Role of nitric oxide in methacholine-induced sweating and vasodilation in human skin

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Lee, Kichang, and Gary W. Mack. Role of nitric oxide in methacholine-induced sweating and vasodilation in human skin. J Appl Physiol 100: 1355–1360, 2006.—The purpose of this study was to determine whether the nitric oxide synthase inhibitor N⁶-nitro-L-arginine methyl ester (l-NAME) demonstrates significant muscarinic-receptor antagonism during methacholine (MCH)-stimulated sweating in human forearm skin. Three intradermal microdialysis probes were placed in the skin of eight healthy adults (4 men and 4 women). MCH in the range of 0.033–243 mM in nine steps was perfused through a microdialysis probe with and without the presence of the nitric oxide synthase inhibitor l-NAME (10 mM) or the l-arginine analog N⁶-monomethyl-L-arginine (l-NMMA; 10 mM). Local sweat rate (sweat rate) and skin blood flow (laser-Doppler velocimetry) were measured directly over each microdialysis probe. We observed similar resting sweat rates at MCh only, MCh and l-NAME, and MCh and l-NMMA sites averaging 0.175 ± 0.029, 0.186 ± 0.034, and 0.139 ± 0.027 mg·min⁻¹·cm⁻², respectively. Peak sweat rate (0.46 ± 0.11, 0.56 ± 0.16, and 0.53 ± 0.16 mg·min⁻¹·cm⁻²) was also similar among all three sites. MCh produced a sigmoid-shape dose-response curve and 50% of the maximal attainable response (0.42 ± 0.14 mM for MCh only) was shifted rightward in the presence of l-NAME or l-NMMA (2.88 ± 0.79 and 3.91 ± 1.14 mM, respectively; P < 0.05). These results indicate that nitric oxide acts to augment MCh-stimulated sweat gland function in human skin. In addition, l-NAME consistently blunted the MCh-induced vasodilation, whereas l-NMMA did not. These data support the hypothesis that muscarinic-induced dilation in cutaneous blood vessels is not mediated by nitric oxide production and that the role of l-NAME in attenuating acetylcholine-induced vasodilation may be due to its potential to act as a muscarinic-receptor antagonist.

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METHODS

Eight healthy subjects (4 men, 4 women; 24.0 ± 2.4 yr, 170.8 ± 2.9 cm, 69.4 ± 3.6 kg) volunteered to participate in this study. On a separate day, before the experiment, all subjects were familiarized with the experimental procedures and written, informed consent was obtained. All studies were approved by the Yale University School of Medicine Human Investigation Committee and started at 9:00 AM.

Dose-response curves for MCh-induced sweating were performed at three skin sites (separated by 2–3 cm) and represented control (MCh only), t-NAME treatment, and t-NMMA treatment.

Experiments were conducted in an environmental chamber controlled at a temperature of 28.0 ± 0.1°C. The subjects rested in the semirecumbent position on a dental chair with skin temperature maintained at ~34°C. Body core temperature was measured using a thermocouple placed under the subject’s tongue. Skin temperature was monitored using surface thermocouples placed at seven sites: abdomen, chest, forehead, forearm, upper arm, anterior thigh, and lateral calf.

Microdialysis probe and instrumentation. All microdialysis probes were constructed in our laboratory. The microdialysis probe consists of polyimide tubing (0.0064-in. diameter Cole-Parmer, Vernon Hills, IL), hollow-fiber microdialysis tubing, PE 10 and 50 tubing, and spring-tempered stainless steel wire (0.002-in. diameter; Alan Baird Industries, Hohokus, NJ). The spring tempered stainless steel wire is used to support the probe during construction and insertion into the tissue. The hollow fiber microdialysis membrane is a regenerated cellulose membrane with an approximate 18,000-Da molecular weight cutoff (216-μm diameter, Spectrum Laboratories, Houston, TX). The polyimide tubing is inserted 1 cm into each end of the hollow fiber microdialysis tubing and fixed with cyanoacrylate glue leaving 2.5 cm of exposed membrane. All connections were verified under a dissecting microscope. The probes were packaged individually and gas sterilized.

