**Original Article**

**Methodological Validity and Feasibility of the Nitric Oxide Clamp Technique for Nitric Oxide Research in Human Resistant Vessels**

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*N*-methyl-1-arginine (L-NMMA) has been widely used for nitric oxide (NO) research, particularly for the assessment of NO-dependent vasodilatation evoked by agonists. However, such experiments may not be straightforward because L-NMMA causes vasoconstriction, which itself must non-specifically affect responses to any vasoactive agents. Therefore, in order to more accurately estimate the roles of NO in human vessels *in vivo*, we developed an NO clamp technique that uses co-infusion of an NO donor with L-NMMA.

To assess the validity and feasibility of this technique, we compared the effects of intra-arterial infusion of L-NMMA on the forearm blood flow responses to vasodilators with and without the NO clamp technique in healthy males. All drugs were intra-arterially infused and changes in forearm blood flow (FBF) were measured by strain-gauge plethysmography. Vasodilatation evoked by atrial natriuretic peptide was significantly attenuated by L-NMMA alone (p = 0.001) but not by the NO clamp technique. L-NMMA significantly attenuated the responses to acetylcholine either with or without the NO clamp technique. However, the ratio of the area under the curve (AUC) of acetylcholine with L-NMMA to that without L-NMMA was significantly higher when the NO clamp technique was not used (AUC ratio: 0.62 ± 0.13 vs 0.48 ± 0.14, respectively; p = 0.031).

The contribution of NO to the FBF responses to vasodilators may be more properly assessed by the co-infusion of L-NMMA with the NO clamp technique than by L-NMMA alone. Our NO clamp technique thus appears to be valid and feasible for human NO research. (Hypertens Res 2004; 27: 351–357)

**Key Words**: N*-methyl-L-arginine, nitric oxide, atrial natriuretic peptide, acetylcholine, nitric oxide clamp

**Introduction**

The roles of nitric oxide (NO) in the regulation of human vascular tone have been extensively investigated using forearm blood flow (FBF) measurement with the intra-arterial infusion of agonists that simulate NO production and of N*-methyl-L-arginine (L-NMMA), which inhibits NO synthase activity (1). The basal vascular NO production can be indirectly represented by the reduction of FBF during the infusion of L-NMMA (2–5). The stimulated NO production of human vessels as a pivotal part of vascular endothelial function can be assessed by the vasodilator response to acetylcholine with L-NMMA (6, 7). The contribution of NO to vasodilatation evoked by any agonist can also be assessed by the co-infusion of vasodilators and L-NMMA (8–10). The results from these experiments, however, should be carefully interpreted, since L-NMMA causes robust vasoconstriction (1) and differences in basal FBF may non-specifically affect responses to vasodilators (11). Stroes et al. first described the NO clamp technique, which pharmacologically restores the reduced FBF induced by L-NMMA in order to avoid the...
non-specific effects of L-NMMA and to assess the contribution of NO as accurately as possible (12). We have also used a modified version of this technique for human NO research (13). In the present study we estimated the methodological validity of this technique and then tested our hypothesis that a reduction in FBF by L-NMMA may non-specifically attenuate the effects of NO-independent vasodilators (e.g., atrial natriuretic peptide (ANP)) and overestimate the NO-dependent part of vasodilatation by acetylcholine if not for NO clamp technique.

Methods

Subjects

Six (protocol 1 and 2), 8 (protocol 3), and 8 (protocol 4) normotensive healthy men between 20 and 24 years of age (mean: 22 years) were studied. All subjects had normal results of routine physical and laboratory examinations. Written informed consent was obtained from each subject after a full explanation of the study. The Yokohama City University Hospital Ethics Committee approved all study protocols.

FBF Measurement

All experiments were performed in a quiet, temperature-controlled room. The subjects fasted overnight and abstained from smoking cigarettes and drinking beverages containing alcohol or caffeine for at least 12 h before the study. FBF was measured by bilateral venous occlusion strain gauge plethysmography. The details of the method and the reproducibility of the results are described elsewhere (14, 15). All drugs were infused into a catheter inserted into the brachial artery of the non-dominant arm.

Baseline FBF was measured at least 30 min after cannulation, after confirming that this variable was stable on 3 consecutive occasions. The drug infusion rate was maintained at 1 ml/min throughout the study.

Study Protocol

Protocol 1: Changes in FBF during L-NMMA Infusion

L-NMMA (Clinalfa, Laufelfingen, Switzerland) was infused intra-arterially at 8 µmol/min for 120 min in each subject. Blood pressure and FBF were measured every 10 min.

