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Superoxide and NADPH oxidase do not modulate skin blood flow in older exercising adults with and without type 2 diabetes

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Abstract

Objective: High aerobic fitness may prevent age-related decrements in cutaneous vasodilation while type 2 diabetes may exacerbate this decline. The mechanisms underlying these responses remain unclear, but may be due to an excess of reactive oxygen species. We hypothesized that superoxide scavenging or NADPH oxidase inhibition would improve cutaneous vasodilation in older adults exercising in the heat, particularly in healthy low-fit individuals and those with type 2 diabetes. **Methods:** Twenty seven older adults were evenly separated into three groups (healthy low-fit: VO_{2peak} = 24.4±2.4 ml·kg^{-1}·min^{-1}, 61±8 years; healthy high-fit: 42.5±9.7 ml·kg^{-1}·min^{-1}, 56±6 years; type 2 diabetes: 30.0±7.6, ml·kg^{-1}·min^{-1}, 58±7 years). The healthy low-fit and type 2 diabetes groups performed two successive 30-min cycling bouts at 65%VO_{2peak} in the heat (35°C), separated by 30-min rest. The high-fit group cycled at the same absolute heat load (and therefore requirement for heat loss) as their healthy low-fit counterparts during the first exercise bout (Ex1) and at the same relative intensity (65%VO_{2peak}) during the second (Ex2). Forearm cutaneous vascular conductance ($CVC_{\%max}$) was measured at microdialysis sites perfused with: 1) lactated Ringer's solution (control); 2) 10 mM N^G-nitro-L-arginine-methyl-ester (L-NAME, nitric oxide synthase inhibitor); 3) 100 μM apocynin (NADPH oxidase inhibitor); 4) 10 μM tempol (superoxide dismutase mimetic), with responses compared at baseline, end-Ex1, and end-Ex2. **Results:** In all groups, L-NAME consistently reduced $CVC_{\%max}$ relative to the other treatment sites by ~16-21% during Ex1 and by ~22-27% during Ex2 (all $P<0.05$). Conversely, superoxide scavenging and NADPH oxidase inhibition did not influence $CVC_{\%max}$ (all $P>0.05$). **Conclusion:** Superoxide and NADPH oxidase do not modulate cutaneous vasodilation in healthy low- or high-fit older adults exercising in the heat, regardless of aerobic fitness level or relative exercise intensity employed, nor do they influence cutaneous vasodilation during an exercise-heat stress in those with type 2 diabetes. However, NOS remains an important modulator of cutaneous vasodilation during exercise in all groups.

Keywords: aging; apocynin; microcirculation; reactive oxygen species; skin blood flow; tempol, thermoregulation

List of Abbreviations

CVC, Cutaneous vascular conductance; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase; ROS, reactive oxygen species; SOD, superoxide dismutase; VO_{2peak}, peak oxygen uptake

INTRODUCTION

Older adults have an impaired ability to dissipate heat (Kenny et al., 2017; Larose et al., 2013) and this effect may be augmented in those with type 2 diabetes (Kenny et al., 2013), making these populations more susceptible to heat-related illness (e.g. heat syncope, heat exhaustion). During exercise-induced heat stress, cutaneous vasodilation is an essential component of dry heat loss to the environment. However, in older adults cutaneous vasodilator function may be attenuated during exercise due to reduced nitric oxide (NO) bioavailability (Fujii et al., 2015; Stapleton et al., 2014). Additionally, individuals with type 2 diabetes may demonstrate further reductions in cutaneous vasodilation compared to their healthy age-matched counterparts (Sokolnicki et al., 2009; Wick et al., 2006). Reactive oxygen species (ROS) can scavenge NO during exercise (Dillard et al., 1978) and aging (Donato et al., 2007), type 2 diabetes (Casoinic et al., 2016; Folli et al., 2011; Restaino et al., 2017) and exercise in hot conditions (Sureda et al., 2015) all independently augment ROS production. Consequently, endogenous antioxidant defenses may be overwhelmed in healthy older adults and those with type 2 diabetes during exercise in the heat. Indeed, oxidative stress can impair cutaneous vasodilation in young adults during high intensity (Meade et al., 2015) and prolonged (McNeely et al., 2017) exercise in the heat and in older adults during passive whole-body heating (Holowatz et al., 2006).

