

Neuronal nitric oxide synthase control mechanisms in the cutaneous vasculature of humans *in vivo*

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The physiological roles of constitutively expressed nitric oxide synthase (NOS) isoforms in humans, *in vivo*, are unknown. Cutaneous vasodilatation during both central nervous system-mediated, thermoregulatory reflex responses to whole-body heat stress and during peripheral axon reflex-mediated, local responses to skin warming in humans depend on nitric oxide (NO) generation by constitutively expressed NOS of uncertain isoform. We hypothesized that neuronal NOS (nNOS, NOS I) effects cutaneous vasodilatation during whole-body heat stress, but not during local skin warming. We examined the effects of the nNOS inhibitor 7-nitroindazole (7-NI) administered by intradermal microdialysis on vasodilatation induced by whole-body heat stress or local skin warming. Skin blood flow (SkBF) was monitored by laser-Doppler flowmetry (LDF). Blood pressure (MAP) was monitored and cutaneous vascular conductance calculated ($CVC = LDF/MAP$). In protocol 1, whole-body heat stress was induced with water-perfused suits. In protocol 2, local skin warming was induced through local warming units at LDF sites. At the end of each protocol, 56 mM sodium nitroprusside was perfused at microdialysis sites to raise SkBF to maximal levels for data normalization. 7-NI significantly attenuated CVC increases during whole-body heat stress ($P < 0.05$), but had no effect on CVC increases induced by local skin warming ($P > 0.05$). These diametrically opposite effects of 7-NI on two NO-dependent processes verify selective nNOS antagonism, thus proving that the nNOS isoform affects NO increases and hence vasodilatation during centrally mediated, reflex responses to whole-body heat stress, but not during locally mediated, axon reflex responses to local skin warming. We conclude that the constitutively expressed nNOS isoform has distinct physiological roles in cardiovascular control mechanisms in humans, *in vivo*.

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Over the last decade, a myriad of functions for nitric oxide (NO) have been elucidated in human physiological and pathophysiological processes. Indeed, the apparent surfeit of functions attributed to NO raises the question of how such a simple molecule can exert such widespread, yet diverse control over numerous bodily functions. It is now appreciated that the plethora of mechanistic roles attributed to NO derives from its generation by the different nitric oxide synthase (NOS) isoforms: neuronal NOS (nNOS, NOS I), inducible NOS (iNOS, NOS II), and endothelial NOS (eNOS, NOS III). These isoforms are found in numerous tissues, in a variety of cellular locations, with multiple regulatory controls (Murad, 2006). Recently, efforts to differentiate the various roles for the NOS isoforms have come to the fore.

For example, work in animal models has revealed that nNOS may be a key component in autonomic control mechanisms (Sears *et al.* 2004); however, to date, the mechanistic involvement of the different NOS isoforms in human physiology, *in vivo*, remains unknown.

The human skin represents a tissue with demonstrated roles for NO generation by constitutively expressed NOS in whole-body thermoregulatory reflexes and in local control of skin vessels (Kellogg, 2006). Thermoregulatory reflex control of the vasculature in non-glabrous regions of human skin is accomplished by dual innervation from the sympathetic nervous system (Lewis & Pickering, 1931; Grant & Holling, 1938). One branch is an active vasoconstrictor system that relies on noradrenaline and neuropeptide Y (NPY) as neurotransmitters and decreases

skin blood flow (SkBF) in response to periods of whole-body cold stress to conserve body heat (Kellogg *et al.* 1989; Stephens *et al.* 2004; Kellogg, 2006). The second branch is a powerful active vasodilator system that is capable of directing 60% of cardiac output, or approximately 81 min^{-1} of blood flow, to skin during periods of whole-body heat stress to dissipate body heat (Fox & Edholm, 1963; Rowell, 1977; Johnson & Proppe, 1996). This vasodilator system is made up of sympathetic cholinergic nerves that release co-transmitters to cause cutaneous active vasodilatation. The neurotransmitters involved in cutaneous active vasodilatation include acetylcholine (ACh), possibly vasoactive intestinal peptide (VIP), as well as others (Kellogg *et al.* 1995; Bennett *et al.* 2003; Wong *et al.* 2004). Studies with non-isoform specific NOS antagonists and direct measurements of NO demonstrated that NO generation by NOS mediates a significant portion of the increased SkBF induced by whole-body heating, demonstrating that cutaneous active vasodilatation is an NO-dependent process (Kellogg *et al.* 1998, 2003; Shastry *et al.* 1998, 2000; Wilkins *et al.* 2003). While it is clear that production of NO by NOS causes at least 30% of cutaneous active vasodilatation during whole-body heat stress (Dietz *et al.* 1994; Kellogg *et al.* 1998, 2003; Shastry *et al.* 1998; Wilkins *et al.* 2003), it is unclear which NOS isoforms are involved in the process.

NO generation by constitutively expressed NOS has also been found to be requisite for the vasodilatation induced by local application of heat to the skin. Local warming of the skin causes a local vasodilatation in two phases: an initial phase that appears to be dependent on antidromic release of neurotransmitter release by afferent sensory nerves (Minson *et al.* 2001) and a second prolonged plateau phase mediated by NO generation (Kellogg *et al.* 1999; Minson *et al.* 2001). The vasodilatation induced by local skin warming thus involves both local axon reflex mechanisms and NO generation by NOS and is capable of increasing SkBF to maximal levels (Pérgola *et al.* 1993; Minson *et al.* 2001; Kellogg, 2006). As with whole-body heat stress, it is unclear which NOS isoforms are involved in the vasodilatation induced by local skin warming because all studies have relied on non-isoform-specific NOS antagonists (Kellogg, 2006).

