Mechanisms of nicotine-induced cutaneous vasodilation and sweating in young adults: roles for K_{Ca} , K_{ATP} , and K_v channels, nitric oxide, and prostanoids

Journal:	<i>Applied Physiology, Nutrition, and Metabolism</i>
Manuscript ID	apnm-2016-0615.R1
Manuscript Type:	Article
Date Submitted by the Author:	14-Dec-2016
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Keyword:	nAChRs, K ⁺ channels, endothelium, ligand-gated ion channels, heat loss responses

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Mechanisms of nicotine-induced cutaneous vasodilation and sweating in young adults: roles for K_{Ca} , K_{ATP} , and K_V channels, nitric oxide, and prostanoids

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ABSTRACT

We evaluated the influence of K^+ channels [i.e., Ca^{2+} -activated K^+ (K_{Ca}), ATP-sensitive K^+ (K_{ATP}), and voltage-gated K^+ (K_V) channels] and key enzymes [(nitric oxide synthase (NOS) and cyclooxygenase (COX)] on nicotine-induced cutaneous vasodilation and sweating. Using intradermal microdialysis, we evaluated forearm cutaneous vascular conductance (CVC) and sweat rate in two separate protocols. In protocol 1 (n=10), four separate sites were infused with 1) lactated Ringer (Control), 2) 50mM tetraethylammonium (K_{Ca} channel blocker), 3) 5mM glibenclamide (K_{ATP} channel blocker), and 4) 10mM 4-aminopyridine (K_V channel blocker). In protocol 2 (n=10), four sites were infused with 1) lactated Ringer (Control), 2) 10mM N^G -nitro-L-arginine (NOS inhibitor), 3) 10mM ketorolac (COX inhibitor), or 4) a combination of NOS+COX inhibitors. At all sites, nicotine was infused in a dose-dependent manner (1.2, 3.6, 11, 33, and 100mM; each for 25 min). Nicotine-induced increase in CVC was attenuated by the K_{Ca} , K_{ATP} , and K_V channel blockers, whereas nicotine-induced increase in sweat rate was reduced by the K_{Ca} and K_V channel blockers ($P \leq 0.05$). COX inhibitor augmented nicotine-induced increase in CVC ($P \leq 0.05$), which was absent when NOS inhibitor was co-administered ($P > 0.05$). In addition, our secondary experiment (n=7) demonstrated that muscarinic receptor blockade with 58 μ M atropine sulfate salt monohydrate abolished nicotine-induced increases in CVC (1.2-11mM) and sweating (all doses). We show that under a normothermic resting state: 1) K_{Ca} , K_{ATP} , and K_V channels contribute to nicotinic cutaneous vasodilation, 2) inhibition of COX augments nicotinic cutaneous vasodilation likely through NOS-dependent mechanism(s), and 3) K_{Ca} and K_V channels contribute to nicotinic sweating. (250/250 words)

Key words: nAChRs, K^+ channels, endothelium, ligand-gated ion channels, heat loss responses, hyperpolarization

INTRODUCTION

Activation of nicotinic receptors can increase cutaneous blood flow (Izumi and Karita 1992; Fujii et al. 2016b) and sweating (Wada et al. 1952; Fujii et al. 2016b). While the mechanisms underpinning these responses remain unclear, extending our understanding of these responses will have important physiological implications. For example, acetylcholine released from cholinergic nerves activates muscarinic receptors that are thought to be important in mediating heat loss responses of cutaneous vasodilation and sweating (Johnson et al. 2014). However, acetylcholine can also activate nicotinic receptors. Delineating nicotinic regulation of cutaneous vasodilation and sweating helps advance our understanding of how nicotinic receptors influence these heat loss responses and therefore body core temperature regulation. In addition, since cigarette smoking can increase nicotine levels in the body, our result may provide some insights into the underlying mechanisms by which cigarette smoking modulates cutaneous vasodilation and sweating during heat exposure (Moyen et al. 2015) as well as microvascular function (Fujii et al. 2013; Fujii et al. 2014a).

A number of recent reports demonstrate that K^+ channels play an important role in regulating cutaneous blood flow (Brunt et al. 2013; Kutz et al. 2015; Fujii et al. 2016a) and sweat secretion (Shamsuddin et al. 2008; Fujii et al. 2016a). In line with this, early work showed that tetraethylammonium, known as a Ca^{2+} -activated K^+ (K_{Ca}) channel blocker, attenuated nicotine-induced sweating (Wada et al. 1952). However, it remains to be determined if K_{Ca} channels are also important in the regulation of nicotinic cutaneous vasodilation. Further, ATP-sensitive K^+ (K_{ATP}) channels reportedly contribute to nicotinic vasodilation as evidenced in thoracic aortae and pial arterioles of rats (Iida et al. 1998; Zou

et al. 2012). However, the extent to which K_{ATP} channels may play a role in mediating nicotinic cutaneous vasodilation and sweating is unknown. Moreover, nicotinic receptors are known as ligand-gated ion channels (Albuquerque et al. 2009) such that they can induce an influx of cation such as Ca^{2+} , resulting in depolarization. This depolarization may provoke voltage-gated K^+ (K_V) channels, thereby mediating cutaneous vasodilation and sweating.