High aerobic fitness levels can counteract age-related impairments in whole-body heat loss during exercise (Flouris et al., 2018; Stapleton et al., 2015). Habitual physical activity can also reduce age-related oxidative stress and enhance endothelium-dependent vasodilation (Pierce et al., 2011; Walker et al., 2014). This effect is associated with increased antioxidant activity via production of superoxide dismutase (SOD), which scavenges superoxide, as well as down-regulation of the pro-oxidant enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Durrant et al., 2009; La Favor et al., 2016). In contrast, type 2 diabetes may be associated with elevated levels of superoxide and NADPH oxidase (Folli et al., 2011; Restaino et al., 2017). Intradermal delivery of superoxide scavengers or NADPH oxidase inhibitors during local heating improves endothelium-dependent vasodilation in the skin under several conditions associated with augmented ROS production (DuPont et al., 2014; Fujii et al., 2014; Hurr et al., 2018;

Kirkman et al., 2018; Medow et al., 2011). Combined, these findings indicate that in older adults high aerobic fitness may attenuate, while type 2 diabetes may amplify age-related impairments in cutaneous vasodilation during an exercise-heat stress relative to healthy low fit individuals, via superoxide and NADPH oxidase.

Therefore, the aim of the present study was to determine if cutaneous vasodilator responses in older adults exercising in the heat would be augmented by continuous delivery of an SOD mimetic (tempol) or NADPH oxidase inhibitor (apocynin) via intradermal microdialysis. First, we evaluated the potential for aerobic fitness to modulate these responses in healthy older adults. Since elevated ROS production is commonly reported at high relative exercise intensities (≥65% VO_{2peak}) (Goto et al., 2003; Lovlin et al., 1987; Seifi-Skishahr et al., 2008; Sureda et al., 2015) we sought to distinguish between cutaneous vasodilator effects due to aerobic fitness *per se*, and the relative exercise intensity employed. As such, responses from healthy low and high fit groups were compared during two distinct exercise bouts. In bout one, groups were evaluated at the same rate of metabolic heat production, but different relative exercise intensities (equivalent to 65% VO_{2peak} of the low fit group) to compare responses at the same thermal drive for heat loss. In bout two, groups were evaluated at the same relative exercise intensity, but different absolute heat loads (equivalent to 65% VO_{2peak} for each respective group) to compare responses at the same percentage of maximum effort.

Second, we compared the contributions of superoxide and NADPH oxidase to cutaneous vasodilation during exercise in the heat between healthy low fit older adults and individuals with type 2 diabetes of comparable age and aerobic fitness during two successive exercise bouts at the same relative intensity (both 65% VO_{2peak}). We hypothesized that delivery of an SOD mimetic or NADPH oxidase inhibitor would augment cutaneous vasodilation 1) in the low fit, but not high fit group during the first exercise bout (same absolute heat load and therefore similar thermal drive for heat loss) due to the greater relative work rate required by low fit individuals under this condition, 2) in both fitness groups during the second exercise bout (same relative intensity) with each performing at the same higher relative exercise intensity and 3) in both exercise bouts for the group with type 2 diabetes performing at the same high relative exercise intensity (both 65% VO_{2peak}) for both bouts.

MATERIALS AND METHODS

Ethical Approval

This study was approved by the University of Ottawa Health Sciences and Science Research Ethics Board in accordance with the Declaration of Helsinki. Written and informed consent was obtained from all volunteers before participation.

Participants

Twenty-seven older adults were evenly separated into healthy low, healthy high fit, and type 2 diabetes groups. Participant characteristics are described in Table 1. All participants were either lifelong non-smokers or had not smoked within 5 years prior to the study and all were free of skin disorders, respiratory disease, heart disease, uncontrolled hypertension and neuropathy. All participants abstained from over-the-counter medications, including vitamins and supplements for 48 h, strenuous physical activity for 24 h, alcohol and caffeine consumption for 12 h, and food consumption for 2 h prior to the experiment. One healthy low fit participant was taking a non-steroidal anti-inflammatory drug and a diuretic. One healthy high fit participant was taking a serotonin reuptake inhibitor. Participants with type 2 diabetes were taking the following medications (number of participants indicated in parentheses): metformin (8), statin (8), sulfonylurea (5), angiotensin converting enzyme inhibitor (3), acetylsalicylic acid (2), insulin (3), angiotensin receptor blocker (2), diuretic (1), 5-alpha reductase inhibitor (1), serotonin reuptake inhibitor (1), vitamin B12 (1).