Of the three isoforms of NOS, two are constitutively expressed in human skin: eNOS and nNOS (Bruch-Gerharz *et al.* 1998; Cas-Grierson & Ormerod, 2004); thus either or both of these isoforms could generate the NO required for active vasodilatation during thermoregulatory responses to heat stress or to local warming of the skin. The eNOS isoform has been found in the endothelial cells of the cutaneous microvasculature (Zancanaro *et al.* 1999). The nNOS isoform has been found co-localized with VIP in cholinergic nerves (Ventura *et al.* 1997) as well as in sweat glands (Zancanaro *et al.* 1999). It is highly unlikely that iNOS mediates any

increase in NO in skin during heat stress because while this isoform has been detected in human skin, it is present in miniscule amounts when compared with nNOS and eNOS (Wang *et al.* 1996). Only inflammatory processes, such as skin burns or wounds, increase iNOS levels to those of nNOS and eNOS (Levin *et al.* 1996). In addition, 24 h or longer are required for sufficient expression of iNOS to generate increases in NO levels (Seo *et al.* 2002). These observations argue against a role for iNOS in cutaneous active vasodilatation. In addition, iNOS is a calcium-independent enzyme that produces large amounts of NO continuously from the time of expression until the enzyme is degraded (Kleinhart *et al.* 2004). This relatively uncontrolled aspect of iNOS contrasts greatly with the highly regulated production of NO through the calcium-activated nNOS and eNOS isoforms (Kleinhart *et al.* 2004). Therefore, of the three NOS isoforms, nNOS and eNOS are the two isoforms with potential vasomotor control roles in human skin.

Prior observations show that abolition of neurotransmitter release from cutaneous nerves by cutaneous nerve blockade or botulinum toxin eliminates cutaneous vasodilatation induced by whole-body heat stress (Pérgola *et al.* 1993; Kellogg *et al.* 1995; Minson *et al.* 2001). In direct contrast, neither cutaneous nerve blockade nor botulinum toxin altered skin vasodilatation induced by local skin warming (Pérgola *et al.* 1993; Kellogg *et al.* 1995; Minson *et al.* 2001). As functioning cutaneous cholinergic nerves are requisite for cutaneous active vasodilatation, but not for increasing SkBF during local skin warming, these vasomotor responses are probably mediated by fundamentally different mechanisms. Given that both cutaneous active vasodilator and local warming responses require NO generation by NOS, it is likely that the NOS isoforms involved in the two distinct types of cutaneous vasodilatation are different. As cutaneous active vasodilatation is mediated by cholinergic nerve co-transmission (Kellogg *et al.* 1995), the foregoing data suggest the involvement of nNOS in cutaneous vascular responses to whole-body heat stress. Since the vasodilatation induced by local skin heating is not mediated by cholinergic nerve co-transmission, the foregoing also suggests that nNOS is not involved in locally mediated, axon reflex responses. We sought to test the hypotheses that the nNOS isoform generates NO during thermoregulatory reflex vasodilatation, centrally mediated responses to whole-body heat stress, but not during increases in SkBF effected by local warming of the skin.

Methods

To test our hypotheses, we examined whether increases in SkBF during heat stress (Protocol 1) and local skin

warming (Protocol 2) were altered by blockade of nNOS with the selective antagonist 7-nitroindazole (7-NI). 7-NI is minimally soluble in purely aqueous solution and therefore it is often dissolved in vehicles that contain dimethyl sulfoxide (DMSO) (Kurihara *et al.* 1998). For example, Shastry *et al.* (Shastry & Joyner, 2002) used the addition of DMSO to microdialysis perfusates to enhance aqueous solubility of geldanamycin successfully, although they did find DMSO to possess minor vasodilating properties. In a series of preliminary experiments, we tested the effect of 5% DMSO in Ringer solution on SkBF under normothermic, cold stress, and heat stress conditions identical to those used subsequently in protocol 1 (see below). Our findings confirmed that DMSO possessed some vasodilating effects in normothermia (Shastry & Joyner, 2002). Under such conditions, the mean SkBF increase induced by DMSO was $10 \pm 2\%$. This concentration of DMSO did not alter reductions in SkBF in response to cold stress. Heat stress responses were also unaltered by the addition of 5% DMSO to the microdialysis perfusate as evidenced by SkBF reaching $65 \pm 6\%$ of maximal cutaneous vascular conductance (%max CVC) at sites perfused with Ringer solution alone and $64 \pm 5\%$ max CVC at sites perfused with 5% DMSO in Ringer solution in our preliminary studies. As 5% DMSO allowed significantly higher concentrations of 7-NI in Ringer solution than were otherwise possible, yet did not alter thermoregulatory reflex responses, we chose to use 5% DMSO in Ringer solution as our vehicle.

Based on our preliminary experiments, we chose a 2 mM concentration of 7-NI for our studies; the maximal concentration of 7-NI possible in solution with 5% DMSO in Ringer solution. We did not increase our concentration of 7-NI further because this would have required increased concentrations of DMSO, consequently increasing the potential for confounding vasodilator effects of this agent on baseline blood flow. The 7-NI was administered by intradermal microdialysis, a technique that permits the local administration of pharmacological agents into a small volume of skin. This technique allows a relatively large local drug concentration to exert purely local effects at the site of administration with no risk of systemic effects. Intradermal microdialysis probes of our own manufacture were used. Each probe was made from polyimide tubing with a 1 cm length of capillary microdialysis membrane (regenerated cellulose, 200 μm diameter, molecular cut-off 20 kDa) with reinforcement by a 51 μm diameter stainless steel wire inserted through the lumen of the membrane and tubing. Microdialysis probes were placed approximately 5 cm apart in the ventral forearm skin so that manipulations at one site did not influence the others. To place the microdialysis probe, a 25 gauge needle was inserted through the dermis using sterile technique after the topical application of ice for anaesthesia. Entry and exit points for each probe were

