Is Flow-Mediated Dilation Nitric Oxide Mediated?

A Meta-Analysis

Daniel J. Green, Ellen A. Dawson, Hans M.M. Groenewoud, Helen Jones, Dick H.J. Thijssen

**Abstract**—Flow-mediated dilation (FMD) is a noninvasive index of endothelial function and vascular health in humans. Studies examining the role of nitric oxide (NO) are not conclusive. In this article, we quantified the contribution of NO in FMD of conduit arteries and explored the effect of the protocol (ie, distal cuff, ≈5-minute ischemia) and method of analysis (ie, automated and continuous edge detection) on the NO dependency of this test. A systematic review and 3-stage meta-analysis of published crossover studies that measured FMD under local infusion of saline or the NO synthase blocker \(N\)\(^\text{\text{-monomethyl-L-arginine (L-NMMA)}}\) was undertaken. Twenty studies met the inclusion criteria for stage 1 (374 individual comparisons). The meta-analyzed outcome was the difference in FMD between infusion of saline (ie, FMD\text{saline}) and NO synthase blocker (ie, FMD\text{L-NMMA}). Overall, FMD\text{saline} was 8.2% (95% confidence interval [CI], 6.8%–9.6%) compared with FMD\text{L-NMMA} of 3.7% (95% CI, 3.1%–4.3%; \(P<0.001\)). Stage 2 analysis focused on studies that used the most commonly adopted approach in healthy volunteers (ie, distal cuff placement, ≈5-minute occlusion), which similarly revealed a significant NO contribution to FMD (FMD\text{saline}, 6.5% [95% CI, 5.7%–7.3%]; FMD\text{L-NMMA}, 0.9% [95% CI, 0.5%–1.3%]; \(P<0.001\)). Stage 3 meta-analyzed the studies that adopted the commonly adopted approach and automated, continuous method of analysis, which also revealed a significant contribution of NO to the FMD (FMD\text{saline}, 6.9% [95% CI, 6.0%–7.8%]; FMD\text{L-NMMA}, 2.4% [95% CI, 1.1%–3.7%]; \(P<0.001\)). This comprehensive analysis demonstrates that FMD of conduit arteries in humans is, at least in part, mediated by NO. (Hypertension. 2014;63:376-382.)

**Key Words:** conduit artery ■ FMD protocol ■ \(N\)\(^\text{\text{-monomethyl-L-arginine ■ nitric oxide ■ shear stress ■ vascular}}\)

Endothelial cells synthesize paracrine hormones such as nitric oxide (NO),\(^{1}\) which have important antiatherogenic properties.\(^{2}\) Early studies in humans that used intra-arterial infusion of endothelial stimulants and inhibitors established that endothelial function is impaired in individuals with cardiovascular diseases and risk factors\(^{3,6}\) and that coronary\(^{7,8}\) and peripheral\(^{9–12}\) endothelial dysfunction can predict clinical events. In 1992, Celermajer et al\(^{13}\) introduced an ultrasound technique involving temporary suprasystolic cuff inflation around the limbs and measurement of conduit artery dilation consequent to postischemic hyperemia and an arterial shear stress stimulus. Owing to its relative simplicity, its noninvasive nature, and prognostic research studies that have consistently suggested clinical utility,\(^{14–20}\) this so-called flow-mediated dilation (FMD) approach has become widely used by both scientists and clinicians.

Despite the popularity of the FMD approach, important questions remained unanswered when it was introduced, and the uptake of the technique largely preceded detailed physiological description of underlying mechanisms. In the watershed article of Celermajer and Deanfield, it was reasonably assumed, on the basis of previous animal data,\(^{21,22}\) that FMD was a flow- and NO-mediated response, despite no evidence being available about its NO dependency. Eventually, the assumption that FMD reflected NO-mediated vascular function was reinforced by physiological experiments that infused NO blockers upstream from an insolated conduit artery and reported that dilator responses to ischemia and shear stress were almost completely abolished.\(^{23–26}\) Nonetheless, there have been some well-designed and controlled studies that have been unable to inhibit FMD using NO blockade.\(^{27,28}\) This disparity in the literature may not only be related to differences in the methodology used to measure FMD, but also to the inclusion of different population groups.

