Neuronal Nitric Oxide Synthase Regulates Basal Microvascular Tone in Humans In Vivo

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Background—Nitric oxide (NO) has a pivotal role in the regulation of vascular tone and blood flow, with dysfunctional release contributing to disease pathophysiology. These effects have been attributed to NO production by the endothelial NO synthase (eNOS); however, recent evidence suggests that a neuronal NO synthase (nNOS) may also be expressed in arterial vessels.

Methods and Results—We undertook a first-in-humans investigation of the role of nNOS in the local regulation of vascular blood flow in healthy subjects. Brachial artery infusion of the nNOS-specific inhibitor S-methyl-L-thiocitrulline (SMTC, 0.025 μmol/min to 0.2 μmol/min) caused a dose-dependent reduction in basal flow, with a 30.1±3.8% decrease at the highest dose (n=10; mean±SE; P<0.01). The effect of SMTC was abolished by coinfusion of the NO synthase substrate L-arginine but was unaffected by D-arginine. A similar reduction in basal flow with the nonsclective NO synthase inhibitor N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA; 37.4±3.1%, n=10) required a 20-fold higher dose of 4 μmol/min. At doses that produced comparable reductions in basal flow, only L-NMMA (4 μmol/min) and not SMTC (0.2 μmol/min) inhibited acetylcholine-induced vasodilation; however, both SMTC and L-NMMA inhibited the forearm vasodilator response to mental stress.

Conclusions—Basal forearm blood flow in humans is regulated by nNOS-derived NO, in contrast to the acetylcholine-stimulated increase in blood flow, which, as shown previously, is mediated primarily by eNOS. These data indicate that vascular nNOS has a distinct local role in the physiological regulation of human microvascular tone in vivo.


Key Words: nitric oxide synthase ▪ blood flow ▪ vasculature ▪ nitric oxide ▪ endothelium ▪ vasodilation

Nitric oxide (NO) has a pivotal role in the regulation of vascular tone and blood flow through its potent vasodilator activity.\textsuperscript{1} It is well established that local intra-arterial infusion of the nonsclective NO synthase (NOS) inhibitor N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA) into the human forearm significantly reduces resting blood flow,\textsuperscript{2} whereas systemic L-NMMA infusion causes a transient increase in blood pressure.\textsuperscript{3} Similar effects are described in animals, including mice lacking the endothelial NOS (eNOS) isoform,\textsuperscript{4} and have generally been attributed to the continuous release of NO from eNOS expressed in the vascular endothelium. The NO-dependent vasodilator effects elicited by increases in flow or by acetylcholine infusion are also thought to involve endothelial eNOS,\textsuperscript{1} and impairment of these responses (known as “endothelial dysfunction”) is a recognized prognostic marker for future cardiovascular events.\textsuperscript{5,6} Interestingly, the impaired flow- or acetylcholine-mediated vasodilation observed in human cardiovascular disease does not always correlate well with basal NO release,\textsuperscript{7} which suggests that regulation of vascular function by NO may be subserved by different mechanisms and possibly different sources of NO. Indeed, recent experimental studies have suggested that neuronal NOS (nNOS)–derived NO can influence vascular tone either by attenuating α-adrenergic vasoconstrictor responses (eg, in skeletal muscle)\textsuperscript{8} or by inducing direct vasodilation (eg, in cerebral arteries).\textsuperscript{9} However, investigation of the potential role of nNOS in regulating human vascular tone in vivo has not hitherto been possible owing to the lack of available inhibitors suitable for use in humans. In the present study, we used intra-arterial infusion of the NOS-selective inhibitor S-methyl-L-thiocitrulline (SMTC)\textsuperscript{10} in healthy male volunteers to investigate the role of nNOS-derived NO in the local regulation of basal blood flow and acetylcholine-mediated vasodilation in vivo.

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Methods

The study was approved by the local Research Ethics Committee after assessment of an independent toxicology report on the first-in-
humans use of SMTC. All subjects included in the study provided written informed consent.

Forty-eight healthy normotensive normcholesterolemic male volunteers (28±1.1 years of age) who were not taking any regular medications were included in the study. Some subjects participated in >1 of the study protocols (Figure 1), which were performed on different days at least 1 week apart. Each inhibitor or vasodilator was studied on a separate occasion. Subjects were asked to abstain from caffeine for at least 12 hours before the studies, which took place in a quiet temperature-controlled room (23°C to 25°C) after at least 30 minutes of rest. Intra-arterial infusions were delivered through a 27-gauge needle catheter inserted into the brachial artery, with a constant-rate pump set at 1 mL/min. Forearm blood flow was measured by venous occlusion plethysmography. During administration of vasoactive drugs, blood flow was measured over the final 2 minutes of infusion, and the mean of 5 measurements was used for analysis.

