Nitric oxide synthase inhibition does not alter the reactive hyperemic response in the cutaneous circulation

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Wong, Brett J., Brad W. Wilkins, Lacy A. Holowatz, and Christopher T. Minson. Nitric oxide synthase inhibition does not alter the reactive hyperemic response in the cutaneous circulation. J Appl Physiol 95: 504–510, 2003. First published April 11, 2003; 10.1152/japplphysiol.00254.2003.—Reactive hyperemia is the sudden rise in skin blood flow after release of an arterial occlusion. Currently, the mechanisms mediating this response in the cutaneous circulation are poorly understood. The purpose of this study was to 1) characterize the reactive hyperemic response in the cutaneous circulation and 2) determine the contribution of nitric oxide (NO) to reactive hyperemia. Using laser-Doppler flowmetry, we characterized reactive hyperemia after 3-, 5-, 10-, and 15-min arterial occlusions in 10 subjects. The total hyperemic response was calculated by taking the area under the curve (AUC) of the hyperemic response minus baseline skin blood flow (SkBF) [i.e., total hyperemic response = AUC – [baseline SkBF as % maximal cutaneous vascular conductance (CVCmax) × duration of hyperemic response in s)]. For the characterization protocol, the total hyperemic response significantly increased as the period of ischemia increased from 5 to 15 min (P < 0.05). However, the 3-min response was not significantly different from the 5-min response. In the NO contribution protocol, two microdialysis fibers were placed in the forearm skin of eight subjects. One site served as a control and was continuously perfused with Ringer solution. The second site was continuously perfused with 10 mM Nω-nitro- L-arginine methyl ester (L-NAME) to inhibit NO synthase. CVC was calculated as flux/mean arterial pressure and normalized to maximal blood flow (28 mM sodium nitroprusside). The total hyperemic response in control sites was not significantly different from L-NAME sites after a 5-min occlusion (3,261 ± 890 vs. 2,907 ± 531% CVCmax·s). Similarly, total hyperemic responses in control sites were not different from L-NAME sites (9,155 ± 1,121 vs. 9,126 ± 1,088%CVCmax·s) after a 15-min arterial occlusion. These data suggest that NO does not directly mediate reactive hyperemia and that NO is not produced in response to an increase in shear stress in the cutaneous circulation.

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reactive hyperemia in the cutaneous microvasculature. Previous studies in the forearm have examined the role of NO after brief periods (≤5 min) of arterial occlusion (1, 7, 15, 26). However, it is possible that NO plays a larger role after longer periods of ischemia (≥10 min) when shear stress is much greater. To this end, we tested the hypothesis that inhibition of NO synthase (NOS) would significantly attenuate the total hyperemic response after a 15-min arterial occlusion but not after 5 min of ischemia. To test this hypothesis, we measured SkBF via noninvasive laser-Doppler flowmetry after 5- and 15-min arterial occlusions in a control site and a NOS-inhibited site.

METHODS

Subjects

Seven men (26 ± 2 yr) and three women (24 ± 4 yr) participated in the characterization protocol. An additional four men (23 ± 1 yr) and four women (20 ± 1 yr) participated in the NO contribution protocol. All subjects were healthy, did not have diabetes, were nonsmokers, had no history of cardiovascular disease, and were not taking any medications other than two of the female subjects who were on oral contraceptives. Because there were no observable differences in the data of the two subjects on oral contraceptives, the data were grouped. The Institutional Review Board of the University of Oregon approved this study, and all subjects gave written and verbal informed consent before participating in the study.

Subject Monitoring

Studies were performed in a thermoneutral laboratory with the subjects in a supine position and the experimental arm at heart level. Subjects’ electrocardiogram and respiration were continuously monitored for the duration of each protocol (Cardiocap, Datex Ohmeda). To ensure that changes in red blood cell (RBC) flux were not due to changes in pressure, each subject’s blood pressure was measured via auscultation during the peak hyperemic response as well as every 3–5 min for the duration of each protocol.

SkBF Measurement

As an index of SkBF, RBC flux was measured by using noninvasive laser-Doppler flowmetry (mooLAB, Moor Instruments, Devon, UK) in both protocols. In the characterization protocol, two integrated probes were used, in conjunction with local skin heaters, to continuously monitor RBC flux at two sites. Two integrated probes were used in the NO contribution protocol to monitor RBC flux at a NOS-inhibited site and at a control site.

