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Endothelial nitric oxide synthase mediates the nitric oxide component of reflex cutaneous vasodilatation during dynamic exercise in humans

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Key points

- Increases in skin blood flow and sweating also occur during exercise; however, it is not known if the mechanisms controlling these responses are the same during passive heat stress and exercise.
- The prevailing thought has been that mechanisms of cutaneous vasodilatation during passive heat stress and sustained dynamic exercise are the same, or very similar.
- Nitric oxide (NO) has been shown to be important for increasing skin blood flow during passive heat stress but it is unknown if this molecule is also involved during sustained dynamic exercise.
- The findings from our study suggest NO is involved in increasing skin blood flow during sustained dynamic exercise in humans but the NO is produced from a different enzyme compared to passive heat stress.
- These findings may help us better understand and aid individuals who have difficulty regulating their body temperature during sustained dynamic exercise (e.g. ageing).

Abstract Recent data suggests neuronal nitric oxide synthase (nNOS) mediates the NO component of reflex cutaneous vasodilatation with passive heat stress. We tested the hypothesis that nNOS inhibition would attenuate reflex cutaneous vasodilatation during sustained dynamic exercise in young healthy humans. All subjects first performed an incremental $\dot{V}_{O_2,peak}$ test to exhaustion on a custom-built supine cycle ergometer. On a separate day, subjects were instrumented with four intradermal microdialysis fibres on the forearm and each randomly assigned as: (1) lactated Ringer's (control); (2) 20 mM N_ω-nitro-L-arginine methyl ester hydrochloride (non-selective NOS inhibitor); (3) 5 mM *N*-propyl-L-arginine (nNOS inhibitor); and (4) 10 mM N^5-(1-iminoethyl)-L-ornithine dihydrochloride [endothelial NOS (eNOS) inhibitor]. Following microdialysis placement, subjects performed supine cycling with the experimental arm at heart level at 60% $\dot{V}_{O_2,peak}$ for a period sufficient to raise core temperature 0.8°C. At the end of cycling, all microdialysis sites were locally heated to 43°C and sodium nitroprusside was perfused to elicit maximal vasodilatation. Mean arterial pressure, skin blood flow via laser-Doppler flowmetry and core temperature via ingestible telemetric pill were measured continuously; cutaneous vascular conductance (CVC) was calculated as laser-Doppler flowmetry/mean arterial pressure and normalized to maximum. There was no significant difference between control ($58 \pm 2\%CVC_{max}$) and nNOS-inhibited ($56 \pm 3\%CVC_{max}$) sites in response to exercise-induced hyperthermia. The increase in CVC at eNOS-inhibited ($41 \pm 3\%CVC_{max}$) and non-selective NOS-inhibited ($40 \pm 4\%CVC_{max}$) sites were significantly attenuated compared to control and nNOS-inhibited ($P < 0.001$ all conditions) but there was no difference between eNOS-inhibited

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and non-selective NOS-inhibited sites. These data suggest eNOS, not nNOS, mediate NO synthesis during reflex cutaneous vasodilatation with sustained dynamic exercise.

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Abbreviations CVC, cutaneous vascular conductance; $\%CVC_{max}$, percentage of maximal cutaneous vascular conductance; DBP, diastolic blood pressure; eNOS, endothelial nitric oxide synthase; HR, heart rate; L-NIO, N^5-(1-iminoethyl)-L-ornithine dihydrochloride; MAP, mean arterial pressure; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NPLA, *N*-propyl-L-arginine; PP, pulse pressure; SBP, systolic blood pressure; SNP, sodium nitroprusside; T_C, core temperature; ΔT_C, change in core temperature; $\dot{V}_{O_2}$, oxygen uptake; $\dot{V}_{O_2,peak}$, peak oxygen uptake.

Introduction

In humans, the primary autonomic response to an increase in core temperature (T_C) is increased blood flow to the skin and sweating. Two branches of the sympathetic nervous system mediate control of skin blood flow and the sweat response, i.e. the adrenergic vasoconstrictor system and cholinergic active vasodilator system. During heat stress, the initial increase in skin blood flow in response to passive heating is achieved by withdrawal of tonic sympathetic vasoconstriction, while further cutaneous vasodilatation and instigation of sweating is achieved via activation of sympathetic cholinergic nerves (Grant & Holling, 1938; Roddie *et al.* 1957).