Protocol 2-a: Co-Infusion of Glycerol Trinitrate with L-NMMA

L-NMMA at 8 µmol/min was infused intra-arterially for 150 to 180 min (L-NMMA alone for 60 min, with glyceryl trinitrate [GTN; Nihon Kayaku, Tokyo, Japan] for 70 to 90 min, and L-NMMA alone again for 30 min). The concomitant infusion of GTN was started 60 min after starting L-NMMA infusion to restore reduced FBF. The dose of GTN was titrated every 10 min from 50 ng/min until FBF returned to the baseline value (NO clamp) (12, 13). The duration of each dose of GTN in this protocol was valid because steady-state FBF during GTN infusion was obtained within 5 min. Once the individual dose was determined, GTN was infused for another 60 min. FBF was measured every 10 min until 30 min after the termination of GTN to confirm the stable, sustained inhibition of NO production.

Protocol 2-b: Repeated NO Clamp with the Individually Determined Doses of GTN

In the 4 subjects participating in protocol 2-a, L-NMMA was intra-arterially infused at 8 µmol/min and GTN at the titrated doses in protocol 2-a was co-infused 20 min after starting L-NMMA. FBF was measured every 10 min.

Protocol 3: Effects of L-NMMA Alone and L-NMMA with GTN on the Vasodilator Response to Acetylcholine in Normal Volunteers

This protocol involved 2 study days. Acetylcholine (Dai-ichi Seiyaku, Tokyo, Japan) was infused intra-arterially at 50, 125, and 250 ng/min (10 min per dose), on two occasions on Day 1. After the first infusion of ANP and a 30-min washout period, the infusion of L-NMMA at 8 µmol/min was started. The second ANP infusion was commenced at least 30 min after starting the L-NMMA infusion. On Day 2, after the first infusion of ANP at the same doses and a 30-min washout period, the infusion of L-NMMA at 8 µmol/min was started, followed by co-infusion of GTN in order to restore reduced FBF (NO clamp). The second ANP infusion was commenced at least 10 min after starting the GTN infusion.

Protocol 4: Effects of L-NMMA Alone and L-NMMA with GTN on the Vasodilator Response to Acetylcholine in Normal Volunteers

This protocol also involved 2 study days. Acetylcholine (Dai-ichi Seiyaku, Tokyo, Japan) was infused intra-arterially at 50, 100, 200, and 400 nmol/min (10 min per dose), on two occasions on Day 1. After the first infusion of acetylcholine and a 30-min washout period, the infusion of L-NMMA at 8 µmol/min was started. The second acetylcholine infusion was commenced at least 30 min after starting the L-NMMA infusion. On Day 2, after the first infusion of acetylcholine at the same doses and a 30-min washout period, the infusion of L-NMMA at 8 µmol/min was started followed by co-infusion of GTN in order to restore reduced FBF (NO clamp). The second acetylcholine infusion was commenced at least 10 min after starting the GTN infusion.

Analysis of FBF Data

All FBF data were obtained via a Mac Lab 4 chart recorder (AD Instruments; Hamstead, London, UK). The percentage change in the ratio of FBF from the baseline value was calculated as \( \frac{F(i)\cdot F(ni)}{F(ni)} \cdot 100\% \), where \( F(i) \) and \( F(ni) \) represent forearm blood flow in the infused arm and non-infused arm, respectively, during baseline measurement (B) and drug infusion (D).
Statistical Analysis

Data are shown as the means ± SD unless otherwise indicated. The dose-response curves of acetylcholine and ANP during co-infusion of L-NMMA with or without the NO clamp technique were compared with those during infusion of saline by repeated measures of analysis of variance (ANOVA). Areas under the dose-response curve (AUC) of acetylcholine alone and acetylcholine with L-NMMA were calculated individually. The ratio of the AUC with L-NMMA to that without the NO clamp technique was compared by paired t-test.

Results

Protocol 1: Effect of L-NMMA on FBF

L-NMMA infusion significantly reduced FBF, and a steady state was achieved approximately 20 min after starting the infusion (Fig. 1). There was no significant change in blood pressure or heart rate during the infusion of L-NMMA. FBF during the L-NMMA infusion remained constant for at least 90 min.
Protocol 2-a: Effect of Concomitant Infusion of GTN (NO Clamp Technique)

Figure 2 shows the changes in FBF during L-NMMA and GTN infusion. L-NMMA significantly decreased blood flow and GTN restored blood flow to the baseline value. Individually titrated doses of GTN for clamp ranged from 50 ng/min to 250 ng/min. FBF during the infusions of L-NMMA and GTN remained constant for 60 min and returned to the value during the infusion of L-NMMA alone after the withdrawal of GTN.