Specific Protocols

Characterization protocol. The purpose of this protocol was to characterize the SkBF response to four different arterial occlusion durations. The SkBF responses to 3-, 5-, 10-, and 15-min arterial occlusions were compared in 10 subjects. Each subject completed a trial for each of the four time periods on separate days. A trial consisted of three arterial occlusions and subsequent reactive hyperemia with a 20-min recovery period between each occlusion.

Arterial occlusion was achieved by inflating a blood pressure cuff placed on the upper arm to 250 mmHg. RBC flux was monitored over two sites on the volar aspect of the nondominant forearm via laser-Doppler flowmetry. When SkBF values returned to baseline after the third occlusion and reactive hyperemia, maximal SkBF was achieved by locally heating each site to 43°C. This is a temperature that has been shown previously to elicit maximal dilation (11, 27).

NO contribution protocol. The purpose of this protocol was to determine the NO contribution to reactive hyperemia in the cutaneous circulation after a 5- and 15-min arterial occlusion. Subjects were randomly assigned to undergo either a 5- or 15-min arterial occlusion. Subjects returned to the laboratory at least 1 wk later to undergo the other occlusion time period. Two microdialysis fibers (MD 2000, Bioanalytical Systems) with a membrane length of 10 mm and a 20-kDa membrane cutoff were placed at least 5 cm apart in the forearm skin of the nondominant arm. Placement of the microdialysis fibers was achieved by inserting a 25-gauge needle through the skin with entry and exit points ∼2.5 cm apart. The microdialysis fiber was then threaded through the lumen of the needle. The needle was withdrawn from the skin, leaving the microdialysis membrane in place.

One of the microdialysis sites was randomly chosen as the experimental site and was perfused with 10 mM Nω-nitro-L-arginine methyl ester (L-NAME; Calbiochem, San Diego, CA) dissolved in Ringer solution at a rate of 2 μl/min for the duration of the protocol to inhibit NOS. Our laboratory has shown previously that 10 mM L-NAME adequately inhibits NOS in the skin (16, 17). The second site served as a control site and was continuously perfused with Ringer solution at a rate of 2 μl/min.

After the insertion trauma had subsided, integrated laser-Doppler probes were placed directly over the microdialysis membranes to continuously measure RBC flux. L-NAME was then infused through the experimental site for at least 20 min. A stable 10-min baseline was recorded before the first arterial occlusion. Arterial occlusion was achieved by inflating a blood pressure cuff placed on the upper arm to 250 mmHg. Arterial occlusion and the resulting reactive hyperemia were performed three times with a 20-min recovery period between each occlusion.

After the third arterial occlusion and reactive hyperemia, RBC flux was allowed to return to baseline, after which maximal RBC flux values were achieved via infusion of 28 mM sodium nitroprusside (SNP; Nitropres, Ciba Pharmaceuticals) for 20–30 min. This concentration of SNP has been found to sufficiently cause maximal vasodilation in the cutaneous circulation (11, 16, 17).

Data Analysis

Data were digitized and saved on a personal computer at 100 Hz using Windaq data acquisition software (Dataq Instruments, Akron, OH). Data were analyzed off-line using signal-processing software. For both protocols, RBC flux values from the laser-Doppler units were divided by mean arterial pressure (MAP) to yield a value of cutaneous vascular conductance (RBC flux × MAP = CVC). RBC flux values were then calibrated to 0 during the period of arterial occlusion and 100 during maximal blood flow (local heating for the characterization protocol or SNP infusion for the experimental protocol). Expression of data in this manner takes into account any changes in blood flow due to changes in blood pressure and also better reflects absolute changes in SkBF. Thus data are presented as a percentage of maximal CVC (%CVCmax).
Calculations and Statistical Analysis

Peak blood flow was defined as the highest blood flow value after release of the pressure cuff, and values were expressed as %CVC_max. The total hyperemic response was calculated by taking the area under the curve (AUC) of the hyperemic response. Baseline SkBF values before arterial occlusion were then multiplied by the time it took for the blood flow to return to baseline levels from the time the pressure cuff was released. This value was subsequently subtracted from the value obtained for the AUC [i.e., total hyperemic response = AUC – (baseline SkBF as %CVC_max × duration of hyperemic response in s)]. Thus differences in baseline between subjects or between L-NAME and control sites were accounted for, and the total hyperemic response was evaluated with regard to the increase in SkBF above baseline. The data for the total hyperemic response were expressed as %CVC_max.