Although several vasodilators have been proposed to be involved in cutaneous active vasodilatation (Bennett *et al.* 2003; Wong *et al.* 2004; McCord *et al.* 2006; Wong & Minson, 2006; Kellogg *et al.* 2010; Wong & Fieger, 2012), it remains unclear how these vasodilators interact and the precise mechanisms responsible for the robust increase in skin blood flow during heat stress. Nitric oxide (NO) has been shown to contribute ∼30–45% to reflex cutaneous vasodilatation during passive heating (Dietz *et al.* 1994; Kellogg *et al.* 1998; Wilkins *et al.* 2003; Kellogg *et al.* 2009). NO has further been shown to have both a direct contribution to reflex cutaneous vasodilatation as well as a synergistic role. That is, not only does NO directly contribute to a portion of reflex cutaneous vasodilation but it also acts synergistically with one or more of the vasodilators implicated in reflex vasodilation. There are several potential source(s) of NO and recent data suggest neuronal NO synthase (nNOS) is the NOS isoform contributing to NO during passive heating (Kellogg *et al.* 2008*b*, 2009). Whether nNOS is responsible for NO production in the cutaneous vasculature during dynamic exercise remains unknown.

During dynamic exercise, a large proportion of muscle mass is engaged and the most abundant byproduct of the muscle contraction and increased metabolism is heat. As a result, T_C increases during dynamic exercise. As with passive heating, the increase in T_C during dynamic exercise results in an increase in skin blood flow and sweating; however, these responses are delayed compared to passive heating (i.e. rightward shift of the T_C–skin blood flow relationship). Compared to passive heat stress, the initial skin blood flow response to dynamic exercise is an adrenergic sympathetic vasoconstriction (Kenney & Johnson, 1992). Although several studies have documented an increase in skin blood flow during exercise and have studied modifiers of the skin blood flow response (e.g. changes in skin temperature, posture; Johnson *et al.* 1974; Brengelmann *et al.* 1977), to our knowledge there have been no studies investigating mechanisms of reflex cutaneous vasodilatation during sustained dynamic exercise, specifically the contribution of NO. The underlying assumption has been mechanisms of reflex cutaneous vasodilatation during sustained dynamic exercise are the same, or, at least very similar, as during passive heat stress. It therefore remains unknown whether any potential NO component to reflex cutaneous vasodilatation stems from increased nNOS or endothelial NOS (eNOS) activity. The purpose of this study was to investigate mechanisms of reflex cutaneous vasodilatation associated with hyperthermia induced by sustained dynamic exercise (i.e. endogenous heat stress). Specifically, we investigated the contribution of eNOS and nNOS to reflex cutaneous vasodilatation during sustained dynamic exercise. We hypothesized that nNOS, not eNOS, would contribute to reflex cutaneous vasodilatation during sustained dynamic exercise, as is the case during passive heat stress. To verify our data further, we performed a follow-up study where a subset of subjects underwent a period of passive heat stress with the same NOS inhibitors as those used during the supine cycling protocol.

Methods

Ethical approval

The Institutional Review Board at Kansas State University approved all protocols. An informed consent was reviewed and signed by each subject before participation in the

study. All protocols conformed to guidelines set forth in the Declaration of Helsinki.

Subjects

Eight male subjects between the ages of 20 and 25 years participated in this study. Before participation, all subjects filled out a health history form to ensure non-smoking status, the absence of metabolic disease and a family history absent of cardiovascular disease. None of the subjects were taking any medications. All subjects were asked to refrain from caffeine, alcohol and strenuous exercise for 12 h before each laboratory visit. Each subject participated in two laboratory visits: incremental supine peak oxygen consumption ($\dot{V}_{O_2,peak}$) test to volitional fatigue and a continuous bout of submaximal supine cycling. All experiments were conducted in a thermoneutral laboratory (~23–24°C).

Determination of $\dot{V}_{O_2,peak}$

$\dot{V}_{O_2,peak}$ was measured with the subjects cycling in the supine position on a custom-built stationary bicycle ergometer. Pulmonary gas exchange ($\dot{V}_{O_2}$) was measured breath-by-breath using a metabolic measurement system (CardiO2; Medical Graphics, St. Paul, MN, USA). Before each testing session, the gas analysers were calibrated using gases of known concentration and the flow transducer was calibrated using a 3.0 litre syringe. After a 5 min warm up at 60 rpm, the workload increased 25 W each minute until the subjects reached volitional fatigue. Subjects were instructed to maintain 60 rpm and were assisted via audio tone from an electronic metronome. Exhaustion was determined when the subject could no longer maintain 60 rpm despite verbal encouragement for a period of 10 s or for ~5 consecutive pedal revolutions. Validation of peak oxygen uptake was performed following a rest period of ~20 min after completion of the peak tests by having the subjects complete a constant power test at 105% of the peak power determined from the ramp test (Poole *et al.* 2008).

