The roles of the Na^+/K^+-ATPase, NKCC, and K^+ channels in the regulation of local sweating and cutaneous blood flow during exercise in humans *in vivo*

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Abstract

Na^+/K^+-ATPase has been shown to regulate the sweating and cutaneous vascular responses during exercise; however, similar studies have not been conducted to assess the roles of the Na-K-2Cl cotransporter (NKCC) and K^+ channels. Additionally, it remains to be determined if these mechanisms underpinning the heat loss responses differ with exercise intensity. Eleven young (24±4 years) males performed three 30-min semi-recumbent cycling bouts at low (30% VO_{2peak}), moderate (50% VO_{2peak}), and high (70% VO_{2peak}) intensity exercise, respectively, each separated by 20-min recovery periods. Using intradermal microdialysis, four forearm skin sites were continuously perfused with either: 1) lactated Ringer solution (Control), 2) 6 mM ouabain (Na^+/K^+-ATPase inhibitor), 3) 10 mM bumetanide (NKCC inhibitor), or 4) 50 mM $BaCl_2$ (non-specific K^+ channel inhibitor); sites at which we assessed local sweat rate (LSR) and cutaneous vascular conductance (CVC). Inhibition of Na^+/K^+-ATPase attenuated LSR compared to Control during the moderate and high intensity exercise bouts (both $P<0.01$), whereas attenuations with NKCC and K^+ channel inhibition were only apparent during the high intensity exercise bout (both $P\leq0.05$). Na^+/K^+-ATPase inhibition augmented CVC during all exercise intensities (all $P<0.01$), whereas CVC was greater with NKCC inhibition during the low intensity exercise only ($P<0.01$) and attenuated with K^+ channel inhibition during the moderate and high intensity exercise conditions (both $P<0.01$). We show that Na^+/K^+-ATPase, NKCC and K^+ channels all contribute to the regulation of sweating and cutaneous blood flow but their influence is dependent on the intensity of exercise.

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ABREVIATIONS

ANOVA, analysis of variance

$BaCl_2$, barium chloride

BK_{Ca}, large conductance calcium-activated potassium channel

CVC, cutaneous vascular conductance

EC_{50}, concentration of agent eliciting 50% of the maximal response

EDH, endothelium-derived hyperpolarization

K^+ channels, potassium channels

K_{ATP}, ATP-sensitive potassium channel

K_{Ca}, calcium-activated potassium channel

K_{IR}, inwardly-rectifying potassium channel

LSR, local sweat rate

Na^+/K^+-ATPase, sodium pump

NaOH, sodium hydroxide

NKCC, Na-K-2Cl cotransporter

PGI_2, prostacyclin

T_{air}, ambient air temperature

T_{skin}, skin temperature

VO_{2peak}, peak rate of oxygen consumption

VSMC, vascular smooth muscle cell

PART ONE

THEORETICAL BACKGROUND

CHAPTER I

INTRODUCTION

1.1 Introduction

Aside from behavioural interventions such as modifications to clothing, activity, and the environment, the human body regulates core temperature by means of producing sweat and increasing blood flow to the skin (*via* the vasodilatation of cutaneous vasculature). Heat is generated during exercise and if an individual cannot adequately dissipate the heat gained, they may be at greater risk for heat-related illnesses such as heat stroke, heat-related syncope, and ultimately death. Despite the importance of sweating and cutaneous vasodilatation as heat loss responses, the physiological mechanisms that regulate them have yet to be fully elucidated, especially during exercise.

To date, the post-synaptic mechanisms underlying the production of sweat are not well understood. It is generally accepted that this process involves the transport of various ions in order to establish electrochemical gradients that promote water movement from the blood across the basolateral membrane into the secretory coil cells of the sweat gland (Sato *et al.*, 1989). This water then moves across the luminal membrane to the lumen of the dermal duct to be secreted to the skin surface as sweat. However, the precise mechanisms determining this water movement are currently unknown. Models that have been developed to describe fluid secretion in other organs have been used to postulate the mechanisms underpinning the production of sweat. For instance, since the Na-K-2Cl cotransporter (NKCC) model had been implicated in the thick ascending limb of Henle's loop in the nephrons of the kidney (O'Grady *et al.*, 1987), it was used to postulate the mechanisms of sweat production (Quinton, 1983; Sato *et al.*, 1989; Saga, 2002). This NKCC model highlights the involvement of various membrane transport proteins, such as the sodium pump (Na^+/K^+-ATPase), NKCC, and K^+ channels.

Following the identification of Na^+/K^+-ATPase in the eccrine sweat gland (Adachi & Yamasawa, 1966; Gibbs, 1967), Sato and colleagues (1969) administered ouabain (a potent

inhibitor of Na^+/K^+-ATPase) *via* intradermal injection into the skin and they observed significantly attenuated local sweat rate during graded exercise. More recently, we showed a 54-60% attenuation in local forearm sweat rate with ouabain administered using the intradermal microdialysis technique, confirming that Na^+/K^+-ATPase plays a role in regulating the sweating response during exercise (Louie *et al.*, 2016). However, the roles of the NKCC and K^+ channels, which are also present in the eccrine sweat gland and have been demonstrated in the production of sweating *in vitro* (Krouse *et al.*, 1989; Henderson & Cuthbert, 1991; Toyomoto *et al.*, 1997; Nejsum *et al.*, 2005; Zhang *et al.*, 2014; Cui *et al.*, 2016), have yet to be studied in humans *in vivo* specifically during exercise.

In our recent study, we also reported a role of Na^+/K^+-ATPase in the regulation of cutaneous vasculature tone during exercise (Louie *et al.*, 2016). We observed an augmented cutaneous vasodilatory response with Na^+/K^+-ATPase inhibition, a response which we determined was related to nitric oxide-dependent mechanisms by simultaneously inhibiting nitric oxide synthase. On the other hand, although there is a lack of human *in vivo* data to support the role of the NKCC in cutaneous vasodilatation, studies utilizing rats have demonstrated that the NKCC regulates tone in other vasculature (Barthelmebs *et al.*, 1994; Wiemer *et al.*, 1994; Liguori *et al.*, 1999; Garg *et al.*, 2007; Orlov, 2007). Specifically, it has been shown that NKCC inhibition *via* the agent furosemide resulted in vasodilatory responses in rat vasculature *in vitro* and in rats *in vivo* (Barthelmebs *et al.*, 1994). In regards to K^+ channels (and its various subtypes) in the cutaneous vasculature of humans *in vivo*, it has been assessed using various methods including the reactive hyperaemic response (Lorenzo & Minson, 2007), local heating (Brunt & Minson, 2012), whole-body passive heating (Brunt *et al.*, 2013), and pharmacological stimulation (Brunt *et al.*, 2015), but not exercise.

It was recently observed that the contribution of certain mechanisms underpinning the sweating and cutaneous vasodilatory responses during exercise did not remain intact between different levels of exercise intensity (Fujii *et al.*, 2014; Meade *et al.*, 2015). This response may be related to the elevated rates of metabolic heat production and therefore greater level of thermoeffector activity (*i.e.* sweat rate and cutaneous vasodilatation). These variations in the mechanisms regulating the heat loss responses require further investigation.

1.2 Rationale and statement of the problem

The *in vivo* contributions of the NKCC and K^+ channels to sweating and cutaneous vasodilatation during exercise have yet to be explored, despite the *in vitro* data that would suggest their involvement. Moreover, it remains to be determined if Na^+/K^+-ATPase, NKCC, and K^+ channels demonstrate exercise intensity-dependent contributions to the heat loss responses.

1.3 Objective

The main objectives of the current study were to:

1. Assess the separate roles of the Na^+/K^+-ATPase, NKCC, and K^+ channels in the regulation of (a) sweating and (b) cutaneous vasodilatation during exercise.
2. Determine whether the contributions of each membrane transport protein to the heat loss responses are consistent throughout exercise at low, moderate, and high intensity exercise (*i.e.* 30, 50, and 70% peak rate of oxygen consumption (VO_{2peak}), respectively).

1.4 Hypotheses

The current thesis tested the hypotheses that:

1a. The separate inhibitions of Na^+/K^+-ATPase, NKCC, and K^+ channels would each attenuate local sweat rate relative to the Control site (*i.e.* perfused with lactated Ringer's solution), thereby indicating their role in the regulation of the sweating response.

1b. The inhibitions of Na^+/K^+-ATPase and NKCC would augment cutaneous vasodilatation whereas K^+ channel inhibition would attenuate the response.

2. The contributions of each membrane transport protein to each heat loss responses would diminish with increasing exercise intensity.

1.5 Relevance of the study

The human heat loss responses of sweating and cutaneous vasodilatation are critical to the regulation of body temperature, especially during exercise in which the rate of metabolic heat production is elevated. However, significant knowledge gaps exist in our understanding of the mechanisms underpinning these heat loss responses during exercise-induced heat stress. The current study addressed this knowledge gap by assessing if, and to what extent, that specific membrane transport proteins (*i.e.* Na^+/K^+-ATPase, NKCC, and K^+ channels) contribute to the regulation of the sweating and cutaneous vasodilatory responses. Our findings demonstrate that the level of exercise intensity and associated factors such as rate of metabolic heat production are important to consider when exploring the underlying mechanisms modulating exercise-induced sweating and cutaneous vasodilatation. Moreover, the current thesis provides direction for future studies studying the regulatory effects of upstream modulators (*e.g.* nitric oxide and cyclooxygenase) and their relationship with membrane transport proteins.

The pharmacological agents administered to inhibit specific membrane transport proteins in the current study are also utilized in clinical settings. For example, as previously discussed in our recent paper (Louie *et al.*, 2016), ouabain is a cardiac glycoside such that the increase in intracellular Ca^{2+} secondary to the inhibition of Na^{+}/K^{+}-ATPase increases contractility strength of the heart and the vagal activity-mediated attenuations in heart rate which are beneficial in treating congestive heart failure and arrhythmias, respectively (Fleckenstein, 1977). As well, the NKCC inhibitor bumetanide is a widely known loop diuretic used to reduce excess levels of fluid in the human body to treat the symptoms of various conditions such as heart failure, liver disease, and kidney disease (Asbury *et al.*, 1972). Administration of Na^{+}/K^{+}-ATPase and NKCC inhibitors (*e.g.* ouabain and bumetanide, respectively) may impose attenuations in whole body heat loss thus exacerbating risk for heat-related illnesses, which may be especially detrimental in an older population since age-related decrements in thermoregulation can occur as early as 40 years of age during exercise (Larose *et al.*, 2013). Although our findings are specific to the local effects of these inhibitors on sweating and cutaneous vasodilatation, these results may provide some perspective on the systemic effects that may result from administration of these agents.

1.6 Delimitations and limitations

The sweating and cutaneous vasodilatory responses observed in the current study were confined to the skin of the dorsal side located at the proximal forearm. Intradermal microdialysis is typically localized to this specific area on the forearm for various reasons including minimization of movement artifact during exercise, sensitivity to the needle insertion, and to study eccrine sweating which is predominantly thermoregulatory (as opposed to apocrine sweating) (Sato *et al.*, 1989; Fujii *et al.*, 2014; Louie *et al.*, 2016; Meade *et al.*, 2016). However, it is possible that

the local mechanisms elucidated in the forearm may not necessarily translate to other areas of the body, such as the chest, axilla, and back, due to regional variability (*e.g.* sweat gland density). Regardless, the insight gained from the forearm responses advance our understanding of the mechanisms underpinning the regulation of heat loss responses.