Under sterile condition, a microdialysis probe was placed intra-dermally at each of three sites on the dorsal aspect of the forearm using a 27-gauge needle to minimize tissue irritation and eliminate the need for local anesthetic. The entrance and exit sites on the skin were separated by at least 2.5 cm. Intramicrodialysis distance was 2.5 cm. Each microdialysis probe was fed through the inside of the needle. The needle was then removed with the probe left within the dermis. After the placement of the probes, each subject began a 150-min recovery period to allow the local skin blood flow to return to baseline levels. During this period the probes were infused with 0.9% saline at a rate of 10 μl/min with a microinfusion pump (model PHD 2000, Harvard Apparatus, Holliston, MA). To assess tissue trauma due to the probe placement, a laser-Doppler imager (Moor LDI, Moor Instruments, Devon, UK) quantified the area and magnitude of the accompanying flare reaction. The experiment was terminated if the laser-Doppler imager revealed excessive skin blood flow indicative of excessive tissue trauma.

After the 150-min recovery period, a sweat rate measurement capsule designed in this laboratory was located directly over the path of each of the hollow-fiber microdialysis probes. A humidity sensor (model HII-3610, Honeywell, Freeport, IL) and a thermocouple were placed inside the sweat rate measurement capsule. Compressed nitrogen gas was continuously perfused into the capsule at a rate of ~120 ml/min. Airflow was controlled using a high-resolution metering valve (model A-03214-81, Cole-Parmer) and continuously monitored using mass flowmeters (FMA-A2105, Omega, Stanford, CT). Subsequently, sweat rate was calculated based on relative humidity, temperature, and airflow.

Skin blood flow was measured directly over each microdialysis site by laser-Doppler flowmetry (FloLab, Moor Instruments) with a DP7a/T laser-Doppler probe consisting of eight collecting fibers on a 2-mm ring with a central delivery fiber. Data were continuously digitized and stored in data files on a personal computer. Relative humidity, temperature, and airflow in 3 capsules and skin blood flow were recorded with a 16-channel computerized data-acquisition system at a sampling rate of 100 Hz (MacLab 8e, ADInstruments, Castle Hill, Australia). Blood pressure was measured on the opposite arm using a noninvasive brachial artery blood pressure monitor (STBP model 780, Colin, Komaki, Japan) and recorded once every 3 min.

Skin blood flow was converted to cutaneous vascular conductance (CVC) by dividing laser Doppler flux (V) by mean arterial pressure (MAP; mmHg). The local sweat capsule and laser-Doppler flow probe were arranged in series directly over the path of the microdialysis probe and covered ~2 cm of the 2.5 cm of intradermal probe length.

At the start of the experiment baseline levels of sweat rate and CVC were established (10-min period) while all probes were perfused with saline at a rate of 10 μl/min. Next, site 2 on the skin was perfused with 10 mM t-NAME (Clinalifa, Läufelfingen, Switzerland) in saline, and site 3 on the skin was perfused with 10 mM t-NMMA (Clinalifa) in saline for 20 min to assess adequate local NOS inhibition. After establishment of NOS inhibition each probe was perfused with a cocktail of NOS inhibitor and MCh (Methapharm, Coral Springs, FL) dissolved in saline. MCh differs from acetylcholine slightly less resistant to hydrolysis by cholinesterases (1), eliminating the need for simultaneous treatment with an acetylcholinesterase inhibitor (27). The functional efficacy of MCh is slightly less than that of acetylcholine and appears to be related to the racemic mixture of the MCh stereoisomers (9). Work by Shibasaki and Crandall (27) established a dose-response relationship for acetylcholine-local sweat rate with an acetylcholine concentration that provoked a response halfway between baseline and maximum (EC50) of 0.0126 mM acetylcholine. Local skin temperature in these experiments was set at 40°C to optimize sweating responses (27). In a thermoneutral environment, we find that local skin temperature tends to equilibrate at ~34°C. Elevated local skin temperature may augment cholinergic sensitivity (6).