Subject monitoring: continuous submaximal cycling

Subjects' heart rate was monitored with a three-lead electrocardiogram (S/5 Light Monitor, Datex-Ohmeda; GE Healthcare, Madison, WI, USA). Blood pressure was monitored beat-by-beat via photoplethysmography (NexfinHD; BMEYE, Amsterdam, The Netherlands) and was verified via automated brachial auscultation (S/5 Light Monitor; Datex-Ohmeda, GE Healthcare) every 5 min. Although blood pressure from the Nexfin device has not been specifically validated during exercise, this system uses similar technology as the Finapres, which has been used previously to measure arterial blood pressure during arm swing exercise (Sheriff *et al.* 2009), where the arterial pressure signal was measured in a moving limb. In addition, blood pressures measured via brachial auscultation during the protocols were in good agreement with those obtained from the Nexfin. Skin blood flow measurements were made from the lateral aspect of the left forearm, while subjects were in the supine position with the experimental arm at heart level for the entire protocol. Subjects' T_C was measured via telemetric ingestible pill (CorTemp Data Recorder and CorTemp Temperature Sensor; Wireless Sensing Systems and Design, Palmetto, FL, USA) every minute.

Subject instrumentation: continuous submaximal cycling

All subjects had four microdialysis fibres placed into the intradermal layer of the skin of the left ventral forearm, which were used to administer drugs to local areas of skin in the forearm. Fibres were placed approximately 3–5 cm apart in the absence of anaesthetics; however, ice was used to numb the skin before placement (Hodges *et al.* 2009). Fibre placement was accomplished by first threading a 23-gauge needle through the skin at each desired site of microdialysis placement. A microdialysis fibre was then threaded through the lumen of the needle, and the needle removed, leaving the membrane in place. To account for insertion hyperaemia, cutaneous blood flow was monitored via laser-Doppler flowmetry as described below and allowed to return to resting values before the start of the protocol (~45–60 min). During this time, all fibres were perfused with lactated Ringer's solution at a rate of 4 μl min^{-1}. The membranes of the microdialysis fibres were 10 mm in length with a 55 kDa molecular mass cut-off (CMA 31 Linear Probe; CMA Microdialysis, Kista, Sweden).

Red blood cell flux measured via laser-Doppler flowmetry was used as an index of skin blood flow. Local heating units (PF5020 local heating units and Peri-Flux 5020 Temperature Unit; Perimed, Jarfalla, Sweden) were placed on the skin centred over each semipermeable membrane. An integrated laser-Doppler probe (Probe 413; Perimed), each with seven emitting and receiving fibres, was placed at each microdialysis site directly over each semipermeable membrane in the centre of each local heating unit to measure skin blood flow.

Continuous submaximal cycling protocol with intradermal microdialysis

Approximately 2–3 h before beginning the continuous cycling protocol, subjects were instructed to swallow the ingestible telemetric core temperature pill. Each microdialysis site was randomly assigned as: (1) control; (2) non-specific eNOS and nNOS inhibition [20 mM

N^G-nitro-L-arginine methyl ester (L-NAME)]; (3) nNOS inhibited [5 mM *N*-propyl-L-arginine (NPLA)]; or (4) eNOS inhibition [10 mM N^5-(1-iminoethyl)-L-ornithine dihydrochloride (L-NIO); see Nitric oxide synthase inhibitors for details]. Baseline data were measured for 10 min before commencement of the continuous exercise protocol. Following the baseline period, endogenous heat stress was accomplished by having the subjects cycle in the supine position, on a custom-built stationary bicycle ergometer, at a workload sufficient to elicit and maintain 60% $\dot{V}_{O_2,peak}$. This relative workload was based on preliminary studies in our laboratory that demonstrated an increase in T_C similar to that achieved with passive heat stress (0.8°C above baseline) that could be achieved in a similar time frame (~45 min). Subjects were instructed to maintain 60 rpm and were assisted via audio tone from an electronic metronome. Cycling at 60% $\dot{V}_{O_2,peak}$ was terminated when T_C increased 0.8°C above baseline (exercise duration range was 35–45 min to reach the desired rise in T_C). Inasmuch as passive heat stress studies elicit an increase in T_C of at least 0.8°C above baseline to investigate mechanisms of reflex cutaneous vasodilatation (Wilkins *et al.* 2003; Wong *et al.* 2004; McCord *et al.* 2006; Wong & Minson, 2006; Wong & Fieger, 2012), our aim was to match the increase in T_C during dynamic exercise as that achieved in passive heat stress studies. Upon completion of the continuous cycling bout, subjects performed unloaded cycling recovery for at least 5–10 min or for as long as deemed necessary by each subject. At the end of the unloaded recovery period, maximal vasodilatation was induced at each site via local heating to 43°C and infusion of 28 mM sodium nitroprusside (SNP). This concentration of SNP and level of local heating has been previously shown to elicit maximal vasodilatation in human skin (Holowatz *et al.* 2005; Smith *et al.* 2011; Bruning *et al.* 2012; Wong & Fieger, 2012).

