Nitric oxide is not obligatory for radial artery flow-mediated dilation following release of 5 or 10 min distal occlusion

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Pyke K, Green DJ, Weisbrod C, Best M, Dembo L, O’Driscoll G, Tschakovsky ME. Nitric oxide is not obligatory for radial artery flow-mediated dilation following release of 5 or 10 min distal occlusion. Am J Physiol Heart Circ Physiol 298: H119–H126, 2010. First published October 30, 2009; doi:10.1152/ajpheart.00571.2009.—This study investigated the nitric oxide (NO) dependence of radial artery (RA) flow-mediated dilation (FMD) in response to three different reactive hyperemia (RH) shear stimulus profiles. Ten healthy males underwent the following three RH trials: 1) 5 min occlusion (5 trial), 2) 10 min occlusion (10 trial), and 3) 10 min occlusion with cuff reinfation at 30 s (10–30 trial). Trials were performed during saline infusion and repeated during N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA) infusion in the brachial artery. RA blood flow velocity was measured with Doppler ultrasound, and B-mode RA images were analyzed using automated edge detection software. Shear rate estimation of shear stress was calculated as the blood flow velocity/vessel diameter. L-NMMA decreased baseline vascular conductance by 35%; L-NMMA infusion did not affect the peak shear rate stimulus ($P = 0.681$) or the area under the curve (AUC) of shear rate to peak FMD ($P = 0.088$). The AUC was significantly larger in the 10 trial vs. the 10–30 or 5 trial ($P < 0.001$). Although percent FMD (%change in diameter) in the 10 trial was larger than that in the 5 trial ($P = 0.035$), there was no significant difference in %FMD between the saline and L-NMMA conditions in any trial: 5 trial; 5.62 ± 1.48 vs. 5.63 ± 1.27%; 10 trial; 9.07 ± 1.16 vs. 11.22 ± 2.21%; 10–30 trial, 6.52 ± 1.43 vs. 7.98 ± 1.51% for saline and L-NMMA, respectively ($P = 0.158$). We conclude the following: 1) RH following 10 min of occlusion results in an enhanced stimulus and %FMD compared with 5 min of occlusion. 2) When the occlusion cuff is reinflated 30 s postrelease of a 10 min occlusion, it does not result in an enhanced %FMD compared with that which results from RH following 5 min of occlusion. 3) The lack of effect of L-NMMA on FMD suggests that NO may not be obligatory for radial artery FMD in response to either 5 or 10 min of occlusion in healthy volunteers.

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IN HEALTHY ARTERIES, an increase in blood flow (and shear stress) results in endothelial-dependent flow-mediated dilation (FMD) (29, 32, 41). FMD can therefore serve as an index of endothelial function and provide insight regarding vascular health (42). Originally published by Celermajer et al. (8), the test most commonly performed in humans increases shear stress in conduit arteries (e.g., the brachial, radial, femoral, or popliteal) via reactive hyperemia following the release of temporary limb ischemia (8). A “standard” technique has emerged that stipulates a 5-min cuff occlusion duration, a cuff position distal to the site of conduit artery diameter measurement, and the absence of exercise performed during occlusion (31). The primary reason for these constraints is the desire to create a largely nitric oxide (NO)-dependent FMD response. NO is of particular interest because of its established athero-protective qualities, including inhibition of smooth muscle proliferation, leukocyte adhesion, and platelet aggregation and adhesion (9).

A small number of previous studies have demonstrated a NO-dependent FMD to the reactive hyperemia following the release of a 5-min distal occlusion in the brachial (11), radial, (20, 27, 35) and femoral arteries (21). These studies reported absent or severely blunted FMD during infusion of the NO synthase (NOS) blocker N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA). It is based on these studies that the common belief is: low FMD reflects a low level of NO bioavailability and therefore reduced NO-mediated vasoprotection (31). Importantly, however, NO is not the only vasodilator that can be released in response to shear stress; prostaglandin (12) and endothelial-derived hyperpolarizing factor (EDHF) (5) involvement have also been observed in human FMD, and these could be important for vascular health both in terms of function and vasoprotection. Furthermore, there is evidence that EDHF may be able to compensate when NO production is compromised (5). Establishing the NO dependence of FMD following specific reactive hyperemia profiles is therefore critical if FMD is to act as a surrogate measure of NO bioactivity.