Therefore, the aim of this study was to systematically review and meta-analyze the literature relating to crossover studies that have compared FMD under local infusion of saline (FMD\text{saline}) with FMD under local infusion of the NO blocker \(N\)\(^\text{\text{-monomethyl-L-arginine (L-NMMA)}}\) (FMD\text{L-NMMA}) to obtain an estimate of the contribution of NO to FMD in human conduit arteries. We used a staged approach and explored the contribution of NO in FMD studies that adopted the standardized approach (ie, using distal cuff placement and ≈5-minute cuff inflation in healthy individuals). Finally, the effect of automated, continuous method of analysis on NO dependency was explored.
Methods

Search Strategy
A systematic review of peer-reviewed studies was conducted, examining FMD of a conduit artery in humans in vivo under intra-arterial infusion of saline and L-NMMA. The literature search was conducted using the PubMed, Scopus, and Web of Science scholarly databases (1992–2013). The keywords and phrases used in the online search included NO-blockade or L-NMMA and flow-mediated dilatation or FMD. Reference lists from published articles were examined to identify any other relevant studies not cited in online databases.

Review Process
The initial search process (FMD or flow-mediated or flow-dependent or flow-related) and NO synthase inhibition or NO synthase inhibition not animals) resulted in the identification of 98 studies that could potentially be included in the analyses (Figure 1). Published literature relating to assessment of FMD under infusion of an NO blocker (eg, L-NMMA) compared with saline infusion was compiled. The importance of inclusion of an FMD baseline test is that it controls for any potential effect of the volume of infusate during protocols. Two members of the research team (D.H.J.T. and D.J.G.) independently selected the studies for inclusion and later met to reach mutual consensus. Based on the abstract, 68 articles did not conform to the inclusion criteria or did not provide relevant data (Figure 1). After reading the full text of the remaining 30 articles, another 10 articles were excluded based on our inclusion criteria (Figure 1). Twenty studies met our inclusion criteria, and a total of 10 articles applied their saline/NO blockade protocol in 2, 3, or 4 distinct groups. This resulted in an overall sample of 374 individual observations.

Criteria
Our inclusion criteria for phase 1 required that studies used an ischemic stimulus (without simultaneous physical or pharmacological procedures), specifically provided information on FMD techniques (cuff position, duration of inflation, and method of analysis), and had no other simultaneous pharmacological intervention or infusion technique. Twenty studies met the initial inclusion criteria and were incorporated into phase 1 of the analysis. Subsequently, a subset of these studies was created by implementing a more stringent inclusion criterion to form phase 2 of our analysis. For this phase of the analysis, 13 studies were selected that used the most commonly adopted approach (ie, distal cuff placement, 5-minute occlusion, healthy volunteers). In phase 3, we examined the effect of using automated, continuous analysis of the diameter by repeating the meta-analysis on a subset of 9 studies using an automated analysis and the commonly adopted approach.

Quality Assessment
A systematic appraisal was devised by the research team to provide an assessment of study quality based on measurement technique and data reporting. The quality assessment was adapted from existing assessment tools and guideline statements to appraise the following items: (1) Is a power calculation reported? (2) Are entry criteria and exclusions clearly stated? (3) Were smokers excluded from participation? (4) Does the study use an ultrasound imaging technique? (5) Is an edge-detection software used? (6) Is the coefficient of variation of the FMD technique reported? (7) Was the dose of L-NMMA reported? (8) Has a placebo/saline control experiment been performed? (9) Is an explanation for missing data given? (10) Was FMD data (ie, % change from baseline) clearly presented with average and SD/SE? Studies were categorized into low quality (0–3 items; n=0), acceptable quality (4–5 items; n=1), adequate quality (6–8 items; n=17), and high quality (9–10 items; n=2; Table).

Statistical Analyses
We conducted a random-effects (DerSimonian–Laird) meta-analysis on the mean difference in FMD between saline and NO blocker infusion at whole study level. To quantify study-to-study heterogeneity, a Q statistic at P<0.05 and an I² statistic >50% were deemed significant. This procedure was repeated in phase 2 for those studies that adopted what has become considered the standardized approach (ie, distal cuff position, 5-minute occlusion). Finally, for phase 3, the mean difference in FMD between saline and NO blocker infusion was examined in a subset of studies that adopted the standardized approach and used an automated analysis. The number of participants in the study was used as the weighted factor. Publication bias was explored with the observation of a funnel plot and Egger linear regression test. A value of P<0.05 was considered significant. Weighted mean difference and forest plots were computed with StatsDirect version 2.7.8.

Results
Phase 1
The pooled mean value of conduit artery FMDsaline was 8.2% (95% confidence interval [CI], 6.8%–9.6%) and FMDL-NMMA
3.7% (95% CI, 3.1%–4.3%; Figures 2 and 3). The pooled mean difference between FMDsaline and FMDL-NMMA was 3.9% (95% CI, 2.6%–5.2%; \(P < 0.001\)). There was evidence of statistically significant heterogeneity in this difference between conduit artery FMD between studies (\(I^2 = 99\%\)) but little evidence of publication bias (Egger intercept=0.38; \(P = 0.88\)).