In addition to K^+ channels, key enzymes such as nitric oxide synthase (NOS) and cyclooxygenase (COX) have been shown to have an important role in modulating cutaneous vascular tone (Holowatz et al. 2005; Kellogg et al. 2005; Lenasi and Strucl 2008; McNamara et al. 2014) and sweating (Fujii et al. 2014c). These enzymes may also be key factors in modulating nicotinic cutaneous vasodilation and sweating. In support of this possibility, if a Ca^{2+} influx mediated by the activation of nicotinic receptors indeed occurs, this can activate NOS (Busse and Mulsch 1990) and phospholipase A2, the latter of which can increase the availability of arachidonic acid, a precursor of COX-derived prostanoids, from phospholipids (Leslie 1997).

In this study, we investigated the roles of K^+ channels (K_{Ca} , K_{ATP} , and K_V channels) as well as the influence of NOS and COX in mediating nicotinic cutaneous vasodilation and sweating in resting normothermic humans in vivo. We evaluated the hypothesis that K_{Ca} , K_{ATP} , and K_V channels as well as NOS and COX contribute to the modulation of nicotinic cutaneous vasodilation and sweating. Muscarinic receptors have been reported to be involved in the nicotinic cutaneous vasodilation and sweating (Low et al. 1992; Schlereth et al. 2005). However, to date, no study has evaluated the extent to which these receptors are involved in the regulation of nicotinic cutaneous vasodilation and

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sweating over different nicotine doses. Therefore, a secondary aim of our study was evaluation of this response.

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MATERIALS AND METHODS

Ethical approval

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

Subjects

Two groups of physically active young males and females participated in two experimental protocols (see below for details). The participants' physical characteristics (means $\pm$ standard deviation) which included body mass, height, and age were as follows: Protocol 1 ($n = 10$, five males and five females), 70.5 ± 13.6 kg, 1.70 ± 0.11 m, and 24 ± 4 years; and Protocol 2 ($n = 10$, five males and five females), 69.4 ± 20.7 kg, 1.67 ± 0.09 m and 25 ± 5 years. Of these participants, three males and three females participated in both protocols. Individuals were excluded from participating in this study if they were taking prescription medications and/or had a history of cystic fibrosis transmembrane conductance regulator mutations, skin disorders, hypertension, heart disease, diabetes, autonomic disorders, cigarette smoking, and/or use of drugs containing nicotine. All females subjects participated in the experimental sessions during their early follicular phases (within 6 days from the beginning of menstruation) or during the placebo phase of contraceptive to minimize the influence of female hormones on cutaneous blood flow and sweating responses (Kuwahara et al. 2005).

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Experimental design

All subjects were instructed to abstain from taking over-the-counter medications (including non-steroidal anti-inflammatory agents and vitamins) for at least 48 h prior to the study, as well as alcohol and caffeine consumption at least 12 h before the study. They were also asked to refrain from performing strenuous exercise the day prior to the study. On the day of the experiment, subjects did not consume any food 2 h prior to and throughout the study. Upon arrival, subjects were then placed in a semi-recumbent position in a thermoneutral room (~23 °C) during which time four intradermal microdialysis fibres (30 kDa cutoff, 10 mm membrane) (MD2000, Bioanalytical Systems, West Lafayette, IN, USA) were implanted on the dorsal side of the left forearm within the dermal layer of the skin. Using an aseptic technique, a 25-gauge needle was first inserted into the unanesthetized skin, and then a microdialysis fibre was threaded through the lumen of the needle. The needle was subsequently withdrawn, leaving only the fibre in place. The entry and exit points were separated by ~2.5 cm. Microdialysis fibres were secured with surgical tape and each fibre was separated by at least 4.0 cm. The inter-site distance of 4.0 cm has been shown to be sufficient to eliminate any between-site contamination of the administered agents (Meade et al. 2016).

Protocol 1

Protocol 1 was employed to investigate the role of K_{Ca} , K_{ATP} , and K_V channels in nicotinic cutaneous vasodilation and sweating. Approximately 10 min after the completion of the fibre insertion, four microdialysis fibres were continuously perfused in a counter balanced manner with 1) lactated Ringer (Control, Baxter, Deerfield, IL, USA), 2) 50 mM

tetraethylammonium (Sigma-Aldrich, St. Louis, MO, USA), a non-selective K_{Ca} channel blocker, 3) 5 mM glybenclamide (Sigma-Aldrich), a specific K_{ATP} channel blocker, or 4) 10 mM 4-aminopyridine (Sigma-Aldrich), a non-specific K_V channel blocker. The concentrations of tetraethylammonium (Lorenzo and Minson 2007; Brunt et al. 2013; Kutz et al. 2015; Fujii et al. 2016a), glybenclamide (Kutz et al. 2015; Fujii et al. 2016a), and 4-aminopyridine (Fujii et al. 2016a) were determined based on previous studies in which an intradermal microdialysis fibre was implanted in human skin. While 10 mM 4-aminopyridine causes a relatively higher baseline cutaneous blood flow, this response subsided with a prolonged (80 ± 3 min with a range of 76-93 min) resting period in this study. Also, this time period (80 ± 3 min) has been shown to be sufficient to ensure that any potential influence of tissue trauma with the insertion of the fibre has subsided (Anderson et al. 1994). In line with this, we observed a stable low cutaneous blood flow for at least 5 min by the end of resolution period in all subjects. Since glybenclamide is water insoluble, it was necessary to utilize 5% of dimethyl sulfoxide (Sigma-Aldrich) dissolved in lactated Ringer solution with a pH of 11 in order to effectively dissolve 5 mM glybenclamide. To increase pH to 11, we added sodium hydroxide (Sigma-Aldrich) to the solution. To account for the potential influence of 5% dimethyl sulfoxide as well as the higher pH (11) on nicotinic cutaneous vasodilation and sweating responses, all four solutions (Control, tetraethylammonium, glybenclamide, and 4-aminopyridine) were dissolved in lactated Ringer containing 5% dimethyl sulfoxide with pH adjusted to 11. Each drug was continuously perfused at a rate of $4.0 \mu\text{l min}^{-1}$ using a microinfusion pump (Model 4004, CMA Microdialysis, Solna, Sweden). Perfusion continued for the entire experimental