The standard brachial artery technique creates a very small stimulus in some subject groups (19, 26), making it somewhat difficult to identify whether a small FMD response is the result of disease or inadequate shear stimulus magnitude. To address this problem, some researchers have tested modifications of the standard FMD technique aimed at increasing the stimulus magnitude (43). Both ischemic handgrip exercise during occlusion and longer duration occlusion increase the duration of the hyperemia upon cuff release and may enhance the peak stimulus (1, 2, 6, 27, 43). However, further studies have indicated that the FMD in response to these enhanced stimuli may not be NO-dependent and therefore may not provide insight specific to NO bioactivity (2, 4, 27). This indicates that, with modest differences in the shear stimulus, FMD can become independent of NO and underscores the importance of understanding the characteristic(s) of the shear stimulus that determine the mechanisms responsible for FMD.
Based on a mechanistic study of FMD and stimulus duration (27), we reasoned that it is the prolonged hyperemia following lengthy cuff occlusion, and not the enhanced early stimulus magnitude, that results in a loss of NO dependence. Therefore, we hypothesized that performing a 10 min occlusion duration (to create a large early stimulus magnitude) and then reinflating the cuff after 30 s of hyperemia (to prevent a long stimulus duration) might enhance the stimulus and FMD response magnitude without compromising the NO dependence of the FMD. This would provide a new procedure with which to examine NO-mediated endothelial function in groups who experience a small stimulus following the release of 5 min of occlusion. The purpose of our experiment was therefore twofold: 1) determine whether 10 min of occlusion with cuff reinflation 30 s postcuff release results in enhanced NO-dependent FMD compared with a standard 5 min occlusion reactive hyperemia test. This is important to determine if this procedure can be used to assess NO-specific endothelial function and 2) establish, within this group of subjects, the NO dependence of the FMD in response to 5 and 10 min of occlusion without cuff reinflation. This is important given a) that a number of vasodilators can be released in response to shear stress, b) the relatively small number of studies that have isolated the NO dependence of released in response to shear stress, and c) that a number of studies have determined that modest changes in the reactive hyperemia profile result in NO-independent responses.

MATERIALS AND METHODS

General Methods

Subjects. Ten nonsmoking healthy young male subjects (mean age 23 ± 2 yr) volunteered to participate. Health status of the subjects was confirmed via verbal interview regarding medical history and fasting blood cholesterol measurements. Subjects with risk factors for endothelial dysfunction or who were taking vasoactive medications were excluded from the study. All subjects provided written consent, and all procedures were approved by the Ethics Committee at Royal Perth Hospital in accordance with the Declaration of Helsinki.

Preparation. Subjects arrived at the hospital in the morning (study start time 7:30–9:30 A.M.) after being requested to observe a minimum 4-h fast. Subjects were also instructed to abstain from caffeine, alcohol, and exercise in the 4 h preceding the study. Studies were performed in a quiet temperature-controlled room.

Cannulation. At the level of the antecubital fossa, a 20-gauge cannula (Arrow) was inserted in the brachial artery of the left arm, under local anesthesia with <2 ml of 1% procaine (procaine hydrochloride injection; Mayne Pharma, Mulgrave, VIC, Australia). This cannula was used to infuse vasoactive agents (l-NMMA, CA-11; Clinalfa) and sterile saline. Saline infusion was administered using an infusion pump at a constant rate of 1 ml/min. After cannulation, saline was infused to maintain patency during an ~30-min stabilization period. During this period, an ~10-ml blood sample was also drawn for analysis of fasting cholesterol.