Male participants were recruited for the current thesis to avoid the potentially confounding influence of known sex-related differences in the underlying mechanisms. Sex-related differences in evaporative heat loss during exercise-induced heat stress have been reported (Gagnon & Kenny, 2011, 2012). This discrepancy in heat loss responses between males and females may be related to differences in the mechanisms regulating end-organ function. Gagnon and colleagues (2013a) demonstrated that the acetylcholine- and methacholine-induced local sweating responses were largely similar (*i.e.* similar log EC_{50}) between the sexes, however maximal achieved sweat rate was attenuated in the females (Gagnon *et al.*, 2013a). It is therefore possible that the mechanisms underpinning the sweating response may differ between males and females at the post-synaptic level of the sweat gland. Additionally, there is evidence demonstrating that female circulating hormones associated with the menstrual cycle, such as estrogen and progesterone, can significantly affect the heat loss responses during exercise (Kuwahara *et al.*, 2005). Thus, future research is warranted to determine the mechanisms that can explain the known differences in heat loss responses between males and females during exercise.

As previously mentioned, there is clear evidence to support age-related impairments in whole body heat dissipation (Larose *et al.*, 2013), which may be explained by specific mechanisms such as diminished nitric oxide-dependent sweating and greater levels of oxidative stress (Holowatz *et al.*, 2003; Holowatz *et al.*, 2006; Stapleton *et al.*, 2014). Indeed, reductions in Na^+/K^+-ATPase activity (Gambert & Duthie, 1983) and K^+ channel expression (Marijic *et al.*,

2001) related to ageing have been previously reported. Therefore, recruitment was limited to younger participants (24 ± 4 years) to minimize the potentially confounding influence of age. However, assessing the roles of membrane transport proteins will be an important direction for future research aimed at determining the mechanisms that explain the age-related attenuations in sweating and cutaneous vasodilatation during exercise.

Healthy participants free of any cardiovascular, respiratory, or metabolic disease(s) were recruited to avoid any potentially confounding effects of chronic disease. For example, in individuals with hypertension, impaired cutaneous vascular function characterized by augmented vasoconstriction and attenuated active vasodilatation has been attributed to mechanisms including the upregulation of rho-kinase activity (Smith *et al.*, 2013), as well as diminished nitric oxide bioavailability related to greater arginase activity (Holowatz & Kenney, 2007b) and oxidative stress (Holowatz & Kenney, 2007a). Thus, healthy participants were recruited to mitigate the effects of differing mechanisms in populations with chronic disease. However, future research can be directed toward determining if the heat loss impairments seen with chronic conditions, such as hypertension, type 1 diabetes (Yardley *et al.*, 2013a) or type 2 diabetes (Yardley *et al.*, 2013b), are related to membrane transport proteins, given their important downstream roles in regulating these heat loss responses.

In the current thesis, participants completed three exercise bouts at low, moderate, and high intensity, respectively, for a total duration of 90 minutes. A potential limitation is that dehydration and the associated electrolyte loss *via* sweating was not assessed, which may have affected the function of the transport membrane proteins given their role in the movement and exchange of ions. It has been demonstrated that hypohydration at a controlled 3, 5, and 7% reduction of total body mass resulted in correlative attenuations in the sweating response despite

increasing elevations in core temperature (Sawka *et al.*, 1985). However, in the current thesis, the participants demonstrated a relatively smaller decrease in body mass following the experimental protocol (1.5 ± 0.4% reduction). Moreover, to mitigate the effects of excessive dehydration, the participants were instructed to arrive to the laboratory euhydrated which was ensured by consuming ≥500 mL of water the night prior as well as two hours before arriving to their experimental session. Upon arriving to the laboratory, the participants provided a urine sample with which urine specific gravity was assessed using a hand-held total solids refractometer. The measured urine specific gravity (average ± standard deviation) was calculated as 1.007 ± 0.002, indicating the participants were indeed adequately hydrated (Sawka *et al.*, 2007).

CHAPTER II

REVIEW OF THE LITERATURE

2.1 Basic thermoregulation

Core temperature is maintained at a temperature of ~37°C *via* the regulation of sweating and cutaneous blood flow. Perturbations in core temperature occur with exercise, since the majority of energy that humans expend during exercise is released endogenously as heat (Gagge & Gonzalez, 2010). As a result, body temperature increases which is sensed by thermoreceptors, located peripherally (*e.g.* skin) and centrally (*e.g.* spine) that send afferent input to the preoptic anterior hypothalamus in the brain (Boulant, 2000). Efferent feedback is then sent back to activate the thermoeffector end-organs, the sweat gland and cutaneous vasculature. The body dissipates heat by means of either dry and/or evaporative heat exchange. This heat exchange is dependent on changes in cutaneous blood flow and/or the evaporation of sweat. During exercise, the heat generated from active muscle is transferred to the blood and is circulated to the core of the body, resulting in increases in core temperature. As well, vasodilatation of cutaneous vasculature permits greater cutaneous vascular conductance and blood volume delivery to the periphery (*i.e.* skin), allowing heat to dissipate *via* conduction, radiation and convection to the environment (*i.e.* dry heat loss) if there is a positive gradient such that the temperature of skin (T_{skin}) is greater than that of the ambient air (T_{air}). However, in situations where T_{air} is greater than T_{skin}, heat will be transferred from the environment to the body (Buskirk, 1977). The heat delivered to the skin also facilitates the evaporation of sweat (*i.e.* evaporative heat loss). The resultant cooler blood circulates back to the core of the body to minimize elevations in core temperature. If total heat gain exceeds that of heat loss, there will be a net heat storage and core temperature will increase.

Below is the conceptual human heat balance equation that summarizes the aforementioned ideas (IUPS Thermal Commision, 1987):

$$M - W = E \pm R \pm C \pm K + S$$

Expressed in watts (W) and can also be standardized for body surface area ($W \cdot m^{-2}$), where:

M = Rate of metabolic energy production

W = Rate of mechanical work

E = Rate of evaporative heat exchange

R = Rate of radiant heat exchange

C = Rate of convective heat exchange

K = Rate of conductive heat exchange

S = Rate of body heat storage

Metabolic heat production, or the heat that is generated within the body, is the difference in metabolic energy production and mechanical work (M – W). Dry heat exchange can either be negative (heat gain) or positive (heat loss) and is the sum of the rates of radiant (R), convective (C), and conductive (K) heat exchange. Heat balance (*i.e.* when the net rate of heat storage (S) is equal to zero) is achieved by increasing thermoeffector activity to match heat loss (E ± R ± C ± K) with the heat gained (M – W). However, during uncompensable heat stress, the amount of heat gained will exceed the body's physiological capacity to dissipate heat and as a result there is a net positive rate of heat storage that will result in a progressive increase in core temperature.

2.2 Sweating

Sweating is the body's greatest potential source of heat dissipation, with a latent heat of vaporization for sweat of approximately 2426 J g^{-1} (Wenger, 1972). Sweating represents the only

avenue of heat loss during exercise in the heat (where T_{air} is greater than T_{skin}). There are roughly 1.6 to 5 million sweat glands on the human body (Saga, 2002) and these are divided into apocrine, apoeccrine, and eccrine sweat glands. Apocrine sweat glands are larger in size, are distributed on the axillae (armpits), areola of the nipples, and genital areas, produce proteinaceous liquid with a distinct odor, and their sweat is secreted *via* the follicles of hair. Sweat glands were identified that shared morphological and functional features of both the apocrine and eccrine gland but could not technically be classified as either, and were therefore labeled as apoeccrine sweat glands (Sato *et al.*, 1987; Sato & Sato, 1987b). However, it is the eccrine glands that primarily contribute to thermoregulation, and thus all discussion of sweating from this point onward will refer to eccrine sweating. Eccrine sweat is secreted to the surface of most glabrous (hairless) skin. In response to elevations changes in the temperature of skin, core or both (Nadel *et al.*, 1971a; Nadel *et al.*, 1971b), eccrine sweating is elicited. Eccrine sweat glands are innervated by sympathetic fibres and predominantly respond to cholinergic stimulation *via* acetylcholine (Sato, 1973). It has also been shown that sweat glands, to a lesser extent, respond to adrenergic stimulation as well (Warndorff, 1972). Anatomically speaking, the eccrine sweat gland is comprised of three major components: the secretory portion, dermal duct, and the acrosyringium (Wilke *et al.*, 2007). Production of precursor fluid, or primary sweat, occurs in the secretory coil portion of the sweat gland and then enters the dermal duct. It is referred to as primary sweat because after leaving the secretory coil (at which point it is isotonic in relation to plasma), it travels toward the skin surface through the dermal duct and acrosyringium where reabsorption of various ions occurs making the solution hypotonic relative to plasma, the end product being typical sweat (Wilke *et al.*, 2007). The composition of eccrine sweat is roughly 99% water and the remainder is solutes including a wide array of organic

compounds as well as ions such as Na^+, Cl^-, K^+, Ca^{2+}, and Mg^{2+}, which offers some insight as to how the sweat is initially produced (Sato *et al.*, 1989).

2.2.1 The Na^+/K^+-ATPase, NKCC, and K^+ channels in sweating

It is generally accepted that the process of sweat production is primarily driven by the transport of ions between the interstitium and secretory coil cells, as well as between the secretory cells and the lumen of the duct. The resultant series of changes in ionic gradients causes water to subsequently move from the blood to secretory coil cell to the lumen of the duct for secretion to the skin surface as sweat. The most widely accepted model for the ionic mechanisms underpinning sweat production is the NKCC model (Quinton, 1983; Sato *et al.*, 1989). This model has been used to describe the movement of ions in other areas of the body such as the thick ascending limb of Henle's loop in the nephrons of the kidney (O'Grady *et al.*, 1987). In this model, initial cholinergic stimulation of the sweat gland causes basolateral membrane K^+ channels and luminal membrane Cl^- channels to open, causing an efflux of KCl from the cytoplasm. The resultant gradient favours K^+ and Cl^- influx from the interstitium *via* the basolateral membrane NKCC which carries one Na^+, one K^+, and two Cl^- into the secretory coil cell in an electroneutral manner. The basolateral membrane Na^+/K^+-ATPase then pumps three Na^+ outwards in exchange for two K^+ resulting in yet another electrochemical gradient, albeit negative. Luminal membrane Cl^- transport then causes a lumen negative transepithelial potential (Sato, 1984), causing interstitial Na^+ to transport into the lumen through an intercellular route (*i.e.* and not a luminal transporter). Finally, Na^+ reabsorption occurs in the dermal duct as the solution travels to the skin surface, resulting in hypotonic sweat.

Na^+/K^+-ATPase has been identified in isolated sweat glands extracted from rhesus monkeys (Adachi & Yamasawa, 1966) and humans (Quinton & Tormey, 1976). To verify the role of the Na^+/K^+-ATPase in humans *in vivo*, Sato and colleagues (1969) injected the potent Na^+/K^+-ATPase inhibitor ouabain into a small area in the upper back of young males and assessed local sweat rate. They showed that exposure to ouabain induced a ~75% attenuation in the sweating response elicited by graded exercise. Similarly, we observed a 54-60% attenuation in local forearm sweat rate with Na^+/K^+-ATPase inhibition *via* intradermal microdialysis during moderate intensity exercise (fixed rate of metabolic heat production of 500 W; ~50% VO_{2peak}) (Louie *et al.*, 2016).

Although the Na^+/K^+-ATPase has been more extensively studied, there remains limited information on the role of the NKCC and K^+ channels in the regulation of sweat production. Since the postulation of the NKCC model (Sato *et al.*, 1989), studies have been conducted to localize and demonstrate the role of these membrane transport proteins in sweat gland cells. For example, NKCC mRNA was demonstrated in rhesus monkey eccrine sweat gland cells (Toyomoto *et al.*, 1997) and more recently has been localized in the human eccrine sweat gland basolateral membrane as assessed *via* immunohistochemical analysis (Nejsum *et al.*, 2005; Zhang *et al.*, 2014) thereby supporting the role of the NKCC in the production of sweat. Bovell and colleagues (2008) utilized the NKCC inhibitor bumetanide in epithelial cell line samples derived from human eccrine sweat gland coils (that are used as a surrogate to study the mechanisms of sweat production given their origin), which was shown to prevent the accumulation of intracellular Cl^- thus reinforcing the involvement of the NKCC for ion transport in sweat production. As well, K^+ channels have been identified in eccrine sweat glands using expression profiling in rodent samples (Kunisada *et al.*, 2009). Although localization of K^+

channels in the eccrine sweat gland has yet to be isolated in humans, given the important nature of cell hyperpolarization typically as a product of K^+ efflux and the relatively high concentration of K^+ in sweat, it is reasonable to assume that K^+ channels are involved in the mechanisms underpinning sweating in humans. In support of the notion that K^+ channels are involved in sweat production, studies have demonstrated relevant patch clamp measurements (Krouse *et al.*, 1989; Henderson & Cuthbert, 1991) and attenuated levels of methacholine-induced sweating (Sato & Sato, 1987a; Henderson & Cuthbert, 1991; Suzuki *et al.*, 1991) in isolated sweat gland cells exposed to K^+ channel inhibitors such as Ba^{2+} and quinidine.