SMTC at a purity >99% was obtained from Calbiochem (Nottingham, United Kingdom). It was formulated in-house as an aqueous solution with an appropriate pH for injectables. After bioburden testing, SMTC was processed as a sterile injection and stored at an external pharmaceutical testing laboratory (Wickham Laboratories, Hampshire, United Kingdom). Each batch was tested for absence of endotoxin according to British Pharmacopoeia standards. Acetylcholine was obtained from CIBA Vision Ophthalmics (Hampshire, United Kingdom), L-NMMA from Clinalfa (Laufelfingen, Switzerland), norepinephrine from Abbott Laboratories (Berkeley, United Kingdom).

Protocol 1: Effect of SMTC or L-NMMA on Basal Flow
Studies in animals suggest that an SMTC concentration of ~10 μmol/L is optimal for inhibition of nNOS-mediated responses without affecting the eNOS-mediated response to acetylcholine, when infused intra-arterially in vivo.12 After saline infusion for 15 minutes, we measured the effects on forearm blood flow of 4 cumulative doses of either SMTC or L-NMMA, each infused for 6 minutes (Figure 1A; n=10 subjects per group). SMTC was infused at 0.025, 0.05, 0.1, and 0.2 μmol/min to achieve estimated local concentrations of 1.25, 2.5, 5, and 10 μmol/L, respectively, and L-NMMA was infused at 0.5, 1, 2, and 4 μmol/min on the basis of previous dose-response studies in the human forearm.11 The first 7 subjects administered SMTC were reviewed at 24 hours and 7 days after the study for clinical assessment and blood biochemistry to ensure that no unanticipated side effects had resulted from this first-in-humans administration; no problems were observed in any subject in the present study.

Protocol 2: Effect of L-Arginine or D-Arginine on Response to SMTC
After 15 minutes of saline infusion, either L-arginine or D-arginine (40 μmol/min; n=6 per group) was infused for 10 minutes before coinfusion of cumulative doses of SMTC as in protocol 1 (Figure 1B). Blood flow was measured during L-arginine or D-arginine infusion and at each dose of SMTC during coinfusion.

Protocol 3: Effect of SMTC or L-NMMA on Vasodilator Responses to Acetylcholine
Saline was infused for 15 minutes, and then the response to acetylcholine (40 and 80 nmol/min; 6 minutes each) was measured. After a 15-minute saline washout, either SMTC or L-NMMA was administered as in protocol 1, and the response to acetylcholine was assessed again during infusion of the highest dose of inhibitor (n=10 each). We also performed this protocol using the NO-donor SNP (3.3 and 10 nmol/min; n=5) instead of acetylcholine in the presence of SMTC (Figure 1C).

Protocol 4: Effect of SMTC or L-NMMA on Vasodilator Response to Mental Stress
Previous human studies have implicated NO as a mediator of the forearm vasodilator response evoked by mental stress, although the NOS isoform that is involved remains unclear. To assess whether SMTC blocks this stimulated response, after 15 minutes of saline infusion, SMTC (0.2 μmol/min; n=10) or L-NMMA (2 μmol/min; n=10) was infused for 7 minutes and then continued during a standardized version of the Stroop color word test, designed to evoke mental stress. In pilot studies, increases in forearm blood flow evoked by mental stress were found to be similar in right and left arms (3.61±0.76 and 3.43±0.77 mL·min⁻¹·100 mL⁻¹ tissue, respectively; n=10; P=NS). We therefore infused SMTC (or L-NMMA) into one arm and then assessed the effect on the mental stress response by comparing blood flow in the infused arm with that in the noninfused arm in each subject. The Stroop color word test was also repeated in the presence of a control vasoconstrictor, norepinephrine (60 pmol/min; n=7), to assess the effect of a reduction in basal flow per se on the vasodilator effects of mental stress.

Statistical Analyses
Data are shown as mean±SEM. Vasoconstrictor responses to SMTC and L-NMMA were calculated as percentage decrease in forearm blood flow. Vasodilator responses were calculated as increases of forearm blood flow above the immediately preceding baseline measurement (mL·min⁻¹·100 mL⁻¹ forearm volume). Data were analyzed by repeated-measures ANOVA or paired t test as appropriate. The effect of SMTC and L-NMMA on vasodilator agonists was evaluated by comparing the response to agonist plus saline.
against that to agonist plus inhibitor. Differences were considered significant at $P<0.05$.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Effect of SMTC and L-NMMA on Basal Flow

Local infusion of SMTC (0.025, 0.05, 0.1, and 0.2 $\mu$mol/min) caused a 9% to 30% dose-dependent reduction in basal blood flow (Figure 2). The top dose of SMTC (0.2 $\mu$mol/min) reduced basal flow from 3.60±0.30 to 2.53±0.28 $\text{mL} \cdot \text{min}^{-1} \cdot 100 \text{mL}^{-1}$ tissue. These effects were reversed after 10 to 15 minutes of washout of SMTC (data not shown). Basal blood flow was also reduced by local infusion of L-NMMA, but substantially higher doses than those for SMTC were required to achieve similar reductions in resting forearm blood flow; L-NMMA 0.5 to 4 $\mu$mol/min caused an 11% to 37% reduction in flow (Figure 2). The highest dose of L-NMMA reduced basal flow from 3.99±0.37 to 2.45±0.19 $\text{mL} \cdot \text{min}^{-1} \cdot 100 \text{mL}^{-1}$ tissue.