Specific Experimental Protocol

Reactive hyperemia. An occlusion cuff was placed around the subject’s wrist to prevent blood flow in the hand and create a reactive hyperemia. The occlusion cuff was inflated to a pressure of 300 mmHg in all trials. All parameters were recorded for 1 min before cuff inflation and, for the final minute of cuff inflation, through to 2 min postcuff release. The following reactive hyperemia trials were performed: 1) 5; 5 min of occlusion (standard test) (n = 10), 2) 10; 10 min of occlusion (n = 10), 3) 10–30; 10 min of occlusion with cuff reinflation after 30 s of hyperemia (n = 10), and 4) 5-R; repeat 5 min of occlusion (repeat standard test) (n = 7). The order of trial 10 and 10–30 was counterbalanced between subjects, but the trial order was the same in saline and l-NMMA infusion conditions. The standard test (5-R) was repeated at the end of the saline and l-NMMA conditions in a subset of seven subjects to assess any affect of repeated trials. A minimum of 10 min was allowed between trials to reestablish baseline conditions.

Blockade of NO production. After completion of the saline trials, l-NMMA (NOS inhibitor) infusion (pump model: 770 IVAC) was initiated with a bolus infusion rate of 99 ml/h for 3 min and then maintained at 30 ml/h. l-NMMA was prepared at a concentration of 16 μmol/ml and therefore infusion at 30 ml/h yielded a constant dose of 8 μmol/min. At least 10 min of infusion at 30 ml/h was performed before the reactive hyperemia protocol commenced (Fig. 1).

Data Acquisition and Analysis

Radial artery blood flow velocity and diameter assessment. Radial artery diameter and blood flow velocity were measured using high-resolution vascular ultrasonography with synchronized Doppler velocity assessment. A 12- to 15-MHz multifrequency linear array probe attached to a high-resolution ultrasound machine (Aspen; Acuson) was used to visualize the artery ~10 cm distal to the antecubital fossa, proximal to the placement of the wrist cuff. Ultrasound parameters...
Table 1. Subject characteristics

<table>
<thead>
<tr>
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<th>Value</th>
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<tr>
<td>Age, yr</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>Height, cm</td>
<td>182 ± 2</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>77.5 ± 0.3</td>
</tr>
<tr>
<td>Blood plasma cholesterol, mmol/l</td>
<td>3.68 ± 0.2</td>
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</table>

Values are means ± SE.

The subjects were then set to optimize longitudinal B-mode images of the artery. Probe position was maintained throughout the study with the assistance of a custom-designed probe clamp equipped with micrometer screws to allow minor adjustments to maintain an optimal image. Continuous Doppler velocity assessment was also performed with the Aspen at an insonation angle of 68 degrees (30) to allow perpendicular arterial wall imaging and thereby optimize image quality, since it is the vessel diameter measurement that demands optimal precision. Standard 3 lead electrocardiogram (ECG) was connected to the ultrasound machine to allow R wave-triggered diameter analysis.

**Arterial blood pressure and distal vascular conductance.** The arterial cannula was used for continuous measurement of intra-arterial pressure (Transpac IV; Abbott Critical Care Systems, Sligo, Ireland, and Hewlett Packard 78342A). Baseline arterial blood pressure and distal vascular conductance (DVC, represents the degree of dilation in resistance vessels downstream of the radial artery measurement site) were taken as an average of the 60-s baseline period. DVC was calculated as follows: radial artery blood flow/arterial blood pressure ×100. Blood flow was calculated as follows: radial artery blood flow velocity ×πr² where r is radial artery radius.

**Radial artery diameter analysis.** Images of the radial artery were analyzed using custom-designed edge-detection software. The video signal was taken directly from the ultrasound machine and saved to a personal computer in DICOM (Digital Imaging and Communications in Medicine) format. Vessel diameter was analyzed using an automated edge-detection software package as described in Woodman et al. (44). This program allows the user to identify a region of interest (ROI) on the portion of the image where the walls are most clear. It then identifies and tracks the walls of the artery via the intensity of the brightness of the walls vs. the lumen of the vessel. The program collects one diameter measurement for every pixel column in the ROI. It calculates the median diameter as the diameter for that frame and collects data at a frequency of ~20–30 frames/s. The program was triggered to the ECG signal and provided a diameter measurement for every R wave (corresponding to end diastole). FMD is reported as the percent change in diameter from the baseline measurement before cuff occlusion (%FMD) and as an absolute change in diameter.