From preliminary studies, it was determined that at a local skin temperature of 34°C, the appropriate doses of MCh were 0.033, 0.1, 0.33, 1.0, 3.0, 9.0, 27.0, 81, and 243 mM. Site 1 did not receive any NOS inhibitor treatment, whereas sites 2 and 3 received a cocktail of their respective NOS inhibitor and MCh. During each dose stage the probes were perfused at a rate of 10 μl/min for 20 min; the first 10 min were discarded to exclude any carryover dose, and the second 10 min were collected for analysis. After the final 10 min of steady-state data were used for further analysis. After the final MCh dose (243 mM MCh), all three microdialysis probes were infused with 28 mM sodium nitroprusside (SNP; Abbott Hospital Products, Chicago, IL) dissolved in saline for 20 min to assess maximal vasodilation. The SNP perfusion did not contain MCh or NOS inhibitors. After perfusion with 28 mM SNP, a brachial arm cuff was inflated to 240 mmHg to occlude blood flow for 4 min and establish the voltage output of the laser-Doppler system during zero skin blood flow.

Data and statistical analysis.

Maximal local sweat rate with 243 mM MCh averaged ~0.458 mg/min. Correcting for the available capsule surface area (0.7 cm²), the average maximal local sweat rate was 0.655 mg·min⁻¹·cm⁻². Preliminary experiments indicate that the 0.7-cm² available surface area under the capsule contained an average of 130 sweat glands. However, only about half of this number of sweat glands (~68 sweat glands) is activated during perfusion of the microdialysis probe with 243 mM MCh. Sweat gland activity in these preliminary experiments was determined using an iodine and starch method described by Kuno (18). These preliminary experiments explain why the average maximal local sweat rate for MCh-induced sweating was lower than that reported for maximal local sweat rate during exercise in the heat (~1.2–1.4 mg·min⁻¹·cm⁻²). All local sweat rate values were normalized to the surface area of skin available for sweating under the capsule (0.7 cm²) but not adjusted for differences in the number of sweat glands activated.

Mean sweat rate and CVC were averaged every 30 s and examined to determine a true steady-state level during the final 10 min of data collection. The steady-state data during the final 10 min of perfusion
were used for further analysis. Dose-response curves between drugs and sweat rate or skin blood flow were constructed using commercially available software (Prism, GraphPad Software). For each condition (control, l-NAME, and l-NMMA) the data were analyzed to determine four standard parameters: baseline, maximal response, the slope (Hill slope), and the MCh EC$_{50}$ using a global-fitting technique. All the data (all 8 subjects and all 3 conditions) were pooled, and a global model was used to test for treatment effects in our series of matched experiments. Because of technical problems during one trial, CVC data from one control skin site was lost. Thus the data are reported for eight subjects with the control site having only seven trials, whereas both l-NAME and l-NMMA sites had eight trials each. We also determined the EC$_{50}$ for each individual skin site and compared the means by ANOVA to confirm our interpretation of the global-fitting analysis. Changes in skin blood flow were evaluated from changes in CVC, calculated by dividing laser-Doppler flux (V) by MAP (mmHg), and normalized to the maximal CVC level achieved during the trial. Maximal CVC was usually observed during perfusion with 28 mM SNP but in some cases maximal CVC occurred during perfusion with the highest concentration of MCh. Physiological and sweat rate responses to each MCh dose were also compared using one-way ANOVA with repeated measures. All values are presented as means ± SE of eight subjects. Statistical significance was accepted at $P < 0.05$.