approximately 2–3 cm apart. The microdialysis probe was threaded through the lumen of the needle that was then withdrawn, leaving the microdialysis probe in place (Kellogg *et al.* 1998). The microdialysis membrane was situated entirely within the dermis, with entry and exit points through the skin via the polyimide tubing. In prior studies, ultrasound measurements revealed that probes placed with such a technique were 0.3–1.0 mm under the epidermal surface, and thus were within the dermal layer (Kellogg *et al.* 1999). The microdialysis probes were perfused with Ringer solution at a rate of $2 \mu\text{l min}^{-1}$ using a microinfusion pump (Harvard Apparatus, South Natick, MA, USA). Subjects then waited 140 min or more to allow for insertion trauma to resolve before additional instrumentation was placed (Anderson *et al.* 1994). After insertion trauma resolved, subjects were placed in the supine position and instrumented to monitor SkBF by laser-Doppler flowmetry (LDF) at all microdialysis sites (Moorlab Flowmeter, Moor Instruments Ltd, Devon, UK). A Finapres cuff was placed on a finger for pulse rate (PR) and mean arterial pressure (MAP) measurement by photoplethysmography (Ohmeda, Madison, WI, USA).

All subjects who volunteered for the protocols were in good health, were non-smokers, and were taking no medications. Prior to participation, written, informed consent was obtained from all subjects. The studies conformed to the standards set by the *Declaration of Helsinki* and institutional ethics committees approved all procedures. Subjects were instructed to forego caffeinated products on the day of the study. The menstrual phase of the female subjects was not assessed. All studies were performed in an air-conditioned laboratory with a constant ambient temperature of 24°C. Studies were done at the same time of day to control for any circadian variations in responses (Aoki *et al.* 2001).

Protocol 1

Ten subjects (3 male, 7 female) participated in the first protocol of the study. Their average age was 34 ± 3 years, average weight was 66 ± 5 kg, and average height was 171 ± 3 cm.

To induce thermoregulatory reflexes, subjects wore a tube-lined suit. The suit was used to control skin temperature (T_{sk}) and hence whole-body temperature by perfusion with water of different temperatures and thus effect periods of normothermia, cold stress and heat stress (Rowell *et al.* 1969; Johnson & Park, 1981). The suit was perfused with 18°C cold water to decrease T_{sk} and induce cold stress, and 48°C warm water to raise T_{sk} to 38–39°C during heating periods. Over the suit, subjects wore a water-impermeable plastic garment to insulate them thoroughly from the normothermic room environment and also to prevent evaporation of sweat.

The suit and garment covered the entire body with the exception of the head and the forearm from which LDF measurements were made. The hands and feet were also uncovered.

Internal temperature (T_{or}) was monitored with a thermocouple placed in the sublingual sulcus. Subjects were instructed not to speak during studies so as not to interfere with this measurement. T_{sk} was recorded as the weighted electrical average from six thermocouples taped on the skin surface (Rowell *et al.* 1969; Johnson & Park, 1981).

After arriving in the laboratory on the morning of the study, two intradermal microdialysis probes were placed on the ventral surface of one forearm. Subjects were prepared for the specific protocol with instrumentation as outlined above. Finally, subjects were placed in a supine position for data collection.

Data collection began with a 5–10 min normothermic control period during which T_{sk} was maintained at a normothermic level and all microdialysis sites were perfused with Ringer solution at $2 \mu\text{l min}^{-1}$. After this period, the perfusates at both microdialysis sites were changed to 5% DMSO in Ringer solution. Perfusion was maintained at $2 \mu\text{l min}^{-1}$ for 30–35 min. Following this period, the perfusate of one microdialysis site was changed to 7-NI (2 mM) dissolved in 5% DMSO and Ringer solution which served as the treated experimental site. Perfusion at a second microdialysis site was continued with 5% DMSO in Ringer solution which served as the

untreated control site for any unanticipated effects of DMSO. Perfusion rates at both sites were maintained at $2 \mu\text{l min}^{-1}$ throughout subsequent periods of cold and heat stress. After perfusing for 30–35 min to allow for the antagonist to enter the intradermal space, T_{sk} was decreased to induce cold stress for 3 min after which T_{sk} was returned to a normothermic level. T_{sk} was then raised to 38–39°C and maintained at that level for 35–50 min to induce heat stress and thus activate the vasodilator system. Whole-body heating was maintained until T_{or} had increased by approximately 1°C and SkBF had stabilized at an elevated level. After heat stress, subjects were cooled to normothermic levels. All microdialysis sites were then perfused with 56 mM nitroprusside (SNP) to effect maximal vasodilatation by an endothelium-independent mechanism at each site (Kreidstein *et al.* 1992; Kellogg *et al.* 2005). SNP perfusion was maintained for 30–40 min until a clear plateau in LDF had been achieved for data normalization. The protocol is summarized in Fig. 1.

Data are presented as group means \pm s.e.m. For data analysis, CVC was indexed as LDF (in mV) divided by MAP (in mmHg). CVC data were normalized to their respective maxima as elicited by SNP at each site. This normalization allowed for comparisons between 7-NI-treated and untreated control sites within and between subjects. The vasomotor and NO responses to heat stress were analysed by comparing the internal temperature thresholds for the initial increases in CVC during whole-body heating at the different