**Blood flow velocity.** Blood flow velocity was measured as a peak envelope of the velocity spectrum using the same custom software utilized for radial artery diameter measurement. This program automatically tracks the peak envelope of the blood flow velocity signal. Velocity measures are stored for each analyzed frame at 20–30 Hz. Postanalysis blood flow velocity was averaged into 3-s average time bins.

**Shear rate.** Shear rate, an estimate of shear stress, was calculated as blood flow velocity/vessel diameter. Vessel diameter measures were plotted over time, and a line of best fit was determined using custom software to minimize the mean squared error and achieve a consistent distribution of residuals above and below the line of best fit. Using the function parameters, another custom program was then applied to provide a corresponding diameter estimate for every 3 s. Thus a diameter measurement and a velocity measurement, time aligned for every 3 s, were obtained and used to calculate shear rate. Shear rate was expressed in the following two ways: as the peak shear rate (highest 3-s average time bin) or as an integral [area under the curve (AUC) until the time of peak diameter measurement (AUC of shear rate to time to peak FMD)].

**Statistics.**

Two-way repeated-measures ANOVA were used to compare both the stimulus (shear rate) and response (%FMD) parameters. For the main subject group (n = 10), the factors were trial (5, 10, and 10–30) and drug (saline vs. L-NMMA). To compare only the standard reactive hyperemia test trials (5 vs. 5-R; n = 7), two-way repeated-measures ANOVA were used with the factors trial (5 and 5-R) and drug (saline vs. L-NMMA). The values reported in the results sections refer to the main subject group (n = 10), and the 5 vs. 5-R (n = 7) comparison is addressed separately. The level for significance was set at P < 0.05, and significant differences for ANOVA were further assessed using Tukey’s post hoc tests. All statistics were calculated using Sigmastat 2.03 (SPSS, Chicago, IL). All data are means ± SE.

**RESULTS**

**Subject Characteristics**

Subject characteristics are displayed in Table 1.

**Baseline Diameter, Shear Rate, Arterial Blood Pressure, Heart Rate, and Radial Artery Conduction**

These variables are summarized in Table 2.

**Radial artery diameter.** Baseline radial artery diameter was significantly smaller in the L-NMMA condition vs. the saline

Table 2. Baseline haemodynamic conditions

<table>
<thead>
<tr>
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<th>Saline Trial</th>
<th>L-NMMA Trial</th>
<th>Significance (P Value)</th>
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<tbody>
<tr>
<td></td>
<td>5</td>
<td>10</td>
<td>10–30</td>
</tr>
<tr>
<td>Baseline diameter, mm</td>
<td>2.46 ± 0.10</td>
<td>2.44 ± 0.09</td>
<td>2.41 ± 0.07</td>
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<td>Baseline shear rate, s⁻¹</td>
<td>29.4 ± 5.5</td>
<td>30.3 ± 5.4</td>
<td>34.9 ± 8.6</td>
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<tr>
<td>Occlusion shear rate, s⁻¹</td>
<td>5.7 ± 0.28</td>
<td>6.1 ± 0.28</td>
<td>5.9 ± 0.47</td>
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<tr>
<td>Baseline MAP, mmHg</td>
<td>94.8 ± 3.1</td>
<td>95.4 ± 3.1</td>
<td>95.6 ± 3.2</td>
</tr>
<tr>
<td>Baseline heart rate, beats/min</td>
<td>52.1 ± 2.4</td>
<td>51.7 ± 2.2</td>
<td>52.3 ± 2</td>
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<tr>
<td>Baseline DVC, ml·min⁻¹·100 mmHg⁻¹</td>
<td>23.5 ± 6.8</td>
<td>19.7 ± 2.8</td>
<td>22.5 ± 4.3</td>
</tr>
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</table>

All values are means ± SE. L-NMMA, Nω-monomethyl-L-arginine; 5, 5 min occlusion; 10, 10 min occlusion; 10–30, 10 min occlusion with cuff reinflation at 30 s; MAP, mean arterial pressure; DVC, vascular conductance of the resistance vessels downstream of the radial artery.
Infusion of L-NMMA still had no effect (no main effect of drug) or the 10 and 10–30 trials (P = 0.947) or between saline and L-NMMA conditions (P = 0.054).

