Bioactive nitric oxide concentration does not increase during reactive hyperemia in human skin

J. L. Zhao, P. E. Pergola, L. J. Roman, and D. L. Kellogg, Jr.

Bioactive nitric oxide concentration does not increase during reactive hyperemia in human skin. J Appl Physiol 96: 628–632, 2004; 10.1152/japplphysiol.00639.2003.—This study examined whether nitric oxide (NO) is involved in the cutaneous response to reactive hyperemia (RH) in the human forearm. We enrolled seven healthy volunteers. NO concentrations were monitored using a NO selective amperometric electrode (ISO-NOP200; World Precision Instruments) inserted into the skin of the forearm. Laser-Doppler flowmetry (Moor Instruments) was used for monitoring skin blood flow (SkBF) at the same site. SkBF and NO levels were monitored and recorded continuously throughout the experiment. An intradermal microdialysis probe was inserted adjacent to the NO electrode for drug delivery. Data collection began 140 min after the NO electrodes and microdialysis probes were inserted. RH was achieved by the inflation of a blood pressure cuff to 25 mmHg above systolic pressure for 7 min after which the pressure in the cuff was abruptly released. Acetylcholine (ACh) was given by microdialysis probe at the end of RH study to verify the ability of the electrode system to detect changes in the NO concentration. SkBF and NO data before RH and immediately, 2, 5, 7, and 10 min after cuff deflation were used for analysis. SkBF increased immediately after release of the occlusion (P < 0.0001) and remained elevated for 2 min. No significant NO changes occurred with the increases in LDF. ACh induced increases in both SkBF and NO (P < 0.000 and P < 0.037, respectively). We conclude that RH increases SkBF by mechanisms that do not require a measurable increase in NO concentrations.

Skin blood flow; vasodilation; amperometric electrode; laser-Doppler flowmetry

Release of an occlusion on a vascular bed results in a rapid, yet ephemeral vasodilation and consequent increase in blood flow. Within seconds of the release of occlusion, the vasodilation and blood flow rise rapidly to a peak. After reaching the peak, there is a decline to preocclusion levels within minutes. This response is known as “reactive hyperemia” and has been described in a variety of tissues, including human skin (8, 16, 19).

The mechanisms that effect reactive hyperemia in the human forearm and human skin have been suggested to involve the release of neuropeptides, prostaglandins, and nitric oxide (NO) (7, 8, 19, 20, 26). Larkin and Williams (19) demonstrated in the human cutaneous circulation that the release of substance P and calcitonin gene-related peptide are required to effect reactive hyperemia. In addition, these authors found that a cyclooxygenase product, probably a vasodilator prostanoïd, was required as well.

This work was expanded on by Meredith et al. (21). These researchers assessed the role of NO during reactive hyperemia in the forearm of healthy humans. They examined the effects of intra-arterial infusion of the NO synthase (NOS) antagonist Nω-nitro-L-arginine methyl ester (L-NMMA) on peak and total hyperemic blood flow after occlusion of forearm blood flow (FBF) as recorded with venous occlusion plethysmography: a technique that measures both skin and muscle blood flow but cannot distinguish between these vascular beds. The effectiveness of their NOS blockade was tested with intra-arterial infusions of acetylcholine (ACh). They found that NO inhibition reduced the FBF response to occlusion and concluded that “endothelial-derived nitric oxide contributes to vasodilatation that follows a period of ischemia in the human forearm” (21).

Work by Bank et al. (3) with L-NMMA confirmed this result by using a similar approach. Engelke et al. (8) reported results suggestive of the involvement of the prostaglandins and NO in reactive hyperemia in humans. These authors used a cyclooxygenase inhibitor to confirm that cyclooxygenase products were involved and the NOS antagonist L-NMMA to show that NO had a “modest” role in the peak FBF achieved during reactive hyperemia. They further discovered that the effect of NOS antagonism was potentiated by simultaneous inhibition of cyclooxygenase activity. They concluded that these two endothelial factors “can contribute to reactive hyperemia in the human forearm” (8).

In two studies in which the authors did not test the effectiveness of NOS blockade, no role for NO was found. Nugent, et al. (23) found no effect of intra-arterial infusions of L-NMMA when compared with placebo infusions on the FBF responses to occlusion. This lead the authors to the conclusion that NO does not contribute significantly to forearm reactive hyperemia. Consistent with these findings, Binggeli et al. (5) found that single-dose intradermal injections of either Nω-nitro-L-arginine methyl ester or Nω-nitro-D-arginine methyl ester failed to alter the reactive hyperemic response to forearm occlusion as recorded with laser-Doppler flowmetry (LDF). They concluded that NO does not have a major role in the response.

Based on the foregoing studies of the mechanisms involved in reactive hyperemia and the observations that both substance...
P and calcitonin gene-related peptide release NO in human skin (6, 11, 14, 15), we hypothesized that bioactive NO levels would increase in skin after release of an occlusion of the human forearm circulation. We tested this hypothesis by monitoring bioactive NO levels and skin blood flow (SkBF) levels from human forearm skin before and during reactive hyperemia.