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protocol until the measurement of maximal cutaneous vasodilation was performed (see below).

Following the 80 ± 3 min period of the administration of each blocker, 10 min of baseline data was collected (Baseline). Subsequently, each microdialysis fibre was perfused with nicotine (nicotine tartrate salt, MP Biomedicals, Santa Ana, CA) in an ascending manner with five different concentrations of 1.2, 3.6, 11, 33, and 100 mM at a rate of $4.0 \mu\text{l min}^{-1}$ (each for 25 min) in combination with the site-specific pharmacological agents with 5% dimethyl sulfoxide and a pH of 11. We found that this combination had no effect on nicotinic cutaneous vasodilation and sweating (see Results section). The range of nicotine concentrations used in the present study was determined based on our pilot work. We limited the concentration of nicotine employed to ≤ 100 mM to avoid potential side effects of nicotine (e.g., nausea, tachycardia, sudden and marked changes in blood pressure, etc.). As presented in the Results section, no such side effects were observed over the course of the treatment protocol.

After completing the last infusion of nicotine (100 mM), 50 mM sodium nitroprusside (Sigma-Aldrich) administration was initiated at each microdialysis site at a rate of $6.0 \mu\text{l min}^{-1}$ to achieve maximal cutaneous vasodilation. The sodium nitroprusside administration lasted for approximately 20-30 min until a stable plateau for a minimum of 2 min was achieved.

Protocol 2

Protocol 2 was designed to determine whether NOS and COX are involved in nicotinic cutaneous vasodilation and sweating. Four microdialysis fibres were assigned in a counter

balanced manner to receive 1) lactated Ringer (Control, Baxter), 2) 10 mM N^G -nitro-L-arginine (Sigma-Aldrich), a non-selective NOS inhibitor, 3) 10 mM ketorolac, a non-selective COX inhibitor (Sigma-Aldrich), or 4) a combination of the NOS and COX inhibitors. These concentrations were determined from previous studies in which intradermal microdialysis was employed in human skin for N^G -nitro-L-arginine (Meadow et al. 2007) and ketorolac (Holowatz et al. 2005; Kellogg et al. 2005). Each drug was continuously perfused at a rate of $4.0 \mu\text{l min}^{-1}$ for at least 75 min to ensure the establishment of each blockade. Perfusion continued for the entire experimental protocol until the measurement of maximal cutaneous vasodilation was conducted (see below).

A 10-min baseline period (Baseline) followed the ≥ 75 min period wherein the inhibitors were administrated. Then nicotine administration was performed in the same manner of protocol 1. Nicotine solutions were made in combination with the site-specific pharmacological agents with a pH of 6.5. After the nicotine administration protocol, 50 mM of sodium nitroprusside (Sigma-Aldrich) administration was initiated to obtain maximal cutaneous vasodilation as with the protocol 1.

Secondary experimental trial

It is thought that nicotinic sweating occurs via acetylcholine release elicited from a cholinergic sudomotor axon reflex (Low et al. 1992; Schlereth et al. 2005). Also, nicotinic cutaneous vasodilation is thought to be largely due to axon reflex (Izumi and Karita 1992). Based on these data, muscarinic acetylcholine receptors may appear to play an important role in modulating nicotinic cutaneous vasodilation and sweating. However, to the best of our knowledge, there is no study evaluating the extent to which muscarinic receptors are

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involved in the nicotinic cutaneous vasodilation and sweating with various nicotine doses. To evaluate this, we performed a secondary experimental trial wherein seven young physically active subjects (4 males and 3 females) were recruited. Their physical characteristics were as follows (mean $\pm$ standard deviation): body mass: 76.8 ± 18.0 kg, height: 1.67 ± 0.03 m, and age: 24 ± 4 years. The protocol procedures were same as protocol 1 and 2 with the exception that two microdialysis fibres were continuously perfused with either 1) lactated Ringers (Control), or 2) $58 \mu\text{M}$ atropine sulfate salt monohydrate (Sigma-Aldrich) to non-selectively block muscarinic receptors. The concentration of atropine was based on previous studies in which intradermal microdialysis was used (Shastry et al. 2000; Meade et al. 2016).