Experimental Design

Participants completed one screening and one experimental session. During the screening session, body mass was measured using a digital weight scale platform (Model CBU150X, Mettler Toledo Inc., OH, USA). Height was measured using an eye-level physician stadiometer (Model 2391, Detecto Scale Company, Webb City, MO, USA). Body density was measured using the hydrostatic weighing technique and used to calculate body fat percentage (Siri, 1956). Peak oxygen uptake was determined via an incremental cycling exercise protocol. Participants were seated on a semi-recumbent cycling ergometer and maintained a consistent pedaling rate of 60-90 rpm. The resistance was set to 60 W for males and 40 W for females, and increased by 20 W every minute until volitional fatigue or if pedaling cadence could not be maintained above 50 rpm. Ventilation and metabolic

158 data were collected with an automated indirect calorimetry system (Medgraphic Ultima;
159 Medical Graphic, St. Paul, MN, USA) and peak oxygen uptake was evaluated as the highest
160 30 s average.
161 The experimental session was performed at least 48 h following the screening
162 session. Upon arrival, participants voided their bladders, after which pre-trial body mass
163 was assessed (Model CBU150X, Mettler Toledo Inc., OH, USA). Next, participants were
164 seated in a semi-recumbent position in a temperate room (25°C). Four microdialysis fibres
165 (MD2000, 30 kDa cutoff, 10 mm membrane; Bioanalytical Systems, West Lafayette, IN,
166 USA) were inserted into the dermal layer of the skin (~4 cm apart) on the left dorsal forearm
167 under aseptic conditions. At each site, a 25-gauge needle was inserted subcutaneously in
168 the skin (~2.5 cm in length). The microdialysis fibre was subsequently threaded through
169 the lumen of the needle, after which the needle was removed, leaving the fibre embedded
170 in the skin. Each fibre was then secured with surgical tape.
171 After fibre insertions, participants were transferred to an adjacent thermal chamber
172 (Can-Trol Environmental Systems, Markham, ON, Canada) that was regulated at 35°C and
173 20% relative humidity where they rested while sitting on a semi-recumbent cycle
174 ergometer. All microdialysis fibres were then perfused at a rate of 4 $\mu l \cdot min^{-1}$ with a
175 microinfusion pump (model 400; CMA Microdialysis, Solna, Sweden). The sites were
176 perfused with either 1) lactated Ringer's (Control) solution (Baxter, Deerfield, IL, USA);
177 2) 10 mM N^G-nitro-L-arginine methyl ester (L-NAME, a specific NO synthase (NOS)
178 inhibitor); 3) 100 μM apocynin (NADPH oxidase inhibitor); or, 4) 10 μM tempol (SOD
179 mimetic). The concentrations of L-NAME (Stapleton et al., 2014), apocynin (Medow et
180 al., 2011) and tempol (Medow et al., 2011) were determined based on previous literature
181 using microdialysis in human skin. Drug infusion continued for at least 60 min to ensure
182 that trauma associated with fibre insertion had subsided (Anderson et al., 1994). During
183 this time each site was instrumented for the measurement of cutaneous blood flow (see
184 below). Drug infusion was maintained throughout the entire experiment.
185 Following 10 min of baseline data collection, all three groups performed two 30
186 min bouts of semi-recumbent cycling, separated by a 30 min recovery period. For the low-
187 fit and type 2 diabetes groups, exercise was performed at 65% of peak oxygen uptake for
188 both bouts. For the high-fit group, the first exercise bout was performed at the same rate of

metabolic heat production as their physically matched low-fit counterparts to compare responses at the same heat load (inducing a similar thermal drive for heat loss but at different relative exercise intensities), while the second bout was performed at 65% of their own peak oxygen uptake in order to compare responses between fitness groups at the same relative exercise intensity (but at different absolute heat loads). For all three groups, at the end of the second exercise bout 50 mM sodium nitroprusside (Sigma-Aldrich) was infused for 20-25 min at all four sites at a rate of 6 $\mu l \cdot min^{-1}$until maximum values for cutaneous perfusion were achieved for at least 2 min. Upon completion of the experiment, body mass was measured again and the difference between pre- and post-measurements were used to assess fluid loss.

Measurements

Cutaneous red blood cell flux (expressed in perfusion units) was measured at all sites by laser-Doppler flowmetry (PeriFlux System 5000, Perimed, Stockholm, Sweden) using integrated seven-laser array laser-Doppler probes (model 413, Perimed, Stockholm, Sweden). Cutaneous vascular conductance (CVC) was then calculated as perfusion units divided by mean arterial pressure and expressed as a percentage of maximum CVC ($CVC_{\%max}$), which was defined as the highest consecutive 2 min interval during sodium nitroprusside infusion. Blood pressure was determined manually every 5 min using a mercury column sphygmomanometer (Baumonometer Standby Model; WA Baum, Copiague, NY, USA) and mean arterial pressure was calculated as diastolic arterial pressure plus one-third the difference between systolic and diastolic pressures.