For a given trial and occlusion time period, the two most consistent responses for each subject were averaged for subsequent analysis. Data for the four different occlusion periods in the characterization protocol were compared by using a one-way ANOVA and are expressed as %CVC_max ± SD. For the NO contribution protocol, data were evaluated by using a two-way ANOVA and are expressed as %CVC_max ± SE. For both protocols, the Student-Newman-Keuls post hoc analysis test was used to identify points of significance once main effects were identified. Significance for both protocols was set at P < 0.05.

RESULTS

Characterization Protocol

Figure 1 is a representative SkBF response to a 15-min arterial occlusion from one subject and depicts the baseline, period of zero flow (i.e., the period of the arterial occlusion), peak blood flow, and the resulting hyperemic response.

AUC. Figure 2A displays the group data for the total hyperemic response to the four different periods of ischemia for the characterization protocol. The between-subject coefficient of variation for all three responses was 23% for the 3-min occlusion, 25% for the 5-min occlusion, 20% for the 10-min occlusion, and 17% for the 15-min occlusion.

The between-subject coefficient of variation was 19% for the 3-min occlusion, 74% for the 5-min occlusion, 39% for the 10-min occlusion, and 40% for the 15-min occlusion. The within-subject coefficient of variation for the two responses used in data analysis was 10, 6, 8, and 11% for the 3-, 5-, 10-, and 15-min occlusions, respectively. The within-subject coefficient of variation for all three responses was 23% for the 3-min occlusion, 25% for the 5-min occlusion, 19% for the 10-min occlusion, and 17% for the 15-min occlusion.

Peak CVC values. The effect of occlusion duration on the peak response is summarized in Figure 2B. Peak

Fig. 1. Representative tracing from 1 subject’s response to a 15-min arterial occlusion. Depicted are the baseline, period of arterial occlusion, peak flow after cuff release, and the resulting reactive hyperemia. CVC_max, maximal cutaneous vascular conductance.

Fig. 2. A: group data for the total hyperemic response to the 4 different periods of ischemia for the characterization protocol. B: group data for the peak blood flow values after 4 different time periods of arterial occlusion for the characterization protocol. Values are means ± SD. AUC, area under the curve; RH, reactive hyperemia; 3, 5, 10, 15 min, respectively. *Significantly different from the 15-min occlusion, P < 0.05. †Significantly different from the 10-min occlusion, P < 0.05. ‡Significantly different from the 3-min occlusion, P < 0.05.
flows after a 3-min occlusion (37 ± 12% CVC_max) were significantly less than those after 5-min (50 ± 11% CVC_max; P < 0.05), 10-min (57 ± 15% CVC_max; P < 0.05), and 15-min (67 ± 14% CVC_max; P < 0.001) occlusions. Similarly, the peak response after a 5-min occlusion was significantly less than after the 15-min occlusions (P < 0.05). However, peak flows after a 10-min period of ischemia were not significantly different from those after 5 or 15 min of ischemia.

The between-subject coefficient of variation for the peak responses was 31, 22, 26, and 20% for the 3-, 5-, 10-, and 15-min occlusions, respectively. For the two responses used in data analysis, the within-subject coefficient of variation for the 3-min occlusion was 9%, for the 5-min occlusion was 8%, for the 10-min occlusion was 10%, and for the 15-min occlusion was 5%. The within-subject coefficient of variation for all three responses was 13, 10, 10, and 7% for the 3-, 5-, 10-, and 15-min occlusions, respectively.

**NO Contribution Protocol**

*Fig. 3A* depicts the differences in the AUC after NOS inhibition for both 5- and 15-min arterial occlusions. Infusion of L-NAME did not alter the baseline SkBF values. After 5-min arterial occlusions, the AUC in control sites averaged 3,261 ± 890% CVC_max’s, which was not significantly different from L-NAME-treated sites (2,908 ± 531% CVC_max’s). The AUC of 15-min control sites averaged 9,155 ± 1,121 vs. 9,126 ± 1,088% CVC_max’s in NOS-inhibited sites. Both the control and NOS-inhibited sites after 15 min of ischemia were significantly larger than the control and NOS-inhibited sites after 5 min of ischemia (P < 0.001 for both conditions).

