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Acetylcholine released from cholinergic nerves contributes to cutaneous vasodilation during heat stress

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Shibasaki, Manabu, Thad E. Wilson, Jian Cui, and Craig G. Crandall. Acetylcholine released from cholinergic nerves contributes to cutaneous vasodilation during heat stress. *J Appl Physiol* 93: 1947–1951, 2002. First published August 23, 2002; 10.1152/japplphysiol.00036.2002.—Nitric oxide (NO) contributes to active cutaneous vasodilation during a heat stress in humans. Given that acetylcholine is released from cholinergic nerves during whole body heating, coupled with evidence that acetylcholine causes vasodilation via NO mechanisms, it is possible that release of acetylcholine in the dermal space contributes to cutaneous vasodilation during a heat stress. To test this hypothesis, in seven subjects skin blood flow (SkBF) and sweat rate were simultaneously monitored over three microdialysis membranes placed in the dermal space of dorsal forearm skin. One membrane was perfused with the acetylcholinesterase inhibitor neostigmine (10 μM), the second membrane was perfused with the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 10 mM) dissolved in the aforementioned neostigmine solution (L-$NAME_{Neo}$), and the third membrane was perfused with Ringer solution as a control site. Each subject was exposed to ~20 min of whole body heating via a water-perfused suit, which increased mean body temperature from 36.4 ± 0.1 to 37.5 ± 0.1°C ($P < 0.05$). After the heat stress, SkBF at each site was normalized to its maximum value, identified by administration of 28 mM sodium nitroprusside. Mean body temperature threshold for cutaneous vasodilation was significantly lower at the neostigmine-treated site relative to the other sites (neostigmine: 36.6 ± 0.1°C, L-$NAME_{Neo}$: 37.1 ± 0.1°C, control: 36.9 ± 0.1°C), whereas no significant threshold difference was observed between the L-$NAME_{Neo}$-treated and control sites. At the end of the heat stress, SkBF was not different between the neostigmine-treated and control sites, whereas SkBF at the L-$NAME_{Neo}$-treated site was significantly lower than the other sites. These results suggest that acetylcholine released from cholinergic nerves is capable of modulating cutaneous vasodilation via NO synthase mechanisms early in the heat stress but not after substantial cutaneous vasodilation.

thermoregulation; skin blood flow; sweat rate; acetylcholinesterase; microdialysis

DURING A HEAT STRESS, skin blood flow (SkBF) increases to facilitate the transfer of heat from the subject's core to the environment. The initial increase in SkBF typically occurs via withdrawal of tonic vasoconstrictor tone. This response is followed by a powerful active cutaneous vasodilator system, which mediates 85–95% of the elevation in SkBF during a heat stress in humans (7). A number of studies have shown that the internal temperature threshold for the onset of active cutaneous vasodilation during a heat stress is modified by factors such as exercise, baroreceptor-loading status, and level of hydration (2, 5, 7, 9). However, the mechanism(s) by which these and other perturbations modulate active cutaneous vasodilation remains unclear.

Prior studies suggest a link between active cutaneous vasodilation and mechanisms associated with sweating, given the similarity of these responses during a heat stress (12, 19). In contrast, Kellogg et al. (11) suggested that the neurotransmitter(s) responsible for active cutaneous vasodilation was not acetylcholine because antagonism of muscarinic receptors, via local atropine administration, did not abolish the elevation in SkBF during the heat stress, although sweating responses were abolished. However, in that study, active cutaneous vasodilation at the atropine-treated site was delayed, and cutaneous vasodilation was inhibited by ~30% compared with the control site. The mechanism of atropine in altering these responses is of interest given recent studies showing that local inhibition of nitric oxide (NO) synthase similarly reduces cutaneous vasodilation by ~30% during a heat stress (8, 16, 17). Because acetylcholine-induced NO production occurs via muscarinic receptors (15), it seems plausible that the delay in the onset of cutaneous active vasodilation and reduction in the elevation in SkBF at atropine-treated sites may be due to attenuation of NO production via inhibition of acetylcholine binding to muscarinic receptors. Therefore, the purpose of the present study was to test the hypothesis that acetylcholine released from cholinergic nerves is capable of contributing to cutaneous vasodilation via NO-related mechanisms.