2.3 Cutaneous vasodilatation

Dry heat exchange between the skin and the environment is a major avenue of heat loss, in which the specific heat capacity of dry air is 1005 J kg_{air}^{-1} K^{-1}. Given the density of capillary loops and subpapillary venous plexuses and their proximity to the epidermis, blood flow to these vessels determines dry heat exchange. This blood flow to the cutaneous vasculature ranges from near zero (maximal vasoconstriction with cooling) to 8 L · min^{-1} or approximately 60% of cardiac output (in extreme heat stress) (Rowell, 1974; Charkoudian, 2010). The rate of cutaneous blood volume delivery is primarily controlled by the connected arterioles. Arteriolar blood flow is dependent on the diameter of the vessel whereby vasoconstriction or vasodilatation permits less or more blood flow, respectively, in response to dual autonomic innervation from cholinergic and noradrenergic neurons (Johnson *et al.*, 2014b). At the onset of exercise there is a transient increase in cutaneous vasoconstriction resulting in a reduction in cutaneous blood flow, but as core temperature increases, cutaneous vasoconstriction is reduced and vasodilatation increases to facilitate heat dissipation (Rowell, 1993).

The supplying arterioles are lined with both endothelial and vascular smooth muscle cells, and so the regulation of cutaneous vasculature diameter is influenced by modulations originating from each of these cell types (Edwards *et al.*, 2010). For example, nitric oxide is derived from the endothelium, produced by nitric oxide synthase located in endothelial cells. By travelling across myoendothelial gap junctions to the vascular smooth muscle cells, nitric oxide ultimately causes relaxation of the smooth muscle, thereby widening the arterioles and permitting greater blood flow (Feletou & Vanhoutte, 2009). However, the vascular smooth muscle can relax independent of the endothelial cells as well. For instance, autonomic stimulation can activate K^+ channels in vascular smooth muscle cells, leading to a rapid efflux of K^+. The resultant hyperpolarization of the vascular smooth muscle cell causes closures of voltage-gated Ca^{2+} channels and reduces Ca^{2+} entry resulting in vasodilatation (Nelson & Quayle, 1995; Ko *et al.*, 2008).

2.3.1 The Na^+/K^+-ATPase, NKCC, and K^+ channels in cutaneous vasodilatation

A role of Na^+/K^+-ATPase in vascular smooth muscle tone has long been established, such that it facilitates active sodium and potassium transport on the endothelial and vascular smooth muscle cells (Woolfson *et al.*, 1990; Woolfson & Poston, 1991). More recently however, we sought to determine the role of this transporter in regulating cutaneous vasodilatation during intermittent exercise (fixed rate of metabolic heat production of 500 W; ~50% VO_{2peak}) (Louie *et al.*, 2016). We observed elevated levels of cutaneous vascular conductance with Na^+/K^+-ATPase inhibition during baseline rest and recovery from exercise, which we attributed to nitric oxide given that this response was abolished with concomitant inhibition of nitric oxide synthase. We postulated that expectedly greater intracellular Ca^{2+} in endothelial cells, secondary to Na^+/K^+-ATPase

inhibition, may have stimulated nitric oxide synthase (Louie *et al.*, 2016). In contrast to our observations during rest and post-exercise recovery we showed that similar levels of cutaneous vasodilatation between the Na^+/K^+-ATPase inhibited and control site during exercise. This was also speculated to in part be related to a reduction in EDH-mediated vasodilatation based on previous reports suggesting that Na^+/K^+-ATPase is a downstream mediator in this pathway (Edwards *et al.*, 2010). Taken together, we proposed that Na^+/K^+-ATPase may act to regulate intracellular Ca^{2+} levels thereby indirectly modulating cutaneous vasodilatation during exercise. In regards to the NKCC, Barthelmebs and colleagues (1994) demonstrated that exposure to the NKCC inhibitor furosemide caused vasodilatory responses in isolated rat vasculature *in vitro* as well as with intravenous injections in rats *in vivo*. These vasodilatory responses with NKCC inhibition have been attributed to a variety of factors including increased synthesis of prostaglandins (Liguori *et al.*, 1999), endothelial kinins (*e.g.* bradykinin), and nitric oxide (Wiemer *et al.*, 1994). Moreover, Garg and colleagues (2007) observed a rapid decline in mean arterial pressure in rats infused intravenously with bumetanide, a NKCC inhibitor. The authors suggested that this effect was dependent on smooth muscle tone in resistance arteries, given they also demonstrated that NKCC inhibition caused a blunted phenylephrine-induced contractile response in mesenteric arteries *in vitro*, similar to previous findings (Lamb & Barna, 1998; Anfinogenova *et al.*, 2004). However, no studies to date have demonstrated whether the NKCC is involved in the regulation of cutaneous vasculature *per se*, especially using a human *in vivo* model. Conversely, the role of K^+ channels in mediating cutaneous vasodilatation has been of particular interest given that EDH predominantly elicits its effects *via* K^+ channel stimulation (Lorenzo & Minson, 2007; Edwards *et al.*, 2010; Brunt & Minson, 2012; Brunt *et al.*, 2013, 2015; Garland & Dora, 2016). This activation of K^+ channels in vascular smooth muscle cells

results in a rapid efflux of K^+ which hyperpolarizes the cell, causing relaxation of the smooth muscle and ultimately leading to vasodilatation. To investigate this notion, a number of studies using the intradermal microdialysis technique in humans *in vivo* have been undertaken. Lorenzo and Minson (2007) showed that the inhibition of large conductance calcium-activated K^+ channels (BK_{Ca}) caused a reduction in the reactive hyperaemic response (by approximately 15.9 $\%CVC_{max}$ from control) following 5 minutes of arterial occlusion. In a follow-up study, local heating was used to raise skin temperature to 42°C at a rate of $0.1°C \cdot s^{-1}$ during which time K_{Ca} channel inhibition was induced *via* the intradermal infusion of tetraethylammonium. This resulted in a blunting of the plateau in cutaneous vasodilatation (by ~15 $\%CVC_{max}$ from control) (Brunt & Minson, 2012). However, Brunt and colleagues (2013) subsequently demonstrated that K_{Ca} channel inhibition, as well as simultaneous blockade of the inwardly-rectifying (K_{IR}) and ATP-sensitive (K_{ATP}) K^+ channels, caused no changes in cutaneous vascular conductance plateau in response to a gradual 1.0°C increase in body core temperature achieved by whole body heating using a water-perfused suit. These findings were in contrast with their hypothesis, which is especially interesting given they demonstrated clear roles for K^+ channels using sole K_{Ca} inhibition in other studies albeit with different methods of activating cutaneous vasodilatation. More recently, however, Brunt and colleagues (2015) induced cutaneous vasodilatation with acetylcholine-mediated cholinergic stimulation, and it was shown that non-specific K_{Ca} inhibition attenuates peak cutaneous vascular conductance during higher concentrations of acetylcholine (*i.e.* 100 mM). Taken together, these findings indicate that K^+ channels and its various subtypes may play key roles in the regulation of cutaneous vasculature based on the type of stimuli. It remains unknown, however, how K^+ channels as well as the NKCC contribute to the regulation of cutaneous vasculature during dynamic exercise.

2.4 Heat loss response mechanisms as a function of thermoeffector activity

In recent work, we demonstrated a role for nitric oxide in modulating cutaneous vasodilatation during moderate intensity exercise (fixed rate of metabolic heat production of 400 W; an equivalent of ~40% VO_{2peak}), a contribution that was not seen during high intensity exercise (fixed rate of metabolic heat production of 700 W; equivalent of ~70% VO_{2peak}) (Fujii *et al.*, 2014). This intensity-dependent contribution of nitric oxide in regulating cutaneous vasodilatation was reiterated in a follow up study, wherein the moderate intensity exercise was employed at a fixed rate of metabolic heat production of 500 W (equivalent to ~52% VO_{2peak}) (Meade *et al.*, 2015). Additionally, Fujii and colleagues (2014) demonstrated that cyclooxygenase (*via* the inhibition with the cyclooxygenase inhibitor ketorolac) contributed to the sweating response during moderate but not high intensity exercise. It is possible that the mechanisms related to nitric oxide and cyclooxygenase that underpin the regulation of heat loss responses are altered as a function of exercise intensity and therefore rate of metabolic heat production. Additionally, it is well known that greater rates of metabolic production are accompanied by elevated thermal drive, which causes an increase in thermoeffector activity (*e.g.* sweating and cutaneous vasodilatation) (Gagnon *et al.*, 2013b; Kenny & Jay, 2013). In a study conducted by Lee & Mack (2006), sweating was induced with incremental doses of methacholine (0.033 to 243 mM), a cholinergic agonist, while simultaneous inhibiting nitric oxide synthase *via* the intradermal microdialysis technique. A rightward shift in local sweat rate revealed that nitric oxide in some way augmented sweat gland activity; however the contribution of nitric oxide was not apparent in the higher doses of methacholine-induced sweating. Further, Metzler-Wilson and colleagues (2014) administered incremental doses of acetylcholine (0.001 to 1000 mM; a cholinergic agonist) while simultaneously lowering interstitial Ca^{2+} with chelation as

well as (at another experimental site) attenuating Ca^{2+} influx with L-type Ca^{2+} channel inhibition. They observed an initial rightward shift in the local sweat rate response with Ca^{2+} chelation and L-type Ca^{2+} channel blockade, thereby indicating the role of Ca^{2+} in the sweating response; however this response did not remain intact in the final stages of acetylcholine administration. Taken together, it is possible that the mechanisms underlying sweating and cutaneous vasodilatation elucidated during low to moderate levels of thermoeffector activity may not remain intact at elevated levels of hyperthermia, which may be associated with greater exercise intensities and therefore metabolic heat production.

PART TWO

METHODS AND RESULTS OF THE THESIS

ARTICLE

The Roles of the Na^+/K^+-ATPase, NKCC, and K^+ Channels in Regulating Local Sweating and Cutaneous Blood Flow during Exercise in Humans *in vivo*

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The Roles of the Na^+/K^+-ATPase, NKCC, and K^+ Channels in Regulating Local Sweating and Cutaneous Blood Flow during Exercise in Humans *in vivo*

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AUTHOR CONTRIBUTIONS

J.L., N.F., R.D.M and G.P.K. conceived and designed experiments. J.L., N.F. and R.D.M. contributed to data collection. J.L. performed data analysis. J.L., N.F., R.D.M. and G.P.K. interpreted the experimental results. J.L. prepared the figures. J.L. drafted the manuscript. J.L., N.F., R.D.M. and G.P.K. edited and revised the manuscript. All authors approved the final version of the manuscript. All experiments took place at the Human and Environmental Physiology Research Unit located at the University of Ottawa.

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Table of contents category: Integrative

Key Points Summary

- Although the role of Na^+/K^+-ATPase in regulating sweating and cutaneous vasculature during exercise has been demonstrated in humans during exercise, little is known about the contribution of NKCC and K^+ channels. Further, it is unclear if the contribution of these mechanisms underpinning the heat loss responses differs with increasing exercise intensity.
- We show that Na^+/K^+-ATPase, NKCC, and K^+ channels contribute to forearm sweating during exercise at moderate (50% VO_{2peak}) and/or only high (70% VO_{2peak}), but not low (30% VO_{2peak}) intensity exercise.
- Na^+/K^+-ATPase inhibition augments cutaneous vasodilatation across all intensities, whereas the influence of NKCC was evidenced at moderate intensity exercise only and the contribution of K^+ channels was seen during the moderate and high intensity exercise bouts.
- This study provides evidence supporting the involvement of Na^+/K^+-ATPase, NKCC, and K^+ channels in regulating sweating and cutaneous vasculature during exercise; an influence which is however dependent on the intensity of exercise.