Measurements

Cutaneous red blood cell flux (expressed in perfusion units) was locally measured at a sampling rate of 32 Hz using the software platform by Perimed (PSW, version 2.5.5), and used as an index of cutaneous blood flow with laser-Doppler flowmetry (PeriFlux System 5000, Perimed, Stockholm, Sweden). Integrated laser-Doppler flowmetry probes with a 7-laser array (Model 413, Perimed) were housed in the center of each sweat capsule over each microdialysis fibre for simultaneous measurement of both local forearm sweat rate and cutaneous red blood cell flux. Manual auscultation was performed using a mercury column sphygmomanometer (Baumanometer Standby Model, WA Baum Co, Copiague, NY, USA) to obtain blood pressures every 10-15 min. Mean arterial pressure was calculated from diastolic arterial pressure plus one-third the difference between systolic and diastolic pressures (i.e., pulse pressure). Cutaneous vascular conductance (CVC) was evaluated as

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cutaneous red blood cell flux divided by mean arterial pressure. Heart rate was estimated from a pulsatile waveform of cutaneous red blood cell flux.

A sweat capsule covering an area of 1.1 cm^2 specifically designed for the use with an intradermal microdialysis probe (Meade et al. 2016) was placed directly over the center of each microdialysis membrane. The sweat capsules were secured to the skin with adhesive rings and topical skin glue (Collodion HV, Mavidon Medical products, Lake Worth, FL, USA). Dry compressed air in gas tanks located in the thermoneutral room ($\sim 23^\circ\text{C}$) was supplied to each capsule at a rate of 0.20 l min^{-1} , while water content of the effluent air from the sweat capsule was measured with high-precision dew point mirrors (Model 473, RH systems, Albuquerque, NM, USA). Vinyl tubes were used for connections between the gas tank and the sweat capsule, and between the sweat capsule and the dew point mirror. Local forearm sweat rate, which was estimated from the difference in water content between influent and effluent air multiplied by the flow rate, was recorded at a sample rate of 0.2 Hz using a self-developed software platform. Sweat rate was normalized for the skin surface area under the capsule ($\text{mg min}^{-1} \text{ cm}^{-2}$).

Data analysis

CVC data were presented as %max. Absolute maximal CVC, which is used to calculate %max, was obtained during sodium nitroprusside administration. All CVC values used for data analysis were obtained by averaging values over the last 5 min of each stage (i.e., Baseline, 1.2, 3.6, 11, 33, 100 mM of nicotine). Peak sweat rate (5-min average) achieved during each nicotine administration was used for data analysis as described in Figure 1B. We elected to use peak sweat rate values given that nicotinic sweating is

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relatively transient and fluctuated (Figure 1B). When we could not detect a visible increase in sweat rate in response to nicotine (such as that which occurred at the nicotinic receptor blocker site), the same period of time used for peak sweat rate at the Control site was chosen. We calculated change in (Δ) CVC and sweat rate from Baseline in response to administration of nicotine. Due to technical difficulties, we were not able to obtain CVC data from one subject in protocol 2 ($n = 9$).

Statistical analysis

All statistical tests were performed using the software package SPSS 23 for Windows (IBM, Armonk, NY, USA). Δ CVC (%max) and Δ sweat rate ($\text{mg min}^{-1} \text{cm}^{-2}$) from Baseline were analyzed using a two-way repeated-measures analysis of variance; with repeated factors of treatment site and stage. One-way repeated-measures analysis of variance with the repeated factors of treatment site was employed to analyze CVC (both perfusion units mmHg^{-1} and %max) and sweat rate ($\text{mg min}^{-1} \text{cm}^{-2}$) obtained at Baseline as well as absolute maximal CVC (perfusion units mmHg^{-1}). When sphericity was not assumed as checked by Mauchly's test, Greenhouse-Geisser correction was employed to adjust degrees of freedom for ANOVA. When a significant interaction or main effect was detected, *post hoc* multiple comparisons were carried out using the Student's paired two-tailed *t*-tests. P values for the multiple comparisons were adjusted using modified Bonferroni tests [i.e., Holm Bonferroni procedure or Hochberg's procedure (Hochberg 1988)]. Furthermore, Student's unpaired two-tailed *t*-tests were employed to compare CVC and sweat rate data between protocol 1 and 2. We employed unpaired *t*-tests for this comparison as the subject participated in protocol 1 and 2 were not same (six out of ten subjects participated in both protocols). We

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also employed Student's paired one-tailed *t*-tests for between-site comparison in the secondary experimental trial. The level of significance for all analyses was set at $P \leq 0.05$. All values are reported with a mean $\pm$ 95% confidence interval. The confidence intervals at 95 % were calculated as $1.96 \times$ standard error of the mean.

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RESULTS

Cutaneous vascular response

Main effects of treatment site and stage, and their interaction for ΔCVC were measured in protocol 1 and 2 (all $P \leq 0.05$). Baseline (all $P > 0.11$) and absolute maximal CVC ($P > 0.56$ for a main effect of treatment site) did not differ between sites for both protocols (Table 1). At the Control site, administration of nicotine increased ΔCVC above Baseline levels at all concentrations tested for both protocol 1 and 2 (all $P \leq 0.05$, Figure 1, 2, and 3). The ΔCVC during nicotine administration was attenuated by all K^+ channel blockers relative to the Control (all $P \leq 0.05$, Figure 2). Although NOS inhibition alone did not influence ΔCVC (all $P > 0.42$), COX inhibition alone augmented ΔCVC in response to nicotine co-infusion in comparison to the Control site (all $P \leq 0.05$, Figure 3). However, this increase was not observed when NOS inhibition was simultaneously applied (all $P > 0.15$, Figure 3). Muscarinic receptor blockade with atropine attenuated ΔCVC at 1.2-11 but not 33-100 mM nicotine (Figure 4).