Passive whole body heat stress verification protocol

To verify the efficacy of our drugs and our findings, a subset of subjects (n = 4 men) participated in a passive whole body heat stress protocol. Subject monitoring, drug administration and instrumentation was the same as described above; however, exogenous hyperthermia was elicited by having subjects wear a nylon tube-lined water-perfused suit (Allen Vanguard, Ottawa, ON, Canada) and pumping 50°C water through the suit. Heat stress was sufficient to raise T_C 0.8°C above baseline.

Nitric oxide synthase inhibitors

All drugs were dissolved in sterile lactated Ringer's solution and obtained from Tocris Bioscience (Ellisville, MO, USA). A 20 mM dose of the L-arginine analog, L-NAME, was used to non-selectively inhibit NOS, which has been shown to be effective at inhibiting NOS in human skin (Hodges *et al.* 2008; Smith *et al.* 2011; Bruning *et al.* 2012). A 5 mM dose of NPLA was used to inhibit nNOS, which has been shown to inhibit nNOS in human skin (Kellogg *et al.* 2009; Smith *et al.* 2011; Bruning *et al.* 2012). A 10 mM dose of L-NIO was used to inhibit eNOS (IC_{50} = 500 nM). Pilot work in our laboratory determined this concentration effectively inhibits the NO-dependent vasodilatation to local heating of the skin, which has been shown to be mediated predominantly by eNOS (Kellogg *et al.* 2008*a*, 2009). All NOS inhibitors were administered for at least 45 min before the start of, and for the duration of, continuous cycling exercise via microdialysis at a rate of 2 μl min^{-1}.

Data collection and analysis

Data were digitized and stored at 100 Hz on a personal computer and were analysed offline using signal-processing software (Windaq; Dataq Instruments, Akron, OH, USA). Skin blood flow data were converted to cutaneous vascular conductance (CVC), calculated as the ratio of skin blood flow to mean arterial pressure (red blood cell flux/MAP). CVC data were expressed as a percentage of maximal vasodilatation (%CVC_{max}) via SNP infusion and local heating to 43°C.

To determine the increase in %CVC_{max} at each treatment site, the final three minutes of data for the continuous cycling protocol for each subject were averaged and used for analysis (corresponding to an increase in T_C of 0.8°C). To determine the onset of reflex cutaneous vasodilatation and the magnitude of increase in %CVC_{max} for a given increase in T_C (ΔT_C), %CVC_{max} data at each microdialysis site during the final minute of each 0.1°C increase in T_C from baseline (ΔT_C = 0.0°C) to the end of the continuous cycling period (ΔT_C = 0.8°C) was used for analysis. The percentage contribution of eNOS and nNOS to the increase in %CVC_{max} was calculated as follows:

$$[(\%CVC_{maxControl} - \%CVC_{maxTreatment}) / \%CVC_{maxControl}] \times 100$$

where %$CVC_{max\ Treatment}$ is the value at L-NAME, NPLA or L-NIO sites.

The group mean increase in %CVC_{max} at the end of continuous cycling exercise (i.e. at a 0.8°C increase in T_C) and the percentage contribution of eNOS and nNOS were analysed using one-way repeated measure ANOVA. A two-way ANOVA with repeated measures was used to compare the effect of drug treatment on the increase in %CVC_{max} for each 0.1°C increase in T_C (drug treatment × %CVC_{max} × ΔT_C). For all ANOVAs Tukey's *post hoc* analysis was used to determine where significant differences occurred. Comparison of haemodynamic data and T_C from rest (baseline) to the end of the continuous cycling bout was performed using a paired t test. All

Table 1. Group mean haemodynamic and core temperature data at baseline and end of exercise

	HR (beats min^{-1})	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	PP (mmHg)	T_c (°C)
Baseline	59 ± 4	112 ± 2	64 ± 1	80 ± 4	49 ± 3	36.51 ± 0.37
Exercise	145 ± 6*	154 ± 11*	70 ± 2*	98 ± 2*	89 ± 7*	37.33 ± 0.37*

Values are means ± S.E.M. HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure; T_C, core temperature. *$P < 0.05$ *vs.* baseline.

statistical analyses were performed using SPSS 20 (IBM Corporation, Armonk, NY, USA). All values are presented as means ± S.E.M. and $P < 0.05$ was considered significant.

Results

Subject demographics and oxygen uptake data

Subject demographics and oxygen uptake data are presented as means ± S.D. Subjects had a mean age of 22 ± 1 years, mean height of 177 ± 5 cm and mean weight of 78 ± 13 kg. Subjects' average absolute oxygen uptake was 3.51 ± 0.59 l min^{-1} and average relative oxygen of 44.87 ± 9.52 ml kg^{-1} min^{-1}. Subjects' peak power achieved during the $\dot{V}_{O_2,peak}$ test averaged 278 ± 34 W and subjects' power during the continuous cycling protocol averaged 147 ± 34 W.

