Acetylcholine-induced vasodilation is mediated by nitric oxide and prostaglandins in human skin

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Kellogg, D. L., Jr., J. L. Zhao, U. Coey, and J. V. Green. Kellogg. J Appl Physiol 98: 629–632, 2005; doi:10.1152/japplphysiol.00728.2004.—Acetylcholine (ACh) can effect vasodilation by several mechanisms, including activation of endothelial nitric oxide (NO) synthase and prostaglandin (PG) production. In human skin, exogenous ACh increases both skin blood flow (SkBF) and bioavailable NO levels, but the relative increase is much greater in SkBF than NO. This led us to speculate ACh may dilate cutaneous blood vessels through PGs and NO independently. We therefore tested the hypothesis that the vasodilation effected by exogenous ACh in skin is due to NO; however, it is not clear whether this is due to NO, prostaglandins (PGs), or other mechanisms.

Several different groups have addressed the ACh-induced vasodilator mechanisms in the human cutaneous circulation and have reported conflicting results. In separate publications, Khan et al. (16) and Noon et al. (18) reported evidence that PGs were responsible for effecting the cutaneous vasodilation caused by exogenous ACh but that NO was not. In contrast, separate papers by Shore and Morris (17) and by Berghoff et al. (2) concluded that PGs were not involved in the vasodilator response of skin blood vessels to ACh.

In the course of investigating the role of NO in thermoregulatory reflex control of skin blood flow (SkBF), we found that exogenous ACh (given by intradermal microdialysis infusion) increased both SkBF and bioavailable NO (15). Whereas it was clear from our results that cutaneous vasodilation involved increased bioavailable NO concentrations, we noted that the relative increase in SkBF was much greater than that of NO. Among the possible explanations for this finding is that ACh could cause vasodilation through NO- and non-NO-dependent mechanisms.

Given the conflicting findings of Khan et al. (16) and Noon et al. (18) with the findings of Shore and Morris (17) and Berghoff et al. (2), and given our finding of a relatively great increase in cutaneous vascular conductance (CVC) with a concomitant small increase in bioavailable NO (15), we sought to clarify the mechanism(s) by which exogenous ACh effects vasodilation in human skin. We therefore tested the hypothesis that exogenous ACh causes vasodilation in human skin by both NO- and PG-dependent mechanisms.

METHODS

The approach we chose to address our hypothesis was to antagonize the generation of NO by NO synthase (NOS) and the generation of PGs by cyclooxygenase (COX). Inhibition of these enzymes was done separately and in combination.

Delivery of all drugs was achieved by intradermal microdialysis, which permitted local administration of the agents directly into the interstitial space within small areas of skin. Drug delivery by microdialysis was combined with local SkBF measurements by laser-Doppler flowmetry (LDF; MBF3D dual-channel flowmeter, Moor Instruments, Devon, UK). This permitted monitoring of SkBF from the same small volumes of skin (~12 mm2) that received the agents. LDF measurements are specific to skin, being unaffected by blood flow in the underlying skeletal muscle tissue (21).

THE ROLE OF ENDOTHELIAL CELLS in the control on human blood vessels is well known. For example, it is widely accepted that nitric oxide (NO) produced by endothelial cells effects vasodilation in normal, healthy human vessels, including those found in the skin (3–5, 7, 9, 11, 15, 22, 23). In addition to NO, endothelial cells also produce vasoactive prostanooids, and endothelium-derived hyperpolarizing factor (EDHF). Administration of exogenous acetylcholine (ACh) to endothelial cells produces these mediators to effect vasodilation; however, which mediator(s) is (are) produced varies from tissue to tissue (20). In the case of human skin, published work makes it clear that exogenous ACh causes vasodilation in human skin vessels (14); however, it is not clear whether this is due to NO, prostaglandins (PGs), or other mechanisms.
Eleven healthy subjects (5 men and 6 women) participated in this study. For male subjects, the average age (± SE) was 30 ± 4 yr, average weight was 73 ± 6 kg, and average height was 172 ± 2 cm. For female subjects, the average age (± SE) was 30 ± 3 yr, average weight was 65 ± 5 kg, and average height was 164 ± 2 cm. All subjects were documented to be in good health by medical history and physical examination. Subjects were taking no medications, and all were nonsmokers. All subjects gave their informed consent to participate in this institutionally approved study. Subjects were instructed to refrain from caffeine intake on the day of the study. The menstrual phase of the female subjects at the time of the study was not assessed, because each subject served as their own control.

