

Cutaneous interstitial nitric oxide concentration does not increase during heat stress in humans

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Crandall, C. G., and D. A. MacLean. Cutaneous interstitial nitric oxide concentration does not increase during heat stress in humans. *J Appl Physiol* 90: 1020–1024, 2001.—Inhibition of cutaneous nitric oxide (NO) synthase reduces the magnitude of cutaneous vasodilation during whole body heating in humans. However, this observation is insufficient to conclude that NO concentration increases in the skin during a heat stress. This study was designed to test the hypothesis that whole body heating increases cutaneous interstitial NO concentration. This was accomplished by placing 2 microdialysis membranes in the forearm dermal space of 12 subjects. Both membranes were perfused with lactated Ringer solutions at a rate of 2 μ l/min. In both normothermia and during whole body heating via a water perfused suit, dialysate from these membranes were obtained and analyzed for NO using the chemiluminescence technique. In six of these subjects, after the heat stress, the membranes were perfused with a 1 M solution of acetylcholine to stimulate NO release. Dialysate from these trials was also assayed to quantify cutaneous interstitial NO concentration. Whole body heating increased skin temperature from 34.6 ± 0.2 to $38.8 \pm 0.2^\circ\text{C}$ ($P < 0.05$), which increased sublingual temperature (36.4 ± 0.1 to $37.6 \pm 0.1^\circ\text{C}$; $P < 0.05$), heart rate (63 ± 5 to 93 ± 5 beats/min; $P < 0.05$), and skin blood flow over the membranes (21 ± 4 to 88 ± 10 perfusion units; $P < 0.05$). NO concentration in the dialysate did not increase significantly during of the heat stress (7.6 ± 0.7 to $8.6 \pm 0.8 \mu\text{M}$; $P > 0.05$). After the heat stress, administration of acetylcholine in the perfusate significantly increased skin blood flow (128 ± 6 perfusion units) relative to both normothermic and heat stress values and significantly increased NO concentration in the dialysate ($15.8 \pm 2.4 \mu\text{M}$). These data suggest that whole body heating does not increase cutaneous interstitial NO concentration in forearm skin. Rather, NO may serve in a permissive role in facilitating the effects of an unknown neurotransmitter, leading to cutaneous vasodilation during a heat stress.

active cutaneous vasodilation; skin blood flow; thermoregulation; acetylcholine

WHOLE BODY HEATING IN HUMANS causes a substantial increase in skin blood flow (SkBF) in nonglabrous skin

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initially because of withdrawal of sympathetic vasoconstrictor activity followed by increases in sympathetic active vasodilator activity (7, 10, 12, 17, 19, 20). The active vasodilator component mediates the majority of the reflex thermoregulatory increase in SkBF (12). However, the mechanism(s), including the neurotransmitter, responsible for elevating SkBF during a heat stress remains unknown.

Previously, we (15) identified that this neurotransmitter was coreleased from cholinergic nerves, because cutaneous active vasodilation was abolished when cholinergic neurotransmission was blocked via local administration of botulinum toxin, whereas blockade of muscarinic receptors, via local administration of atropine, only partially inhibited cutaneous vasodilation associated with a heat stress. Recently, we (13) and others (21, 22) reported that nitric oxide (NO) was required for the full manifestation of cutaneous active vasodilation during a heat stress. This finding was revealed after local inhibition of NO synthase (NOS) during a heat stress, either via microdialysis or intra-arterial administration of a NOS inhibitor, which reduced the elevation in cutaneous vascular conductance by $\sim 30\%$.

In contrast to humans, blockade of NOS in the rabbit ear completely abolished elevations in SkBF during a heat stress (24). In a series of follow-up experiments, Farrell and Bishop (8, 9) revealed that NO-dependent cutaneous vasodilation in the rabbit ear did not result from an increased NO concentration associated with the heat stress. Rather, NO served in a permissive role, meaning that an unknown neurotransmitter was solely responsible for causing active vasodilation in heated rabbits but that it could due so only in the presence of a basal pool of NO.