Key words: Na^+/K^+-ATPase, NKCC, K^+ channels, heat loss, exercise, sweat gland, microcirculation

Abbreviations CVC, cutaneous vascular conductance; K_{ATP}, ATP-sensitive K^+ channel; K_{Ca}, Ca^{2+}-activated K^+ channel; K_V, voltage-gated K^+ channel; LSR, local sweat rate; NKCC, Na-K-Cl cotransporter; PGI2, prostacyclin; VSMC, vascular smooth muscle cell

Abstract

Na^+/K^+-ATPase has been shown to regulate the sweating and cutaneous vascular responses during exercise; however, similar studies have not been conducted to assess the roles of the Na-K-2Cl cotransporter (NKCC) and K^+ channels. Additionally, it remains to be determined if these mechanisms underpinning the heat loss responses differ with exercise intensity. Eleven young (24±4 years) males performed three 30-min semi-recumbent cycling bouts at low (30% VO_{2peak}), moderate (50% VO_{2peak}), and high (70% VO_{2peak}) intensity exercise, respectively, each separated by 20-min recovery periods. Using intradermal microdialysis, four forearm skin sites were continuously perfused with either: 1) lactated Ringer solution (Control), 2) 6 mM ouabain (Na^+/K^+-ATPase inhibitor), 3) 10 mM bumetanide (NKCC inhibitor), or 4) 50 mM $BaCl_2$ (non-specific K^+ channel inhibitor); sites at which we assessed local sweat rate (LSR) and cutaneous vascular conductance (CVC). Inhibition of Na^+/K^+-ATPase attenuated LSR compared to Control during the moderate and high intensity exercise bouts (both $P<0.01$), whereas attenuations with NKCC and K^+ channel inhibition were only apparent during the high intensity exercise bout (both $P\leq0.05$). Na^+/K^+-ATPase inhibition augmented CVC during all exercise intensities (all $P<0.01$), whereas CVC was greater with NKCC inhibition during the low intensity exercise only ($P<0.01$) and attenuated with K^+ channel inhibition during the moderate and high intensity exercise conditions (both $P<0.01$). We show that Na^+/K^+-ATPase, NKCC and K^+ channels all contribute to the regulation of sweating and cutaneous blood flow but their influence is dependent on the intensity of exercise.

INTRODUCTION

The physiological mechanisms regulating the heat loss responses of sweating and cutaneous vasodilatation during exercise have yet to be fully elucidated. It is generally accepted that the production of sweat is facilitated by the transport of various ions (*e.g.* Na^+, K^+, Cl^-) to establish a series of electrochemical gradients across the basolateral and luminal membranes on the sweat gland (Sato *et al.*, 1989). The process of sweat production has been explained by the Na-K-2Cl cotransporter (NKCC) model (Quinton, 1983; Sato *et al.*, 1989; Saga, 2002), which highlights the involvement of various membrane transport proteins including the sodium pump (Na^+/K^+-ATPase), NKCC, and K^+ channels. Indeed, Na^+/K^+-ATPase has been demonstrated to regulate sweating in humans during exercise, evidenced by large attenuations in sweat rate following local administration of the Na^+/K^+-ATPase inhibitor ouabain (Sato & Dobson, 1969; Sato *et al.*, 1969; Louie *et al.*, 2016). In contrast, despite the NKCC and K^+ channels being implicated in the production of sweat *in vitro* (Sato & Sato, 1987; Reddy & Quinton, 1991; Suzuki *et al.*, 1991; Samman *et al.*, 1993; Sato *et al.*, 1993; Toyomoto *et al.*, 1997; Reddy & Quinton, 1999; Bovell *et al.*, 2008), it is unknown if these findings can be extended to the responses in humans *in vivo*.

In our recent study, we demonstrated that Na^+/K^+-ATPase inhibition augmented cutaneous vasodilatation during exercise in the heat (Louie *et al.*, 2016). We had determined that the augmented cutaneous vasodilatory response seen with Na^+/K^+-ATPase inhibition was nitric oxide-dependent *via* the simultaneous inhibition of the enzyme nitric oxide synthase (Louie *et al.*, 2016). Furthermore, although studies have demonstrated that NKCC inhibition results in relaxation of vascular smooth muscle (Liguori *et al.*, 1999; Garg *et al.*, 2007; Orlov, 2007) and K^+ channel inhibition typically causes an attenuated cutaneous vascular response (Hojs *et al.*,

2009; Brunt & Minson, 2012; Brunt *et al.*, 2013; Kutz *et al.*, 2015), none have addressed their roles in cutaneous vasodilatation in humans during exercise.

It was recently observed that key modulators of the sweating and cutaneous vasodilatory responses, such as those associated with the influence of nitric oxide synthase, varied as a function of the exercise intensity (Fujii *et al.*, 2014; Meade *et al.*, 2015). Although the underlying mechanism(s) to explain these variations remain unknown, there exist several factors that may affect the regulation of sweating and cutaneous vasodilatation that have been demonstrated to increase in production or activation in parallel with the level of exercise intensity, such as those associated with aldosterone and vasopressin (Montain *et al.*, 1997), heat shock proteins (*i.e.*, HSP70) (Milne & Noble, 2002), endothelin-1 (Maeda *et al.*, 1994), and oxidative stress (Goto *et al.*, 2003). However, given the fundamental nature of the downstream regulators Na^+/K^+-ATPase, NKCC, and K^+ channels and their likely contribution to the regulation of the heat loss responses as hypothesized above, these regulators may also demonstrate similar intensity-dependent responses as previously observed (Fujii *et al.*, 2014; Meade *et al.*, 2015).

The purpose of this study was to investigate the roles of the Na^+/K^+-ATPase, NKCC, and K^+ channels in regulating the local sweating and cutaneous vasodilatory responses during exercise. Moreover, we sought to determine whether the contributions to the heat loss responses of each of these transporters varied depending on the level of exercise intensity and therefore rate of metabolic heat production. We hypothesize that inhibition of the Na^+/K^+-ATPase, NKCC and K^+ channels will result in attenuations in local sweat rate; whereas Na^+/K^+-ATPase and NKCC inhibition will augment cutaneous vasodilatation and K^+ channel inhibition will attenuate this response. Further, we hypothesized that the contributions of each transporter would be attenuated with greater levels of exercise intensity. These data will advance our knowledge of the

mechanisms underpinning sweating and cutaneous vasodilatation; the physiological heat loss responses which are critical for the maintenance of a stable core temperature. Failure to maintain core temperature increases the risk for heat-related illnesses such as heat stroke and ultimately death. Moreover, our findings will provide direction for future studies aimed at determining the underlying physiological mechanisms that explain why certain populations, such as those with chronic conditions or the elderly, have impaired heat loss responses that put them at greater risk for heat-related illnesses.

METHODS

Ethical Approval

The current study obtained approval from the University of Ottawa Health Sciences Ethics Board and conformed to the guidelines set forth by the *Declaration of Helsinki*. Written and informed consent were acquired prior to involvement in the study.

Participants

Eleven young, healthy, and physically active (2-5 days · $week^{-1}$ of structured physical activity; $\geq$ 30 min · day^{-1}) males were recruited to participate in this study. Participants were normotensive, non-smoking, non-heat acclimatized, and were excluded if they had any history of cardiovascular, respiratory, and/or metabolic diseases. Participants' characteristics (mean ± standard deviation) were as follows: age, 24 ± 4 years; height, 1.78 ± 0.07 m; mass, 75.7 ± 10.5 kg; body surface area, 1.9 ± 0.2 m^2; body fat percentage, 14 ± 4%; peak rate of oxygen consumption (VO_{2peak}), 48 ± 5 mL O_2 · kg^{-1} · min^{-1}.

Experimental Procedures

Preliminary testing session

Participants were required to undergo a preliminary testing session. During this time, their anthropometric and VO_{2peak} data were collected for screening purposes. Participants were asked to refrain from food (≥2 hr prior to the session), alcohol, caffeine, high intensity exercise (≥12 hr) and over-the-counter and/or prescriptions medications (including supplements such as vitamins and minerals) (≥24 hr) prior to this session. We measured body height and mass using a stadiometer (Detecto, model 2391, Webb City, MO, USA) and digital high-performance weighing terminal (model CBU150X, Mettler Toledo Inc., Mississauga, ON, Canada), respectively, and these measurements were subsequently used to determine body surface area (Du Bois & Du Bois, 1989). The hydrostatic weighing technique was utilized to determine body density, and from this body composition was estimated (Siri, 1956). We assessed VO_{2peak} using an incremental exercise protocol until exhaustion on a semi-recumbent cycle ergometer (Corival Recumbent, Lode, Groningen, Netherlands). The initial workload was set to 100 W, increasing by 20 W · min^{-1}. Participants were instructed to maintain a pedaling cadence of 60-90 revolutions · min^{-1} and the test concluded when the participant reached volitional fatigue or could not maintain ≥50 revolutions · min^{-1}. Expired air was concomitantly assessed using an automated indirect calorimetry system (MCD Medgraphics Ultima Series, MGC Diagnostics, MN, USA) and VO_{2peak} was taken as the greatest average oxygen uptake over a period of 30 sec.

Experimental testing session: intradermal microdialysis fibre placement

On a separate day (≥ 48 hours from the preliminary testing session), participants underwent the experimental testing session. Participants were asked to refrain from the same items outlined

above for the preliminary testing sessions prior to arriving to the laboratory on this day. Participants were instructed to adequately hydrate prior to the experimental testing session by consuming ≥500 mL of water the night prior and roughly 2 h before arriving to the laboratory. Urine samples were collected to assess urine specific gravity (1.007 ± 0.002) to ensure the participants were euhydrated before commencing the experimental testing session (Sawka *et al.*, 2007). Following a measurement of nude body mass, participants were seated semi-recumbently in a thermoneutral room (25°C). Four intradermal microdialysis fibres (30 kDa cutoff; MD2000, Bioanalytical Systems, West Lafayette, IN, USA) were instrumented in aseptic conditions to the dermal layer of skin on the dorsal side of the left forearm. This was accomplished using a 25-gauge needle inserted into the non-anaesthetized skin which traveled ~2.5 cm before exiting. Following needle placement, the microdialysis fibre was threaded through the needle's lumen. By carefully withdrawing the needle, the 10 mm semi-permeable membrane of the microdialysis fibre was situated in the forearm skin. The fibre was then secured in place to the skin using surgical tape. This process was repeated for placement of the remaining three fibres, each being placed ≥4 cm apart from one another.