**RESULTS**

**Temperature and cardiovascular responses.** Body core, mean skin, and forearm skin temperature near the probe insertion site averaged 36.87 ± 0.13, 34.32 ± 0.14, 33.96 ± 0.12°C, respectively, and remained at these levels throughout the testing session. In addition, systolic (111 ± 6 mmHg), diastolic (68 ± 2 mmHg), and mean arterial blood pressure (82 ± 4 mmHg), and heart rate (66 ± 3 beats/min) did not change at any time during the testing session.

**Sweating and skin blood responses.** Baseline skin blood flow was similar at all skin sites and averaged 34.0 ± 6.7, 31.7 ± 5.5, and 27.8 ± 6.7% of maximal CVC for MCh only, MCh and l-NAME, and MCh and l-NMMA, respectively. Before the start of construction of the MCh dose-response curve, the microdialysis probes were perfused with saline or saline plus l-NAME or l-NMMA. Resting skin blood flow decreased 33 ± 5 and 39 ± 6% ($P < 0.05$) after the 20 min of perfusion with l-NAME and l-NMMA, respectively (Fig. 1). Skin blood flow at the saline only site was unchanged during this same time.

Administration of the lowest dose of MCh (0.033 mM) produced a marked increase in skin blood flow to 65.1 ± 7.9, 54.6 ± 2.0, and 59.3 ± 6.1% of maximal CVC at MCh only, MCh and l-NAME, and MCh and l-NMMA, respectively. The increase in skin blood flow during MCh perfusion was always attenuated ($P < 0.05$) at the skin site treated with l-NAME (Fig. 2). At the highest MCh dose (243 mM), CVC averaged 88.2 ± 4.3% of maximal CVC at the MCh only site. At the MCh and l-NAME and MCh and l-NMMA sites, CVC was significantly lower ($P < 0.05$), averaging 75.0 ± 4.1, and 76.4 ± 4.75% of maximal CVC, respectively. Because of the large increase in skin blood flow with the lowest dose of MCh, we were unable to obtain a reliable estimate of the EC$_{50}$ or the Hill slope for the CVC data set. At the MCh only skin site, perfusion of the microdialysis probe with 28 mM SNP did not produce a significant increase in CVC (87.2 ± 2.6% of maximal CVC). In contrast, at MCh and l-NAME and MCh and l-NMMA skin sites, 28 mM SNP increased CVC to 92.8 ± 2.0 and 83.8 ± 5.5% of maximal CVC, respectively.

Local sweat rate during perfusion of the microdialysis probes with saline was similar at all skin sites and averaged 0.175 ± 0.029, 0.186 ± 0.034, and 0.139 ± 0.027 mg·min$^{-1}$·cm$^{-2}$ at the MCh only, MCh and l-NAME, MCh and l-NMMA skin sites, respectively. MCh significantly increased local sweat rate and the highest dose of MCh produced a local sweat rate of 0.46 ± 0.11, 0.56 ± 0.16, and 0.53 ± 0.16 mg·min$^{-1}$·cm$^{-2}$ at MCh only, MCh and l-NAME, MCh and l-NMMA, respectively. The dose-response relationship between MCh and local sweat rate showed a typical sigmoid shape (Fig. 3). On the basis of the analysis of the pooled data, the EC$_{50}$ for MCh-induced sweating was 0.42 ± 0.14 mM MCh. Administration of either l-NAME or l-NMMA caused a significant ($P < 0.05$) rightward shift of MCh-sweat rate dose-response curve and an increase in the EC$_{50}$ to 2.88 ± 0.79 and 3.91 ± 1.14 mM MCh, respectively (Fig. 3). The estimated Hill slope for each dose-response curve was similar for all conditions. The rightward shift in the sigmoid dose-response curve was similar for NOS inhibition by l-NAME or l-NMMA. After perfusion with 28 mM SNP, a brachial arm cuff was inflated to 240 mmHg to occlude blood flow for 4 min. Local sweat rate declined during the occlusion period to 53.3 ± 3.3% of maximum sweat rate at all skin sites.