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METHODS

Seven subjects (3 women and 4 men) participated in this study. Their age, height, and weight were 29.7 ± 3.9 yr, 170.6 ± 6.1 cm, and 67.4 ± 8.1 kg, respectively. Each subject was informed of the purpose and risks of this study before providing their written consent. The consent form was approved by the University of Texas Southwestern Medical Center at Dallas and Presbyterian Hospital of Dallas.

On entering the laboratory (room temperature: 22–23°C), each subject was instrumented for the measurement of mean skin temperature from the weighted average of six thermocouples placed on the skin (20). The subject was then dressed in a tube-lined suit that permitted the control of skin temperature. The suit covered the entire body surface except for the head, feet, and forearms. A thermistor was placed in the sublingual solcus to provide an index of internal temperature. Mean body temperature was calculated by the following equation

$$\text{Mean body temperature} = 0.8 \times \text{sublingual temperature} + 0.2 \times \text{averaged skin temperature}$$

Heart rate was obtained from the electrocardiogram, and blood pressure was obtained via auscultation of the brachial artery (Tango, Suntech Medical Instruments).

Each subject rested in the supine position, while three microdialysis probes were placed in the dermal space of dorsal forearm skin. Each probe was placed at least 3 cm apart. The depth of probe placement was not identified, although similar procedures report a depth of 0.3–1.0 mm (10). The probes were constructed in our laboratory from a semipermeable cellulose membrane (18,000 molecular weight cutoff; Spectrum) glued between two polyimide tubes (MicroLumen) and reinforced with a 51-μm-diameter stainless steel wire placed in the lumen of the membrane and tubes (1, 10). The membrane window for each probe was 10 mm. The probes were placed by piercing a 25-gauge needle in the dermal space without anesthesia 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 then withdrawn, leaving the probe in place. After placement, the probes were perfused with Ringer solution at a rate of 2 μl/min. Chambers having a small window (10 × 5 mm, i.e., surface area of 0.5 cm^2) were positioned over each membrane to measure sweat rate by the ventilated-capsule method, using compressed nitrogen as the perfusion gas delivered at a rate of 150 ml/min. Location of capsule placement was aided through the use of markings on the polyimide tubing that indicated the center of the membrane portion of the microdialysis probe. Humidity of the effluent gas was measured via a humidity-temperature probe (model HMP 35E, Vaisala) that was positioned 1 m from the capsule on the skin. The humidity-temperature probe connected to a humidity data processor (model HMI 38, Vaisala) that calculated absolute humidity from the relative humidity and temperature of the effluent nitrogen. In addition, the chamber used to measure sweat rate also housed an integrating laser-Doppler flow probe (model PF413, Perimed) such that sweat rate and SkBF (model PF4000, Perimed) were assessed from the same location directly over the microdialysis membrane. Approximately 60–120 min after microdialysis membrane placement, once the hyperemic response associated with membrane placement subsided, an acetylcholinesterase inhibitor (10 μM of neostigmine) dissolved in Ringer solution and a NO synthase inhibitor [10 mM of N^G-nitro-L-arginine methyl ester (L-NAME)] dissolved in the 10 μM neostigmine solution (L-$NAME_{Neo}$) were administered through each of two microdialysis membranes. The third microdialysis membrane continued to be perfused with Ringer solution.

Protocol. To confirm the effectiveness of neostigmine in inhibiting acetylcholinesterase, each subject underwent a brief heating protocol (<10 min) in which the water perfusing the tube-lined suit was elevated. Support for this method of testing the effectiveness of acetylcholinesterase inhibition is discussed in our prior paper (18). Once sweating was detected at the neostigmine-treated sites, but not at the control site, the subject was returned to thermoneutral conditions by decreasing the temperature of the water perfusing the suit. Once stable thermal conditions were evident, a 30-min resting period ensued. This resting period was followed by a period of whole body heating by perfusing 46°C water through the tube-lined suit until substantial increases in SkBF were observed at all sites. After the heat stress, maximal SkBF at each site was identified via administration of 28 mM sodium nitroprusside through the microdialysis membranes (10).