On arrival in the laboratory, each subject had four microdialysis probes made from polyimide tubing with a 1-cm length of capillary microdialysis membrane (200-μm diameter, molecular mass cutoff 20 kDa) placed on the ventral aspect of one forearm. A 25-gauge needle was inserted through the dermis using sterile technique with entry and exit points ∼2.5–3 cm apart. A microdialysis probe was threaded through the lumen of the needle that was then withdrawn, leaving the probe in place with the microdialysis membrane remaining entirely within the dermis. Probes placed with this technique lie 0.3–1 mm under the epidermal surface (11, 25). After insertion of the probes, subjects waited for at least 2 h to permit resolution of insertion trauma (1, 8, 11, 13, 22).

Mean arterial pressure (MAP) was recorded continuously from a finger (Finapres BP Monitor, Ohmeda, Madison, WI). LDF probes were held in special probe holders that permit LDF measurements and control of local skin temperature. Local skin temperature was controlled at 34°C. Room temperature was kept constant at 22°C.

For the study, subjects were placed in the supine position and instrumented. Data collection began with a 5- to 10-min baseline period during which the microdialysis probes were perfused with Ringer solution at a rate of 5 μL/min by using a microinfusion pump (Harvard Apparatus, Holliston, MA). After this baseline period, one microdialysis probe was perfused with 10 mM Nω-nitro-l-arginine methyl ester (l-NAME) dissolved in Ringer's solution to inhibit NOS (9, 11). This agent was chosen as it is a potent antagonist of all NOS isoforms (5, 9, 11, 22). A second probe was perfused with 10 mM ketorolac (Keto) dissolved in Ringer solution to inhibit PG formation. This nonselective cyclooxygenase inhibitor was chosen because both COX type 1 and COX type 2 have been found in normal human skin (6). Keto concentrations were chosen on the basis of preliminary studies. On the basis of these studies, 10 mM Keto was chosen because it was the greatest concentration that caused no consistent increase in baseline SkBF. Higher concentrations of Keto consistently caused a progressive increase in SkBF above untreated basal SkBF levels (data not shown). A third probe was perfused with a combination of both 10 mM l-NAME and 10 mM Keto dissolved in Ringer solution. The fourth probe perfused with Ringer solution for the duration of the study. These solutions were perfused for 45 min.

After the foregoing solutions had been perfused through the microdialysis probes for 45 min, a 16 mM solution of ACh was perfused through all four probes. ACh was perfused for 25 min, the time required for all sites to reach steady states on the basis of preliminary studies. After ACh perfusion, all microdialysis probes were perfused with 56 mM nitroprusside [sodium nitroprusside (SNP); Sigma Chemical, St. Louis, MO] in Ringer solution for 20–40 min to effect maximal vasodilation (9, 11). SNP was used to dilate cutaneous vessels to maximal levels no different from those achievable by raising the local temperature of the skin to 42°C (9, 11). CVC values were normalized to their respective maximal levels as achieved with SNP for data analysis (9, 12).

For data analysis, CVC was indexed as LDF (in V) divided by MAP (in mmHg). Vasomotor responses were analyzed by comparing the average levels of CVC over the last 5 min of each period. CVC responses were analyzed by repeated-measures ANOVA. Statistical significance was assessed at the 5% level. Data are presented as means ± SE.