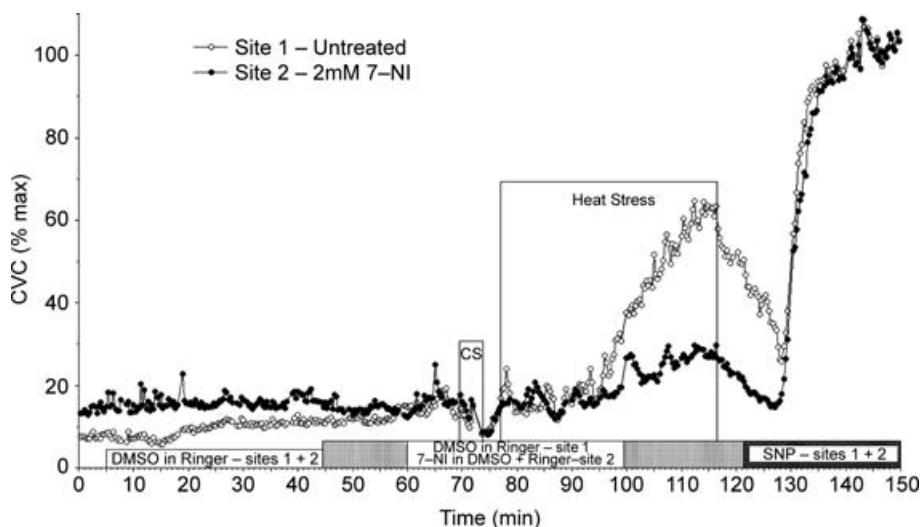


Figure 1. Protocol 1. Example of nNOS antagonism and whole-body heat stress

Two microdialysis sites were used. After an initial normothermic control period when both microdialysis sites were perfused with Ringer solution, both perfusates were changed to 5% DMSO in Ringer solution. Perfusate at one microdialysis site was then changed to a 2 mM solution of 7-NI in 5% DMSO in Ringer solution while 5% DMSO in Ringer solution was maintained at the other site that served as an untreated control. After 30–35 min of perfusion with the 7-NI, skin temperature (T_{sk}) was decreased to induce whole-body cold stress for 3 min. T_{sk} was then increased to induce whole-body heat stress for 35–50 min. Finally, subjects were cooled to normothermia and the perfusates at both sites changed to 56 mM nitroprusside to cause maximal vasodilatation.

microdialysis sites. The internal temperature threshold for the onset of vasodilatation for each site was defined as the level of T_{or} at which a sustained increase in CVC began, after T_{sk} had been increased to 38°C. T_{or} thresholds were chosen from individual graphs of CVC versus T_{or} by an investigator blinded as to the condition, subject and antagonist treatment. The thresholds for cutaneous vasodilatation were compared by paired *t* test that compared T_{or} thresholds at untreated and 7-NI-treated sites. For each site, the rate of rise of CVC with T_{or} during heat stress was calculated as the CVC change from onset of vasodilatation to the peak of heat stress divided by the corresponding T_{or} change. CVC values during normothermia, the final minute of cold stress, and the final 3 min of heat stress were compared by ANOVA followed by planned means comparisons (Kellogg *et al.* 1998). *P* values < 0.05 were deemed statistically significant.

Protocol 2

Seven subjects participated in the local skin warming protocol (4 male, 3 females). Their average age was 39 ± 3 years, average weight was 75 ± 5 kg, and average height was 169 ± 2 cm. Subjects were fully clothed with the exception of the instrumented forearm and the head so as to be comfortably normothermic throughout the experiment.

Upon arrival in the laboratory, each subject had three microdialysis probes placed on the ventral aspect of one forearm and were instrumented for LDF and MAP monitoring as previously described. Unlike in protocol 1, each LDF probe was equipped with a special probe holder equipped with heating elements to permit simultaneous

LDF measurements and control of T_{loc} at each site (Kellogg *et al.* 1999). After the LDF probes were placed, the T_{loc} at each site was increased to 34°C to ensure identical local thermal conditions at the start of the study. Perfusion of the microdialysis probes was then initiated with Ringer solution, each at a rate of $2 \mu\text{l min}^{-1}$.

Data collection began with a 5–10 min control period with T_{loc} maintained at 34°C. The perfusate at one site was then changed to 5% DMSO in Ringer solution and served as a control for any unanticipated effects of DMSO on local warming responses. Another probe's perfusate was changed to 2 mM 7-NI in the 5% DMSO in Ringer vehicle. Perfusion with Ringer solution was maintained at the third site, which served as an untreated control site. T_{loc} was maintained at 34°C for at least 45 min during perfusion with these solutions, after which T_{loc} was increased to 41.5°C at all sites for 45 min. This temperature was chosen to preclude activation of pain fibres as can happen with warming to greater temperatures and probably causes vasodilatation through mechanisms other than NO alone (Kellogg *et al.* 1999). Finally, the perfusates at all sites were changed to 56 mM sodium nitroprusside (SNP) in Ringer solution for 30–40 min to effect the maximal vasodilatation mechanism for data normalization (Kreidstein *et al.* 1992; Kellogg *et al.* 2005). SNP perfusion was maintained for 30–40 min until a clear plateau in LDF had been achieved. The protocol is summarized in Fig. 2.

Data are presented as group means \pm s.e.m. For data analysis, CVC was calculated as LDF/MAP to control for any fluctuations in blood pressure during data collection. Vasomotor responses were analysed by comparing the mean levels of CVC during the final 3 min with a T_{loc} of 34°C with the final 3 min of the 41.5°C T_{loc}