Given these findings in the rabbit, the aforementioned observations that NOS inhibition in humans reduces the elevation in SkBF during a heat stress is insufficient to conclude that this perturbation increases NO concentration in the skin. Thus the pur-

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pose of this study was to test the hypothesis that whole body heating increases cutaneous interstitial NO concentration. This purpose was accomplished through the use of intradermal microdialysis to sample cutaneous interstitial fluid for changes in NO concentrations before a heat stress (normothermic conditions) and after SkBF was substantially elevated due to whole body heating.

METHODS

Twelve subjects (7 women and 5 men) participated in the study. The subjects' average age was 31 ± 2 yr, and all were of normal weight (73 ± 4 kg) and height (173 ± 2 cm). Each subject was informed of the purpose and risks of this institutionally approved study before providing written consent.

Instrumentation. Each subject was instrumented for the measurement of sublingual temperature (T_{sl}) with a thermistor and mean skin temperature (T_{sk}) from the electrical average of six thermocouples attached to the skin. The subject then dressed in a tube-lined suit that permitted the control of T_{sk} by changing the temperature of the water perfusing the suit. The suit covered the entire body with the exception of the head, feet, and one arm. The arm not exposed to the tube-lined suits was used to assess SkBF during the heat stress. Thus any changes in SkBF during the heat stress were not related to mechanisms associated with local heating. Heart rate was obtained from the electrocardiogram via a cardiometer.

Each subject had two microdialysis probes placed in the dermal space of the dorsal aspect of the forearm that was not exposed to the tube-lined suit. The probes were constructed in our laboratory from a semipermeable cellulose membrane (18,000 molecular weight cutoff; Spectrum) glued between two polyimide tubes (Polymicro Technologies) and reinforced by a 51- μ m-diameter stainless steel wire placed in the lumen of the membrane and polyimide tubes. The membrane window for each probe was 10 mm. The probes were placed by piercing a 25-gauge needle in the dermal space and then having the needle exit 20–25 mm away from the point of entry. The microdialysis probe was inserted through the lumen of the needle. The needle was withdrawn, leaving the probe in place. After placement, the probes were perfused with lactated Ringer solution at a rate of 2 μ l/min. A multi-fiber laser-Doppler flow probe (Perimed) was placed directly over each microdialysis membrane to assess SkBF. Laser-Doppler flow probe placement was aided through the use of markings on the polyimide tubing that indicated the center of the membrane portion of the microdialysis probe.

Experimental protocol. After instrumentation, the subjects rested for a minimum of 60–90 min before data collection to allow the hyperemic response associated with microdialysis placement to subside. Before whole body heating, dialysate draining both microdialysis membrane was collected in a

single vial for a duration of 12 min. T_{sk} was then elevated to $\sim 38.8^\circ\text{C}$ by perfusing the tube-lined suit with warm water (46°C). Once sublingual temperature was elevated ~ 0.8 – 1.3°C , a second 12-min dialysate collection period ensued. T_{sk} was then returned to normothermic levels by initially perfusing the suit with cool water ($\sim 25^\circ\text{C}$) followed by perfusing the suit with neutral water ($\sim 34^\circ\text{C}$).

After NO concentration was assayed from the first 6 subjects, it became apparent that a test was necessary to identify whether a stimulus that is known to increase NO release would result in increases in NO concentration in the dialysate. Thus, in a second group of six subjects, after the heat stress and subsequent cooling period, the membranes were perfused with a 1 M acetylcholine solution while dialysate was collected for another 12-min period. Previously, our laboratory reported that this dose of acetylcholine elicited near-maximal sweating (23) and that this was the dose at which SkBF was saturated on an acetylcholine-SkBF dose-response curve (C. G. Crandall and M. Shibasaki, unpublished observations). Dialysate from each condition was immediately frozen via dry ice after each collection period and subsequently placed in a -80°C freezer after each study. Dialysate from each trial was assayed in triplicate via the chemiluminescence technique (280 NOA, Sievers Instruments). This device uses the Radical Purger method to measure NO and its oxidation products. The coefficient of variation of these triplicate samples was 4.9%. In addition, the perfusate for each period was similarly assayed to confirm that the detection system did not artificially detect elevated NO in the Ringer or Ringer plus acetylcholine solutions.