Experimental testing session: exercise protocol

After placement of the intradermal microdialysis fibres, participants were directed to a thermal chamber located in an adjacent room (Can-Trol Environmental Systems, Markham, ON, Canada) regulated to 25°C and 20% relative humidity and seated on a semi-recumbent cycle ergometer. They remained in this seated position for the remainder of the experimental testing session. In a counter-balanced manner, the microdialysis fibres were perfused with one of the following pharmacological agent solutions: 1) lactated Ringer solution (Control), 2) 6 mM ouabain

(Ouabain; Sigma-Aldrich, St Louis, MO, USA), Na^+/K^+-ATPase inhibitor, 3) 10 mM bumetanide (Bumetanide; Cayman Chemical, Ann Arbor, MI), NKCC inhibitor, or 4) 50 mM $BaCl_2$ ($BaCl_2$; Sigma-Aldrich), non-specific K^+ channel inhibitor at a rate of 4 μL · min^{-1} for the remainder of the trial using a microinfusion pump (Model 400, CMA Microdialysis, Solna, Sweden). An habituation period (≥60 min) of drug perfusion prior to baseline data collection was undertaken at all four skin sites to ensure a complete blockade was established. The concentration of ouabain (*i.e.* 6 mM) was chosen based on a previously conducted study in our lab that used this agent (Louie *et al.*, 2016). Given that the pharmacological agents bumetanide and $BaCl_2$ had not been utilized with the intradermal microdialysis technique as NKCC and non-specific K^+ channel inhibitors, respectively, it was necessary to conduct pilot work to determine the appropriate concentrations of these drugs. Insight was gleaned from previous studies utilizing 10 mM bumetanide (Garg *et al.*, 2007) and 2 μM-10 mM $BaCl_2$ (Sato & Sato, 1987; Nelson & Quayle, 1995; Brunt *et al.*, 2013). Moreover, although Brunt and colleagues (2013) had previously administered 100 μM $BaCl_2$ *via* intradermal microdialysis, they chose this lower concentration to inhibit only the inwardly rectifying (K_{IR}) and ATP-sensitive (K_{ATP}) K^+ channels, whereas other K^+ channels, such as the calcium-activated (K_{Ca}) and voltage-gated (K_V) K^+ channels likely remained active. The pilot work for the current study involved the placement of microdialysis fibres, as previously described, perfused with lactated Ringer solution (Control), or various low, medium, or high concentrations of either bumetanide or $BaCl_2$ (*i.e.* separate trials). To elicit increases in sweating and cutaneous vasodilatation, participants exercised at a constant rate of metabolic heat production of 500 W until a steady state in the responses was attained (approximately 30-45 min). For bumetanide it was determined that 10 mM (dissolved in lactated ringers and 20 mM NaOH) was the highest concentration able to be made and resulted in the

largest attenuation in local sweat rate (a decrease of ~1.05 mg · min^{-1} · cm^{-2} relative to Control) at the end of the exercise bout. In regards to $BaCl_2$, it was observed that 50 mM $BaCl_2$ attenuated the end-exercise cutaneous vasodilatory response to a similar extent as the administration of 100 mM of $BaCl_2$ (a reduction of ~33% of maximal cutaneous vascular conductance (CVC_{max}) relative to Control), both of which were greater than 5 mM $BaCl_2$ (a decrease of ~6% CVC_{max} relative to Control).

Following the habituation period of the experimental testing session, the exercise protocol consisted of a 5 min baseline period. Participants then performed three successive 30-min bouts of exercise performed at a low (30% VO_{2peak}), moderate (50% VO_{2peak}) and high (70% VO_{2peak}) intensity exercise (equivalent to a rate of metabolic heat production of 316 ± 36, 497 ± 45, and 715 ± 70 W, respectively; an equivalent external workload of 60 ± 8, 111 ± 9, and 149 ± 13 W, respectively). Each exercise bout was followed by a 20-min recovery period. After completion of the last recovery period, the final stage of the experimental protocol was employed to elicit maximal cutaneous vasodilatation. All fibres were perfused with 50 mM sodium nitroprusside (Sigma-Aldrich) at a rate of 6 μL · min^{-1} for the remainder of the ~20 min period until a stable 2-min plateau in cutaneous blood flow measurements was established. Blood pressure was measured after cessation of this final period and was used to calculate maximal cutaneous vascular conductance (CVC_{max}). Lastly, the fibres were removed from the forearm skin and a final nude body mass was measured.

Measurements

Local forearm sweat rate (LSR) was measured with the ventilated capsule technique in which each skin site was designated a sweat capsule covering a surface area of 1.1 cm^2, specially

designed for use with intradermal microdialysis to account for the diffusion distance of the pharmacological agents (Meade *et al.*, 2016). These capsules were placed directly over the semi-permeable membrane of the fibre and were secured to the skin with adhesive rings and topical glue (Collodion HV, Mavidon Medical Products, Lake Worth, FL, USA). Long vinyl tubes connected each compressed anhydrous air tank to a flow rate monitor (Omega FMA-A2307, Omega Engineering, Stamford, CT, USA), which was then connected to each capsule, and subsequently to a capacitance hygrometer (model HMT333, Vaisala, Helsinki, Finland). Moreover, the aforementioned equipment was located in the thermal chamber to ensure internal gas temperatures were equilibrated to near room temperature (25°C). The anhydrous air was delivered at a flow rate of 0.2 $L \cdot min^{-1}$. Every 5 sec LSR was calculated using the difference in water content between the influent and effluent air, multiplied by flow rate, and normalized for the skin surface area under the capsule (expressed as $mg \cdot min^{-1} \cdot cm^{-2}$).

Cutaneous red blood cell flux (expressed as perfusion units) was measured at a 32 Hz sampling rate with laser Doppler flowmetry (PeriFlux System 5000, Perimed, Stockholm, Sweden). An integrated laser Doppler flowmetry probe with a seven-laser array (Model 413, Perimed) was housed in each specially designed sweat capsule, positioned on the skin directly over the semi-permeable membrane of each fibre. This setup allowed for the simultaneous measurement of LSR and cutaneous red blood cell flux at each site. CVC was calculated as cutaneous red blood cell flux divided by mean arterial pressure and is presented as a percentage of the CVC_{max} acquired during the maximal skin blood flow protocol. Mean arterial pressure (calculated as diastolic pressure plus one-third of systolic minus diastolic pressure [*i.e.* pulse pressure]) was determined at 5-min intervals using manual auscultation with a validated mercury

column sphygmomanometer (Baumanometer Standby Model, WA Baum Co, Copiague, NY, USA).

Heart rate was measured continuously using a Polar coded WearLink and transmitter, Polar RS400 interface and Polar Trainer 5 software (Polar Electro, Kempele, Finland). Oesophageal temperature was measured using a paediatric thermocouple probe (~2 mm diameter; Mon-a-therm; Mallinckrodt Medical, St Louis, MO, USA) inserted ~40 cm past the entrance of the nostril. Mean skin temperature was measured using thermocouples (Concept Engineering, Old Saybrook, CT, USA) at four skin sites and weighted to the following regional proportions: upper back, 30%; chest, 30%; quadriceps, 20%; and calf, 20%. Oesophageal and skin temperature were collected at a sampling rate of 15 sec using a data acquisition module (Model 34970A; Agilent Technologies Canada, Mississauga, ON, Canada), displayed and recorded using LabVIEW software (National Instruments, Austin, TX, USA). Using these data, mean body temperature was determined as (0.9 × core temperature) + (0.1 × skin temperature).

The rate of metabolic heat production was assessed as the difference between metabolic rate and external workload (Kenny & Jay, 2013). Metabolic energy expenditure was measured using indirect calorimetry in which electrochemical gas analysers (AMETEK model S-3A/1 and CD3A, Applied Electrochemistry, Pittsburgh, PA, USA), calibrated using reference gas mixtures of known concentrations, were used to assess the oxygen and carbon dioxide of expired gas. Moreover, ventilation rate was measured with a turbine ventilometer that was calibrated using a 3 L syringe. In order to collect expired air, subjects wore a full face mask (Model 7600 V2, Hans-Rudolph, Kansas City, MO, USA), connected to a two-way T-shape non-rebreathing valve (Model 2700, Hans-Rudolph) and the respired gases were averaged over periods of 30 sec.

Data Analysis

The values for LSR, CVC, heart rate, as well as oesophageal, mean skin, and mean body temperature were obtained by averaging the measurements made over the last 5 min of each time period (*i.e.* exercise and recovery), whereas baseline values represent an average of the 5 min prior to the first exercise bout. Blood pressure data were calculated as the average of the two measurements taken during 10-min intervals. During the CVC_{max} period, the plateau was defined as the greatest CVC values averaged over 2 min. The differences (Δ) in LSR and CVC from Control were calculated for the Ouabain, Bumetanide, and $BaCl_2$ sites at the end of each exercise bout.

Statistical Analysis

LSR and CVC were analysed with separate two-way repeated measures analyses of variance (ANOVAs) using the factors of time (7 levels: Baseline, Low Intensity Exercise, Recovery 1, Moderate Intensity Exercise, Recovery 2, High Intensity Exercise, and Recovery 3) and treatment site (4 levels: Control, Ouabain, Bumetanide, and $BaCl_2$). Similarly, ΔLSR and ΔCVC from Control at the end of each exercise bout were analysed using separate two-way repeated measure ANOVAs with the factors of exercise period (3 levels: Low, Moderate, and High Intensity Exercise) and treatment site (3 levels: Ouabain, Bumetanide, and $BaCl_2$). Oesophageal, mean skin, mean body temperatures, mean arterial pressure, and heart rate were analyzed using separate one-way repeated measures ANOVAs with the factor of time (7 levels: Baseline, Low Intensity Exercise, Recovery 1, Moderate Intensity Exercise, Recovery 2, High Intensity Exercise, and Recovery 3). Absolute CVC_{max} (expressed in perfusion units $mmHg^{-1}$) was analysed with a one-way repeated measures ANOVA using the factor of treatment site (4 levels:

Control, Ouabain, Bumetanide, and $BaCl_2$). *Post hoc* analyses were carried out using two-tailed paired samples *t* tests adjusted for multiple comparisons using the Holm-Bonferroni procedure when a significant main effect was observed. Statistical analyses were completed using the software package SPSS 23.0 for Windows (IBM, Armonk, NY, USA). For all analyses, $P \leq 0.05$ was considered statistically significant. Values are presented as the mean ± 95% confidence intervals, unless otherwise indicated, calculated as 1.96 × standard error of the mean.

RESULTS

Sweating Response

LSR was similar to Control at all treatment sites during Baseline (all $P \geq 0.17$; Fig 1) as well as at the end of the low intensity exercise bout (all $P \geq 0.06$). At the end of the moderate exercise bout, LSR was attenuated at the Ouabain site ($P < 0.01$) compared to Control (interaction of treatment site and time, $P < 0.01$) but similar at the Bumetanide and $BaCl_2$ sites (both $P \geq 0.24$). LSR was reduced from Control during high intensity exercise at all treatment sites (all $P \leq 0.05$). At the end of each recovery period, LSR was similar to Control at all treatment sites (all $P \geq 0.07$). ΔLSR from Control at the Ouabain site was greater during both moderate and high intensity exercise compared to low (both $P < 0.01$; Fig 2), and greater during the high compared to the moderate intensity exercise bout ($P < 0.01$). At the Bumetanide and $BaCl_2$ sites, ΔLSR from Control was greater during high compared to both low and moderate intensity exercise (all $P \leq 0.05$).

Cutaneous Vascular Response

CVC was elevated at the Ouabain site at all time periods (all $P < 0.01$; Fig 3) in comparison to Control (interaction of treatment site and time, $P < 0.01$). At the Bumetanide site, CVC was

elevated at the end of the low intensity exercise and Recovery 1 in comparison to Control (both $P \leq 0.05$) but similar during the other time periods (all $P \geq 0.07$). Perfusion of $BaCl_2$ resulted in similar levels of CVC compared to Control at Baseline and the end of the low intensity exercise (both $P \geq 0.13$) but attenuated CVC at the remaining time periods (all $P < 0.05$). ΔCVC from Control at the Ouabain site was similar during each exercise bout (all $P \geq 0.23$; Fig 4). Bumetanide administration resulted in greater ΔCVC from Control during both the moderate and high compared to the low intensity exercise bout (both $P < 0.05$). At the $BaCl_2$ site, ΔCVC from Control was greater during high compared to low and moderate intensity exercise (both $P < 0.05$). CVC_{max} was similar at all treatment sites ($P \geq 0.16$).

Cardiovascular and temperature responses

Heart rate was elevated compared to Baseline values at the end of each exercise and recovery period (all $P < 0.01$; Table 1) with the exception of Recovery 1 ($P = 0.12$). At the end of the moderate and high intensity exercise, as well as Recovery 2 and 3, heart rate was increased compared to their respective previous time periods (all $P < 0.01$). Mean arterial pressure was only elevated at the end of the moderate and high intensity exercise bout compared to baseline (both $P < 0.05$), and greater at the end of the high compared to moderate intensity exercise ($P < 0.01$).