Aural canal temperature was used as an index of core temperature and was measured every 5 min with an infrared tympanic thermometer (Welch Allyn Braun ThermoScan Pro 6000, Braun GmbH, Kronberg, Germany). Skin temperature was measured continuously at four sites (calf, quadriceps, chest, bicep) using thermocouple disks (Concept Engineering, Old Saybrook, CT, USA) attached to the skin with adhesive rings and surgical tape. Mean skin temperature was estimated as the weighted mean of local calf (20%), quadriceps (20%), chest (30%), and bicep (30%) temperatures (Ramanathan, 1964). Skin temperature data were recorded at 15 s intervals with a data acquisition module (model 34970A; Agilent Technologies Canada, Mississauga, ON, Canada) and displayed and recorded with LabVIEW software (National Instruments, Austin, TX, 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).

Data Analysis

Baseline resting values were determined by averaging the data collected over the final 5 min prior to the start of the first exercise bout. CVC at each skin site, mean skin temperature, and heart rate were evaluated by averaging data collected during the final 5 min of each 10 min interval throughout the protocol. Due to technical issues, some measurements were not recorded for some participants (number of participants excluded for data analysis are indicated in parentheses): core temperature (low fit, n=1; high fit, n=1), mean skin temperature (low fit, n=1), and heart rate (type 2 diabetes, n=1).

Statistical Analysis

For each exercise bout, $CVC_{\%max}$ was analyzed using a two-way mixed design analysis of variance (ANOVA) with the factors of treatment site (Within-group, 4 levels: Control, L-NAME, apocynin, and tempol) and group (Between-group, 2 levels: low fit and high fit OR low fit and type 2 diabetes). Cardiovascular and body temperature variables (heart rate, mean arterial pressure, and aural canal and mean skin temperatures) were each analyzed using a two-way mixed design ANOVA with the factors of time (Within-group, 3 levels) and group (2 levels: low fit and high fit OR low fit and T2D). Maximum absolute cutaneous vascular conductance was analyzed using a two-way mixed design ANOVA with the factors of treatment site (4 levels) and group (2 levels: low fit and high fit OR low fit and T2D). When a significant main effect or interaction was detected, multiple comparisons tests were performed using the Bonferroni procedure. Student's paired and unpaired t-tests (two-tailed) were used where appropriate. The level of significance for all analyses was set at $P<0.05$. All values are reported as mean ± standard deviation. Statistical analyses were conducted using Prism v. 8.0.2 (GraphPad, San Diego, CA, USA).

RESULTS

Participant Characteristics

Age and height were not different between any groups (all $P>0.05$). Body fat percentage was lower in the high-fit group relative to the low fit ($P<0.0001$). By design, relative peak oxygen uptake was higher in the high-fit group relative to low fit ($P<0.0001$). Body mass was higher in the type 2 diabetes group relative to healthy low fit ($P=0.0499$). All values are presented in Table 1.

Cutaneous Vascular Responses: healthy low fit versus healthy high fit

At baseline, there was a main effect of treatment site on $CVC_{\%max}$ ($P=0.002$, Fig. 1), but not for group ($P=0.853$) or the treatment site by group interaction ($P=0.843$). Multiple comparisons following the main effect of treatment site revealed that L-NAME was significantly lower than control and apocynin (both $P<0.05$), but not tempol ($P>0.05$).

For the first exercise bout ($CVC_{\%max}$ as a function of similar heat load but differing exercise intensities) there was a main effect of treatment site on $CVC_{\%max}$ ($P<0.0001$, Fig. 1), but not for group ($P=0.748$) or the treatment site by group interaction ($P=0.530$). Multiple comparisons following the main effect of treatment site revealed that L-NAME was significantly lower than control, apocynin, and tempol (all $P<0.05$).

For the second exercise bout ($CVC_{\%max}$ as a function of similar relative exercise intensity but differing heat load) there was a main effect of treatment site on $CVC_{\%max}$ ($P<0.0001$, Fig. 1), but not for group ($P=0.177$) or the treatment site by group interaction ($P=0.933$). Multiple comparisons following the main effect of treatment site revealed that L-NAME was significantly lower than control, apocynin, and tempol (all $P<0.05$).

No main effects were identified for treatment site ($P=0.489$), group ($P=0.379$), or the treatment site by group interaction ($P=0.997$) for maximum absolute CVC (Table 2).

Cutaneous Vascular Responses: healthy low fit versus type 2 diabetes

At baseline, there were no significant differences in $CVC_{\%max}$ for the main effects of treatment site ($P=0.076$, Fig. 2), group ($P=0.423$) or the treatment site by group interaction ($P=0.493$).