**Mean arterial blood pressure.** Baseline mean arterial blood pressure (MAP) was modestly higher (~4 mmHg) in the L-NMMA vs. saline conditions (P = 0.031), but it was not different between trials (P = 0.275).

**Heart rate.** There were no significant differences in baseline heart rate between trials (P = 0.787) or between saline and L-NMMA conditions (P = 0.965).

DVC: Vascular conductance of resistance vessels downstream of the radial artery imaging site. Baseline DVC was not significantly different between trials (P = 0.772); however, it was lower in the L-NMMA condition (P = 0.006). To assess the effect of L-NMMA delivery on baseline conductance more closely, we specifically examined the baseline conductance in the last saline trial and the first L-NMMA trial, since these are the most closely temporally associated with only the intervening time of drug administration. Baseline conductance in the last saline trial was 18.35 ± 4.12 ml·min⁻¹·100 mmHg⁻¹, and baseline conductance in the first L-NMMA trial was 9.93 ± 1.14 ml·min⁻¹·100 mmHg⁻¹. This represents an average 35.20% decrease in conductance (range −6 to −57%). Baseline flow decreased over this period from 17.7 ± 3.87 to 9.68 ± 1.14 ml/min.

**Shear Rate Stimulus**

Two components of the reactive hyperemia shear rate profile were quantified.

**Peak shear rate.** There was no effect of L-NMMA infusion on the peak shear rate stimulus (P = 0.681). Ten minutes of occlusion resulted in a larger peak shear rate vs. 5 min of occlusion [10–30 and 10 trial vs. 5 trial (P < 0.001 and 0.006, respectively); Fig. 2A].

**AUC of shear rate to peak FMD.** L-NMMA infusion had no effect on the AUC of shear rate to peak FMD (P = 0.088). The AUC of shear rate to peak FMD was significantly larger in the 10 trial vs. the 10–30 or 5 trials (P < 0.001) (Fig. 2B).

**FMD**

Infusion of L-NMMA did not impair peak %FMD in any of the trials [no main effect of drug (P = 0.158)]. The %FMD was larger in the 10 trial vs. the 5 trial (P = 0.029), but there was no significant difference between the 5 and 10–30 trials (P = 0.578) or the 10 and 10–30 trials (P = 0.196) (Fig. 3A). When FMD was measured as the absolute diameter change (mm), infusion of L-NMMA still had no effect (no main effect of drug, P = 0.235). As with %FMD, the absolute change in diameter was only significantly larger in the 10 trial vs. the 5 trial (P = 0.021) (Fig. 3B). Response normalization (%FMD/shear rate AUC to peak FMD) also did not reveal any effect of L-NMMA infusion (data not shown).

**Time to peak diameter measurement.** Infusion of L-NMMA did not have an effect on the time to peak diameter measurement (P = 0.101). However, the 10 and 5 trials had a significantly longer time to peak than the 10–30 trial (P = 0.002 and 0.038, respectively) (Fig. 4).

**Trial 5 vs. 5-r.** When only the 5 vs. 5-R trials were compared (n = 7), L-NMMA infusion again failed to have an impact on the %FMD response (P = 0.733). In addition, there was no significant difference in %FMD between trials (P = 0.895) (5 saline 5.83 ± 2.13%; 5-R saline 6.04 ± 1.34%; 5 L-NMMA 5.31 ± 1.71%; and 5-R L-NMMA 5.56 ± 0.95%). Repeat L-NMMA infusion also had no impact on the absolute change in diameter (P = 0.644), and there was no significant difference in the absolute change in diameter between trials (P = 0.781) (5 saline 0.13 ± 0.05 mm; 5-R saline 0.14 ± 0.03 mm; 5 L-NMMA 0.11 ± 0.04; and 5-R L-NMMA 0.12 ± 0.02 mm). For stimulus magnitude values, see Table 3. There was a significant difference in peak shear rate between trials only in the saline condition (saline 5-R greater than saline 5, P < 0.001; main effect of trial, P = 0.01; interaction between drug and trial, P = 0.028).
DISCUSSION