Compared to Baseline values, oesophageal, mean skin, and mean body temperatures were elevated during all exercise and recovery periods (all $P < 0.01$; Table 1). Oesophageal, mean skin, and mean body temperatures were greater at the end of moderate and high intensity exercise compared to their respective previous bouts, as well as greater at the end of Recovery 2

and 3 compared Recovery 1 and 2, respectively (all $P \leq 0.05$) (with the exception of mean skin temperature at the end of the high compared to the moderate intensity exercise bout ($P = 0.06$)).

DISCUSSION

We demonstrated that the Na^+/K^+-ATPase, NKCC, and K^+ channels are involved in the regulation of the sweating and the cutaneous vasodilatory response during exercise, and that their apparent contribution varied based on the intensity of exercise. Local sweat rate was unaffected during the low intensity exercise at all treatment sites in comparison to Control. However, Na^+/K^+-ATPase inhibition resulted in a marked attenuation in the sweating response during the moderate and high intensity exercise bouts, whereas NKCC and K^+ channel inhibition attenuated sweat rate during the high intensity exercise bout only. In regards to the regulation of cutaneous vasculature, Na^+/K^+-ATPase inhibition resulted in an augmented cutaneous vasodilatory response throughout the incremental intermittent exercise protocol. Moreover, NKCC inhibition augmented cutaneous vasodilatation during low intensity exercise only whereas K^+ channel inhibition attenuated the response in the moderate and high intensity exercise bouts. These findings suggest that exercise intensity is an important factor to take into consideration when studying the physiological mechanisms underpinning sweating and cutaneous vasodilatation. Moreover, the mechanisms elucidated using protocols such as local heating or methacholine administration may not necessarily reflect the mechanisms that regulate the heat loss responses during exercise.

Sweating Response

In line with previous findings, we demonstrated an attenuation in sweat rate with ouabain administration (Sato & Dobson, 1969; Sato *et al.*, 1969; Louie *et al.*, 2016), reiterating the role of the Na^+/K^+-ATPase in regulating sweating during exercise. Although we previously reported a role for Na^+/K^+-ATPase during exercise, this was examined under moderate intensity exercise only (Louie *et al.*, 2016). In the present study we show that this response is only evident at exercise intensities of moderate or higher levels, while the role of Na^+/K^+-ATPase in the regulation of sweating is absent during low intensity exercise. For the first time, we show that the NKCC and K^+ channels play an important regulatory role in modulating the sweating response in humans during exercise. By inhibiting these transporters we observed marked attenuations in the sweating response when compared to the Control site, but their influence is limited to exercise intensities that result in higher rates of metabolic heat production. Although speculative, it is possible that during the lower intensities of exercise we failed to see a marked attenuation in the sweat rate with the inhibition of a specific membrane transport protein, since other non-inhibited transporters may have been compensating for their lack of function. Regardless, our findings support other studies, that showed the inhibition of the NKCC and K^+ channels (*via* bumetanide and Ba^{2+}, respectively) caused a rapid cessation in methacholine-induced sweat rate in isolated sweat glands *in vitro* (Sato & Sato, 1987). Given we did not observe a complete suppression in sweating with NKCC inhibition, our findings support that other transporters located on the sweat gland basolateral membrane likely contribute to the influx of Cl^- such as the Cl^-/HCO_3 exchanger (Sato & Sato, 1987; Wilson & Metzler-Wilson, 2015).

Cutaneous Vascular Response

Consistent with our previous study (Louie *et al.*, 2016), Na^+/K^+-ATPase inhibition resulted in augmented levels of CVC compared to Control during the Baseline and all recovery periods; the latter response occurring irrespective of the greater exercise-induced rates of metabolic heat production. By simultaneously inhibiting nitric oxide synthase we had previously determined that this augmented cutaneous vasodilatory response was nitric-oxide dependent (Louie *et al.*, 2016). However, we also showed that CVC was similar at the Na^+/K^+-ATPase inhibited and Control sites as assessed during two successive bouts of moderate (~50% VO_{2peak}) intensity exercise (Louie *et al.*, 2016). On the other hand, in the current study we observed an augmented CVC response during the three successive exercise bouts performed at increasing intensities. These contrasting findings may be explained by the warm environment (35°C) employed in the previous study, resulting in greater levels of mean skin temperature (Louie *et al.*, 2016) compared to those measured in the current study. It is well known that the underlying mechanisms regulating cutaneous vasodilatation in response to elevations in local skin temperature alone can differ compared to those observed during whole-body heat stress and exercise (Johnson *et al.*, 2014). However, it is unclear to what extent differences in skin temperature between exercise in thermoneutral conditions and in the heat can explain the discrepancy in the mechanisms underpinning cutaneous vasodilatation.

We observed that inhibition of the NKCC with bumetanide resulted in greater levels of CVC compared to Control during the low intensity exercise bout. This elevated CVC response has been demonstrated in rats, using both isolated vasculature *in vitro* and intravenous injection *in vivo*, in which vasodilatory responses were seen following the administration of the NKCC inhibitor, furosemide (Barthelmebs *et al.*, 1994). Liguori and colleagues (1999), using human

endothelial cells *in vitro* as well as intravenous infusion in humans *in vivo*, demonstrated that exposure to furosemide resulted in greater release of prostacyclin (PGI_2), a potent vasodilator. However, we recently demonstrated that non-selective inhibition of cyclooxygenase, an enzyme responsible for the production of prostaglandins including PGI_2, did not have any effect on the cutaneous vascular response during both moderate- and high-intensity exercise in the heat (Fujii *et al.*, 2014). Taken together, the augmented CVC response seen in the current study with bumetanide administration may not be related to prostaglandin synthesis *per se*, but a result of other vasodilators such as endothelial kinins (*e.g.* bradykinin) and/or nitric oxide that have been shown to elevate with furosemide exposure in bovine aortic endothelial cells (Wiemer *et al.*, 1994).

The attenuated CVC response during exercise with $BaCl_2$ administration in the current study builds upon recent observations demonstrating the regulatory function of K^+ channels in the regulation of cutaneous vasculature tone. Activation of K^+ channels located on endothelial cells results in hyperpolarization which can travel to vascular smooth muscle cells (VSMCs) *via* the gap junction. Additionally, activation of K^+ channels on the VSMC itself can result in hyperpolarization. The hyperpolarization of VSMCs induces a relaxation of smooth muscle, thereby resulting in vasodilatation (Feletou & Vanhoutte, 2009; Edwards *et al.*, 2010). Thus, the inhibition of K^+ channels may have attenuated the hyperpolarization of the VSMC thus diminishing cutaneous vasodilatation. However, Brunt and colleagues (2013) observed that during whole-body passive heating, K_{Ca} blockade did not attenuate the cutaneous vasodilatory response. Based on the study by Brunt and colleagues (2013) and the present findings, K^+ channels other than K_{Ca} channels may play a role in the cutaneous vasodilatation during whole-

body heating, such as K_{ATP} (Hojs *et al.*, 2009) and/or K_V (Ferrer *et al.*, 1999; Gupta *et al.*, 2008) channels.

Exercise Intensity-dependent Contributions

As previously discussed, there exist a number of factors that may affect the sweating and cutaneous vasodilatory response which increase in production or activation with the level of exercise intensity, such as aldosterone and vasopressin (Montain *et al.*, 1997), heat shock proteins (*i.e.*, HSP70) (Milne & Noble, 2002), endothelin-1 (Maeda *et al.*, 1994), and oxidative stress (Goto *et al.*, 2003). For instance, oxidative stress is known to influence the function of various membrane transport proteins including the Na^+/K^+-ATPase, NKCC, and K^+ channel (Elliott & Schilling, 1992; Elliott & Koliwad, 1995; Liu & Gutterman, 2002). Given that previous work demonstrating elevated levels of an oxidative stress marker, malondialdehyde, following 30 min of high (*i.e.*, 75% VO_{2peak}) but not moderate (*i.e.* 50% VO_{2peak}) intensity exercise (Goto *et al.*, 2003), we had previously conducted a study to assess the effects of ascorbate, an anti-oxidant, on the cutaneous vasodilatory response during exercise. We observed that local administration of ascorbate resulted in elevated levels of CVC compared to a control site during high intensity exercise (71 ± 8% VO_{2peak}); a response which was not seen during moderate intensity exercise (52 ± 6% VO_{2peak}) (Meade *et al.*, 2015). Further evaluation is warranted to determine if the exercise intensity-dependent increases in oxidative stress (or other factors such as those listed previously) can explain the varying contributions of the membrane transport proteins to the sweating and cutaneous vasodilatory responses observed in the current study.

Limitations

A primary limitation of the intradermal microdialysis technique employed in the current study is that we can only administer the inhibitory agents in a non-specific fashion. It is not currently possible to target specifically the basolateral, luminal, or ductal membrane of the sweat gland in humans *in vivo* without affecting the other membranes. The development of pharmacological agents to target specific locations and membranes can greatly advance our knowledge of these underlying physiological mechanisms. Similarly, pertaining to the cutaneous vasculature, the Na^+/K^+-ATPase, NKCC and K^+ channels were likely inhibited on both the endothelial and vascular smooth muscle cells. Additionally, the influence of the pharmacological agents administered on surrounding cells (*e.g.* keratinocytes, antigen-presenting cells, mast cells, etc.) cannot be directly determined. Thus the sweating and cutaneous vasodilatory data must be interpreted as the response to non-specific inhibition.

Greater levels of exercise intensity and therefore rates of metabolic heat production are accompanied by elevated thermal drive leading to increased thermoeffector activity and thus rate of heat loss (Gagnon *et al.*, 2013; Kenny & Jay, 2013). A potential limitation of the experimental design is that the successive exercise bouts can lead to progressively greater increases in thermal drive, which may have been more pronounced than if we had opted to have the participants perform each exercise intensity on separate days. However, the microdialysis technique is limited in the sense that performing a multiple day experiment may not be feasible since the insertions and equipment would have to be placed at the exact same locations (*i.e.* to avoid potential differences due to regional variation). The responses observed in the current study may have been influenced by increases in cumulative heat storage with each successive exercise bout and therefore thermal drive, a response that would not be seen with the separate day protocol.

Taken together, our findings should be interpreted carefully and future studies employing non-exercise passive heating models are warranted to determine if the observations made in the current study are the results of changes in the thermoeffector activity (*i.e.* magnitude of sweat rate and cutaneous vascular conductance) associated with a greater core temperature, and not exercise intensity *per se*.

Perspectives

The findings of the current study further our knowledge pertaining to the underlying mechanisms that regulate cutaneous vasodilatation and sweating during exercise. These data can provide insight for future research that aims to determine which specific end-organ mechanisms (*e.g.* Na^+/K^+-ATPase, NKCC, and K^+ channels) that upstream modulators, such as nitric oxide and cyclooxygenase, modulate their influence on the heat loss responses. Moreover, for the first time we demonstrated a key role for K^+ channels in regulating cutaneous vasodilatation during exercise using non-specific K^+ channel inhibition (*i.e.* $BaCl_2$ administration). As previously mentioned, given that inhibitors are available for safe use in humans to study the specific K^+ channel subtypes (*e.g.* K_{Ca}, K_{ATP}, K_V) (Ferrer *et al.*, 1999; Gupta *et al.*, 2008; Hojs *et al.*, 2009; Brunt *et al.*, 2013), it remains to be determined which subtype(s) can explain the cutaneous vasodilatory and sweating responses seen in the current study.

Conclusion

We demonstrated the regulatory influence of the Na^+/K^+-ATPase and, for the first time, the NKCC and K^+ channels on the sweating and cutaneous vascular responses during exercise.

Furthermore, their contributions to these responses differed as a function of exercise intensity (*i.e.* 30, 50, and 70% VO_{2peak}) and therefore rates of metabolic heat production.

COMPETING INTERESTS

None.