Haemodynamic data

Group mean haemodynamic data for the continuous exercising protocol are summarized in Table 1 and in Table 2 for the passive heat stress protocol. Importantly, note the increase in pulse pressure from baseline during the exercise protocol but the lack of an increase in pulse pressure during the passive heat stress protocol (see Discussion).

Cutaneous vascular conductance data

The mean data for the increase in %CVC$_{max}$ elicited following a 0.8ºC increase in T_C during continuous cycling at each treatment site are summarized in Fig. 1. There were no differences between control (58 ± 2%CVC$_{max}$) and NPLA (nNOS inhibition: 56 ± 3%CVC$_{max}$) sites. The increase in CVC at L-NIO (eNOS inhibition: 41 ± 3%CVC$_{max}$) and L-NAME (non-specific NOS inhibition: 40 ± 5%CVC$_{max}$) sites was attenuated compared to control and NPLA ($P < 0.001$ all conditions) sites. There was no difference between L-NIO (eNOS inhibition) and L-NAME (non-specific NOS inhibition).

Figure 2 shows the increase in %CVC$_{max}$ as a function of increasing T_C from baseline to the end of the exercise in 0.1ºC increments. A significant increase in %CVC$_{max}$ above baseline occurred with an increase in T_C of 0.4ºC at all treatment sites.

The percentage contribution of eNOS and nNOS to the increase in %CVC$_{max}$ is shown in Fig. 3. The contribution of eNOS (30 ± 5%CVC$_{max}$) was greater than the contribution of nNOS (2 ± 5%CVC$_{max}$) ($P < 0.05$).

The data in Fig. 4 are from the four subjects who completed the passive whole body heat stress protocol.

Table 2. Group mean haemodynamic data for the passive heating protocol

	HR (beats min^{-1})	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	PP (mmHg)
Baseline	66 ± 7	117 ± 3	66 ± 2	79 ± 3	51 ± 3
Passive heating	95 ± 8*	124 ± 2	67 ± 3	77 ± 4	56 ± 5

Values are means ± S.E.M. HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure. *$P < 0.05$ *vs.* baseline.

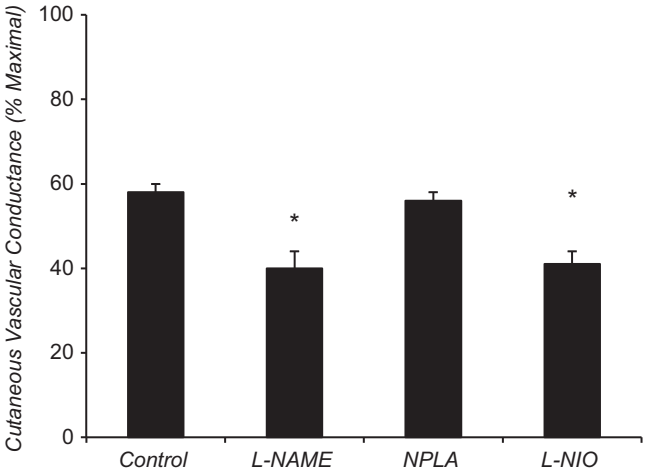

Figure 1. Increase in cutaneous vascular conductance during supine cycling exercise
Increase in percentage of maximal cutaneous vascular conductance in response to a 0.8°C increase in core temperature during supine cycling exercise was similar between control and NPLA sites. The increase in cutaneous vascular conductance was attenuated at the L-NIO and L-NAME sites compared to both control and NPLA sites, suggesting a significant role for endothelial nitric oxide synthase to reflex cutaneous vasodilatation during dynamic exercise. *$P < 0.05$ *vs.* control and NPLA. Data are means ± S.E.M. L-NIO, N^5-(1-iminoethyl)-L-ornithine dihydrochloride; L-NAME, N_ω-nitro-L-arginine methyl ester hydrochloride; NPLA, *N*-propyl-L-arginine.

In contrast to the exercise data, there were no differences between control (61 ± 5%CVC_{max}) and L-NIO (eNOS inhibition: 59 ± 6%CVC_{max}) sites while the increase in CVC was attenuated at NPLA (nNOS inhibition: 44 ± 6%CVC_{max}) and L-NAME (non-specific NOS inhibition: 41 ± 5%CVC_{max}) sites.