Phase 2

When we limited our analysis to studies that use distal cuff placement and \(\approx 5\)-minute cuff occlusion in healthy volunteers, the pooled mean value of conduit artery FMDsaline was 6.5% (95% CI, 5.7%–7.3%) and FMDL-NMMA 0.9% (95% CI, 0.5%–1.3%), with a pooled difference of 4.7% (95% CI, 3.8%–5.6%; \(P < 0.001\)).

Phase 3

Here, studies were limited to the standardized approach and automated analysis of the conduit artery diameter. We found that the pooled mean value of conduit artery FMDsaline was 6.9% (95% CI, 6.0%–7.8%) and FMDL-NMMA 2.4% (95% CI, 1.1%–3.7%), with a significant pooled difference of 4.6% (95% CI, 3.6%–5.5%; \(P < 0.001\)).

Discussion

This is the first meta-analysis investigating the contribution of NO to the FMD response. It included 20 published studies and 374 individual comparisons. It provides compelling evidence that at least half of the FMD response in human
conduit arteries is mediated by NO. This in-depth and staged analysis provides a more precise estimate and novel insight into the highly relevant topic of NO dependency of FMD than individual studies that have typically involved small sample sizes and disparate methodology. We conclude that conduit artery FMD is, at least partly, mediated by NO even in young healthy individuals. This should be taken into consideration, especially given the clinical relevance that is given to NO and its antithrombotic properties.

Our analysis provides a more precise estimate and novel insight into the highly relevant topic of NO dependency of FMD than individual studies in this field that have typically involved small sample sizes and disparate methodology. For example, Wray et al44 recently reported that brachial artery FMD was not NO mediated. This conclusion was based on a sample size of 8 individuals and focused the interpretation of results on FMD responses that were ratio normalized to shear rate, an approach that is statistically flawed47 and not supported by consensus guidelines.46 In contrast, when all extant data are taken into account, the current analysis leads to a conclusion that brachial artery FMD is, at least partly, mediated by NO even in young healthy individuals.

We used a staged meta-analysis approach to examine the potential effect of differences in the methodology adopted. When all studies were included, regardless of technique or methodology, the contribution of NO to the FMD was ≈47%. However, many of these studies adopted technical approaches that have subsequently been reported as largely NO independent. For example, Mullen et al24 found that 5 minutes but not 15 minutes of occlusion led to an NO-mediated dilation, whereas Doshi et al25 found that the FMD response was fully blocked using L-NMMA when a distal (98%) but not proximal cuff placement was adopted (35%). We, therefore, repeated our meta-analysis on a subgroup of studies for which we used more stringent criteria to include only those that adopted the most common contemporary approach (ie, 5 minutes of distal cuff occlusion in healthy volunteers). This analysis revealed a similarly high degree of NO dependency, with ≈72% of the dilation being mediated by NO.

One important methodological issue that we have explored in phase 3 of our analysis is the method of analysis used to determine changes in arterial diameter. Early FMD studies typically adopted manual placement techniques and had low temporal resolution, whereas more recent experiments moved to automated and continuous edge-detection and wall-tracking software that is largely devoid of observer bias. Our findings suggest that the effect of L-NMMA on FMD remains substantial in studies that adopted the automated analysis, with an average contribution of NO to the response in these studies of ≈67%. It is, therefore, important to acknowledge that, even when automated techniques are adopted, there remains a significant contribution of NO to the FMD response so long as distal cuff placement and an appropriate cuff occlusion duration (≤5 minutes) are adopted.

Our observation that NO explains approximately half of the FMD response raises questions about the role of other vasoactive substances in the FMD response and the possibility of redundancy between mechanisms, that is the compensatory overlap between vasodilator mechanisms to maintain vasodilator integrity in the face of knockout of any 1 pathway.46,49 For example, increases in shear stress are known to lead to the corelease of NO and prostacyclin,50 and Osanai et al51 found that inhibition of endothelial NO synthase enhanced the production of prostacyclin in response to shear stress. Bellien et al52,53 also demonstrated a role for endothelium-derived hyperpolarizing factor (EDHF) during a sustained shear stress stimulus (ie, hand warming) because this vasodilator response is attenuated to a greater extent when EDHF inhibition is performed in combination with NO blockade compared with selective inhibition. This supports the presence of cross-talk between NO and EDHF52,53 and suggests a role for EDHF in the FMD response. Furthermore, increased activity of EDHFs is apparent in individuals with hypertension44 or coronary artery disease55 compared with healthy controls, suggesting that the cross-talk between NO and EDHF may differ between groups. Shear stress also affects endothelin-1, a potent vasoconstrictor that impairs NO production.56 A recent study found that endothelin A receptor blockade improves the FMD response in patients with heart failure, suggesting a role for endothelin-1 in the FMD response.57 Data on angiotensin II, another vasoconstrictor, demonstrate conflicting results about the effect on the FMD response.29,33 Finally, increased activity of the sympathetic nervous system has been shown to impair the conduit artery FMD response in humans in some studies.58–60 Taken together, these studies suggest that interplay between various vasodilator and vasoconstrictor stimuli is likely to explain the total FMD response.