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Table 1. Temperature and cardiovascular responses during baseline, exercise and recovery periods.

	Baseline	LOW (30% VO_{2peak})	Recovery 1	MODERATE (50% VO_{2peak})	Recovery 2	HIGH (70% VO_{2peak})	Recovery 3
Oesophageal temperature, ˚C	36.8 ± 0.2	37.2 ± 0.2*	37.0 ± 0.2*	37.6 ± 0.1*†	37.1 ± 0.2*†	38.2 ± 0.1*†	37.4 ± 0.2*†
Mean skin temperature, ˚C	31.4 ± 0.3	32.4 ± 0.4*	32.3 ± 0.3*	33.5 ± 0.3*†	32.9 ± 0.4*†	33.8 ± 0.3*	33.2 ± 0.4*†
Mean body temperature, ˚C	36.2 ± 0.1	36.8 ± 0.1*	36.6 ± 0.2*	37.2 ± 0.1*†	36.7 ± 0.2*†	37.7 ± 0.2*†	37.0 ± 0.2*†
Mean arterial pressure, mmHg	90 ± 3	97 ± 4	89 ± 3	101 ± 7*	89 ± 5	108 ± 8*†	86 ± 5
Heart rate, bpm	65 ± 7	95 ± 7*	69 ± 7	125 ± 9*†	79 ± 8*†	160 ± 7*†	89 ± 7*†

Presented values (n = 11) are mean ± 95% confidence interval. Oesophageal, mean skin and mean body temperatures, as well as heart rate values represent an average of the final 5 min for the corresponding time period. Mean arterial pressure values represent an average of two measurements from the final 10 min for each corresponding time period. Baseline values represent 5 min prior to the first exercise bout. LOW/MODERATE/HIGH, Low/Moderate/High intensity exercise bout; VO_{2peak}, peak oxygen consumption; bpm, beats per minute. *Significantly different vs. Baseline. †Significantly different vs. previous period (*i.e.* exercise vs. exercise; recovery vs. recovery) (all $P \leq 0.05$).

FIGURE LEGENDS

Figure 1: Local sweat rate at the end of each time period during intermittent 30-min graded intensity exercise bouts separated by 20-min recovery periods (n = 11). Four forearm skin sites were continuously perfused with: 1) lactated Ringer solution (Control, white), 2) 6 mM ouabain (Na^+/K^+-ATPase inhibitor, light grey), 3) 10 mM bumetanide (NKCC inhibitor, medium grey), or 4) 50 mM $BaCl_2$ (non-specific K^+ channel inhibitor, dark grey). Values are presented as mean ± 95% confidence interval. Baseline values represent the 5 min prior to the first exercise bout. All other values represent the final 5 min of the corresponding period. BL, baseline; LOW/MODERATE/HIGH, Low/Moderate/High intensity exercise bout; Rec, recovery period; VO_{2peak}, peak rate of oxygen consumption. * Significantly different from Control; $P \leq 0.05$.

Figure 2: The difference (Δ) in local sweat rate from Control at the end of each exercise bout (n = 11). Four forearm skin sites were continuously perfused with: 1) lactated Ringer solution (Control), 2) 6 mM ouabain (Na^+/K^+-ATPase inhibitor, light grey), 3) 10 mM bumetanide (NKCC inhibitor, medium grey), or 4) 50 mM $BaCl_2$ (non-specific K^+ channel inhibitor, dark grey). Values are presented as mean ± 95% confidence interval. Values represent the final 5 min of the corresponding period. LOW/MODERATE/HIGH, Low/Moderate/High intensity exercise bout; VO_{2peak}, peak rate of oxygen consumption. † significantly different from LOW, ‡ HIGH significantly different from MODERATE; all $P \leq 0.05$.

Figure 3: %Maximal cutaneous vascular conductance (%CVC_{max}) at the end of each time period during intermittent 30-min graded intensity exercise bouts separated by 20-min recovery periods (n = 11). Four forearm skin sites were continuously perfused with: 1) lactated Ringer solution (Control, white), 2) 6 mM ouabain (Na^+/K^+-ATPase inhibitor, light grey), 3) 10 mM bumetanide (NKCC inhibitor, medium grey), or 4) 50 mM $BaCl_2$ (non-specific K^+ channel inhibitor, dark grey). Values are presented as mean ± 95% confidence interval. Baseline values represent the 5 min prior to the first exercise bout. All other values represent the final 5 min of the corresponding period. BL, baseline; LOW/MODERATE/HIGH, Low/Moderate/High intensity exercise bout; Rec, recovery period; VO_{2peak}, peak rate of oxygen consumption. * Significantly different from Control; $P \leq 0.05$.

Figure 4: The difference (Δ) in %maximal cutaneous vascular conductance (%CVC_{max}) from Control at the end of each exercise bout (n = 11). Four forearm skin sites were continuously perfused with: 1) lactated Ringer solution (Control), 2) 6 mM ouabain (Na^+/K^+-ATPase inhibitor, light grey), 3) 10 mM bumetanide (NKCC inhibitor, medium grey), or 4) 50 mM $BaCl_2$ (non-specific K^+ channel inhibitor, dark grey). Values are presented as mean ± 95% confidence interval. Values represent the final 5 min of the corresponding period. LOW/MODERATE/HIGH, Low/Moderate/High intensity exercise bout; VO_{2peak}, peak rate of oxygen consumption. † significantly different from LOW, ‡ HIGH significantly different from MODERATE; all $P \leq 0.05$.

FIGURES

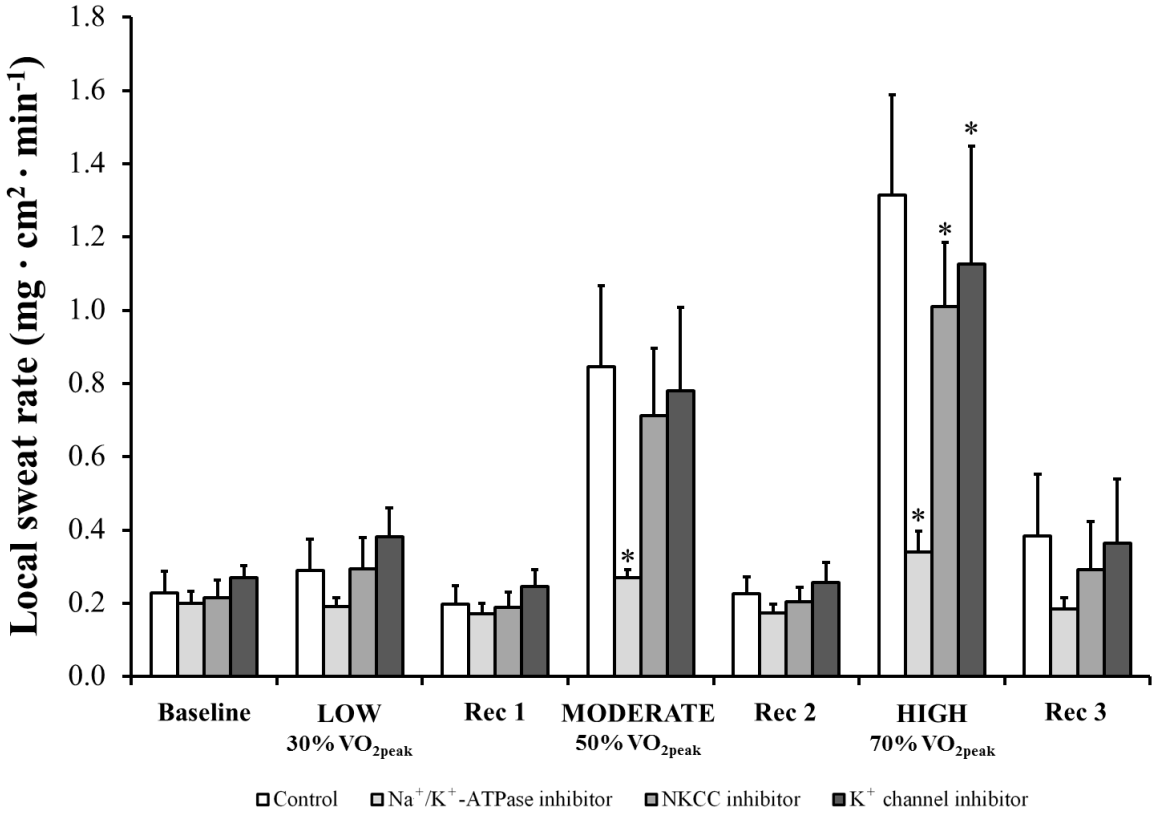

Figure 1

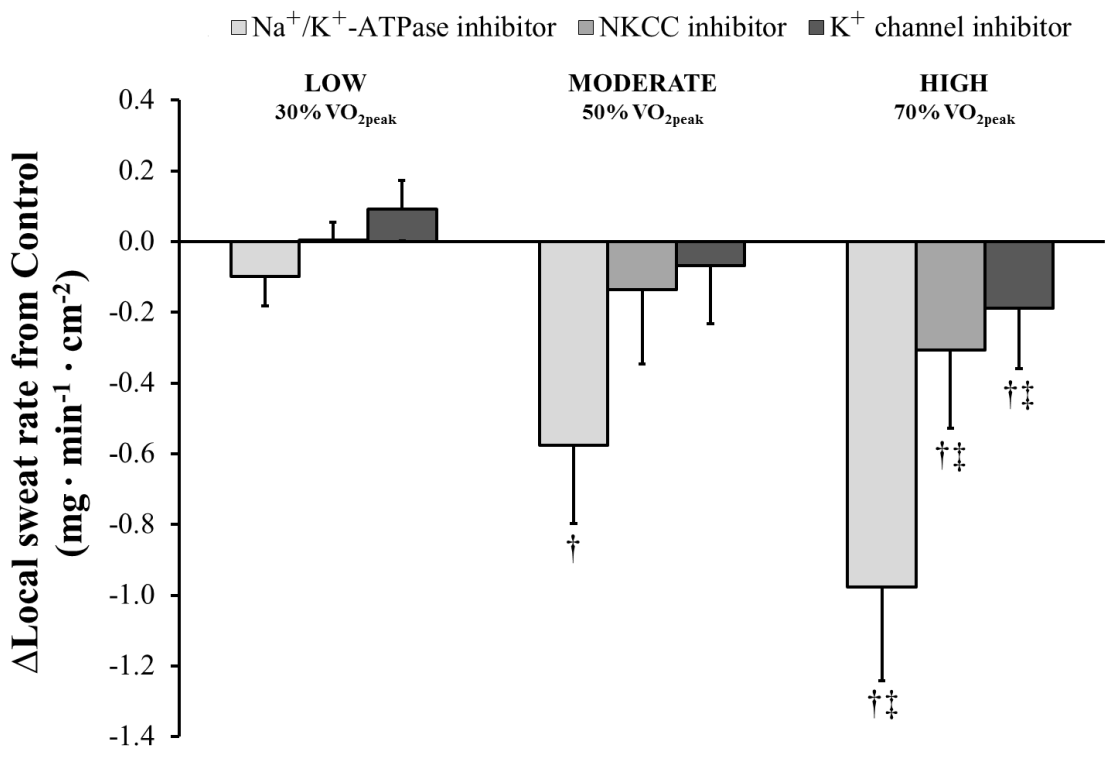
□Na+/K+-ATPase inhibitor
■NKCC inhibitor
■K+ channel inhibitor
LOW
30% VO2peak
MODERATE
50% VO2peak
HIGH
70% VO2peak
ΔLocal sweat rate from Control
(mg · min-1 · cm-2)
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
†
††‡
††‡
††‡

