Sciences REB

Principal Investigator / Supervisor / Co-investigator(s) / Student(s)

First Name	Last Name	Affiliation	Role
Glen	Kenny	Health Sciences / Human Kinetics	Principal Investigator
Sheila	Davis	Health Sciences / Human Kinetics	Research Assistant
Andrew	D'Souza	Health Sciences / Human Kinetics	Research Assistant
Brian	Friesen	Health Sciences / Human Kinetics	Research Assistant
Dallon	Lamarche	Health Sciences / Human Kinetics	Research Assistant
Brendan	McNeely	Health Sciences / Human Kinetics	Research Assistant
Robert	Meade	Health Sciences / Human Kinetics	Research Assistant
Martin	Poirier	Health Sciences / Human Kinetics	Research Assistant

File Number: H10-04-04B

Type of Project: Professor

Title: Heat stress for workers in the electrical power industry/ Defining age-specific heat exposure limits for workers: from laboratory to field

Renewal Date (mm/dd/yyyy)	Expiry Date (mm/dd/yyyy)	Approval Type
01/31/2017	01/30/2018	Approved

Special Conditions / Comments:
N/A

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File Number: H10-04-04B

Date (mm/dd/yyyy): 12/01/2016

Université d'Ottawa
Bureau d'éthique et d'intégrité de la recherche

University of Ottawa
Office of Research Ethics and Integrity

This is to confirm that the University of Ottawa Research Ethics Board identified above, which operates in accordance with the Tri-Council Policy Statement (2010) and other applicable laws and regulations in Ontario, has examined and approved the ethics application for the above named research project. Ethics approval is valid for the period indicated above and subject to the conditions listed in the section entitled "Special Conditions / Comments".

During the course of the project, the protocol may not be modified without prior written approval from the REB except when necessary to remove participants from immediate endangerment or when the modification(s) pertain to only administrative or logistical components of the project (e.g., change of telephone number). Investigators must also promptly alert the REB of any changes which increase the risk to participant(s), any changes which considerably affect the conduct of the project, all unanticipated and harmful events that occur, and new information that may negatively affect the conduct of the project and safety of the participant(s). Modifications to the project, including consent and recruitment documentation, should be submitted to the Ethics Office for approval using the "Modification to research project" form available at: http://www.research.uottawa.ca/ethics/forms.html

Please submit an annual report to the Ethics Office four weeks before the above-referenced expiry date to request a renewal of this ethics approval. To close the file, a final report must be submitted. These documents can be found at: http://www.research.uottawa.ca/ethics/forms.html

If you have any questions, please do not hesitate to contact the Ethics Office at extension 5387 or by e-mail at: ethics@uOttawa.ca.

Signature:

Danika Barleben
Ethics Coordinator
For Daniel Lagarec, Chair of the Health Sciences and Sciences REB

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