Data collection and analysis. Data were recorded at 200 Hz (Biopac, Santa Barbara, CA) and reduced to 1-s averages. One-minute-averaged responses were then obtained from the following three periods: before the heat stress (baseline), early in the heat stress (defined as the period just before measurable increases in SkBF at the L-$NAME_{Neo}$-treated site), and at the end of heating. Mean body temperature, as opposed to sublingual temperature, was used for the identification of the threshold for cutaneous vasodilation, because at the neostigmine-treated sites sweating and cutaneous vasodilation occurred with the elevation in skin temperature but before measurable changes in internal temperature. Differences in responses between sites were compared for each level of heating via a one-way repeated-measures ANOVA, followed by a Scheffé's test when a significant main factor was identified. Moreover, the duration of time before the onset of sweating and cutaneous vasodilation, and the mean body temperature threshold for the onset of vasodilation and sweating were also compared between sites via a one-way repeated-measures ANOVA, followed by a Scheffé's test when a significant main factor was identified. Finally, the effects of the heat stress (i.e., before heating vs. end of heating) on skin, internal, and mean body temperatures were statistically compared via a paired *t*-test. All data are expressed as means ± SE. The level of statistical significance was set at $P < 0.05$.

RESULTS

Whole body heating significantly increased skin temperature from 34.6 ± 0.1 to 38.6 ± 0.2°C ($P < 0.05$), sublingual temperature from 36.9 ± 0.1 to 37.3 ± 0.1°C ($P < 0.05$), and mean body temperature from 36.4 ± 0.1 to 37.5 ± 0.1°C ($P < 0.05$). The average duration of whole body heating was 20.0 ± 1.5 min (range from 16.5 to 26 min). The onset of cutaneous vasodilation occurred significantly earlier at the neostigmine-treated site (80 ± 36 s; $P < 0.05$), relative to the onset of vasodilation at the control (246 ± 57 s) and L-$NAME_{Neo}$-treated (353 ± 42 s) sites (see Table 1). Also, the mean body temperature threshold for the onset of cutaneous vasodilation was significantly lower at the neostigmine-treated site relative to the other sites (see Fig. 1 and Table 1), whereas no statistical

Table 1. *Duration of heating before onset of sweating and cutaneous vasodilation as well as mean body temperature threshold for onset of cutaneous vasodilation and sweating*

	Onset of Sweating		Onset of Cutaneous Vasodilation	
	Time of heating, s	Body temperature threshold, °C	Time of heating, s	Body temperature threshold, °C
Control	233 ± 39*†	36.8 ± 0.1*†	246 ± 57*	36.8 ± 0.1*
Neostigmine	35 ± 11	36.4 ± 0.1	80 ± 36	36.5 ± 0.1
L-NAME$_{Neo}$	26 ± 12	36.4 ± 0.1	353 ± 42*	37.0 ± 0.1*

Values are means ± SE. L-NAME$_{Neo}$, N^G-nitro-L-arginine methyl ester plus neostigmine. *Significantly different from neostigmine-treated site, $P < 0.05$. †Significantly different from L-NAME$_{Neo}$-treated site, $P < 0.05$.

differences in this variable were observed between control and L-NAME$_{Neo}$-treated sites. Increases in sweating were observed at both the neostigmine- and L-NAME$_{Neo}$-treated sites within 35 s after the onset of heating. In contrast, the onset of sweating at the control site did not occur until 233 s after the onset of heating. Similarly, the mean body temperature threshold for the onset of sweating was significantly greater at the control site relative to the neostigmine- and L-NAME$_{Neo}$-treated sites (see Table 1).