Sweating

When analyzing sweating, there were main effects of treatment site and stage and their interaction in protocol 1, whereas only a main effect of stage was detected in protocol 2 (all $P \leq 0.05$). Baseline sweat rate did not differ between sites in both protocols (all $P > 0.08$, Table 1). Administration of nicotine increased Δsweat rate from Baseline levels at all concentrations employed for both protocol 1 and 2 (all $P \leq 0.05$, Figures 1, 5, and 6). Infusion of K_{Ca} and K_{V} channel blockers attenuated nicotine-induced increase in Δsweat rate relative to the Control site (both $P \leq 0.05$, Figure 5). However, administration of the

K_{ATP} channel blocker (Figure 5) as well as separate and combined inhibition of NOS and COX (Figure 6) had no effect on Δ sweat rate as compared to the Control site (all $P > 0.06$). Atropine-induced muscarinic receptor blockade attenuated Δ sweat rate at all nicotine doses tested (Figure 7).

Heart rate and blood pressure

Heart rate and mean arterial pressure did not change throughout both protocol 1 and 2 (all $P > 0.06$ for a main effect of stage, Table 2). The lack of a change in heart rate and mean arterial pressure indicates that there was no systematic nicotinic influence.

Influence of pH and dimethyl sulfoxide on nicotinic responses

All CVC and sweat rate data evaluated at the Control site (presented in Table 1 and Figures 2, 3, 5, and 6) did not differ between protocol 1 and 2 (all $P > 0.16$). Specifically, the use of a 5% dimethyl sulfoxide maintained at higher pH of 11 employed in protocol 1 did not influence nicotinic cutaneous vasodilation and sweating response relative to that measured using a dimethyl sulfoxide-free solution with a pH of 6.5 employed in protocol 2.

DISCUSSION

We show for the first time that the infusion of K_{Ca} , K_{ATP} , and K_V channel blockers attenuated the nicotine-induced cutaneous vasodilation, whereas inhibition of COX augmented this cutaneous vasodilation; a response likely due to NOS-dependent mechanism(s). We also showed that nicotine-induced sweating was attenuated by K_{Ca} and K_V channel blockers. Lastly, the present study suggests that muscarinic receptors largely mediate nicotinic cutaneous vasodilation and sweating. However, these receptors do not have a functional role in modulating nicotinic cutaneous vasodilation with high doses of nicotine.

Cutaneous vasodilation

As our study findings demonstrate, K_{Ca} , K_{ATP} , and K_V channels all contribute to the nicotinic-induced cutaneous vasodilation (Figure 2). The present study also shows that muscarinic receptors exclusively mediate nicotinic cutaneous vasodilation in response to the administration of 1.2-11 mM nicotine (Figure 4). Therefore, it appears that K^+ channel-component of nicotinic cutaneous vasodilation observed at 1.2-11 mM nicotine is associated with muscarinic receptors. In line with this, we recently showed that the same K^+ channels all contribute to cutaneous vasodilation induced by muscarinic receptor agonist, methacholine (Fujii et al. 2016a).

Our results also demonstrated that nicotinic cutaneous vasodilation induced by high doses of nicotine (33-100 mM) occurs independently of muscarinic receptors (Figure 4). Hence this response may be due to the activation of nicotinic receptors expressed on the endothelial cells of cutaneous blood vessels (Hagforsen et al. 2002) and/or possibly

vascular smooth muscle cells. It is well documented that nicotinic receptors work as ligand-gated ion channels (Albuquerque et al. 2009). It is plausible therefore that the activation of nicotinic receptors may allow cations such as Ca^{2+} and Na^{+} to move into the intracellular space via nicotinic receptors in human skin. An increase in intracellular Ca^{2+} can activate K_{Ca} channels located in the endothelial and smooth muscle cells, thereby causing a subsequent hyperpolarization associated with K^{+} efflux. This can subsequently induce cutaneous vasodilation. With respect to K_v channels, an influx of cations would induce a depolarization in the vascular smooth muscle cells of the skin vessels. This could in turn activate K_v channels, resulting in hyperpolarization mediated cutaneous vasodilation. Finally, nicotine may activate K_{ATP} channels through increasing superoxide production as demonstrated under in vitro conditions (Hanna et al. 2005).

While we observed that COX inhibition alone augmented nicotinic cutaneous vasodilation, this response did not occur with the simultaneous inhibition of NOS (Figure 3). Our findings indicated that nicotine appears to activate COX, which in turn reduces NOS-derived increases in NO thereby limiting nicotinic-mediated cutaneous vasodilation. It is likely that the nicotine infusion activated nicotinic receptors located on endothelial cells of the cutaneous blood vessels (Hagforsen et al. 2002), which subsequently activated COX also located in the endothelial cells (Muller-Decker et al. 1999). The activation of COX by nicotine may increase superoxide production from COX itself (Egan et al. 1976), or NADPH oxidase (Zhang et al. 2011). An increase in superoxide, if any, could reduce NO bioavailability as superoxide is known to bind with NO (Pryor and Squadrito 1995).