Discussion

This study investigated mechanisms underlying reflex cutaneous vasodilatation in response to hyperthermia induced by dynamic exercise in humans. Our data suggest eNOS is required for the NO-mediated cutaneous vasodilatation attending dynamic exercise in thermoneutral conditions. This finding is contrary to our hypothesis, which supposed NO-mediated cutaneous vasodilatation would be nNOS-dependent as is the case during passive heating (Kellogg *et al.* 2008*b*, 2009). This supposition was based on the observation that cutaneous vascular responses to the thermal load of dynamic exercise and to passive heat stress have historically been viewed as similar effector responses, mediated through identical pathways, with subtly different manifestations in the overall response (Johnson & Proppe, 1996; Simmons *et al.* 2011). Thus, the divergence noted in the present study with regard to the mechanisms underlying cutaneous NO generation during active *vs.* passive thermal stress is surprising. Based on our current data and those of Kellogg *et al.* (2008*b*, 2009), it appears that different mechanisms and NOS isoforms are involved in reflex cutaneous vasodilatation during passive heat stress and the endogenous heat stress associated with dynamic exercise. The data from this study further suggest NO directly contributes ∼30% to the increase in skin blood flow during dynamic exercise, which is in agreement with several studies that have shown NO directly contributes ∼30–45% to cutaneous active vasodilatation during passive whole body heating (Dietz *et al.* 1994; Kellogg *et al.* 1998; Wilkins *et al.* 2003).

Several studies heave clearly shown a robust cutaneous vasodilatation during exercise (Blair *et al.* 1961; Johnson *et al.* 1974; Brengelmann *et al.* 1977; Kellogg *et al.* 1991, 1993); however, mechanistic data specific to the NO pathway underlying this cutaneous vasodilatation is lacking. To date, only one study by Blair *et al.* (1961) has provided evidence that the increase in skin blood flow during exercise may be of reflex origin. It has often been assumed that mechanisms are similar during dynamic exercise and passive whole body heating. Data from our current study suggest these mechanisms may differ depending on the means by which hyperthermia is induced.

Our data clearly suggest eNOS mediates the NO component of cutaneous vasodilatation during dynamic exercise; however, it is unclear what is responsible for activating eNOS during dynamic exercise. There are at least two possible explanations for the participation of eNOS rather than nNOS in the cutaneous vasodilatation attending dynamic exercise.

First, it is possible that NO generation in the cutaneous vasculature during thermal stress occurs via nNOS or eNOS activity depending on local haemodynamic conditions. Several local haemodynamic signals have been shown to regulate eNOS activity at the vascular level, including shear stress, cyclic circumferential vascular strain, pressure and others (Laughlin *et al.* 2008). Although blood flow (and presumably shear stress) in the cutaneous circulation is typically higher during passive heating compared to dynamic exercise at a given T_C (Kenney & Johnson, 1992; Simmons *et al.* 2011), pulse pressure and therefore cyclic circumferential vascular wall strain, would tend to be higher during exercise than passive

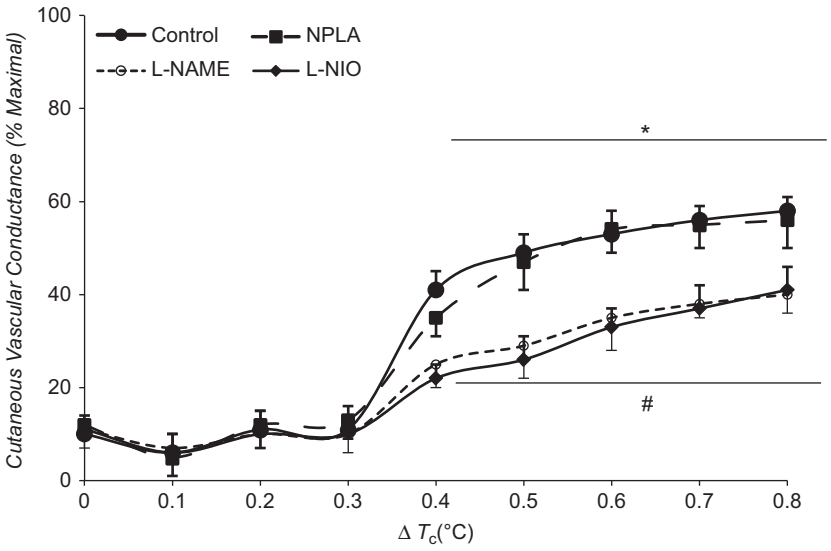

Figure 2. Increase in cutaneous vascular conductance as a function of increasing core temperature
The increase in percentage of maximal cutaneous vascular conductance at each microdialysis site is shown for each 0.1°C increase in core temperature from rest (T_C = 0°C) to end of exercise (T_C = 0.8°C). Significant increases in cutaneous vascular conductance above baseline occurred at the same increase in core temperature (0.4°C) at all treatment sites. *$P < 0.05$ *vs.* baseline for all treatment sites; #$P < 0.05$ for L-NIO and L-NAME *vs.* control and NPLA. Data are means ± S.E.M.L-NIO, N^5-(1-iminoethyl)-L-ornithine dihydrochloride; L-NAME, N_ω-nitro-L-arginine methyl ester hydrochloride; NPLA, *N*-propyl-L-arginine.