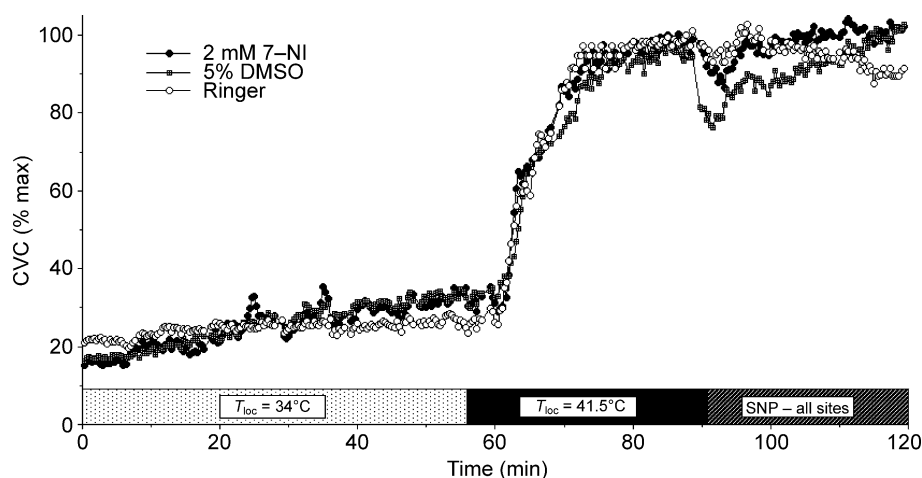


Figure 2. Protocol 2. Example of nNOS antagonism and local skin warming

Three microdialysis sites were used. One site was perfused with Ringer solution alone. A second site was perfused with 5% DMSO in Ringer. The third site was perfused with 7-NI in 5% DMSO in Ringer. During the initial part of the protocol, the local temperature (T_{loc}) of all microdialysis sites was held at 34°C. Local skin warming was then performed to raise T_{loc} to 41.5°C and maintained at that level for 45 min. Finally, the perfusates at all sites changed to 56 mM nitroprusside to cause maximal vasodilatation.

period. CVC responses were analysed by repeated measures ANOVA, followed by specific means comparisons (Kellogg *et al.* 1999). P values < 0.05 were deemed statistically significant.

Abbreviations

ACh, acetylcholine; CVC, cutaneous vascular conductance; DMSO, dimethyl sulfoxide; eNOS, endothelial nitric oxide synthase, NOS 3; HSP90, heat shock protein 90; iNOS, inducible nitric oxide synthase, NOS 2; LDF, laser-Doppler flowmetry; L-NAME, N^G -nitro-L-arginine-methyl ester; L-NMMA, N^G -monomethyl-L-arginine; MAP, mean arterial pressure; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase, NOS 1; NPY, neuropeptide Y; PR, pulse rate; SkBF, skin blood flow; SNP, sodium nitroprusside; T_{loc} , local skin temperature; T_{or} , oral temperature; T_{sk} , skin temperature; VIP, vasoactive intestinal peptide; 7-NI, 7-nitroindazole.

Results

The physical characteristics (age, height and weight) of the subject groups in protocols 1 and 2 were not statistically different. Within each protocol, no differences

were noted between responses of male or female subjects, therefore data from the two were combined for statistical analysis.

Protocol 1

Under normothermic conditions at the beginning of the protocol, T_{or} averaged $36.67 \pm 0.07^\circ\text{C}$ while T_{loc} at the LDF microdialysis sites averaged $32 \pm 1^\circ\text{C}$. During this period, when all microdialysis sites were perfused with Ringer solution only, there were no statistical differences between CVC values at the two microdialysis sites (14 ± 2 versus $18 \pm 3\%$ max CVC, $P > 0.05$). CVC increased to 24 ± 3 and $28 \pm 3\%$ max CVC at the two respective microdialysis sites during perfusion with 5% DMSO in Ringer solution ($P < 0.05$ Ringer only versus DMSO), a result consistent with our preliminary findings and those of Shastry *et al.* (Shastry & Joyner, 2002). The addition of 7-NI to the 5% DMSO in Ringer solution did not cause any significant change in CVC ($P > 0.05$) (see Fig. 3).

In response to whole-body cooling, CVC fell at both microdialysis sites. Average CVC at untreated sites fell to $17 \pm 3\%$ max and to $15 \pm 3\%$ max at 7-NI-treated sites ($P < 0.05$ versus pre-cold stress, control versus 7-NI-treated sites). These responses did not differ between the two sites ($P > 0.05$).

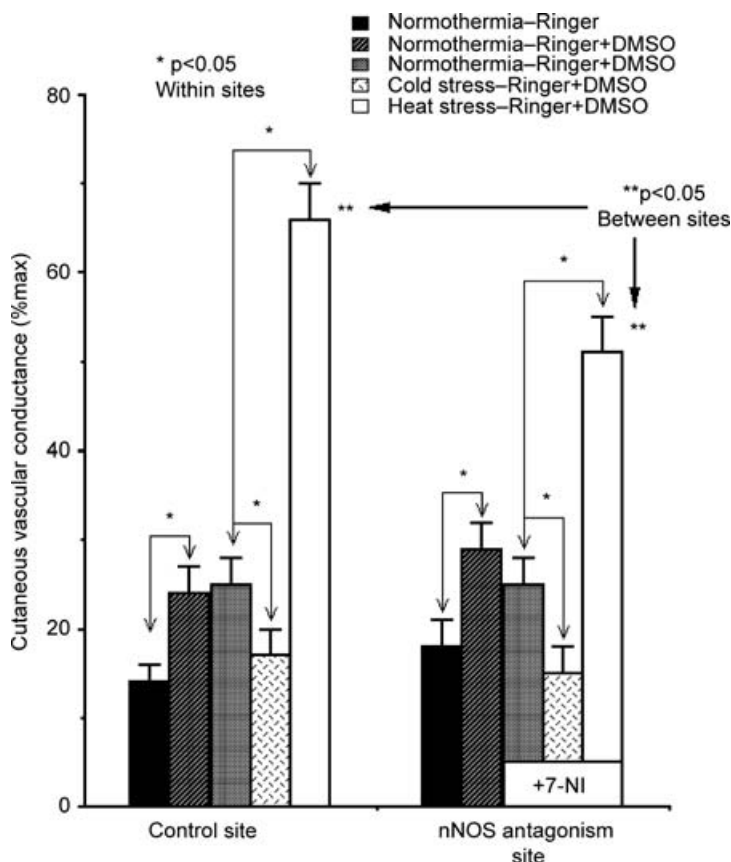


Figure 3. Cutaneous vascular conductance (CVC) responses to whole-body heat stress with and without nNOS antagonism

During normothermia, when all sites were perfused with Ringer solution only, CVC did not differ between microdialysis sites ($P > 0.05$ between sites). During perfusion of 5% DMSO in Ringer, these values increased at both sites ($P > 0.05$ between sites). Perfusion of the latter site with 2 mM 7-NI in 5% DMSO in Ringer did not change CVC values significantly ($P > 0.05$ 7-NI versus 5% DMSO in Ringer). No such trend was present at the untreated site where 5% DMSO perfusion was maintained. During whole-body cold stress, CVC fell at both sites. The CVC responses to cold stress did not differ between sites ($P > 0.05$ between sites). During whole-body heating, CVC rose at both sites. The CVC responses to whole-body heat stress differed significantly between untreated and 7-NI-treated sites, thus nNOS antagonism attenuated the cutaneous active vasodilator response to whole-body heat stress. (* $P < 0.05$ within sites; ** $P < 0.05$ between sites).