Sweating

Consistent with a previous human study in vivo (Wada et al. 1952), we confirmed that tetraethylammonium, a K_{Ca} channel blocker, almost entirely abolished sweating in response to nicotine administration (Figure 5). Importantly, we demonstrated for the first time that K_V but not K_{ATP} channels contribute to nicotine-induced sweating as observed at the lower doses of nicotine administration (Figure 5). Our results also demonstrated that nicotinic sweating is exclusively due to activation of muscarinic receptors, as nicotinic sweating was not clearly observed at atropine-treated site (Figure 7). This is in keeping with the notion that nicotine-induced sweating is due to acetylcholine release via a cholinergic sudomotor axon reflex (Low et al. 1992; Schlereth et al. 2005). This exclusive role of muscarinic receptors in nicotinic sweating is in contrast to nicotinic cutaneous vasodilation wherein there is a muscarinic-insensitive component of nicotinic cutaneous vasodilation (see above). This difference may infer that nicotinic receptors located on sweat glands (Hagforsen et al. 2002) do not functionally modulate sweating whereas those on skin vessels (Hagforsen et al. 2002) modulate cutaneous vasodilation.

Given the importance of muscarinic receptors in nicotinic sweating, the K_V channel contribution to nicotinic sweating is likely a result of muscarinic receptor-dependent mechanisms. Along these lines, we recently reported that K_V channels are involved in the muscarinic induced sweating (Fujii et al. 2016a). Alternatively, K_V channels may be involved in augmenting acetylcholine release from cholinergic nerves associated with sudomotor axon reflex (pre-muscarinic receptor level). Specifically, depolarization associated with the activation of nicotinic receptors (White et al. 2014) propagates to cholinergic nerve terminals, which in turn induces the activation of K_V channels and thus hyperpolarization associated with K^+ efflux. This hyperpolarization may increase Ca^{2+}

influx in an electrochemical gradient-dependent manner; a response comparable to that observed during hyperpolarization associated with the stimulation of K_{Ca} channels (Pattillo et al. 2001). Given that Ca^{2+} influx in nerve terminals is critical for the release of neurotransmitters (Stanley 1993), an increase in Ca^{2+} influx would therefore facilitate acetylcholine release at the sudomotor nerve endings thereby inducing the production of sweat.

Another important new finding of our study was our observation that both NOS and COX are not involved in nicotinic sweating (Figure 6). Noteworthy, we previously observed NOS- and COX-dependent sweating during exercise in the heat (Fujii et al. 2014c) wherein acetylcholine released from cholinergic nerves mediates sweating. Since acetylcholine can activate both muscarinic and nicotinic receptors, the observed NOS- and COX-dependent sweating during exercise in the heat (Fujii et al. 2014c) may be the result of the activation of muscarinic and/or nicotinic receptors. However, our results do not support the involvement of nicotinic receptors in NOS- and COX-dependent sweating. Since previous studies reported that muscarinic receptors are essential in mediating the sweating response during heat stress (Kellogg et al. 1995; Shibasaki et al. 2002), one might conclude that the observed NOS- and COX-dependent sweating during exercise in the heat is associated with muscarinic receptors. However, we previously reported that muscarinic receptor activation by methacholine did not exhibit NOS- and COX-dependent sweating under a normothermic state (Fujii et al. 2014b). It may be that an exercise-mediated increase in body temperature (skin and/or core body temperature) may be required to induce the NOS- and COX-dependent sweating during exercise in the heat.

It is important to highlight that nicotinic sweating is not dose-dependent such that Δ sweat rate was similar across the different doses tested (Figure 5, 6). We do not know why this dose-independent nicotinic sweating was measured. It may be that nicotinic sweating shows tachyphylaxis, attenuating subsequent nicotinic sweating. Alternatively, no dose-dependent nicotinic sweating potentially reflects some modulation of nicotinic receptors located on sweat glands (Hagforsen et al. 2002) in sweating. Clearly, future studies are warranted to delineate the mechanisms underpinning this dose-independent nicotinic sweating.

Perspectives and significance

While this study was not designed to evaluate role of nicotinic receptors in the activation of heat loss responses of cutaneous vasodilation and sweating per se, our findings highlight the possible influence that these receptors may play a role in the regulation of body core temperature during heat stress. While muscarinic receptor activation by acetylcholine is thought to be a major mechanism for cholinergic cutaneous vasodilation and sweating during heat stress (Kellogg et al. 1995; Shibasaki et al. 2002), it is well accepted that acetylcholine can also activate nicotinic receptors. Hence, nicotinic receptors may also contribute to the regulation of the heat loss responses.

To the best of our knowledge, we are the first to demonstrate the extent to which the activation of nicotinic receptors by nicotine induces changes in the level of cutaneous vasodilation and sweating. Specifically, we show that nicotinic receptor activation can increase CVC and sweating by as much as 30 %max and $0.25 \text{ mg min}^{-1} \text{ cm}^{-2}$, respectively; levels which correspond to those measured for forearm cutaneous vasodilation (Brunt et al.

2013) and sweating (Machado-Moreira et al. 2012) during a moderate heat stress condition (resulting in a ~ 0.5 °C increase in core body temperature) induced by whole-body heating. Therefore, our results lend support to the notion that nicotinic receptors are capable of modulating cutaneous blood flow and sweating during heat stress. Given our observation that nicotine-induced cutaneous vasodilation and sweating is a result of K_{Ca} , K_{ATP} , and/or K_V channel activation, nicotinic receptors may therefore play a role in K^+ channel-dependent cutaneous vasodilation and sweating during heat stress. Further research is warranted to elucidate this mechanism during an exercise-induced heat stress.