In the early heat stress period, the increase in SkBF above baseline at the neostigmine-treated site (12.8 ± 3.2% of maximum) was significantly greater than at the other sites (Table 2), whereas no differences were observed in this variable between the control (7.6 ± 2.7% of maximum) and the L-NAME$_{Neo}$ (3.6 ± 1.0% of maximum) sites. The greater increase in SkBF early in the heat stress at the neostigmine-treated site was likely due to NO-related mechanisms, because this increase in SkBF was absent when the NO synthase inhibitor L-NAME was coinfused with neostigmine (L-NAME$_{Neo}$-treated site). It is interesting to note that increases in SkBF were not detected at the L-NAME$_{Neo}$ site until obvious cutaneous vasodilation and sweating responses were observed at the control site (Table 1).

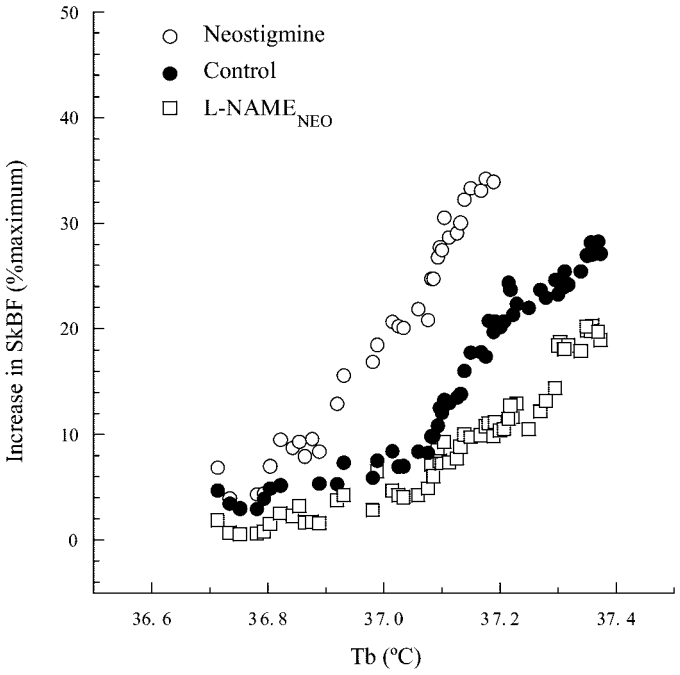

Fig. 1. Skin blood flow (SkBF) responses from a representative subject at neostigmine, control, and N^G-nitro-L-arginine methyl ester plus neostigmine (L-NAME$_{Neo}$) sites as a function of mean body temperature (T_b). At the neostigmine-treated site, the elevation in SkBF occurred at a significantly lower T_b relative to the other sites.

As the heat stress continued, sweat rate at the neostigmine- and L-NAME$_{Neo}$-treated sites remained significantly elevated relative to the control site. However, by the end of the heat stress, there were no significant differences in sweat rate between sites (neostigmine site: 1.23 ± 0.18 mg·cm^{-2}·min^{-1}, L-NAME$_{Neo}$: 0.99 ± 0.07 mg·cm^{-2}·min^{-1}, and control site: 0.92 ± 0.11 mg·cm^{-2}·min^{-1}; $P > 0.05$). Although the increase in SkBF at the neostigmine-treated site occurred significantly earlier relative to the control site, differences in SkBF between these sites were not significantly different at the end of the heat stress (Table 2). Despite similarities in sweating responses between the neostigmine- and L-NAME$_{Neo}$-treated sites, SkBF at the L-NAME$_{Neo}$ remained significantly less than at the other two sites throughout the heat stress.

DISCUSSION

The primary finding of this study is that acetylcholine released from cholinergic nerves is capable of modulating active cutaneous vasodilation during whole body heating, especially in the early phase of the heat stress. This finding is demonstrated by a significant increase in SkBF at the neostigmine-treated site early in the heat stress before measurable increases in SkBF at the L-NAME$_{Neo}$-treated site. However, a lack of significant difference in SkBF between the neostigmine-treated site and the control site at the end of heating suggests that acetylcholine released from cholinergic nerves does not contribute to active cutaneous vasodilation after a marked rise of SkBF.