During whole-body heating, CVC began to rise at untreated sites when T_{or} reached $36.86 \pm 0.10^\circ\text{C}$. CVC began to increase at 7-NI-treated sites when T_{or} reached $36.87 \pm 0.10^\circ\text{C}$. These T_{or} values did not differ between sites ($P > 0.05$). At the peak of heat stress, T_{or} achieved a maximal level of $37.82 \pm 0.18^\circ\text{C}$ ($P < 0.01$ versus normothermia).

Under normothermic conditions, MAP averaged 75 ± 3 mmHg and tended to fall during heating (71 ± 2 mmHg, $P > 0.10$, normothermia versus heat stress). PR averaged 62 ± 8 in normothermia and increased significantly to 97 ± 6 over the course of whole-body heating ($P < 0.01$, normothermia versus heat stress).

As heat stress responses developed, CVC rose significantly at both untreated and 7-NI-treated sites ($P < 0.05$, normothermia versus heat stress within sites). The rate of rise was $55 \pm 7\%$ max CVC $^\circ\text{C}^{-1}$ at untreated sites and $33 \pm 6\%$ max CVC $^\circ\text{C}^{-1}$ at 7-NI-treated sites. The rate of rise was significantly attenuated at the 7-NI-treated sites ($P < 0.01$ between sites). At the peak of heat stress, CVC had risen to $66 \pm 4\%$ max CVC at the untreated site, but only to $51 \pm 4\%$ max CVC at the 7-NI-treated site ($P < 0.05$ untreated heat stress versus 7-NI). The overall CVC results are summarized in Fig. 3.

Protocol 2

With the skin warmed to a T_{loc} of 34°C , CVC at three microdialysis sites averaged $27 \pm 5\%$ max CVC at sites perfused with Ringer only, $28 \pm 5\%$ max CVC at sites perfused with 5% DMSO in Ringer, and $28 \pm 5\%$ max CVC at sites that received 2 mM 7-NI in 5% DMSO and Ringer. There were no significant differences between these sites at a T_{loc} of 34°C ($P > 0.05$ between sites).

During local warming of the skin to 41.5°C , CVC increased significantly to plateaus at all sites ($P < 0.05$, 34°C versus 41.5°C for all sites). At a T_{loc} of 41.5°C , CVC averaged $83 \pm 9\%$ max at sites perfused with Ringer only, $88 \pm 7\%$ max CVC at sites perfused with 5% DMSO in Ringer, and $85 \pm 5\%$ max CVC at sites that received 2 mM 7-NI in 5% DMSO in Ringer. There were no significant differences between these sites with T_{loc} values of 41.5°C ($P > 0.05$ between sites). These results are summarized in Fig. 4.

Discussion

Selective blockade of the nNOS isoform attenuated cutaneous active vasodilatation induced by whole-body heat stress, but did not alter the vasodilatation induced by local warming of the skin. These results clarify how different constitutively expressed NOS isoforms are used to control specific vasomotor responses in humans, *in vivo*:

the nNOS isoform is responsible for NO generation during centrally mediated, reflex cutaneous active vasodilatation in whole-body heat stress, but is not involved in increasing SkBF in response to axon reflex-mediated, local warming of the skin.

The results of protocol 1 show that the administration of 7-NI by intradermal microdialysis did not alter the cutaneous vasoconstriction induced by whole-body cold stress. This demonstrates that 7-NI did not measurably alter the function of noradrenergic vasoconstrictor nerves. The preservation of this response also demonstrates that 7-NI did not alter the responsiveness of skin vessels to neural inputs, thus obviating any concerns that reduced vascular responsiveness could explain attenuation of cutaneous active vasodilatation during whole-body heat stress.

While the extent of cutaneous active vasodilatation was attenuated by 7-NI treatment, the internal temperature threshold at which that active vasodilator system was first activated was unaltered by nNOS antagonism. The T_{or} at which CVC began to rise during whole-body heating did not differ between sites treated with 7-NI and untreated control sites. This finding shows that nNOS activation coincides with the initiation of cutaneous active vasodilatation. This finding is consistent with prior studies that used non-isoform-specific NOS antagonists, such as N^G -nitro-L-arginine-methyl ester (L-NAME) and N^G -monomethyl-L-arginine (L-NMMA), that found the initiation of cutaneous active vasodilatation was not altered by NOS blockade (Dietz *et al.* 1994; Kellogg *et al.* 1998; Shastry *et al.* 1998; Wilkins *et al.* 2003).