Cutaneous vasodilation induced by pharmacological manipulation (e.g., acetylcholine and methacholine) and or local heating is commonly employed to study microvascular function (Minson 2010; Hellmann et al. 2015). Additionally, the administration of cholinergic agents to assess the sweating response can be used to detect neurophysiological disorder such as peripheral neuropathy (Low 2004; Schlereth et al. 2005; Illigens and Gibbons 2009). In the context of our study findings, the evaluation of nicotinic cutaneous vasodilation and sweating may help in identifying changes in microvascular and peripheral nervous functions associated with K^+ channels.

Limitations

For protocol 1, we employed a solution containing 5% dimethyl sulfoxide with a pH of 11. This adjustment was necessary to dissolve the water-insoluble compound glybenclamide. We believe this elevated pH with 5% dimethyl sulfoxide had a minimal influence, if any, on nicotinic cutaneous vasodilation and sweating. This is supported by our observation that CVC and sweat rate responses evaluated at the Control site in protocol 1 did not differ in

comparison to those obtained in protocol 2 wherein we employed a solution without dimethyl sulfoxide and a pH of 6.5. Furthermore, it is important to note that all four skin sites in protocol 1 received nicotine solutions similarly dissolved in lactated Ringer containing 5% dimethyl sulfoxide with a pH of 11. Therefore, any between-site difference in CVC and sweat rate observed in protocol 1 should have been a result of specific K^+ channel blockade.

We employed 4-aminopyridine to block K_v channels. However, 4-aminopyridine has been shown to augment acetylcholine release from the nerve terminals (Vizi et al. 1977). Since nicotine-induced sweating is mediated through sudomotor axon reflex induced increase in acetylcholine release (Schlereth et al. 2005), nicotinic sweating (and possibly nicotinic cutaneous vasodilation) may have been augmented with 4-aminopyridine administration in this study. That is, we may have underestimated the role of K_v channels in the regulation of nicotinic sweating, as any decreasing effect of K_v channel blockade on sweating could be dampened by augmented sweating associated with 4-aminopyridine induced increase in acetylcholine release. Nevertheless, we still observed a lower sweat rate with 4-aminopyridine, thus our conclusion pertaining to K_v channels is still valid.

The magnitude of increases in sweat rate in reaction to nicotinic receptor activation in the secondary study (Figure 7) were small relative to those obtained in protocol 1 and 2 (Figure 5 and 6). This could be due to the fact that nicotinic sweating has a large individual variation (Fujii et al. 2016b), and our 7 subjects in the secondary study exhibited relatively smaller responses. We do not know what causes this large inter-individual variation, and a future study is warranted to elucidate this response. In the present study, our primary focus is however the between-site comparison and thus we

believe the smaller magnitude of nicotinic sweating in the secondary study is not a major issue.

CONCLUSION

In normothermic resting humans, we show that 1) K_{Ca} , K_{ATP} , and K_V channels contribute to nicotinic cutaneous vasodilation, 2) inhibition of COX augments nicotinic cutaneous vasodilation possibly by activating NOS, 3) K_{Ca} and K_V channels contribute to nicotinic sweating, and 4) muscarinic receptors are mainly responsible for nicotinic cutaneous vasodilation and sweating, with the exception that nicotinic cutaneous vasodilation occurring with high doses of nicotine.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

ACKNOWLEDGEMENTS

This study was supported by the Natural Sciences and Engineering Research Council of Canada (Discover grant, RGPIN-06313-2014 and Discovery Grants Program - Accelerator Supplement, RGPAS-462252-2014; funds held by Dr. Glen P. Kenny). G. P. Kenny was supported by a University of Ottawa Research Chair Award. N. Fujii was supported by the Human and Environmental Physiology Research Unit. J. C. Louie was supported by a Queen Elizabeth II Graduate Scholarship in Science and Technology. We thank My-An Tran, Sarah Yan Zhang, and Jessica Elizabeth Szlapinski for their assistance with the study.

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Table 1. Cutaneous vascular conductance and sweat rate measured during Baseline (before starting nicotine administration) and absolute maximal cutaneous vascular conductance obtained during sodium nitroprusside administration.

	Cutaneous vascular conductance			Sweat rate
	Baseline		Absolute maximum	Baseline
	(perfusion units mmHg ⁻¹)	(%max)	(perfusion units mmHg ⁻¹)	(mg min ⁻¹ cm ⁻²)
Protocol 1				
Control	0.20 ± 0.05	13 ± 3	1.68 ± 0.50	0.10 ± 0.02
K _{Ca} channel blockade	0.21 ± 0.05	14 ± 4	1.67 ± 0.35	0.10 ± 0.02
K _{ATP} channel blockade	0.16 ± 0.05	12 ± 3	1.36 ± 0.20	0.11 ± 0.02
K _V channel blockade	0.22 ± 0.08	16 ± 3	1.44 ± 0.44	0.13 ± 0.02
Protocol 2				
Control	0.17 ± 0.09	13 ± 5	1.28 ± 0.23	0.11 ± 0.01
NOS inhibition	0.17 ± 0.10	10 ± 2	1.62 ± 0.51	0.11 ± 0.01
COX inhibition	0.22 ± 0.10	16 ± 4	1.33 ± 0.52	0.12 ± 0.01
NOS + COX inhibition	0.19 ± 0.12	13 ± 5	1.33 ± 0.36	0.13 ± 0.03

All values are expressed as means ± 95 % confidence interval (n = 10 for both protocols except n = 9 for cutaneous vascular conductance data in protocol 2). K_{Ca}, Ca²⁺-activated K⁺; K_{ATP}, ATP-sensitive K⁺; K_V, voltage-gated K⁺; NOS, nitric oxide synthase, COX, cyclooxygenase. There was no between-site difference in any variable (all P > 0.08).