In this study, we used L-NMMA to block endothelial (eNOS) and neuronal (nNOS) NOS during various reactive hyperemia protocols. The major findings of our experiments were as follows: 1) NO is not obligatory for FMD in response to the reactive hyperemia following 5 or 10 min of occlusion in young healthy males and 2) release of a 10-min occlusion followed by cuff reinflation after 30 s of hyperemia does not result in enhanced dilation compared with that following the release of 5 min of occlusion.

**FMD Following a 5-min Occlusion Duration**

Contrary to our hypothesis, we found that NO was not obligatory for normal FMD in our 5-min occlusion trials (5 and 5-R). This is a surprising finding in light of the few published studies in the brachial (11), radial (20, 27, 34), and superficial femoral (21) arteries, which report severely blunted or absent FMD following the release of ≤5 min of distal occlusion during L-NMMA infusion. The methodology employed by Mullen et al. (27) in particular is virtually identical to that employed in the present study (although Mullen et al. report a lower dose of 4 μmol/min with no bolus dose vs. 8 μmol/min in the current study) as is the radial FMD reported during saline infusion (5.3 ± 1.2 vs. 5.6 ± 1.5% in Mullen et al. and the present study, respectively). Close scrutiny of the potential alternate explanations reinforce our conclusion that NO is not necessarily obligatory for FMD in healthy young males.

**L-NMMA blockade efficacy.** If we had used a dose significantly lower than that reported by others, or if we had observed no physiological consequence of L-NMMA infusion, it might be suggested that inadequate blockade could explain our results. However, the L-NMMA dose used in the current study (8 μmol/min) is the same as that used by Joannides et al. (20) (8 μmol/min), twice that of Mullen et al. (27) (4 μmol/min), and four times that of Seddon et al. (34) (2 μmol/min), all of whom report abolition of FMD with L-NMMA infusion. Furthermore, the current study preinfused the L-NMMA (before performing a test) for an equal or greater duration than the studies mentioned above.

With respect to the physiological evidence for blockade; NO is known to contribute to baseline resistance vessel tone. We observed a similar reduction in baseline blood flow (indicative of resistance vessel tone) with L-NMMA infusion as Joannides et al. (20) and Seddon et al. (34) [from 17.7 to 9.68 ml/min vs. 24 to 13 ml/min in the present study and that of Joannides et al. (20), respectively]. Baseline conductance also represents the state of dilation of the resistance vessels, and, in the present study, it decreased 35% from the last saline trial to the first L-NMMA trial. This is in agreement with Ishibashi et al. (18) who found a similar reduction in baseline conductance with L-NMMA infusion in healthy subjects (34.5%). Furthermore, recent work by Seddon et al. (34, 40) indicates that forearm resistance vessel tone is influenced by NO derived from nNOS in addition to eNOS. In these studies, they also demonstrated that an L-NMMA (nonselective NOS antagonist) dose of 2 μmol/min [4 μmol/min (less than the current study)] is sufficient to block both nNOS and eNOS. Taken together, the findings in the literature and our observations support the position that our NOS blockade was as effective as these previous studies.

**Fig. 4.** The time to peak diameter measurement. Black bars, saline infusion conditions; gray bars, L-NMMA infusion conditions. No significant differences between saline and L-NMMA conditions. Trial effects: significantly less than the 5 trial (#). Error bars represent the SE.