For the first exercise bout there was a main effect of treatment site on $CVC_{\%max}$ ($P=0.0001$, Fig. 2), but not for group ($P=0.151$) or the treatment site by group interaction ($P=0.821$). Multiple comparisons following the main effect of treatment site revealed that L-NAME was significantly lower than control and apocynin (all $P<0.05$).

For the second exercise bout there was a main effect of treatment site on $CVC_{\%max}$ ($P<0.0001$, Fig. 2), but not for group ($P=0.400$) or the treatment site by group interaction ($P=0.717$). Multiple comparisons following the main effect of treatment site revealed that L-NAME was significantly lower than control, apocynin, and tempol (all $P<0.05$).

No main effects were identified for treatment site ($P=0.731$), group ($P=0.819$), or the treatment site by group interaction ($P=0.782$) for maximum absolute CVC (Table 2).

Cardiovascular and body temperature responses: healthy low fit versus healthy high fit

All cardiovascular and body temperature responses are summarized in Table 3. For heart rate, significant main effects were found for time and the time by group interaction while for mean arterial pressure, aural canal temperature, and mean skin temperature, significant main effects were observed for time (all $P<0.05$).

Cardiovascular and body temperature responses: healthy low fit versus type 2 diabetes

For heart rate, significant main effects were found for time, group, and the time by group interaction while for mean arterial pressure, aural canal temperature, and mean skin temperature, significant main effects were observed for time (all $P<0.05$).

DISCUSSION

The aim of this study was to determine if cutaneous vasodilation in healthy low fit and high fit older adults and those with type 2 diabetes would be augmented by continuous local delivery of a superoxide scavenger (tempol) or NADPH oxidase inhibitor (apocynin) to the skin during exercise in the heat. In healthy older adults we found that neither superoxide scavenging nor NADPH oxidase inhibition measurably influenced cutaneous vasodilation, regardless of aerobic fitness level or the relative exercise intensity employed. Additionally, we found similar cutaneous vasodilator responses in those with type 2 diabetes, with no measureable effects of superoxide scavenging or NADPH oxidase inhibition. However, we did show that NOS is an important regulator of cutaneous vasodilation in all three groups under these conditions.

During rest in the heat, application of neither tempol nor apocynin altered cutaneous vasodilation relative to the untreated control site in healthy low and high fit older adults and in those with type 2 diabetes. These findings indicate that superoxide and NADPH oxidase do not appreciably influence local forearm cutaneous vessel tone in older adults

resting in the heat. However, NOS inhibition significantly attenuated resting cutaneous perfusion relative to control and apocynin treated sites in healthy older adults, although this effect was not observed when comparing the healthy low fit and type 2 diabetes groups. While not definitive, this finding may indicate a reduction in NO bioavailability during rest in the heat for individuals with type 2 diabetes.

During exercise in the heat, cutaneous vasodilation was not measurably influenced by local tempol or apocynin administration in healthy low or high fit older adults, regardless of whether exercise was performed at the same fixed heat load or the same high relative intensity. These findings suggest that in healthy older adults, aerobic fitness *per se* does not alter the influence of oxidative stress on cutaneous vasodilation during exercise in the heat. In addition, superoxide scavenging and NADPH oxidase inhibition do not appear to independently alter the cutaneous vasodilator response to exercise at a high relative intensity (~65% VO_{2peak}) in healthy older adults, regardless of aerobic fitness level. Further, despite typically elevated levels of superoxide and NADPH oxidase in individuals with type 2 diabetes (Folli et al., 2011; Restaino et al., 2017), inhibition of these mechanisms was not associated with augmented cutaneous vasodilation in this population during rest or exercise in the heat. These findings are in stark contrast to responses observed during local heating of the skin wherein intradermal delivery of superoxide scavengers or NADPH oxidase inhibitors consistently improves endothelium-dependent cutaneous vasodilation under several conditions associated with augmented ROS production (DuPont et al., 2014; Fujii et al., 2014; Hurr et al., 2018; Kirkman et al., 2018; Medow et al., 2011). However, this discrepancy may be due to the fact that local skin temperatures are typically much higher during local heating protocols (42°C) compared to exercise in the heat (~35°C).