RESULTS

The overall results of this study are illustrated in Fig. 1. Comparisons of CVC during initial untreated, baseline period of the study, when all sites were perfused with Ringer solution only, with CVCs during treatment with l-NAME, Keto, or the combination of these two agents, showed no significant change in CVC (P > 0.05 for all sites vs. untreated, baseline period). There was a trend for an increase at the Keto treated site; however, this did not reach statistical significance.

Perfusion of ACh increased CVC to 79 ± 4% CVCmax at untreated control sites (P < 0.05 vs. pre-ACh). At l-NAME-treated sites, ACh increased CVC to 46 ± 6% maximal CVC (P < 0.05 vs. pre-ACh). At sites treated with Keto, ACh increased CVC to 55 ± 7% maximal CVC (P < 0.05 vs. pre-ACh). Comparison of average CVC at the l-NAME-treated site with average CVC at the Keto site showed a trend for a greater reduction at the l-NAME-treated site; however, this did not reach statistical significance (P < 0.05 between sites).

At sites that were treated with a combination of l-NAME and Keto, ACh increased CVC to 31 ± 5% maximal CVC (P < 0.05 vs. pre-ACh). Comparison of the responses at sites treated with both l-NAME and Keto in combination with sites treated with the agents separately showed that the combination of the two agents attenuated the vasodilation induced by ACh to a greater extent than either agent administered separately (P < 0.05 for both comparisons).

DISCUSSION

The major finding of our study is that exogenous ACh effects vasodilation in human skin by mechanisms that involve...
both NO and PG production. This finding is based on the observations that 1) inhibition of NOS generation of NO by l-NMMA attenuated the increases in CVC caused by ACh, 2) inhibition of COX production of PG by Keto attenuated the increases in CVC caused by ACh, and 3) simultaneous inhibition of NO and PG production by the combination of l-NMMA and Keto attenuated the increase in CVC caused by ACh to a greater extent that either antagonist separately. On the basis of our present and prior findings (9, 14, 15), in the human cutaneous circulation, ACh effects vasodilation by binding to muscarinic receptors that, in turn, causes an increase in NO generation by NOS and an increase in PG production by COX. Thus both NO- and PG-dependent mechanisms are involved in ACh-induced cutaneous vasodilation.

Our results with the microdialysis technique demonstrate that both NO and PG play a role in ACh-induced vasodilation in human skin and contrast with several prior studies. Those studies used iontophoresis to deliver exogenous ACh to skin in combination with systemic antagonist administration.

In 1996, Morris and Shore (17) found that a single oral dose of aspirin did not attenuate the SkBF response to exogenous ACh. This led them to conclude that PG were not involved in the response.

Subsequent work by Khan et al. (16) and by Noon et al. (18) employed systemic administration of the NOS inhibitor Nω-monomethyl-l-arginine (L-NMMA) or aspirin to clarify the roles of NO and PGs in the cutaneous vascular response to ACh. Both groups found attenuation of ACh-induced vasodilation with aspirin, but not with l-NMMA, and concluded that exogenous ACh mediates vasodilation in skin by causing the production of vasodilator prostanoids rather than by NO production.

In 2002, Berghoff et al. (2) administered four different oral doses of aspirin with a maximal dose of 1,944 mg given before ACh administration by iontophoresis. These authors found no effect of any aspirin dose and concluded that PGs did not contribute to ACh induced vasodilation in skin.

In comparing the design of the above studies with our design, two major differences are apparent. First, all of the prior studies relied on systemic administration of antagonists in an attempt to inhibit the local administration of ACh. We used local intradermal microdialysis to deliver our antagonists. By using a localized delivery of drug, rather than a systemic delivery, we were able to achieve a sufficiently high concentration of antagonist to attenuate the effects of ACh. Prior studies that relied on systemic drug administration were unable to achieve the necessary local concentration of antagonist in the skin because of the whole body distribution of their antagonists.