**Peak CVC values.** Figure 3B shows the effect of NOS inhibition on peak blood flow in response to a 15- and 5-min arterial occlusion. Peak blood flow values after 5 min of arterial occlusion in the control sites averaged 53 ± 4% CVC_max. Blockade of NOS with L-NAME had no effect on peak flows (51 ± 2% CVC_max). Similarly, peak flows in control sites (71 ± 4% CVC_max) were not significantly different from L-NAME-treated sites (69 ± 4% CVC_max) after a 15-min occlusion.

**DISCUSSION**

The purpose of this study was twofold: 1) to systematically characterize the reactive hyperemic response in the cutaneous circulation in response to different periods of arterial occlusion and 2) to determine the contribution of NO to reactive hyperemia in the microvasculature of the skin in response to brief (5 min) and more prolonged (15 min) periods of arterial occlusion. The major new finding of this study is that NO does not play a significant role in the development or maintenance of reactive hyperemia in the cutaneous circulation. That is, inhibition of NOS did not significantly diminish the peak response or the total hyperemic response. Moreover, NOS inhibition had no significant effect on reactive hyperemia regardless of the length of the arterial occlusion. Thus NO does not appear to be a mediator of reactive hyperemia in the cutaneous circulation after brief or more prolonged periods of arterial occlusion (Figs. 2 and 3).

**Magnitude of Reactive Hyperemia and Duration of Arterial Occlusion**

In this study we characterized the reactive hyperemic response to arterial occlusions of varying durations, which, to date, has not been thoroughly described, despite the fact that macrovascular and microvascular changes associated with diabetes and other pathological conditions have been assessed by using the reactive hyperemic response to varying periods of arterial occlusion (6, 12, 28, 30). In the human forearm, Patterson and Whelan (22) demonstrated that as the duration of the arterial occlusion increased, the total hyperemic response also increased but that the peak flows remained relatively unchanged. Our results for the characterization protocol are in agreement with

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Patterson and Whelan in that there is a significant increase in the total hyperemic response as the duration of the arterial occlusion increases in the microvasculature of the skin. In contrast, we did observe progressively greater peak flows with increased occlusion time with the use of laser-Doppler flowmetry. These differences could be due to different techniques used to measure blood flow. Patterson and Whelan used venous occlusion plethysmography, which measures changes in blood flow to both muscle and skin vascular beds. Thus it cannot be determined whether the changes occurred in the muscle, the cutaneous circulation, or both. Furthermore, when venous occlusion plethysmography is used, measurements can only be taken every 10–15 s, and it is difficult to quantify extremely high flow levels.

The method by which our data were analyzed is important in light of the fact that reactive hyperemia is used as a clinical tool to assess microvascular function. There has been considerable variation in the literature with regard to the reported hyperemic response to a given occlusion duration in both healthy controls as well as in individuals with diabetes and other patient groups (6, 12, 28, 30). When reactive hyperemia between subjects or between studies is compared, differences in the hyperemic response to a given period of ischemia are impossible to assess because of reporting of data in terms of laser-Doppler flux units, which vary significantly between subjects and even between sites of the same subject due to spatial variations in the number of blood vessels under the laser-Doppler probe. Furthermore, changes in blood pressure and baseline SkBF values can impact the calculation of hyperemic values. Expression of data as a %CVC_max and subtracting baseline values takes into account these variations and allows for a more consistent comparison between subjects and between studies.

Although we achieved maximal SkBF by using two different methods (local heating and SNP infusion), it has been shown previously that these two methods cause maximal dilation in the skin (11, 16, 17, 27). When reactive hyperemia between subjects or between studies is compared, differences in the hyperemic response to a given period of ischemia are impossible to assess because of reporting of data in terms of laser-Doppler flux units, which vary significantly between subjects and even between sites of the same subject due to spatial variations in the number of blood vessels under the laser-Doppler probe. Furthermore, changes in blood pressure and baseline SkBF values can impact the calculation of hyperemic values. Expression of data as a %CVC_max and subtracting baseline values takes into account these variations and allows for a more consistent comparison between subjects and between studies.

Clinically, reactive hyperemia has been used to assess microvascular function. Thus our goal in the characterization protocol was to improve and standardize the methods used to analyze the reactive hyperemic response in the skin. Because of the high degree of between-subject variability, we used the two most consistent hyperemic responses in subsequent data analysis to determine how well we could improve the method of analysis. Despite our improved analysis method and the use of the two most consistent responses from each subject, the total hyperemic response still showed a high degree of variability between and within subjects. Hence, evaluation of the reactive hyperemic response may not be an adequate tool to assess microvascular function in the skin. The large range of variability between subjects would make it difficult to determine whether a given response is normal or affected by a pathological condition.