**Fig. 3.** A: FMD in % change from baseline. B: FMD in absolute change in diameter from baseline. Black bars, saline infusion conditions; gray bars, L-NMMA infusion conditions. No significant differences between saline and L-NMMA conditions. Trial effects: significantly greater than the 5 trial (*). Error bars represent the SE.
NITRIC OXIDE IS NOT OBLIGATORY FOR RADIAL FMD

Table 3. Comparison of first and second (5 vs. 5-R) 5 min occlusion reactive hyperemia trials with saline and l-NMMA

<table>
<thead>
<tr>
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<th>Saline 5</th>
<th>Saline 5-R</th>
<th>l-NMMA 5</th>
<th>l-NMMA 5-R</th>
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<td>Peak shear rate, s⁻¹</td>
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<td>Saline 5-R &gt; 5, &lt;0.01</td>
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<td></td>
<td></td>
<td>Drug 0.090</td>
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<td></td>
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<td>Trial 0.945</td>
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<tr>
<td>AUC of shear rate</td>
<td>10,329±1,841</td>
<td>11,128±2,876</td>
<td>7,486±1,214</td>
<td>6,857±1,022</td>
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</table>

All values are means ± SE. AUC, area under the curve; FMD, flow-mediated dilatation.

Reduced baseline vessel diameter in the l-NMMA condition. We report a reduced baseline diameter in the l-NMMA condition (consistent with efficacious blockade of eNOS and nNOS). The slightly more constricted baseline radial diameter in l-NMMA might at first be viewed as a potential confound that may have “masked” an obligatory role for NO in radial FMD in this study (i.e., a “nonspecific” effect of vasoconstriction on the impact of eNOS blockade). However, for this to have been the case, it would have to affect the relationship between smooth muscle relaxation and the index of vessel diameter change in a specific way, namely that the same vessel diameter change occurs with the loss of an obligatory vasodilator mechanism (NO in this case), when the baseline condition is one of slightly more constriction. This is a highly unlikely scenario. Furthermore, we observed the same percentage and absolute change in radial diameter in saline and l-NMMA, so it is not a case of a “mathematical artifact” where a given dilation from a different baseline diameter results in a different percent dilation simply because of the baseline starting point. Therefore, although the absence of a condition in this study where FMD is measured under conditions of an NO-independent constritor remains a limitation, we do not believe that this compromises the study findings that NO was not obligatory for radial FMD.

Shear stimulus magnitude in the l-NMMA vs. saline conditions. There was a trend toward lower shear stimulus AUC in the l-NMMA vs. saline condition, but this did not reach statistical significance (P = 0.088). This could reflect a type II error with respect to identifying that maintenance of resistance vessel dilation during reactive hyperemia following occlusion release is NO dependent. However, this does not confound the conclusion that NO is not obligatory for radial artery FMD, since preservation of FMD under NO blockade conditions that we observed was not due to any enhancement of shear stimulus AUC. When the shear rate AUC was calculated as a delta to take into account the lower baseline shear rate before occlusion in the l-NMMA condition, the pattern of stimulus magnitudes was the same as presented in Fig. 2B.

Hemodynamic parameters in the l-NMMA vs. saline conditions. The MAP was modestly higher (4 mmHg) following l-NMMA infusion. Importantly, although it did not reach statistical significance, baseline MAP was also elevated by 4 mmHg in the l-NMMA vs. saline conditions in the study by Joannides et al. (20) in which l-NMMA abolished the FMD response in the radial artery. Because it is possible that sympathetic activation may attenuate FMD (16), increased MAP resulting in a baroreflex-mediated decrease in sympathetic tone in the l-NMMA condition might have masked a blunting of FMD in the l-NMMA condition. However, heart rate was virtually identical between the l-NMMA and saline conditions. This strongly suggests that reflex changes in autonomic control as a result of a minor increase in MAP do not explain the failure of l-NMMA infusion to alter FMD.

Evidence for Multiple Endothelial Phenotypes?