Data collection and analysis. Heart rate and temperature data were obtained during the 12-min periods in which dialysate was collected (pre-heat stress, heat stress, and post-heat stress). These data were sampled at 10 Hz through a data acquisition system (Biopac) and averaged over the last 3 min of each period. For each period, SkBF values over both membranes were averaged to a single value. All data were statistically analyzed using one-way analysis of variance followed by a Student-Newman-Keuls multiple-comparison test when significant main-factor differences were identified. Data are expressed as means \pm SE. The level of statistical significance was set at $P \leq 0.05$.

RESULTS

Hemodynamic and temperature data are presented in Table 1. Whole body heating increased T_{sk} from 34.6 ± 0.2 to $38.8 \pm 0.2^\circ\text{C}$ and returned to $34.8 \pm 0.6^\circ\text{C}$ after the heat stress. The heat stress caused a significant elevation in T_{sl} from 36.4 ± 0.1 to $37.6 \pm 0.1^\circ\text{C}$. During the heat stress, heart rate increased from 63 ± 5 to 93 ± 5 beats/min ($P < 0.05$) and then returned toward pre-heat stress levels after the end of heating (68 ± 6 beats/min). SkBF increased significantly dur-

Table 1. Temperature and hemodynamic responses during baseline, whole body heating, and acetylcholine administration after the end of the heat stress

	Pre-Heat Stress ($n = 12$)	Heat Stress ($n = 12$)	Acetylcholine Administration ($n = 6$)
Skin temperature, $^\circ\text{C}$	34.6 ± 0.2	$38.8 \pm 0.2^*$	34.8 ± 0.6
Sublingual temperature, $^\circ\text{C}$	36.4 ± 0.1	$37.6 \pm 0.1^*$	
Skin blood flow, perfusion units	21 ± 4	$88 \pm 10^*$	$128 \pm 6^{*\dagger}$
Heart rate, beats/min	63 ± 5	$93 \pm 5^*$	$68 \pm 6^{*\dagger}$

Values are means \pm SE; n , no. of subjects who performed that procedure. *Significantly different from pre-heat stress stage, $P < 0.05$. \dagger Significantly different from heat stress stage, $P < 0.05$.

ing the heat stress from 21 ± 4 to 88 ± 10 perfusion units. Administration of 1 M acetylcholine through the microdialysis membrane further increased SkBF to 128 ± 6 perfusion units ($P < 0.05$ relative to preheat stress and heat stress values). Dialysate concentration of NO did not increase significantly during the heat stress (Fig. 1). However, administration of acetylcholine through the microdialysis membrane virtually doubled the concentration of NO in the dialysate ($P < 0.05$ relative to preheat stress and heat stress periods).

DISCUSSION

The primary finding from this study is that cutaneous interstitial NO concentration, as detected by intradermal microdialysis, does not increase during whole body heating sufficiently to increase T_{sk} $\sim 1.2^\circ\text{C}$ and increase SkBF approximately fourfold. However, we are confident that increases in cutaneous interstitial NO concentration can be detected using intradermal microdialysis, as evidenced by an approximately twofold increase in dialysate NO concentration when NO production was stimulated with acetylcholine.

The use of intradermal microdialysis to quantify cutaneous interstitial NO concentration in normothermic humans was previously performed by Clough and colleagues (5, 6). They demonstrated that basal NO concentration in the dialysate was $0.5\text{--}0.6 \mu\text{M}$ and that this concentration was reduced by 31% when the NOS inhibitor *N*-nitro-*L*-arginine methyl ester (*L*-NAME) was administered in the perfusate. Moreover, intradermal injection of histamine increased the concentration of NO in the dialysate by two- to threefold.