**DISCUSSION**

A major finding of the present study is that NOS inhibition, sufficient to reduce resting skin blood flow by over 30%, caused a rightward shift in the dose-response relationship of MCh-local sweat rate and a significant increase in the EC$_{50}$ for MCh-induced sweating. Both l-NAME and l-NMMA caused a rightward shift of the dose-response curve. As such, the shift in the sweating response cannot be attributed to muscarinic-receptor antagonism by l-NAME, but rather it reflects an impact of NOS inhibition on MCh stimulated sweat gland function. These data indicate that NO may act to augment local sweat gland activity in humans.
Buxton et al. (2) reported that l-NAME, but not l-NMMA, significantly shifted acetylcholine-produced concentration-dependent smooth muscle contraction to the right. The authors were also able to demonstrate that the effect of l-NAME was to “reduce the potency” of acetylcholine-induced contraction of vascular smooth muscle. In addition, l-NAME successfully competed for binding on muscarinic receptors of the M2 and M3 subtype and could competitively displace carbochol from the muscarinic receptor. They concluded that l-NAME possessed some competitive antagonism with acetylcholine at the muscarinic receptor. On the basis of this hypothesis, we would expect that l-NAME would also antagonize MCh-induced sweating and dilation by competitively blocking muscarinic receptors. However, we observed that both l-NAME and l-NMMA shifted MCh-stimulated sweating to the right. One interpretation of these data is that NOS inhibition reduces local sweat rate because it blocks NO production and thereby removes the augmenting action of NO on MCh-stimulated sweating.

Our data do support an antimuscarinic action of l-NAME. Figure 2 demonstrates that l-NAME but not l-NMMA significantly limits MCh-induced cutaneous vasodilation. However, the ability of l-NMMA to inhibit sweat gland function to the same degree (similar shift in the EC50 for MCh-stimulated sweating) indicates that the antimuscarinic action of l-NAME is not the dominant pathway by which sweat gland function is modulated by NOS inhibition. Although we can state that NO acts as to augment local sweat gland activity, we cannot identify the site of action of the NOS inhibitors. Specifically, we do not know whether NOS inhibitors acted by blocking an intrinsic NOS system within eccrine sweat gland cells (29, 32) or reduced NO production in other adjacent tissues, such as cutaneous vascular endothelium (25).

Elevated local skin temperature is reported to increase cholinergic sensitivity of sweat glands (6). However, an alternative hypothesis is that elevated local skin temperature augments sweating because of an increase in NO production. Mild local skin heating (without stimulation of cutaneous nociceptors) causes a biphasic dilation of the skin (21). The second peak in the skin blood flow response to local heating is mediated by NO and is blocked by administration of l-NAME (21). It is likely that NO production during changes in skin temperature would contribute to both cutaneous dilation and augmented sweating.