heating (see Tables 1 and 2). Because circumferential strain has been shown to increase eNOS activation independently (Awolesi *et al.* 1994, 1995; Laughlin *et al.* 2008), it is possible the elevation of this signal in the cutaneous vasculature during dynamic exercise caused eNOS activation, and subsequent NO generation, that reduced the requirement for nNOS activation. Notably, elevations in pulse pressure develop quickly after the onset of dynamic exercise, preceding cutaneous vasodilatation at times by several minutes (Blair *et al.* 1961). Therefore, it is likely the vascular transduction of active vasodilator nerve activity takes place amidst a very different local haemodynamic environment during dynamic exercise compared to passive heat stress.

Second, it is possible nNOS activation is dependent on the firing frequency of neural signals carried along sympathetic active vasodilator nerves, in a manner analogous to the stimulation frequency dependence of neuropeptide Y release from noradrenergic nerves, a phenomenon regulated by subcellular processes that operate in a neural firing frequency-dependent manner (Lundberg *et al.* 1989). If this is the case, then any variation in the afferent inputs (e.g. skin temperature) that collectively determine active vasodilator outflow might, through effects on active vasodilator nerve firing frequency, alter the relative activation state of nNOS and therefore its contribution to NO generation during thermal stress. In this regard, the increase in skin blood flow during thermal stress is sensitive to alterations in mean skin temperature (Johnson & Proppe, 1996), suggesting that mean skin temperature represents an important afferent input to the hypothalamic regions that regulate efferent active vasodilator outflow. This, coupled with observed differences in mean skin temperature during passive heating compared to dynamic exercise, suggests it is at least possible that active vasodilator neural firing frequency-dependent mechanisms are responsible for the variation in NOS isoform contribution to NO generation during exercise compared to non-exercise thermal stress.

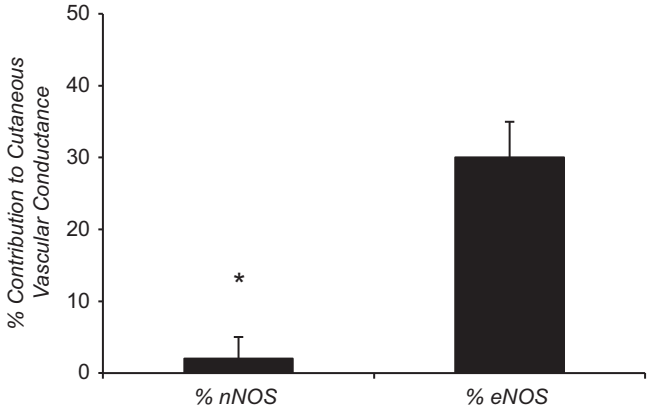

Figure 3. Percentage contribution of eNOS and nNOS to reflex cutaneous vasodilatation
Contribution of nNOS to reflex cutaneous vasodilatation during supine cycling exercise was significantly less than the contribution of eNOS. $^*P < 0.05$ *vs.* eNOS. Data are means ± S.E.M. eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase.

Passive whole body heat stress verification protocol

To ensure the data obtained during dynamic exercise were not due to non-specific effects of our eNOS and nNOS inhibitors, we performed a validation study with passive