In contrast to tempol and apocynin, we found that NOS inhibition with L-NAME significantly attenuated CVC relative to the control site for both groups during each exercise bout. This effect is consistent with previous reports examining healthy older adults under similar conditions (Fujii et al., 2017; Fujii et al., 2016). However, in the present study we extend these prior findings to show that the magnitude of the NOS contribution in older adults is not influenced by aerobic fitness during exercise in the heat. Further, we show that NOS also contributes to cutaneous vasodilation during exercise in the heat in those with

type 2 diabetes. This is consistent with prior work examining the NOS contribution to cutaneous vasodilation during exercise in the heat (Fujii et al., 2017) and during passive whole-body heating using a water perfused suit (Sokolnicki et al., 2009). These combined findings indicate that while type 2 diabetes may reduce resting NO bioavailability in human skin, it does not blunt NOS-dependent cutaneous vasodilation in response to either passive or exercise-induced heat stress. However, it should be noted that we evaluated individuals with well-controlled type 2 diabetes and it is certainly possible that individuals diagnosed with peripheral neuropathy or other diabetic complications may present with attenuated responses.

Given the clear role of NO in mediating cutaneous vasodilation during an exercise-induced heat stress in older adults, and the fact that excess ROS production is generally associated with reduced NO bioavailability during exercise (Dillard et al., 1978) and with aging (Donato et al., 2007), it is somewhat surprising that antioxidant strategies were ineffective at augmenting cutaneous vasodilation in healthy older adults and those with type 2 diabetes during exercise in the heat in the current study. One reason for this could be that while increased superoxide production can result from an upregulation of NADPH oxidase activity, the concomitant production of other free radicals such as peroxynitrite (Pacher et al., 2007) or the hydroxyl radical (Datla and Griendling, 2010), may counteract any beneficial effects of superoxide scavenging or NADPH oxidase inhibition on cutaneous vasodilation during exercise in the heat. Indeed, in addition to NADPH oxidase, ROS derived from other enzymatic pathways such as the xanthine oxidase pathway are important modulators of cutaneous vasodilation during local skin heating (Medow et al., 2011), and hydrogen peroxide may be produced by this enzymatic process at a much higher rate than superoxide under physiological conditions (Kelley et al., 2010). While hydrogen peroxide promotes cutaneous vasodilation during local skin heating (Medow et al., 2011), it may also be rapidly converted to the highly potent hydroxyl radical under various conditions (Datla and Griendling, 2010), potentially negating any positive effects of superoxide scavenging or NADPH oxidase inhibition during exercise in the heat. As such, future work is necessary to further describe the complex interplay between various oxidative stress pathways that may influence cutaneous vasodilation in healthy older adults and those with type 2 diabetes during exercise in the heat.

In young adults we previously demonstrated that the generalized antioxidant ascorbate can augment cutaneous vasodilation during both high intensity (Meade et al., 2015) and prolonged (McNeely et al., 2017) exercise in the heat. Conversely, we did not identify any effects of ascorbate on cutaneous vasodilation in healthy older adults and in those with type 2 diabetes during high intensity exercise in the heat (Fujii et al., 2017). However, that approach did not allow for the elucidation of the specific ROS pathways involved in mediating the cutaneous vasodilator response to an exercise-induced heat load. Additionally, while superoxide may attenuate cutaneous vasodilation, formation of hydrogen peroxide can have the opposite effect in human skin (Medow et al., 2011). As such, the use of a non-selective antioxidant such as ascorbate may have negligible effects on cutaneous vasodilation depending on the relative concentrations of these competing ROS being produced, with each mechanism producing opposing effects on cutaneous vessel tone. Further, while *in vitro* studies show that ascorbate can scavenge ROS such as superoxide, it is important to note that direct evidence for this effect in humans is limited (Margaritelis, 2016). In addition, specific antioxidant enzymes often display more favorable reaction kinetics with various types of ROS that may influence cutaneous vasodilation such as superoxide, NO, hydrogen peroxide and peroxynitrite (Cobley et al., 2015). As such, the antioxidant effects of an agent such as ascorbate may be negligible in human skin in vivo, and any of its previously observed effects during exercise in young adults (McNeely et al., 2017; Meade et al., 2015) or in older adults during passive whole-body heating (Holowatz et al., 2006) may actually be independent of its antioxidant properties (Cobley et al., 2015).

Another plausible explanation for observing negligible effects on cutaneous perfusion following superoxide scavenging and NADPH oxidase inhibition is that our current and past protocols may have failed to sufficiently upregulate ROS production to levels necessary to cause observable reductions in cutaneous vasodilation. Consistent with this possibility, in habitually active young men we previously showed an augmented NO-dependent cutaneous vasodilation with local ascorbate administration during high (~71% VO_{2peak}), but not moderate (~52% VO_{2peak}) intensity exercise in the heat (Meade et al., 2015). However, given the comparable durations (30 min) and relative exercise intensities used for the high-intensity bouts in our current (~65% VO_{2peak}) and prior (~70% VO_{2peak})

studies in older adults, coupled with the fact that advanced age and type 2 diabetes are both associated with augmented ROS production, we believe that the exercise stimulus used in the current study should have been adequate to observe an effect of superoxide scavenging and NADPH oxidase inhibition if present.