Protocol 2-b: Repeated NO Clamp with the Individually Determined Doses of GTN

GTN at the individually determined doses in protocol 2-a successfully restored FBF during L-NMMA infusion in the same subjects (3.26 ± 0.78 ml/min/dl before L-NMMA, 1.73 ± 0.41 after L-NMMA, 3.21 ± 0.63 after GTN).

Protocol 3: Effects of L-NMMA Alone and L-NMMA with GTN (NO Clamp Technique) on the Vasodilator Response to ANP in Normal Volunteers

Figure 3 shows the effects of L-NMMA alone (Day 1) and L-NMMA with the NO clamp technique (Day 2) on the vasodilator response to ANP. Although the FBF before the second infusion of ANP was significantly lower than that before the first infusion on Day 1 (L-NMMA only) (2.06 ± 0.60 vs. 3.70 ± 1.14 ml/min/dl, p < 0.0005), the FBF before each infusion of acetylcholine was comparable on Day 2 (L-NMMA with the NO clamp technique) (3.59 ± 1.05 vs. 3.43 ± 0.87 ml/min/dl). Although co-infusion of L-NMMA significantly attenuated the response to acetylcholine on both study days (p < 0.0001 on Day 1, p = 0.0014 on Day 2) the attenuation of the response to acetylcholine by L-NMMA was significantly smaller with the NO clamp technique than that without the NO clamp technique (AUC ratio: 0.62 ± 0.13 vs. 0.48 ± 0.14, p = 0.031).

Discussion

Validity of the NO Clamp Technique

We showed that the intra-arterial infusion of L-NMMA significantly reduced FBF by approximately 50%, and the co-infusion of GTN successfully restored FBF to the baseline values. Although Stroes et al. (12) introduced the NO clamp technique, we here validated this technique with respect to
several methodological points for the first time. First, reduced FBF during the infusion of L-NMMA was achieved 20 min after starting the infusion, suggesting that infused L-NMMA may maximally inhibit NO production of forearm vessels at this point. This time-dependent effect of infused L-NMMA on FBF was first shown by Duffy et al. (16) and reproduced by our present study. This result suggests that the infusion of GTN to restore reduced FBF should be started approximately 20 min after starting L-NMMA infusion, and thus that Stroes et al. (12) started sodium nitroprusside (5 min after L-NMMA infusion) too quickly. Second, we also showed that reduced FBF by L-NMMA was successfully restored by GTN and that FBF during the co-infusion of L-NMMA and GTN at individually titrated doses was kept constant for 60 min. Although the doses of GTN ranged from 50 to 250 ng/min, the titration period was not time-consuming, since the steady-state response to GTN could be achieved within 5 min. Third, FBF after the withdrawal of GTN was comparable with that before GTN, suggesting constant inhibition of NO production during the NO clamp technique. Finally, we showed that the individually determined dose could be used for another experiment in the same subjects. All these findings support the validity of our NO clamp technique.

One would question, however, if using an NO donor (GTN) could be justified for NO clamp. We chose GTN for several reasons. First, GTN acts directly through vascular smooth muscle cells and does not affect endothelial function. Second, supplying exogenous NO with L-NMMA infusion to restore FBF inhibits stimulated NO production but leaves the amount of basal NO unchanged, which may be suitable for the assessment of the contribution of NO to the vasodilating effect of agonists. Finally, no other appropriate vasodilators are available. For example, a steady-state response to hydralazine is achieved more than 1 h after the start of infusion (Ueda S and Wada A, unpublished data).

Feasibility of the NO Clamp Technique

We demonstrated that the intra-arterial infusion of L-NMMA alone significantly attenuated changes in FBF during the infusions of ANP as previously shown by Sugamori et al. (17), which seems to suggest an NO-dependent vasodilating effect of ANP. Costa et al. also demonstrated an NO-dependent effect of ANP (18). They showed that the hypotensive effect of ANP was abolished by the infusion of L-NMMA in rats. However, we also demonstrated that the infusion of L-NMMA with the NO clamp technique had no significant effect on the FBF responses to ANP, which suggests that the vasodilating effect of ANP is not NO dependent in human forearm resistant vessels and the attenuated response to ANP during the infusion of L-NMMA alone may be due to the reduction of basal FBF. Although the NO-dependency of ANP may differ among species or among different types of vascular beds, the hypothesis that ANP elicits vasorelaxation through NO production in human forearm resistant vessels was adequately rejected. This result is consistent with the reports by Winquist et al., which showed endothelium-independent vasorelaxation by ANP (19), and Honing et al., which showed hyperpolarization-dependent, NO-independent vasorelaxation by a C-type natriuretic peptide (20) that shares natriuretic peptide receptor-B with ANP (21).