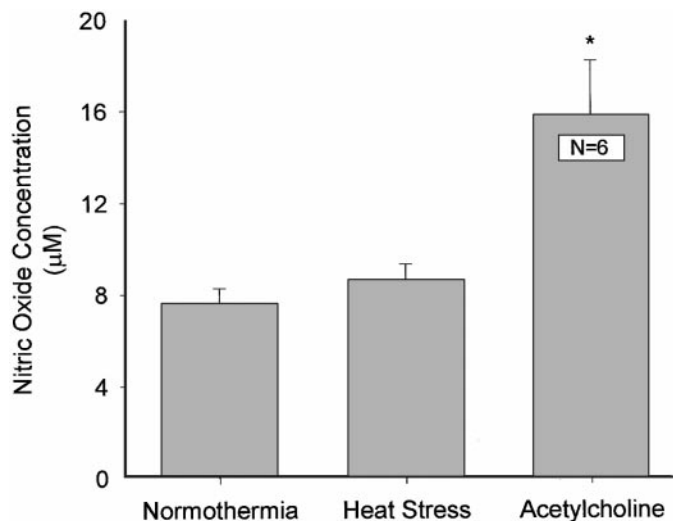


Fig. 1. Nitric oxide concentration as measured via intradermal microdialysis during normothermia, heat stress, and administration of 1 M acetylcholine in the perfusate after the heat stress. Whole body heating did not increase the concentration of nitric oxide in the dialysate from microdialysis membranes placed in forearm skin. However, after the heat stress, in 6 subjects dialysate concentration of nitric oxide was significantly elevated because of the administration of acetylcholine relative to both the normothermic and heat stress periods ($*P < 0.05$). These data suggest that cutaneous interstitial nitric oxide concentration does not increase during whole body heating in humans. Values are means \pm SE; *n*, no. of subjects.

In the present experiment, the basal concentration of NO in the dialysate was an order of magnitude greater than that observed by Clough and colleagues. Differences in basal concentration of NO in the dialysate between the present and cited studies could be due to differences in flow through the microdialysis membrane (2 vs. $5 \mu\text{l}/\text{min}$, respectively), differences in the technique used to reduce nitrate and nitrite (reaction products of NO) to NO, and/or differences in the method of analysis (chemiluminescence vs. amperometry, respectively). Nevertheless, the relative increase in dialysate concentration of NO because of the addition of acetylcholine in the perfusate was similar to that observed by Clough and colleagues in studies in which histamine was injected intradermally near the microdialysis membrane. Taken together, it is clear that intradermal microdialysis is a useful tool in assessing cutaneous interstitial NO concentration and that this technique is useful in assessing increases in NO production during perturbations such as acetylcholine and histamine administration.

In both humans (3, 7, 10, 17) and rabbits (8, 9, 25), increases in SkBF during a heat stress are primary due to an active neurogenic mechanism because nerve blockade proximal to the area where SkBF is monitored abolishes the majority of cutaneous vasodilation. In addition, inhibition of NOS prevents the elevation in SkBF in the rabbit ear (8, 9, 24) while decreasing the magnitude of elevation in SkBF in the human (13, 21, 22) during a heat stress. These findings could lead to the conclusion that NO release is increased during a heat stress and, because of its vasodilatory properties, is responsible for or contributes to the elevation in SkBF. However, findings by Farrell and Bishop (8, 9) in the rabbit ear are counter to this hypothesis. In these studies, inhibition of NOS via intra-arterial administration of *L*-NAME in heated rabbits returned ear blood flow to pre-heat stress levels. Subsequent infusion of a small dose of the NO donor sodium nitroprusside while the rabbits remained heated elevated ear blood flow to levels observed before *L*-NAME administration. However, that same dose of sodium nitroprusside administered in normothermic rabbits was insufficient to increase ear blood flow. These investigators concluded that NO played a permissive role in active vasodilation, meaning that an unknown neurotransmitter was solely responsible for causing active vasodilation in heated rabbits, but it could do so only in the presence of a basal pool of NO (8).

In humans, we (13) and others (21, 22) showed that inhibition of NOS via intra-arterial or microdialysis administration of NOS antagonists reduces the magnitude of cutaneous vasodilation during a heat stress by $\sim 30\%$. However, these data do not identify whether NO actively participates in cutaneous vasodilation or serves solely in a permissive role with an unknown neurotransmitter, as was shown in the rabbit (8, 9). The present observation of a lack of increase in cutaneous interstitial concentration of NO in heated humans, coupled with the aforementioned observation in heated rabbits (8, 9), strongly suggests a permissive

role of NO in facilitating cutaneous active vasodilation during a heat stress in humans.