Table 2. Heart rate and mean arterial pressure measured during Baseline (before starting nicotine administration) and the administration of each nicotine dose

		Nicotine (mM)				
	Baseline	1.2	3.6	11	33	100
Protocol 1						
Heart rate, beats min ⁻¹	59 ± 5	58 ± 6	58 ± 5	61 ± 6	60 ± 6	58 ± 7
Mean arterial pressure, mmHg	88 ± 3	88 ± 2	88 ± 3	88 ± 3	86 ± 3	89 ± 3
Protocol 2						
Heart rate, beats min ⁻¹	68 ± 5	66 ± 5	66 ± 5	67 ± 5	67 ± 6	70 ± 5
Mean arterial pressure, mmHg	91 ± 5	91 ± 6	92 ± 6	92 ± 5	93 ± 5	94 ± 5

All values are expressed as means ± 95 % confidence interval (n = 10 for both protocols). Both heart rate and mean arterial pressure remained unchanged throughout both experiments (all P > 0.06 for a main effect of stage).

FIGURE CAPTIONS

Figure 1: An example of cutaneous blood flow (panel A) and sweat rate (panel B) during baseline (BL) and intradermal nicotine administration. For data analysis, cutaneous blood flow was averaged over the last 5 min period, whereas the highest sweat rate averaged over 5 min was evaluated (denoted as grey squares in panel B) at each nicotine dose.

Figure 2: Change (Δ) in cutaneous vascular conductance from Baseline at each dose of nicotine (mean $\pm$ 95 % confidence interval, $n = 10$). The responses were evaluated at the four sites treated with either: 1) lactated Ringer (Control, circles), 2) Ca^{2+} -activated K^+ (K_{Ca}) channel blockade (squares), 3) ATP-sensitive K^+ (K_{ATP}) channel blockade (triangles), or 4) voltage-gated K^+ (K_{V}) channel blockade (diamonds). *, Control vs. K_{Ca} channel blockade ($P \leq 0.05$). †, Control vs. K_{ATP} channel blockade ($P \leq 0.05$); ‡, Control vs. K_{V} channel blockade ($P \leq 0.05$).

Figure 3: Change (Δ) in cutaneous vascular conductance from Baseline at each dose of nicotine (mean $\pm$ 95 % confidence interval, $n = 9$). The responses were evaluated at the four sites treated with either: 1) lactated Ringer (Control, circles), 2) nitric oxide synthase (NOS) inhibition (squares), 3) cyclooxygenase (COX) inhibition (triangles), or 4) NOS + COX inhibition (diamonds). †, Control vs. COX inhibition ($P \leq 0.05$).

Figure 4: Change (Δ) in cutaneous vascular conductance from Baseline at each dose of nicotine (mean $\pm$ 95 % confidence interval, $n = 7$). The responses were evaluated at the two sites treated with either: 1) lactated Ringer (Control, circles), or 2) muscarinic receptor blockade (squares). *, Control vs. muscarinic acetylcholine receptor blockade ($P \leq 0.05$).

Figure 5: Change (Δ) in sweat rate from Baseline at each dose of nicotine (mean $\pm$ 95 % confidence interval, $n = 10$). The responses were evaluated at the four sites treated with either: 1) lactated Ringer (Control, circles), 2) Ca^{2+} -activated K^+ (K_{Ca}) channel blockade (squares), 3) ATP-sensitive K^+ (K_{ATP}) channel blockade (triangles), or 4) voltage-gated K^+ (K_{V}) channel blockade (diamonds). *, Control vs. K_{Ca} channel blockade ($P \leq 0.05$); ‡, Control vs. K_{V} channel blocker ($P \leq 0.05$).

Figure 6: Change (Δ) in sweat rate from Baseline at each dose of nicotine (mean $\pm$ 95 % confidence interval, $n = 10$). The responses were evaluated at the four sites treated with either: 1) lactated Ringer (Control, circles), 2) nitric oxide synthase (NOS) inhibition (squares), 3) cyclooxygenase (COX) inhibition (triangles), or 4) NOS + COX inhibition (diamonds). No between-site difference in Δ sweat rate was measured ($P > 0.81$ for a main effect of treatment site and an interaction between treatment site and stage).

Figure 7: Change (Δ) in sweat rate from Baseline at each dose of nicotine (mean $\pm$ 95 % confidence interval, $n = 7$). The responses were evaluated at the two sites treated with either: 1) lactated Ringer (Control, circles), or 2) muscarinic receptor blockade (squares). *, Control vs. muscarinic acetylcholine receptor blockade ($P \leq 0.05$).

Figure 3

Figure 7