It is well documented that NO can mediate vasodilation induced by acetylcholine (10, 25). In the skin, NO modulates cutaneous vasodilation associated with thermal stress (5, 14, 15, 17). NOS inhibition reduces resting skin blood flow (23) and attenuates active cutaneous vasodilation during heat stress (14, 26). In the present study, several observations appear important to note. First, we observed that resting skin blood flow was reduced by over 30% after perfusion of the microdialysis probes with 10 mM l-NAME or 10 mM l-NMMA. Thus we confirm the findings of earlier work that this concentration of NOS inhibitor is sufficient to inhibit NO production and reduce resting skin blood flow (14, 15). With l-NAME administration, the reduction in skin blood flow persisted throughout the construction of the MCh dose-response curve, whereas NOS inhibition with l-NMMA had little impact on MCh-induce dilation. The mechanism of action of acetylcholine-induced dilation in cutaneous blood vessels is somewhat unclear. At present, three possible mechanisms may contribute. The first is a muscarinic-receptor-mediated activation of NOS (16). The second is the production of vasoactive prostanoids (7, 12, 13, 24). A third possibility is the production of an endothelium-derived hyperpolarization factor (3, 11). The literature...
is divided as to the importance of the NOS system in acetylcholine-mediated dilation in the skin. However, Fig. 2 clearly shows that NOS inhibition with L-NMMA had little impact on MCh-induced dilation, whereas L-NAME produced a consistent downward shift in the MCh-induced dilation, resulting in a reduction in peak dilation. Both these NOS inhibitors produced a similar reduction in resting skin blood flow and may be viewed as equipotent in their ability to block resting NO production in the skin. The data in Fig. 2 support the hypothesis that L-NAME competes with MCh for the muscarinic receptor and thereby limits MCh-mediated dilation. This novel observation provides significant motive to reevaluate earlier work on acetylcholine-induced dilation in the skin, especially work using L-NAME as the specific NOS inhibitor. In previous work, the ability of L-NAME to attenuate acetylcholine-induced dilation may be related to either its inhibition of NO production or its antimuscarinic activity.

Second, although we were unable to analyze the dose-response curve for MCh-induced cutaneous dilation, it is clear that the EC50 for dilation is below our initial dose of MCh (0.003 mM). In addition, this low dose of MCh, which produced a CVC of >50% maximum, is much smaller than the MCh EC50 for sweating. A difference in the EC50 for dilation and sweating might be expected because MCh might diffuse a farther distance to activate sweat glands in the lower horizontal plexus than to dilate terminal arterioles in the upper horizontal plexus of the skin. Third, we noted that during peak sweating that a reduction in skin blood flow resulted in a marked decrease in local sweat rate, reaching a nadir 50% of the peak local sweat rate in ~4 min of arterial occlusion. The dependency of sweating during heat stress on skin blood flow has been demonstrated previously (19, 30). On the basis of early work, it has been suggested that the reduction in “thermal sweating” during arterial occlusion is due to a problem in neuroglandular function (8). In the present experiment, the attenuation of MCh-induced sweating indicates that the mechanism must be more closely associated with sweat gland function rather than the ability of the sudomotor nerve to communicate with the sweat gland. The available data support the hypothesis that some “level” of blood flow to the skin is required to maintain a high level of sweating during heat stress (19, 30) or, as in the present study, MCh-induced sweating. However, these data should not be interpreted to mean that sweat gland function is related to the level of skin blood flow.

Looking at the skin blood flow data in Fig. 2, one might wonder whether our dose of NOS inhibitors effectively blocks the NOS system in the cutaneous vasculature. In baseline conditions, resting skin blood flow was reduced after infusion of 10 mM L-NAME or 10 mM L-NMMA. This CVC response is consistent with some studies that have shown reductions in resting skin blood flow with NOS inhibition (23). Not all studies have demonstrated reductions in resting skin blood flow with NOS inhibition (14). Perfusion of the MCh-only probe with 28 mM SNP did not increase CVC above that measured during the highest MCh concentration (243 mM). In contrast, 28 mM SNP increased CVC above that seen with 243 mM MCh perfusion at the L-NAME-treated site. On the basis of these data, we conclude that we observed NOS inhibition at rest similar to previously reported studies using similar concentrations of L-NAME or L-NMMA. These concentrations of NOS inhibitors were clearly insufficient to blunt the majority of the MCh-induced dilation in our study.

In conclusion, in human skin muscarinic-receptor-mediated sweating is reduced by NOS inhibition. The observed reduction in sweating by both L-NAME and L-NMMA supports the hypothesis that NO acts to augment local sweat gland activity. We believe that it is unlikely that this modulation of sweating by L-NAME is related to its proposed antimuscarinic activity because L-NMMA produced a similar rightward shift in the MCh-local sweat rate dose-response curve.

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GRANTS

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