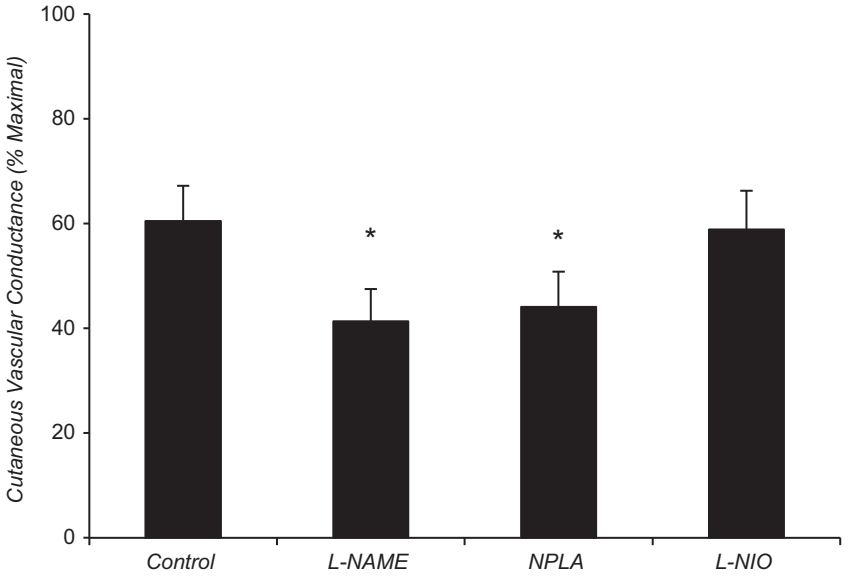

Figure 4. Increase in cutaneous vascular conductance during passive whole body heat stress
In contrast to dynamic exercise (Fig. 1), the increase in percentage of maximal cutaneous vascular conductance during passive heat stress was significantly attenuated at NPLA and L-NAME sites, suggesting a significant contribution of neuronal nitric oxide synthase to reflex cutaneous vasodilatation during passive heat stress. $^*P < 0.05$ *vs.* control and L-NIO. Data are means ± S.E.M.L-NIO, N^5-(1-iminoethyl)-L-ornithine dihydrochloride; L-NAME, N_ω-nitro-L-arginine methyl ester hydrochloride; NPLA, *N*-propyl-L-arginine.

whole body heating on a subset of subjects using the same concentrations of L-NIO and NPLA. Consistent with previous data (Kellogg *et al.* 2009), we found nNOS is the primary NOS isoform responsible for the NO component of reflex cutaneous vasodilatation during passive heating with no effect observed at sites where eNOS was inhibited. The conclusion that eNOS, but not nNOS, contributes to reflex cutaneous vasodilatation during dynamic exercise is substantiated.

Limitations

There are four possible limitations to our study. First, mechanisms underlying cutaneous active vasodilatation during dynamic exercise remain unresolved. Our data suggest a role for eNOS to the reflex vasodilatation associated with dynamic exercise; however, whether proposed mechanisms that have been shown to contribute to reflex cutaneous vasodilatation during passive heating (e.g. vasoactive intestinal polypeptide, H_1 histamine receptors, etc.) also contribute during dynamic exercise remains unresolved.

Second, physical conditioning/training has been shown to increase eNOS expression and our subjects were all normally active to moderately trained individuals (Table 1). The data from this study show eNOS, not nNOS, contributes to reflex cutaneous vasodilatation during dynamic exercise and these mechanisms may be altered with physical training or detraining.

Third, this is the first *in vivo* human study to use L-NIO to inhibit eNOS specifically. It is possible the development of more specific eNOS inhibitors would reveal a greater eNOS-mediated NO synthesis. Inasmuch as our passive whole body heating data are consistent with observations of a role for nNOS, but not eNOS (Kellogg *et al.* 2009), it appears L-NIO was selective for eNOS during dynamic exercise. While these limitations may underestimate our observed eNOS contribution they would not negate the main conclusions of this study.

Fourth, due to technical limitations, we were only able to measure skin temperature in two of our subjects. Measurement of skin temperature may provide more insight into mechanisms underlying the differences in reflex cutaneous vasodilatation between passive heat stress and dynamic exercise.

Perspectives

It has generally been assumed the mechanisms attending the cutaneous vasodilatation associated with exercise-induced hyperthermia are similar, if not the same, as the mechanisms associated with passive whole body heat stress. Despite similar increases in T_C, CVC and calculated contributions of NO to reflex cutaneous vasodilatation during dynamic exercise and passive heat stress, the present data in conjunction with those of others suggest not all forms of hyperthermia are similar and, in fact, yield increases in skin blood flow via different NOS mechanisms. As discussed above, it remains unknown whether vasodilators that have shown to contribute to reflex cutaneous vasodilatation during passive heat stress (e.g. vasoactive intestinal polypeptide, H_1 histamine receptors) are also involved during dynamic exercise. Yet, with regards to NO and NOS, it would appear that the magnitude of the contribution of NO to reflex cutaneous vasodilatation is the same during both dynamic exercise and passive heat stress but the increase in NO occurs via different enzymatic activity.

Conclusion

In this study, we found eNOS, not nNOS, is the NOS isoform responsible for the NO component of reflex cutaneous vasodilatation during dynamic exercise. This finding is in contrast to previous data demonstrating a role for nNOS to reflex cutaneous vasodilatation during passive whole body heat stress (Kellogg *et al.* 2008*b*, 2009). These data suggest mechanisms underlying reflex cutaneous vasodilatation may differ depending on whether the increase in T_C is via endogenous heat stress (dynamic exercise) or exogenous heat stress (passive whole body heating).

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Additional information

Conflict of interest

None.

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None.

Author contributions

This study was completed in the Department of Kinesiology at Kansas State University. T.C.M. was responsible for data collection, data analysis, interpretation of the data and drafting the manuscript. J.T.K. assisted with data collection and analysis and edited drafts of the manuscript. G.H.S. was responsible for experimental design, data interpretation and editing drafts of the manuscript. L.M.A. was responsible for experimental design, data interpretation and editing drafts of the manuscript. B.J.W. was responsible for experimental design, data collection, data analysis and interpretation, and editing drafts of the manuscript. All authors approved the final version of the manuscript.

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