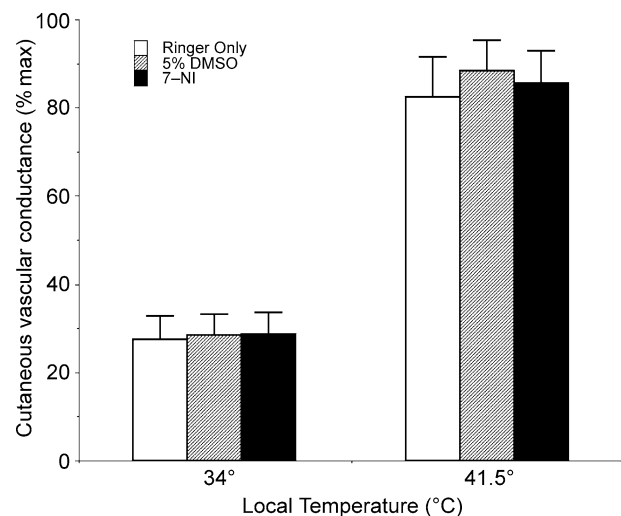


Figure 4. Cutaneous vascular conductance (CVC) responses to local heating of the skin with and without nNOS antagonism With a T_{loc} of 34°C , CVC did not differ between sites ($P > 0.05$). With local warming to 41.5°C , CVC increased significantly at all sites ($P < 0.05$, 34°C versus 41.5°C). At a T_{loc} of 41.5°C , CVC did not differ between sites ($P > 0.05$), thus nNOS antagonism failed to alter the vasodilatation induced by local warming of the skin.

While non-isoform-specific antagonists also attenuated cutaneous active vasodilatation during prolonged heat stress, it was unclear whether nNOS, eNOS or both isoforms were involved in the process. Given that 7-NI attenuated cutaneous active vasodilatation during prolonged heat stress, but did not alter the initiation of cutaneous active vasodilatation, our results show that the nNOS isoform generates NO from the very initial phase of cutaneous active vasodilatation and continues during prolonged whole-body heat stress.

Protocol 2 tested the hypothesis that nNOS is involved in increasing SkBF in response to local skin warming. In contrast to protocol 1, the results of protocol 2 show that nNOS antagonism did not alter the vasodilatation induced by local skin warming and leads us to conclude that the nNOS isoform is not involved in the generation of the NO required for increasing SkBF during local warming. Our finding in protocol 2 contrasts directly with prior studies that used the non-isoform-specific NOS antagonist L-NAME and found non-isoform-specific NOS antagonism greatly attenuated the extent of local, axon reflex vasodilatation during local skin warming (Kellogg *et al.* 1999; Minson *et al.* 2001, 2002; Houghton *et al.* 2007). Given that iNOS is not constitutively expressed in skin and that nNOS antagonism was without effect, our results strongly suggest that eNOS is the isoform that mediates peripheral, axon reflex vasodilatation during local skin heating.

The increase in CVC by 5% DMSO in normothermia for protocol 1 was not observed in protocol 2 with T_{loc} maintained at 34°C. This difference is attributable to T_{loc} being slightly lower in protocol 1 (where local LDF/heater probe holders were not used) than in protocol 2. The slightly warmer T_{loc} in protocol 2 masked the increase in CVC that was observed with 5% DMSO in protocol 1. Whole-body T_{sk} may also have differed slightly between the normothermic period of protocol 1 and the initial period of protocol 2. This difference is unlikely to confound the separate results of the two protocols because the vasodilator effect of increased T_{loc} is known to be unaltered by whole-body cooling or warming (Wenger *et al.* 1985; Pérgola *et al.* 1993).

Our conclusions regarding the physiological roles of the nNOS isoform in vasomotor control mechanisms in humans, *in vivo*, are predicated on the specificity of nNOS isoform blockade as administered by intradermal microdialysis. Since pharmacological agents such as acetylcholine can activate both nNOS and eNOS, there is no pharmacological method to definitively characterize the extent to which selective nNOS blockade was achieved (Moore *et al.* 1993*b*). The selectivity of 7-NI for the nNOS isoform lies in the differential uptake of this inhibitor into neurons, but not endothelial cells (Babbage *et al.* 1993; Moore *et al.* 1993*a,b*; Yoshida *et al.* 1994; Southan & Szabo, 1996; Jiang *et al.* 1999). Although *in vitro* studies

on the isolated enzymes have demonstrated that 7-NI can inhibit both the eNOS and nNOS, *in vivo* studies have consistently shown no evidence of an effect of 7-NI on eNOS (Babbage *et al.* 1993; Moore *et al.* 1993*a,b*; Yoshida *et al.* 1994; Southan & Szabo, 1996; Jiang *et al.* 1999). For example, *in vivo*, 7-NI has no effects on blood pressure or on endothelium-dependent relaxation of blood vessels (Babbage *et al.* 1993; Moore *et al.* 1993*a,b*; Yoshida *et al.* 1994; Southan & Szabo, 1996; Jiang *et al.* 1999).

Beyond the above evidence for the selectivity of 7-NI as an *in vivo* nNOS antagonist, the dichotomous results of protocols 1 and 2 verify the specificity of nNOS antagonism in our studies. We found the same concentration of 7-NI that significantly attenuated cutaneous active vasodilatation during whole-body heat stress failed to have any effect on skin vasodilatation induced by local skin warming. Both of these responses are known to be dependent on NO generation by NOS based on studies with non-isoform-specific antagonists (Kellogg *et al.* 1998, 1999, 2003; Shastry *et al.* 1998, 2000; Minson *et al.* 2001, 2002; Houghton *et al.* 2007). The divergent findings of an attenuating effect of 2 mM 7-NI in whole-body heat stress, but yet no effect of 2 mM 7-NI in local skin warming shows that selective nNOS had been achieved. If this were not the case, and only non-selective NOS blockade had been achieved, the vasodilatations induced by both whole-body heat stress and local skin warming would have been attenuated.

Immunohistochemical studies of human skin have found nNOS in cutaneous nerves around dermal microvessels (Ibba-Manneschi *et al.* 2006). In addition, nNOS has been found to be co-localized with VIP in cholinergic nerves (Ventura *et al.* 1997) such as those that mediate cutaneous active vasodilatation through a co-transmitter system (Kellogg *et al.* 1995). Considering the results of the present study, these observations suggest that nNOS located in cutaneous cholinergic nerves is activated to generate NO during cutaneous active vasodilatation.