On excitation, cholinergic nerves release acetylcholine and cotransmitters, which are likely peptides (6, 11, 13). Acetylcholine causes sweating through binding to muscarinic receptors on sweat glands, whereas the cotransmitter(s) released from these nerves may mediate active cutaneous vasodilation (6, 11). However, this latter point remains a matter of speculation because it is unclear whether sweating and active cutaneous vasodilation are mediated by the same cholinergic nerve. On administration of the acetylcholinesterase inhibitor neostigmine, the hydrolysis of acetylcholine is inhib-

Table 2. *SkBF before heating (baseline) and increase in SkBF from baseline during early phase of heating and at end of heat stress*

	Baseline	Early Heating; Increase From Baseline	End of Heat Stress; Increase From Baseline
Control	19.5 ± 3.1	7.6 ± 2.7	39.4 ± 2.9†
Neostigmine	29.2 ± 4.7*†	12.8 ± 3.2*†	40.0 ± 4.3†
L-NAME$_{Neo}$	13.0 ± 1.5	3.6 ± 1.0	20.3 ± 2.1

Values are means ± SE, measured as %maximum skin blood flow (SkBF). *Significantly different from control site, $P < 0.05$. †Significantly different from L-NAME$_{Neo}$-treated site, $P < 0.05$.

ited, resulting in measurable effects of increased interstitial acetylcholine concentration (18). Thus, during a heat stress, the concentration of acetylcholine at the neostigmine-treated site will increase to a greater relative magnitude compared with the increase in the cotransmitter(s) responsible for active cutaneous vasodilation, which is not affected by inhibition of acetylcholinesterase. Given that acetylcholine is a potent stimulator of NO, coupled with the present findings that early vasodilation at the neostigmine-treated site was blocked when the perfusate contained the NO synthase inhibitor (L-NAME), these data suggest that acetylcholine released from cholinergic nerves during a heat stress is capable of modulating SkBF via NO-dependent mechanisms.

Previously, Kellogg et al. (11) reported that the onset of cutaneous vasodilation was delayed during whole body heating at a site in which muscarinic receptors were blocked via local administration of atropine. In that study, the mechanism for this delay was not investigated. The observation in the present study that NO released from cholinergic nerves during a heat stress is capable of modulating cutaneous vasodilation suggests that the delay in cutaneous vasodilation at the atropine-treated site in the study of Kellogg et al. (11) may have been due to the effects of atropine in reducing acetylcholine-induced NO production.

Conversely, Shastry and colleagues (16, 17) locally administered atropine (via the brachial artery and intradermal microdialysis) during a heat stress after substantial cutaneous vasodilation was evident and found that inhibition of muscarinic receptors late in the heat stress did not significantly reduce cutaneous vascular conductance. They concluded that the contribution of NO in mediating cutaneous vasodilation was not due to acetylcholine spillover from sudomotor nerves. In agreement with those findings, as the heat stress continued in the present study, the effects of inhibiting acetylcholinesterase on modulating cutaneous vasodilation were no longer evident, because SkBF at the control and neostigmine-treated sites were not statistically different later in the heat stress. Although speculative, this finding suggests that acetylcholine does not contribute to cutaneous vasodilation later in the heat stress, perhaps because the neurotransmitter(s) responsible for active cutaneous vasodilation overrides a possible contribution from acetylcholine in facilitating cutaneous vasodilation.

Our laboratory (3) and others (4) reported that NO may contribute to active cutaneous vasodilation via enhancing the effects of the unknown neurotransmitter, as opposed to NO directly causing vasodilation. Thus a possible hypothesis leading to the present findings is that increases in SkBF at the neostigmine-treated site early in the heat stress were due to this enhanced facilitation of an unknown neurotransmitter secondary to increased NO production as a result of elevated acetylcholine concentrations. However, this hypothesis remains speculative because our laboratory (3) previously was unable to identify increases in cutaneous interstitial NO concentrations near the end of a heat stress, although NO concentrations were not measured early in the heat stress in that study. Moreover, early in the heat stress when vasodilation was observed at the neostigmine-treated site, the active vasodilator system (and thus the release of the unknown neurotransmitter) was probably not engaged because SkBF at the control site had yet to increase at this time. Another possible mechanism, and perhaps the simplest explanation for the early elevation in SkBF at the neostigmine-treated site, is the result of elevated acetylcholine concentrations leading to increased NO-mediated vasodilation.