Figure 2

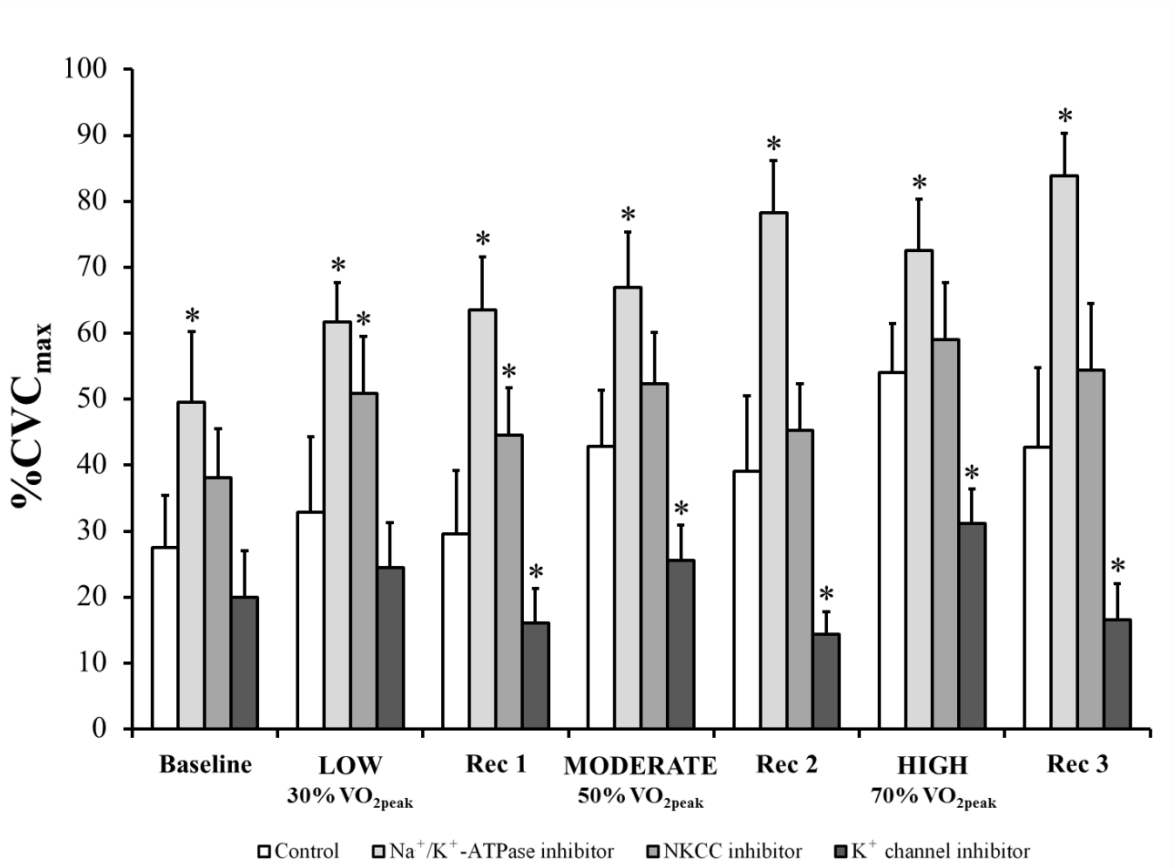
100
90
80
70
60
50
40
30
20
10
0
%CVC$_{max}$
Baseline
LOW
30% VO_{2peak}
Rec 1
MODERATE
50% VO_{2peak}
Rec 2
HIGH
70% VO_{2peak}
Rec 3
Control
Na^+/K^+-ATPase inhibitor
NKCC inhibitor
K^+ channel inhibitor

Figure 3

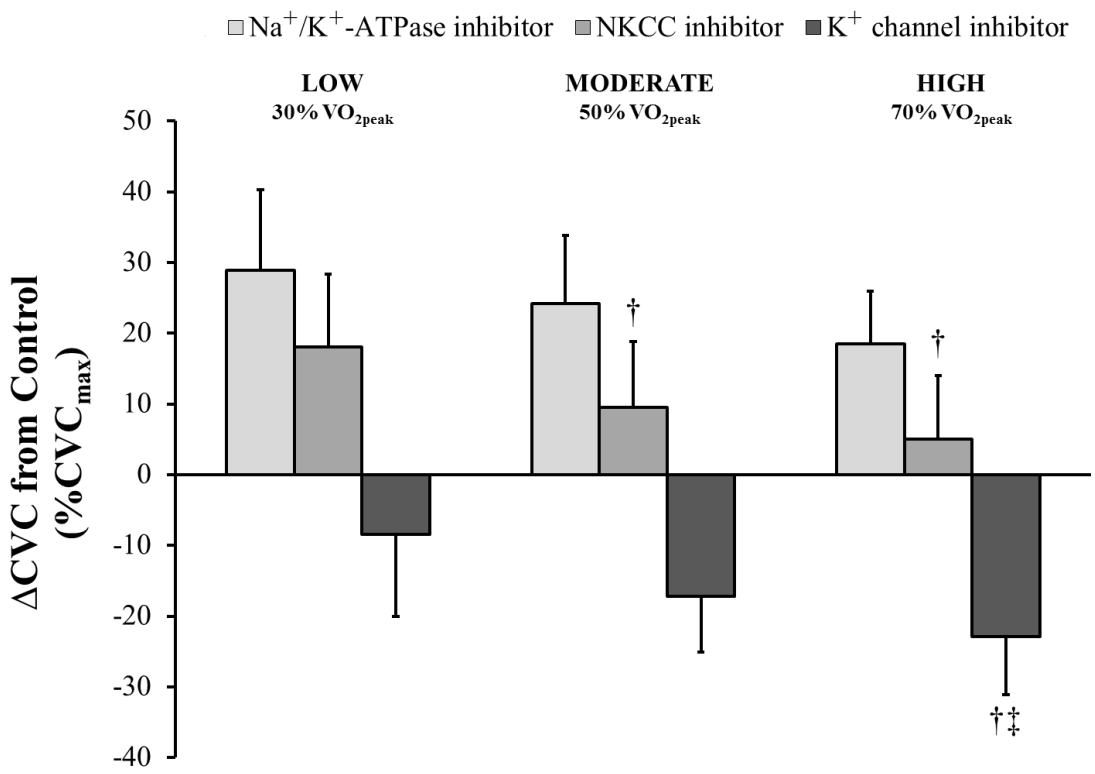
Na+/K+-ATPase inhibitor
NKCC inhibitor
K+ channel inhibitor
LOW
30% VO2peak
MODERATE
50% VO2peak
HIGH
70% VO2peak
ΔCVC from Control
(%CVCmax)
50
40
30
20
10
0
-10
-20
-30
-40
†
†
†‡

Figure 4

PART THREE

GENERAL CONCLUSIONS OF THE THESIS

Despite our advanced understanding of time-dependent changes in sweating and cutaneous vasodilatation during exercise, there remains a lack of information related to the physiological mechanisms that regulate these responses. The Na-K-2Cl cotransporter (NKCC) model was proposed to explain the production of sweat, which highlights the involvement of membrane transport proteins such as the Na^+/K^+-ATPase, NKCC, and K^+ channels (Sato *et al.*, 1989; Saga, 2002). We recently demonstrated the role of the Na^+/K^+-ATPase during exercise in mediating sweating and cutaneous vasodilatation (Louie *et al.*, 2016), however no studies of similar design exist to assess the involvement of NKCC and K^+ channels. Moreover, we recently observed that upstream modulators such as nitric oxide and cyclooxygenase would vary in their degree of regulating the heat loss responses during exercise, based on the level of exercise intensity (Fujii *et al.*, 2014; Meade *et al.*, 2015). Thus the current thesis sought to demonstrate the involvement of the Na^+/K^+-ATPase, NKCC, and K^+ channels in regulating sweating and cutaneous vasodilatation during exercise, and to determine if these responses were dependent on the level of exercise intensity.

Inhibitors of Na^+/K^+-ATPase, NKCC, and K^+ channels (*i.e.* ouabain, bumetanide, and $BaCl_2$, respectively) were perfused *via* the intradermal microdialysis technique to assess how local forearm sweat rate and cutaneous vascular conductance would be affected in young, healthy males during low, moderate, and high intensities (*i.e.* 30, 50, and 70% VO_{2peak}) of intermittent exercise in thermoneutral conditions (25°C). It was demonstrated that each of these membrane transport proteins do indeed play roles in regulating the heat loss responses. However, their apparent contributions varied as a function of exercise intensity and associated factors such as the rate of metabolic heat production. Specifically, Na^+/K^+-ATPase inhibition attenuated the sweating response during moderate and high, but not low intensity exercise, whereas NKCC and

K^+ channel inhibition resulted in attenuations during the high intensity exercise bout only. It was also observed that Na^+/K^+-ATPase inhibition augmented the cutaneous vasodilatory response at all exercise intensities employed, which contrasts our previous findings that revealed an augmented response during baseline and post-exercise recovery only (Louie *et al.*, 2016). The discrepancy in these findings may be ascribed to the higher ambient temperature (35°C) employed in our previous study. Furthermore, NKCC inhibition resulted in greater levels of cutaneous vasodilatation during the low intensity exercise bout only. This response is likely not related to prostaglandin synthesis (Liguori *et al.*, 1999), as suggested by Fujii et al. (2014) who demonstrated no role for cyclooxygenase, an enzyme responsible for the production of prostaglandins. On the other hand, K^+ channel inhibition caused marked attenuations in the cutaneous vasodilatory response during the moderate and high intensity exercise bouts. Future research should be directed toward determining which specific K^+ channel subtype(s) is responsible for this response during exercise, such as calcium-activated (K_{Ca}) (Brunt *et al.*, 2013), ATP-sensitive (K_{ATP}) (Hojs *et al.*, 2009), and/or voltage-gated (K_V) (Ferrer *et al.*, 1999; Gupta *et al.*, 2008) K^+ channels.

In conclusion, the findings from the current thesis further advance our knowledge of the physiological end-organ mechanisms that regulate the sweating and cutaneous vasodilatory responses during exercise. Moreover, careful consideration to exercise intensity is warranted to future studies employing exercise models, given that elevated rates of metabolic heat production and therefore thermoeffector activity may be necessary to reveal the role(s) of specific mechanisms. Lastly, our findings also provide direction for subsequent investigations aimed at determining the underlying physiological mechanisms that can explain chronic disease- and age-related attenuations in whole body heat loss.

PART FOUR

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PART FIVE

APPENDIX

File Number: H12-11-04 **Date (mm/dd/yyyy):** 10/20/2015

Université d'Ottawa University of Ottawa
Bureau d'éthique et d'intégrité de la recherche Office of Research Ethics and Integrity

Ethics Approval Notice

Health Sciences and Science REB

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

First Name	Last Name	Affiliation	Role
Glen	Kenny	Health Sciences / Human Kinetics	Principal Investigator
Pierre	Boulay	Health Sciences / Human Kinetics	Co-investigator
Naoto	Fujii	Health Sciences / Human Kinetics	Co-investigator
Ronald	Sigal	Medicine / Medicine	Co-investigator
Pegah	Akbari	Health Sciences / Human Kinetics	Research Assistant
Sheila	Dervis	Health Sciences / Human Kinetics	Research Assistant
Imane	Foudil-Bey	Health Sciences / Human Kinetics	Research Assistant
Brian	Friesen		Research Assistant
Lyra	Halili	Health Sciences / Human Kinetics	Research Assistant
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Martin	Poirier	Health Sciences / Human Kinetics	Research Assistant
Sarah	Singh	Health Sciences / Human Kinetics	Research Assistant

File Number: H12-11-04

Type of Project: Professor

Title: Body Heat Storage during Work in the Heat in Type 2 Diabetes Mellitus/Heat Stress in Older Adults and Individuals with Type 2 Diabetes

Renewal Date (mm/dd/yyyy)	Expiry Date (mm/dd/yyyy)	Approval Type
02/27/2015	02/26/2016	Ia

(Ia: Approval, Ib: Approval for initial stage only)

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Special Conditions / Comments:
N/A

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://research.uottawa.ca/ethics/submissions-and-reviews.

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://research.uottawa.ca/ethics/submissions-and-reviews.

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.

Jasmine Sarazin
Ethics Coordinator
For Daniel Lagarec, Chair of the Health Sciences and Sciences REB

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