The vasodilatation evoked by acetylcholine is, albeit not in all vascular beds, deemed NO-dependent because co-infusion of L-NMMA significantly attenuated acetylcholine-induced vasodilatation in healthy volunteers (6, 8). However, we demonstrated that co-infusion of L-NMMA resulted in significantly less inhibition of the acetylcholine response when the NO clamp technique was used than did L-NMMA alone, which may be due to differences in basal FBF, because Chowienczyk et al. showed that vasodilator responses to acetylcholine were significantly dependent on resting FBF (22). Therefore, the contribution of NO to vasodilatation by acetylcholine may have been overestimated without the NO clamp technique and may be less than we previously thought. In fact, Coats et al. recently showed that NO accounted for only 20% of the maximum relaxation response to acetylcholine in human subcutaneous resistance-size arteries (23). Urakami-Harasawa also similarly showed that endothelium-dependent vasorelaxation to acetylcholine was significantly inhibited by L-NMMA and indomethacin in large arteries but not in microvessels (24). Our study suggests that the “true” L-NMMA-sensitive, NO-dependent component may have been approximately 40% of the maximum response to acetylcholine in the forearm-resistant vessels of our subjects. However, it should be noted that the compensatory activation of other endogenous vasoactive systems (e.g., endothelium-derived hyperpolarizing factor [EDHF] (25)) was not taken into account in our experiment.

Our study also showed that non-specific effect of L-NMMA on vasodilator response to acetylcholine was smaller than that to ANP and to bradykinin (BK) particularly at higher doses because of NO dependency. Therefore comparison of NO dependent part of vasodilatation of acetylcholine before and after treatment may be possible without NO clamp, albeit not so accurate as with NO clamp, as long as FBF after L-NMMA is comparable.

Vascular endothelial function in humans has been frequently assessed by the response to acetylcholine of the forearm resistant vessels (5–7, 26, 27). Blunted responses to acetylcholine have been considered to indicate endothelial dysfunction in patients with several diseases, including hypertension (6), hyperlipidaemia (28), and diabetes mellitus (7). However, our results together with the report by Chowienczyk et al. (22) caution that any reduction in basal blood flow could non-specifically blunt the responses to acetylcholine, which cannot be regarded as endothelial dysfunction.

It has also been shown that NO mediates the vasodilating effects of several other vasoactive agents, including BK and serotonin. While the vasodilating effect of serotonin was
completely abolished by L-NMMA even with the NO clamp technique (29), the vasodilating effect of BK, like that of ANP in the present study, was attenuated by L-NMMA alone (30, 31) but not by L-NMMA with the NO clamp technique (13, 29) in human forearm resistant vessels. These results suggest that the vascular action of serotonin depends purely on NO, while that of BK depends on other factors, for example EDHF (29).

Advantage and Disadvantage of the NO Clamp Technique

As discussed above, FBF reduction during the infusion of L-NMMA non-specifically affects the activities of vasodilators, which can be avoided by using the NO clamp technique. Therefore, the effects of inhibition of stimulated NO production on the vascular actions of drugs or peptides may be more accurately evaluated with an NO clamp, which is the apparent advantage of this experimental technique. Although this also can be a disadvantage, because the effect of reduced basal NO cannot be evaluated with an NO clamp, our results suggest that substantial changes in basal FBF may lead to less accurate results.

In conclusion, the contribution of NO to the FBF responses to vasodilators may be more correctly assessed by the co-infusion of L-NMMA with the NO clamp technique than by infusion of L-NMMA alone, which may overestimate the role of NO in agonist-induced vasodilatation. Our results also suggest that adequate comparison of the vasodilator properties of any agonist, as for example between patients and controls, can be made only when the baseline flow of the vascular beds is comparable.

References

22. Chowienczyk PJ, Cockcroft JR, Ritter JM: Blood flow responses to intra-arterial acetylcholine in man: effect of...