METHODS

The approach we chose to test our hypothesis was to simultaneously monitor SkBF and biologically active NO concentrations from skin during a preocclusion baseline period, a 7-min suprasystolic arm occlusion, and a 10-min recovery period after release of that occlusion. SkBF was indexed by LDF (Moorlab, Moor Instruments, Devon, UK). LDF provides a continuous index of SkBF and is uninfluenced by underlying skeletal muscle blood flow (25). Bioactive NO was monitored by a selective-membrane, amperometric electrode technique (ISO-NO Mark II, World Precision Instruments, Sarasota, FL) and is based on the diffusion of NO through a selective membrane that coats the electrode’s surface (2, 4, 18, 28). The NO that reaches the electrode’s surface is oxidized and generates an electric current. The current generated is directly proportional to the diffusible NO concentration (2, 4, 18, 28).

Seven subjects (4 women, 3 men) participated in this study. Their average age was 29 ± 9 yr, average weight was 75 ± 20 kg, and average height was 168 ± 11 cm. All subjects were healthy and were taking no medications. All subjects gave their informed consent before participation in this institutionally approved study.

On arriving in the laboratory on the day of the study, each subject had a NO-selective electrode placed into the cutaneous interstitial space of one forearm. This was accomplished by using a 25-gauge needle to create a tract in the skin. The needle was removed and the electrode inserted down the tract. An intradermal microdialysis probe of our own manufacture was placed into the dermis adjacent to the NO-selective electrode (17). The microdialysis probe was of a linear design and had a molecular mass cutoff of 20 kDa. The microdialysis probe was used to infuse ACh into the skin adjacent to the NO-selective electrode to verify the ability of the electrode system to detect changes in NO levels (18). After insertion of the electrode and microdialysis fiber, subjects waited 140 min or longer to allow insertion trauma to resolve (1, 18).

After resolution of insertion trauma, subjects were placed in a seated position and a LDF probe put within 1–2 mm of the NO-selective electrode and adjacent to the microdialysis probe. A blood pressure cuff was placed on the upper arm, well above the aforementioned instrumentation.

Data collection began with a 5- to 15-min baseline period. The blood pressure cuff was then inflated to suprasystolic levels to occlude all blood flow into the instrumented arm. The occlusion was maintained for 7 min, after which the cuff was deflated completely to effect reactive hyperemia. Data collection continued for 10 min after release of the occlusion to monitor the recovery phase of the response. Finally, a 16 mM solution of ACh in Ringer solution was perfused through the microdialysis fiber to cause NO production. This was done for 20–30 min to verify the ability of the NO-selective electrode to detect changes in NO levels. The study was then ended.

After the study, the NO electrode was calibrated for NO concentrations. NO concentrations were calculated from standard curves by using the protocol given by the manufacturer. Final calculated NO concentrations were derived from these standard curves. The electrode output is linear across the entire range of NO concentrations from 25 to 1,600 nM. Averaged calibration curves are illustrated in Fig. 1.

Data are presented as means ± SE. For data analysis, baseline values for LDF and NO levels were compared with those levels achieved at the peak of the reactive hyperemic response (immediately after blood pressure cuff release) and those levels recorded at 2, 5, 7, and 10 min after release of the occlusion. Peak values achieved for LDF and NO during ACh infusion were compared with baseline levels to verify our ability to detect changes in these variables. Responses were analyzed by repeated-measures ANOVA followed by Dunnett’s test. A P value of 0.05 was considered significant.

RESULTS

The protocol and data from one subject are illustrated by Fig. 2. The overall results of the study for LDF and NO levels are summarized in Fig. 3.

During the baseline period, LDF and NO levels remained steady at 597 ± 133 mV and 655 ± 71 nM, respectively. After the occlusion, LDF rose to a peak value of 2,518 ± 277 mV (P < 0.05 vs. baseline) and remained elevated for 2 min after release of the occlusion at 1,254 ± 213 mV (P < 0.05 vs. baseline). By the fifth minute after release of the occlusion, LDF had returned to 594 ± 97 mV, a value no different from baseline levels (P > 0.05 vs. baseline). LDF remained at levels no different from those of the preocclusion baseline period throughout the remainder of the postocclusion recovery period (P > 0.05 vs. baseline).

In contrast to LDF values, NO levels never changed from baseline levels after release of the occlusion (P > 0.05 vs. baseline). NO levels remained statistically no different from the preocclusion baseline during the entire recovery period (P > 0.05 vs. baseline).

During the infusion of ACh by microdialysis, LDF values increased significantly from 597 ± 133 to 5,440 ± 271 mV (P < 0.05 vs. baseline). NO levels also increased significantly in response to ACh from 655 ± 71 to 805 ± 104 nM (P < 0.05 vs. baseline).
DISCUSSION

The major finding of this study is that no change in bioactive NO levels occurs in human skin at any point in the reactive hyperemia response. Despite significant increases in SkBF as indexed by LDF, we found no significant change of NO levels from preocclusion levels at any point in the postocclusion period. Administration of exogenous ACh, an endothelium-dependent vasodilator, produced significant increases in both LDF and NO levels and thus verified the ability of our system to detect simultaneous changes in both. Based on our results that no increase in NO levels occurs in the cutaneous interstitial space during reactive hyperemia, we conclude that NO does not play a direct vasodilatory role in the skin as part of this response.