Based on previous data, a smaller contribution of NO to the FMD response might be expected in individuals with cardiovascular risk factors or diseases.32–34,38,40,41,62 Although a formal meta-analysis on the comparison between groups with different health status was limited by the modest sample size available at this time, the data in the Table suggest a substantial effect of L-NMMA on FMD in both healthy subjects and subjects with cardiovascular risk or disease. Hence, one potential moderating factor that may affect the question of NO dependency in distinct patient groups relates to the lower baseline FMD in subjects with established cardiovascular disease or risk. A related issue to the effect of differences in baseline FMD between groups is
that L-NMMA infusion reduces blood flow and may, therefore, lead to smaller shear-mediated diameter. This smaller baseline artery diameter may, in itself, lead to arithmetically driven overestimation of the FMD% during L-NMMA infusion. In any event, such an effect would lead to an underestimation of the contribution of NO to the conduit artery dilation.63

A strength of our study was that we included 20 studies involving 374 individual within-subject comparisons. However, some limitations are germane. The number of studies in which invasive cannulation for L-NMMA delivery has been performed limits our capacity to investigate the effect of numerous potential moderating variables. For example, it is conceivable that different arteries possess distinct degrees of NO dependency. Previous studies have typically examined brachial or radial artery responses, making it somewhat difficult to extrapolate these findings to other conduit arteries, especially given the heterogeneity in endothelial NO synthase expression level64 and vasodilator responsiveness65 between different arterial beds. That said, the only extant femoral artery study demonstrated a significant effect of L-NMMA on the FMD response.36 Our primary outcome was the percentage change in diameter from a resting baseline to a hyperemic peak value. This approach to expressing change in diameter is ubiquitous across studies, which enabled the meta-analysis to be undertaken, yet this approach might not always be the most appropriate. Percentage change and ratio statistics have been criticized in many research contexts because they may not scale consistently over the full range of diameter sizes.63 There

Figure 3. Forrest plot demonstrating the individual study differences in flow-mediated dilation (FMD)saline compared with FMDL-NMMA to show contribution of nitric oxide to the FMD response. Also shown in the final row (open symbol) is the random-effects estimate of the mean difference between FMDsaline and FMDL-NMMA.
are other approaches to expressing change while controlling for general anatomic size, involving ANCOVA and allometric techniques. At present, these approaches have only been applied to a few recent FMD studies.

Perspectives

Our meta-analysis demonstrates that NO contributes, at least in part, to the FMD response of conduit arteries in humans. Furthermore, we found that although the method of analysis may alter the contribution of NO to FMD, studies involving both manual placement techniques and automated analysis exhibited substantial NO dependency. The finding that FMD is partly NO mediated is of clinical importance because FMD is often used in large clinical trials and NO possesses potent antiatherogenic properties. Taken together, our findings suggest that FMD is a valid measure of endothelium-dependent vasomotor function in humans, which is partly NO mediated.

Sources of Funding

D.H.J. Thijssen is recipient of the E. Dekker stipend (Netherlands Heart Foundation, 2009/0764). D.J. Green is funded by the Australian Research Council (DP 130103793).

Disclosures

None.

References

What Is New?

- Studies examining the role of nitric oxide (NO) in flow-mediated dilation (FMD) are not conclusive.
- We quantified the contribution of NO to FMD in all extant studies involving arterial cannulation and NO blockade.

What Is Relevant?

- FMD of conduit artery is frequently used to examine endothelial function and is strongly related to cardiovascular risk.

Summary

Approximately half of the dilation of the FMD response of conduit arteries is mediated by NO. A large contribution of NO to the FMD response was apparent in studies that adopted the standardized approach (ie, distal cuff, 5 minutes), a finding independent of the method of analysis.
Is Flow-Mediated Dilation Nitric Oxide Mediated?: A Meta-Analysis
Daniel J. Green, Ellen A. Dawson, Hans M.M. Groenewoud, Helen Jones and Dick H.J. Thijssen

Hypertension. 2014;63:376-382; originally published online November 25, 2013;
doi: 10.1161/HYPERTENSIONAHA.113.02044
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/63/2/376

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/