Another possibility is that nNOS located in sweat glands is activated to generate NO as an adjunct to sweat production. The nNOS isoform has been found in myoepithelial cells in eccrine sweat glands in the dermis (Zancanaro *et al.* 1999). Activation of nNOS in this location could also contribute to NO generation during heat stress. Increases in NO near sweat glands could effect periglandular hyperaemia and thus contribute not only to cutaneous vasodilatation, but also to increased fluid delivery to the sweat glands to maintain sweat production. This possibility is consistent with the longstanding idea that cutaneous active vasodilatation and sweat gland activation are mechanistically linked (Grant & Holling, 1938; Brengelmann *et al.* 1981; Sugeno *et al.* 1995, 1998; Shibasaki *et al.* 2002; Kamijo *et al.* 2005).

When combined with findings of prior studies, the finding that nNOS is mechanistically involved in cutaneous active vasodilatation has a number of implications. The observation that treatment of skin with botulinum toxin abolishes cutaneous active vasodilatation shows that the neurotransmitters required for the initiation and maintenance of active vasodilatation must be stored in small and large vesicles within cholinergic nerve terminals (Kellogg *et al.* 1995; Morris *et al.* 2001). NO cannot be stored in vesicles in nerve terminals and hence, nitrergic transmission by NO is insensitive to botulinum toxin. These observations mean that the generation and release of NO is not the primary neurotransmitter mechanism for initiating or maintaining cutaneous active vasodilatation; rather, one or more neurotransmitters stored in vesicles within cutaneous cholinergic nerve terminals must be released to initiate and maintain the process. If nitrergic transmission by NO directly initiated and maintained cutaneous active vasodilatation, cutaneous active vasodilatation would be preserved after botulinum treatment, which it is not (Kellogg *et al.* 1995).

Given the foregoing findings, one or more neurotransmitters stored within the vesicles of cholinergic nerve terminals must be released to initiate and maintain cutaneous active vasodilatation. One such stored cholinergic neurotransmitter is ACh. ACh might cause NO generation through nNOS activation via pre-junctional muscarinic receptors on skin nerves or on sweat gland myoepithelial cells; however, prior observations obviate such a role for ACh. In the present study, we found that although nNOS antagonism attenuated the extent of cutaneous active vasodilatation during whole-body heating, NOS blockade did not delay the initiation of the process; this was also found in studies using non-isoform-specific antagonists (Dietz *et al.* 1994; Kellogg *et al.* 1998; Shastry *et al.* 1998; Wilkins *et al.* 2003). In addition, bioavailable NO measurements increase simultaneously with the onset of active vasodilatation during whole-body heating (Kellogg *et al.* 2003). In contrast, blockade of muscarinic receptors with atropine before whole-body heating delays the initiation of active vasodilatation (Roddie *et al.* 1957; Fox & Hilton, 1958; Kellogg *et al.* 1995), but has no effect once cutaneous active vasodilatation is established and SkBF has risen to high levels (Shastry *et al.* 2000). If ACh release and muscarinic receptor activation were involved in NO generation, both atropine and NOS antagonism should delay the onset of active vasodilatation and attenuate established active vasodilatation. Instead, the effects of atropine and NOS antagonists are dichotomous, therefore ACh activation of NOS isoforms via muscarinic receptors cannot be involved in cutaneous active vasodilatation; ACh-dependent mechanisms and NO-dependent mechanisms clearly

must be separate. This suggests that either one or more co-transmitters released from cholinergic nerves activates nNOS with consequent NO generation during whole-body heat stress and raises the possibility that ACh contributes to cutaneous active vasodilatation through other mechanisms, that perhaps include prostaglandin production (McCord *et al.* 2005).

While it is clear from the foregoing discussion that NO does not directly initiate or maintain cutaneous active vasodilatation, our findings strongly suggest that rather than acting as a direct mediator of vasodilatation, NO acts to increase synergistically the vasodilating effects of other neurotransmitters. NO generated by nNOS in cholinergic nerves and/or sweat glands could increase pre-junctional neurotransmitter release or magnify the post-junctional effects of released neurotransmitters as has been found with VIP and other peptide transmitters (Grider *et al.* 1992; Jia & Stamler, 1999; Toda & Herman, 2005). The existence of synergistic effects between NO and the neurotransmitters that cause cutaneous active vasodilatation during heat stress in humans is consistent with the foregoing model of nNOS mechanisms.

Finally, could eNOS have a role in centrally mediated reflex cutaneous active vasodilatation? The non-selective NOS antagonist, L-NAME, attenuates the increase in SkBF induced by whole-body heat stress by about 30–40% (Kellogg *et al.* 1998; Shastry *et al.* 1998, 2000). With the selective nNOS antagonist 7-NI, we were able to attenuate SkBF increases by approximately 30%. The similarity of attenuation reported for L-NAME and our finding with 7-NI suggests the possibility that nNOS is the sole NOS isoform involved in NO generation during centrally mediated reflex vasodilatation as effected by whole-body heat stress. However, in contrast to the nNOS isoform, no specific inhibitor of the eNOS isoform is available for *in vivo* studies at present. We therefore cannot exclude a role for eNOS in thermoregulatory control of the cutaneous circulation at this time.

In summary, we found that nNOS antagonism with 7-NI significantly attenuated cutaneous active vasodilatation during heat stress, but did not attenuate the vasodilatation induced by local warming of the skin. Collectively, these results show that NO generation required for cutaneous active vasodilatation in heat stress is mediated by the nNOS isoform. This result demonstrates a specific role for nNOS activation in centrally mediated thermoregulatory reflex cutaneous vasodilatation in humans. Whether only this isoform effects NO increases during cutaneous active vasodilatation remains to be proven. In contrast, generation of NO to effect the vasodilatation induced by local skin warming is not through the nNOS isoform. Whether the eNOS isoform effects NO increases during local skin warming remains to be proven. These results demonstrate a specific mechanistic role for nNOS

activation in the vasomotor control of the human vasculature.

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