A second difference is in the way ACh was delivered into the skin. All of the prior studies used local iontophoresis, an essentially noninvasive technique, to deliver ACh. We used local intradermal microdialysis, a minimally invasive technique, to deliver ACh. Both of these techniques can be used to achieve a sufficiently high local drug concentration of ACh to effect a cutaneous vasodilation while avoiding the effects of systemic drug administration (9, 10); however, the variability of SkBF responses to ACh has been reported to be problematic with iontophoretic administration. In 2002, Ramsay et al. (19) investigated whether variations of the electrical resistance characteristics of skin between persons could result in variability in the cutaneous vascular responses to ACh administration by iontophoresis. These authors used oral administration of aspirin to alter PG production followed by ACh iontophoresis, a design quite similar to the foregoing studies. They found an apparent attenuation of ACh-induced vasodilation by aspirin; however, this apparent effect was lost after inter-subject differences in skin electrical resistance were accounted for. These findings suggest that electrical characteristics of human skin can confound iontophoretic drug doses. Our use of microdialysis to administer ACh obviated this problem.

Whereas our results show that the NO and PG mechanisms both mediate the cutaneous vasodilation caused by exogenous ACh, because of the possibility of incomplete inhibition of NOS and COX by the doses of antagonists we chose, we are unable to exclude a vasodilatory role for EDHF in skin. That is, our inability to abolish the vasodilation induced by ACh with the combination of l-NMMA and Keto could be due to either incomplete inhibition of NOS and COX at the concentrations we used, or due to the involvement of other mediators, in particular, EDHF (19).

Our results have significant implications in regard to tests of endothelial function. A frequently adopted approach to assess the physiological state of the endothelium, i.e., endothelial function, is to monitor the vasodilation caused by exogenous ACh. This pharmacological test is based on the observation that activation of muscarinic receptors by ACh causes the release of NO from endothelial cells, which, in turn, leads to relaxation of vascular smooth muscle, vasodilation, and a recordable increase in blood flow. This pharmacological test has been used in vivo, in humans, including studies done in the cutaneous circulation, despite an incomplete understanding of the physiological mechanisms involved. Given our results, the administration of ACh in human skin does not represent a simple test of the endothelial NO system; rather, it is a simultaneous test of cholinergic-muscarinic activation of at least NO and PG production by endothelial cells. Other mediators such as EDHF may also be involved (19). The pharmacological test of comparing endothelium-dependent vasodilations caused by exogenous ACh with endothelium-independent vasodilations caused by SNP is clearly not simple to interpret in the human cutaneous circulation. Although such comparisons do represent a test of “endothelial function,” this function is not limited to the NO system in human skin.

In addition to the foregoing implication, our study suggests that PGs could have a heretofore unrecognized role in the reflex cutaneous vasodilation effect by whole body heat stress. It is known that ACh release from cutaneous sympathetic nerves effects part of the SkBF increases during heat stress, along with one or more cholinergic cotransmitters (14). In addition, Shibasaki et al. (24) have reported that ACh released from cholinergic nerves contributes to cutaneous vasodilation via NOS mechanisms during early heat stress. Our finding that ACh also vasodilates through PG mechanism suggests that vasodilator prostanoids may have a role in increasing SkBF during heat stress. It is thus possible that, during heat stress, ACh released from cutaneous nerves causes thermoregulatory cutaneous active vasodilation in part by PG production and in part by NO production (14, 15). This suggestion clearly warrants further study.

In summary, we found that the cutaneous vasodilation induced by exogenous ACh can be attenuated by NOS inhibition...
with L-NAME and inhibition of COX by Keto. The combination of NOS and COX inhibition produced an even greater attenuation than inhibition of either separately. We conclude that the vasodilation induced by exogenous ACh in human skin is mediated in part by NOS activation and consequent NO production and in part by COX activation and consequent PG production.

**GRANTS**

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**REFERENCES**