Contribution of NO to Reactive Hyperemia

The observation that NOS inhibition did not diminish the total hyperemic response agrees with the results from Engelke et al. (7), who found that, in the forearm, inhibition of NOS did not attenuate the total or peak hyperemic response when changes in baseline as a result of intra-arterial infusion of Nω-mono-methyl-L-arginine (L-NMMA) were taken into consideration. Contrary to our findings and those of Engelke et al., Meredith et al. (15) found that NO inhibition significantly attenuated the total hyperemic response in the human forearm. The discrepancy in results between Engelke et al. (7) and Meredith et al. (15) may be due to the fact that different doses of L-NMMA were used. In agreement with Meredith et al., Tagawa et al. (26) observed an attenuated total hyperemic response in the forearm with L-NMMA; however, they did not account for changes in baseline due to the arterial infusion of L-NMMA. In light of our findings, the results from Meredith et al. (15) and Tagawa et al. (26) suggest that NO may contribute to reactive hyperemia in skeletal muscle but not in skin. The effect of NO on peak flows after an arterial occlusion yield similar conflicting results in the literature as those obtained for the total hyperemic response. Engelke et al. (7) and Tagawa et al. (26) found that NO does not play a role in the development of the peak hyperemic response. However, Meredith et al. (15) found that inhibition of NOS significantly reduced the peak response.

In terms of the cutaneous circulation, it has been shown that an intra-arterial infusion of L-NMMA is inconsistent, at best, in inhibiting NO (24, 25). Our laboratory shown previously that 10 mM L-NAME adequately inhibits NOS in the skin (16, 17). Using the microdialysis technique, we were able to deliver a relatively high concentration of L-NAME to a small area of skin. This technique ensures that we are adequately blocking NO, and the use of laser-Doppler flowmetry allows us to directly measure changes that occur solely in the skin. On the basis of this, our results provide evidence that NO does not directly mediate any phase of reactive hyperemia in the cutaneous microvasculature.

Although we observed that NO does not directly mediate reactive hyperemia in the skin, the possibility still exists that NO could act in conjunction with one or more vasodilators to mediate reactive hyperemia. In the study by Engelke et al. (7), inhibition of prostaglandins with ibuprofen significantly reduced the peak hyperemic response but had no effect on the total hyperemic response. However, when ibuprofen and L-NMMA were administered simultaneously, the total hyperemic response was significantly reduced. These data suggest that prostaglandin inhibition may unmask a role for NO in reactive hyperemia. Moreover, Larkin and Williams (14) found that inhibition of sensory
nerves with EMLA cream (2.5% lidocaine and 2.5% prilocaine ointment; Astra USA, Westborough, MA) and inhibition of prostaglandins with indomethacin significantly reduced, but did not abolish, the reactive hyperemic response in the skin. Taken together, these data may suggest prostaglandins, sensory nerves, and NO act in conjunction to mediate reactive hyperemia. However, the fact that no studies have completely abolished the response suggests that other vasodilator substances may also contribute to reactive hyperemia. Clearly, further studies are warranted to elucidate the mechanism of reactive hyperemia in both skeletal muscle and cutaneous vascular beds.

**Perspectives**

It has been shown that NO is released from the endothelium in response to shear stress (4, 23). In this context, it has been suggested that the NO-dependent portion of cutaneous active vasodilation during whole body heating may be due to an increase in flow-mediated production of NO (9, 10, 24). In this construct, the release of an unknown neurotransmitter(s) in response to activation of sympathetic cholinergic nerves in the skin stimulates an increase in flow leading to production of NO from the endothelium (10). Similarly, it has been suggested that shear stress may stimulate NO production during local heating of the skin (16, 17). However, our finding in this study that NOS inhibition did not significantly attenuate the total hyperemic response or the peak hyperemic response after either a 5- or 15-min arterial occlusion argues against shear stress-mediated NO production in the cutaneous endothelium. Thus it appears unlikely the NO-dependent portion of cutaneous active vasodilation and the SkBF response to local heating are due to an increase in NO production by a shear stress mechanism. A limitation to this interpretation of our data is that we cannot rule out a possible time-dependent factor in which flow must be elevated for a sustained period of time before NO is produced in response to shear stress during local and whole body heating. Thus the possibility remains that there is a time-dependent factor associated with an increase in NO production in response to an increase in shear stress.

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**DISCLOSURES**

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