Our study also demonstrates the utility of the selective-membrane, amperometric technique in vivo, in humans. Both LDF and the NO concentration increased when exogenous ACh was delivered to the skin by intradermal microdialysis, demonstrating that ACh increases NO in skin. This finding contrasts prior reports that ACh effects vasodilation in human skin solely by a prostaglandin-dependent mechanism (22). It is interesting to note that LDF values increased ~9-fold, whereas NO concentrations increased 1.2-fold. This suggests either that the relationship between SkBF and NO concentrations is highly nonlinear or that ACh vasodilated cutaneous vessels by more than one mechanism.

Our results were unexpected given the prior reports (3, 8, 21) that suggest a mechanistic role for NO in the vasodilatory response to limb occlusion, but they are consistent with reports by others (5, 23). One possible explanation for this discrepancy of results is the different techniques used in the various studies. Our study used LDF and NO-selective electrodes to specifically study the cutaneous vascular response to reactive hyperemia. These two techniques are highly specific for the skin circulation. The earlier studies used venous occlusion plethysmography to measure FBF (which includes both SkBF and muscle blood flow) in combination with intra-arterial infusions of NOS antagonists. It is possible that the mechanistic role of NO differs between the skin and muscle vascular beds. According to this possibility, NO would play no role in the cutaneous vasodilation during reactive hyperemia, but it would play a role in the skeletal muscle vasodilator response to occlusion.

Recent work has suggested that reactive hyperemia can be used to assess endothelial function in humans (12). This proposal is based on the reduction in the FBF response by NOS antagonists during reactive hyperemia. The results of Meredith et al. (21) and Engelke et al. (8) support the use of reactive hyperemia in conjunction with FBF measurements to assess endothelial function in humans (12). Studies using the cutaneous vascular response during reactive hyperemia to assess endothelial function have been also published (13, 24, 27). In light of our finding that NO concentrations do not increase in skin during reactive hyperemia, the use of reactive hyperemia in conjunction with the skin-specific blood flow measurements by LDF or laser-Doppler imagery to assess endothelial function is problematic. Use of vascular occlusion and reactive hyperemia to assess general endothelial function in the skin appears to be supported by published studies. However, given the uncertainty of the role of NO in the skin response to occlusion, use of this approach to study the endothelial NO system in the skin is premature.

Our work demonstrates that measurement of NO tissue concentrations during reactive hyperemia by the amperometric electrode technique is sensitive enough to detect small changes in NO levels. ACh was used to test the ability of our technique to detect changes in NO concentrations. NO is a potent vasodilator as demonstrated by the significant increase in SkBF associated with relatively small increase in NO concentration. Although other vasodilator substances have been suggested to be involved in the response to ACh, NO seems to play a significant role in the cutaneous response.

The lack of detectable increase in cutaneous NO concentrations by the amperometric electrode technique above the normal cutaneous NO levels (~650 nM) could still allow NO to play a role in the vasodilatory response to reactive hyperemia. Farrell and Bishop (9, 10) proposed that NO can act as a...
permissive factor that must be present in the tissue to facilitate or permit the action of other vasodilator substances. This proposal is not supported by the work of others published while our original manuscript was under review (29).

During the period that our work was under review, a study was published using a different technique to address the same question we examined: does NO play a role in the cutaneous vascular response to reactive hyperemia (29)? These researchers used intradermal microdialysis to deliver the NOS antagonist N\(^{G}\)-nitro-l-arginine methyl ester into the forearm skin of human volunteers. SkBF responses were recorded with LDF at the microdialysis sites. Forearm occlusions of 5- and 15-min duration were performed to effect reactive hyperemia. They found no significant alteration in the reactive hyperemic response by NOS blockade and concluded “that NO does not directly mediate reactive hyperemia” in skin. The findings and conclusions by Wong et al. (29) using a NOS antagonist are consistent with our findings and conclusions using a NO-selective amperometric technique. The reports by Wong et al. and ourselves provide complementary verification with different techniques that NO generation by NOS does not directly effect reactive hyperemia in human skin.

In conclusion, we combined microdialysis, LDF, and amperometric measurements of NO concentrations to simultaneously monitor SkBF and bioactive NO responses to reactive hyperemia. Consistent with our laboratory’s prior experience (18), we found that the amperometric technique was useful to directly measure bioactive NO concentrations and detect in vivo changes in humans and can be combined with indexes of blood flow, such as LDF, to monitor the vascular consequences of alterations in NO levels. We found that bioactive NO levels in the cutaneous interstitial space did not increase despite increases in SkBF during reactive hyperemia in humans. This finding suggests that NO is not a direct effector of the cutaneous vasodilation that occurs during reactive hyperemia. Given the foregoing, it is clear that 1) cutaneous NO concentrations do not increase during reactive hyperemia and 2) NO appears to play no role in the vasodilation after ischemia in the skin.
REFERENCES