Conclusions

In healthy older adults we showed that neither aerobic fitness *per se*, nor relative exercise intensity, influenced cutaneous vasodilation during exercising in the heat via superoxide or NADPH oxidase dependent mechanisms. In addition, we showed that inhibition of these oxidant pathways failed to improve cutaneous vasodilation in individuals with type 2 diabetes relative to their healthy low fit counterparts. However, in all three groups of older adults, NOS contributed to the cutaneous vasodilator response to an exercise-heat stress.

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affiliation of N. Fujii is the University of Tsukuba, Faculty of Health and Sport Sciences, Tsukuba City, Japan.

COMPETING INTERESTS

None.

AUTHOR CONTRIBUTIONS

N.F. and G.P.K. conceived and designed the experiments. N.F., G.W.M., B.D.M., and K.H. performed data collection. G.W.M. performed data analysis. G.W.M., N.F., T.N., R.J.S., P.B., and G.P.K. interpreted the results. G.W.M. prepared figures and drafted the manuscript. All authors 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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552

Table 1. Participant characteristics.

	low fit	high fit	type 2 diabetes
Number of participants	9	9	9
Sex (men/women)	4/5	4/5	8/1
Age (years)	61 ± 8	56 ± 6	58 ± 7
Height (m)	1.64 ± 9.3	1.69 ± 6.4	1.71 ± 6.3
Body mass (kg)	73.8 ± 19.1	67.2 ± 9.6	91.5 ± 16.2*
Body fat (%)	35.3 ± 7.0	20.1 ± 4.5*	33.1 ± 9.7
Peak oxygen uptake ($ml \cdot kg^{-1} \cdot min^{-1}$)	24.4 ± 2.4	42.5 ± 9.7*	30.0 ± 7.6
HbA1c (%)	-	-	7.2 ± 0.5
Duration of diabetes (years)	n/a	n/a	7.7 ± 4.5

All values are presented as mean ± standard deviation (body fat: low fit, n=8, high fit, n=8; HbA1c, n=7).
*$P<0.05$ vs. low fit.

Table 2. Maximum absolute cutaneous vascular conductance during sodium nitroprusside infusion.

	Cutaneous Vascular Conductance (perfusion units · $mmHg^{-1}$)			
	Control	L-NAME	Apocynin	Tempol
high fit	2.63 ± 1.34	2.79 ± 1.89	2.78 ± 0.98	2.27 ± 0.58
low fit	2.15 ± 0.87	2.23 ± 0.77	2.42 ± 1.17	2.01 ± 0.93
type 2 diabetes	2.21 ± 1.06	2.00 ± 0.77	2.17 ± 0.92	2.10 ± 0.69

All values are presented as mean ± standard deviation, n=9 per group. There were no significant main effects (all $P>0.05$).

Table 3 Cardiovascular and temperature responses at Baseline, End-Exercise 1 and End-Exercise 2

		Baseline	End-Exercise 1	End-Exercise 2
Heart rate (bpm)	high-fit	65 ± 5	102 ± 21*	134 ± 20*†
	low-fit	75 ± 12	119 ± 14*	128 ± 19*†
	type 2 diabetes (n=8)	80 ± 13	144 ± 28*	152 ± 21*
Mean arterial pressure (mmHg)	high-fit	88 ± 7	98 ± 11	104 ± 11*
	low-fit	96 ± 12	107 ± 14*	107 ± 17*
	type 2 diabetes	94 ± 6	114 ± 12*	112 ± 14*
Aural canal temperature (°C)	high-fit (n=8)	37.1 ± 0.3	37.4 ± 0.6	37.9 ± 0.5*†
	low-fit (n=8)	37.0 ± 0.3	37.5 ± 0.6*	37.7 ± 0.6*†
	type 2 diabetes	37.1 ± 0.2	37.9 ± 0.2*	38.0 ± 0.2*†
Mean skin temperature (°C)	high-fit	34.86 ± 0.49	35.47 ± 0.46*	35.77 ± 0.53*
	low-fit (n=8)	34.82 ± 0.67	35.61 ± 0.38*	35.67 ± 0.79*
	type 2 diabetes	34.51 ± 0.58	35.53 ± 0.43*	35.38 ± 0.48*

All values are presented as mean ± standard deviation, n=9 per group unless stated otherwise. All values represent the average over the final 5 min of each time interval. *P<0.05 vs. Baseline; †P<0.05 vs. End-Exercise 1.