As stated previously, the methodology reported here and in previous radial artery studies (20, 27, 34) is very similar and therefore does not account for the essentially opposite findings. An alternate explanation for our findings is that multiple endothelial phenotypes exist in humans. In one phenotype, EDHF (or another vasodilator released in response to shear stress) may compensate for an acute absence of NO and allow an uncompromised FMD response. This is supported by observations in both rats (17) and humans (demonstrated during a sustained, distal limb-heating induced shear stress stimulus) (5) where EDHF appears to compensate for the loss of NO-mediated dilation. It is possible that previous radial artery studies (20, 27) and the present study selected subjects with distinct phenotypes (without and with EDHF compensatory capacity, respectively). Although not statistically significant, perhaps because of power limitations, there was a trend toward a shorter time to peak diameter in the l-NMMA condition (Fig. 4). This could reflect a faster action of the compensating vasodilator(s). Future blockade studies investigating the role of EDHF after NOS inhibition and during reactive hyperemia are required to test this hypothesis.

FMD Following a 10-min Occlusion Duration

The observation of a large NO-independent FMD in our 10 trial is consistent with the observations of others in the radial (27) and superficial femoral (21) arteries; however, Bellien et al. (4) noted a modest blunting of radial FMD following the release of 10 min of occlusion. Mullen et al. (27) found that, compared with 5 min of occlusion, 15 min of wrist occlusion resulted in prolonged shear stimulus duration and an enhanced FMD response magnitude that was not blunted with the infusion of l-NMMA. This was in contrast to their observation that NO was obligatory for FMD in response to a short-duration shear stimulus and led them to hypothesize that short- and long-duration shear stimuli evoke mechanistically distinct FMD responses.

This conclusion inspired the design of the 10–30 trial in our study. We reasoned that if the NO independence with long-duration occlusion reactive hyperemia was the result of prolonged shear stimulus duration, reinfusion of the cuff after a short interval would preserve the NO dependence of the response but take advantage of an enhanced early shear stimulus magnitude. A caveat to employing the longer cuff inflation duration is increased discomfort with negative consequences...
regarding patient compliance; however, that was not a problem in this study. We did not observe an increase in FMD in the 10–30 trial vs. the 5 trial despite the elevated early shear stimulus. This suggests that the elevated stimulus beyond 30 s was important to the response development in the 10 trial. Finally, with regard to the mechanisms of FMD with enhanced shear stimulus duration, the lack of effect of NOS blockade on the FMD within the 10–30 trial argues against an obligatory role for NO in the FMD response to this shear stimulus profile.

**Trial 5 vs. 5-R**

This experiment required that we perform several trials in succession. This created concern regarding the possibility of an effect of repeated exposure to reactive hyperemia-evoked shear stimulus. To address this we performed a second standard reactive hyperemia test (trial 5-R) at the end of the saline and L-NMMA protocols in a subset of seven subjects. We found no effect of repeated trials on the %FMD or the absolute change in diameter, indicating that there was no effect of repeated exposure to hyperemia on the reactivity of the endothelium.

**FMD as a Bioassay of Endothelial Function and Health**

FMD is assumed to reflect NO bioavailability, but if its contribution to the response is not obligatory (as reported here) and can be compensated for, then the degree to which FMD reflects NO bioactivity is highly variable. Therefore, the use of FMD as a specific bioassay for NO may need to be reconsidered. For example, when FMD is diminished in an individual, it could be that it reflects a problem with other FMD mechanisms, either in concert with NO (no vasodilators functioning) or independently (NO functioning but not compensating for other vasodilator function that is impaired).

Given that there is clearly a relationship between vascular health and FMD (3, 7, 8, 10, 13, 25, 27, 28, 33), this suggests that NO bioavailability (and its associated anti-atherogenic properties) is not the only mechanism responsible for the connection. Importantly, FMD can provide direct clinical benefit independent of the vasoprotective effects of the vasodilatory mechanism. In the coronary circulation, FMD is an important part of the exercise response, ensuring adequate myocardial perfusion (12, 14, 15). However, diseased coronary arteries may experience impaired FMD or even vasoconstriction in response to increased flow (12, 15). This exacerbates any existing ischemia that may be caused by vessel narrowing. Thus a preserved or improved FMD response can provide direct clinical benefit to patients at risk for ischemia that may be caused by vessel narrowing.

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