Consistent with our prior findings (18), early in the heat stress, the elevation in sweat rate was greater at the sites receiving neostigmine relative to the control site. Moreover, the inhibition of NO synthase did not alter sweat rate throughout the heat stress, compared with the neostigmine-treated site, despite a clear suppression of cutaneous vasodilation. This latter finding is consistent with that of Kellogg et al. (11) in which sweating responses during whole body heating were also not affected by local administration of L-NAME. These data indicate that NO does not play a role in modulating sweating responses in humans, which is in contrast to that observed in the exercising horse in which systemic administration of L-NAME significantly reduced sweating (14).

Limitation of this study. Depending on environmental temperatures, the initial increase in SkBF during a heat stress may or may not be due to withdrawal of tonic sympathetic vasoconstrictor tone (7). In the present experiment, the laboratory temperature was between 22 and 23°C, which is cool enough to see some withdrawal of tonic vasoconstrictor tone early in the heat stress. In this study, early in the heat stress, SkBF increased to a greater magnitude at the neostigmine-treated site relative to the other sites (see Table 2). It is unlikely that this greater increase in SkBF was completely reliant on withdrawal of tonic vasoconstrictor tone, because if this were the case, similar magnitudes of vasodilation should have been observed at all sites. Nevertheless, we did not separate the effects of withdrawal of tonic vasoconstrictor tone from active cutaneous vasodilation, and thus we recognize that a component of the increase in SkBF at all sites during

the early phase of the heat stress may have been caused by withdrawal of tonic vasoconstrictor tone.

In the present experiment, each subject was exposed to two heat stresses. The first heat stress was brief (<10 min) and was used to test the effectiveness of neostigmine. For that heat stress, skin temperature was returned to normothermic levels once slight increases in sweating were observed at the neostigmine-treated site. However, we also observed a slight increase in SkBF at the neostigmine-treated site during this first heat stress. On the return of skin temperature to normothermic levels, although sweat rate returned to pre-heat stress levels, SkBF at the neostigmine-treated site remained slightly elevated relative to the period before this initial heat stress. Thus SkBF at the neostigmine-treated site was significantly elevated relative to the other sites before the onset of the second heat stress (see Table 2). However, we do not believe this slight elevation in SkBF at the neostigmine-treated site negatively affects the interpretation of the data. In contrast, the observation that SkBF remained elevated before the second heat stress at the neostigmine-treated site, but not at the NO synthase-inhibited site (L-$NAME_{Neo}$), which also received neostigmine, supports our hypothesis that acetylcholine released from cholinergic nerves is capable of contributing to cutaneous vasodilation via NO mechanisms.

We recognize that the location of acetylcholine released at the sweat gland (i.e., sudomotor nerves) may be outside the proposed range of measurement of the laser-Doppler device. However, it is likely that acetylcholine "spillover" near sweat glands dilates blood vessels via NO mechanisms upstream from the area sampled by the laser device. Thus the laser would detect the consequence of NO-mediated vasodilation near the sweat gland, because dilating this area would result in an increase in SkBF within the area sampled by the laser. Moreover, it remains unknown whether the source of acetylcholine resulting in NO-mediated cutaneous vasodilation is from sudomotor nerves or from other cholinergic nerves that may innervate vessels within the area sampled by the laser.

In conclusion, inhibition of acetylcholinesterase (via neostigmine) results in earlier and greater elevations in SkBF during the heat stress relative to a control site. Furthermore, when acetylcholinesterase was inhibited in combination with NO synthase inhibition (L-NAME), the aforementioned early elevation in SkBF was abolished. This latter finding suggests that the elevation in SkBF early in the heat stress may be affected by NO-mediated vasodilation, presumably because of release of acetylcholine from cholinergic nerves. In contrast, later in the heat stress, acetylcholine released from cholinergic nerves does not modulate SkBF.

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