FIGURE LEGENDS

Figure 1. Cutaneous vascular conductance evaluated during the last 5 min of baseline and the first (Ex1) and second (Ex2) 30 min exercise bouts in both low (black bars) and high fit (gray bars) older adults. Four skin sites were continuously treated with either 1) lactated Ringer (control); 2) 10 mM N^G-nitro-L-arginine methyl ester (L-NAME, nitric oxide synthase inhibitor); 3) 100 μM apocynin (NADPH Oxidase inhibitor); or, 4) 10 μM tempol (superoxide dismutase mimetic). All values are presented as mean ± standard deviation; n=9. All values represent the average over the final 5 min of each time interval. *P<0.05 vs. control; †P<0.05 vs. apocynin; δP<0.05 vs. tempol.

Figure 2. Cutaneous vascular conductance evaluated during the last 5 min of baseline and the first (Ex1) and second (Ex2) 30 min exercise bouts in both low fit older adults (black bars) and those with type 2 diabetes (gray bars). Four skin sites were continuously treated with either 1) lactated Ringer (control); 2) 10 mM N^G-nitro-L-arginine methyl ester (L-NAME, nitric oxide synthase inhibitor); 3) 100 μM apocynin (NADPH Oxidase inhibitor); or, 4) 10 μM tempol (superoxide dismutase mimetic). All values are presented as mean ± standard deviation; n=9. All values represent the average over the final 5 min of each time interval. *P<0.05 vs. control; †P<0.05 vs. apocynin; δP<0.05 vs. tempol.

Figure 1.

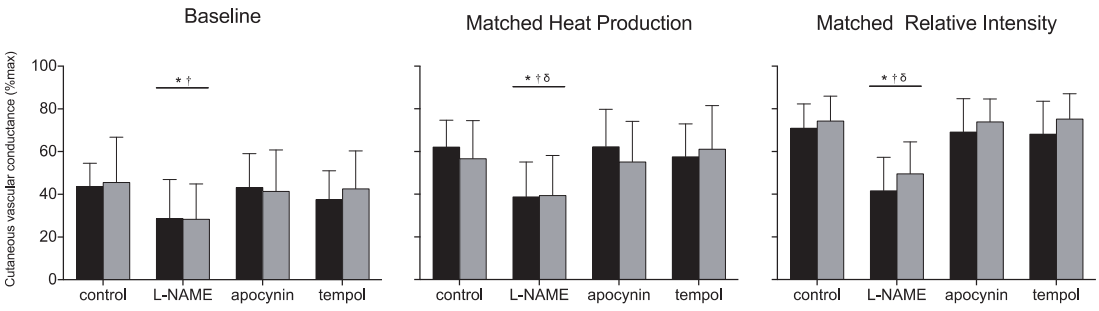
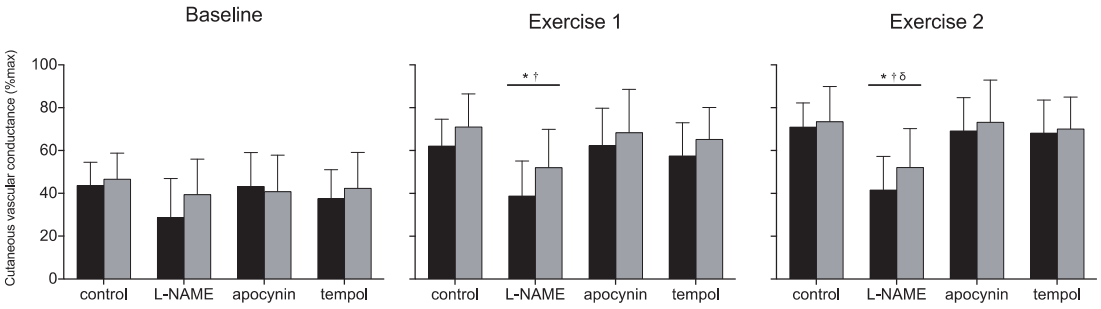

Baseline
Matched Heat Production
Matched Relative Intensity
Cutaneous vascular conductance (%max)
100
80
60
40
20
0
* †
* † δ
* † δ
control
L-NAME
apocynin
tempol

Figure 2.


Baseline
Exercise 1
Exercise 2
Cutaneous vascular conductance (%max)
100
80
60
40
20
0
* †
* † δ
control
L-NAME
apocynin
tempol