Cutaneous vascular conductance in humans during local heating is reduced by ~70% when NOS is inhibited via intradermally administered L-NAME (14). This is in contrast to ~30% reduction in the magnitude of cutaneous vasodilation in the unheated arm during a heat stress in humans in which NOS is inhibited (13, 21, 22). The mechanism resulting in cutaneous vasodilation during local heating is different from that observed during whole body heating because local heating-induced cutaneous vasodilation is preserved in botulinum toxin-treated skin, whereas active cutaneous vasodilation is abolished (15). It is currently unknown whether cutaneous interstitial NO concentration increases during local heating. If local heating increases NO concentration, being that in the present experiment T_{sk} over the majority of the body is locally heated to 38.8°C during the heat stress, it is possible that systemic NO concentrations increase during the heat stress. However, even if this were to occur, it is clear from the present study that the concentration of NO released would be insufficient to cause measurable increases in cutaneous interstitial NO concentration, or its metabolites, in nonheated but actively vasodilated skin. Thus it is unlikely that increases in systemic NO concentration, if present due to local heating under the water-perfused suit, contribute to cutaneous vasodilation in unheated skin.

Limitations to the interpretation of the data. Microdialysis is a viable method of assessing the concentration of substances in the cutaneous interstitium (1, 2, 4–6, 11, 16, 18). However, whereas 100% of the substance is not diffused into the membrane, estimates of the concentration of a substance in the interstitium cannot be made without identifying the relative recovery of that substance. In the present experiment, we did not quantify the relative recovery of NO and thus we do not know the absolute concentration of NO within the interstitium. Moreover, we assumed that the relative recovery of the microdialysis membrane did not change during the course of the experiment, which is consistent with the findings of others (2). Nevertheless, identification of relative recovery was not required to address the question of interest, since the hypothesis to be tested was whether cutaneous interstitial concentration of NO increases during a heat stress in nonheated skin. If interstitial NO concentration increased during the heat stress, this would result in an increase in NO concentration in the dialysate. Thus we are confident that cutaneous interstitial NO concentration within the area measured did not increase during a heat stress. Moreover, we are confident that, had cutaneous interstitial NO concentration increased, we would have been able to identify this increase because we were able to detect increases in interstitial NO concentration during acetylcholine administration.

The technique of microdialysis uses the principle of diffusion to sample the concentration of substances in the interstitial space. If a substance is released in the

vascular space and does not readily diffuse into the interstitial space, then microdialysis cannot be used to assess changes in the release of that substance. For example, NO production may increase during a heat stress from endothelial cells on the luminal side of the vasculature and cause cutaneous vasodilation from that compartment without altering interstitial NO concentration. Moreover, it is possible that increased washout of endothelial derived NO, because of increased skin blood flow during the heat stress, limits the diffusion gradient of NO between vascular smooth muscle and cutaneous interstitium. Work by Clough and colleagues (5, 6) demonstrated that cutaneous interstitial NO concentration can be quantified using intradermal microdialysis under normothermic conditions and after stimulation of NO production via intradermal injection of histamine. In addition, we showed in the present study that cutaneous interstitial NO concentration increases in response to acetylcholine administration. Thus it is clear that NO released from the vascular endothelium is capable of being detected in the cutaneous interstitium using microdialysis. Nevertheless, the possibility remains that, during a heat stress, NO is released in sufficient quantity to elicit cutaneous vasodilation but, because of washout or diffusion limitations, does not result in detectable increases in interstitial NO concentration.

In the present experiment, we did not inhibit NOS during either the heat stress or acetylcholine administration. Previously we (13) and others (22) showed that inhibition of NOS, via inclusion of L-NAME in the perfusate of a microdialysis probe placed in the skin, inhibited the degree of cutaneous vasodilation during a heat stress. Moreover, Clough (5) showed that inhibition of NOS reduced the concentration of cutaneous interstitial NO in the dialysate in normothermic humans and during intradermal administration of histamine. Thus, to investigate the primary question in the present investigation, i.e., whether cutaneous interstitial NO concentration increases during whole body heating, it was not necessary to repeat our work, or the work of others, in demonstrating the effectiveness of NOS inhibition in reducing cutaneous vasodilation and cutaneous interstitial NO concentration.

In conclusion, whole body heating does not increase cutaneous interstitial NO concentration as measured by intradermal microdialysis. This observation, coupled with prior observations that inhibition of NOS reduces the magnitude of cutaneous vasodilation during a heat stress (13, 21, 22), raises the possibility that NO is not directly involved in cutaneous active vasodilation but rather that a basal pool of NO acts permissively with an unknown neurotransmitter to elicit active cutaneous vasodilation.

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