Effect of L-Arginine and D-Arginine on Response to SMTC

The reduction in basal forearm blood flow induced by SMTC was fully abolished by coinfusion of the NOS substrate L-arginine (Figure 3). However, coinfusion of d-arginine failed to affect the SMTC response (Figure 3), which indicates that the effects of SMTC were mediated by stereospecific inhibition of the L-arginine/NO pathway. Neither L-arginine nor d-arginine caused any change in blood flow per se (data not shown).

Effect of SMTC and L-NMMA on Vasodilator Responses to Acetylcholine

Because SMTC is a more potent nNOS inhibitor than L-NMMA,\textsuperscript{10} the above results suggested that the reduction in basal blood flow observed with SMTC infusion may be mediated via nNOS inhibition. To assess whether SMTC might affect eNOS-mediated responses, we next compared the effect of local SMTC versus L-NMMA infusion on acetylcholine-induced increases in forearm blood flow. As shown in Figure 4A, the highest dose of SMTC studied that reduced basal blood flow (ie, 0.2 $\mu$mol/min) had no significant effect on the vasodilator response to acetylcholine. In contrast, L-NMMA significantly reduced the increase in flow induced by acetylcholine by $\approx 60\%$ (Figure 4B), in agreement with previous reports.\textsuperscript{11} The vasodilator response to SNP was not affected by local infusion of SMTC (Figure 4C).

Effect of SMTC and L-NMMA on Vasodilator Responses to Mental Stress

To assess whether SMTC had an effect on stimulated increases in flow that may involve nNOS, we studied the responses to mental stress. None of the drugs that were studied altered basal flow in the noninfused arm. SMTC (0.2 $\mu$mol/min) reduced basal forearm blood flow in the infused arm by 23.1±3.5% and inhibited the response to mental stress by 53.5±7.6% (an increase of 3.05±0.58 $\text{mL} \cdot \text{min}^{-1} \cdot 100 \text{mL}^{-1}$ tissue in the noninfused arm versus 1.29±0.27 $\text{mL} \cdot \text{min}^{-1} \cdot 100 \text{mL}^{-1}$ tissue in the infused arm; $P<0.005$; Figure 5A and 5B). L-NMMA (2 $\mu$mol/min) comparably reduced basal forearm blood flow by 20.8±3.8% and inhibited the response to mental stress by 62.4±9.7% (4.08±0.46 $\text{mL} \cdot \text{min}^{-1} \cdot 100 \text{mL}^{-1}$ tissue in the noninfused arm versus 1.61±0.46 $\text{mL} \cdot \text{min}^{-1} \cdot 100 \text{mL}^{-1}$ tissue in the infused arm; $P=0.0009$; Figure 5A and 5C). The control vasoconstrictor, norepinephrine (60 $\text{pmol}/\text{min}$), reduced basal flow by 32.3±6.5% but did not significantly alter the vasodilator response evoked by mental stress (Figure 5A and 5D).

Discussion

We have undertaken the first investigation of the possible role of nNOS-derived NO in the local regulation of blood flow in humans using SMTC, an nNOS inhibitor that has a 17-fold selectivity over eNOS.\textsuperscript{10} Local infusion of SMTC into the brachial artery of healthy males resulted in a significant dose-dependent reduction in basal blood flow. The reduction in resting blood flow induced by SMTC at an estimated local concentration of 1.25 to 10 $\mu$mol/L was completely abolished in the presence of excess L-arginine (the substrate for NOS).
but was unaffected by d-arginine, which indicates that the effects of SMTC were mediated by stereospecific inhibition of the L-arginine/NO pathway. Although SMTC has a broadly similar structure and molecular weight to L-NMMA (ie, 278 and 248 kDa, respectively), we found that it reduced basal blood flow at a substantially lower (\(\times 10\) fold) concentration than L-NMMA; the dose response to L-NMMA was similar to that reported previously, 11 and doses were chosen to achieve comparable reduction in blood flow to that seen with SMTC. These results are consistent with previous in vitro data that indicate that a comparable nNOS inhibition in the rat brain was achieved with much lower concentrations of SMTC than of L-NMMA (IC\(_50\) for nNOS was 0.31 \(\mu\)mol/L with SMTC versus 4.1 \(\mu\)mol/L with L-NMMA). 10 Likewise, a comparison of the effects of systemic infusion of SMTC versus the nonselective NOS inhibitor L-nitroarginine methyl ester in rats in vivo showed that SMTC increased blood pressure and reduced renal blood flow at doses that did not induce such effects with L-nitroarginine methyl ester. 17

Although the above results suggested that local SMTC infusion reduced basal blood flow through the inhibition of nNOS, this assumption is based on the selectivity of SMTC for nNOS over eNOS reported by Furfine et al 10 and its greater potency at reducing basal forearm flow than L-NMMA. The original studies by Furfine et al 10,18 were performed on in vitro preparations, including rodent isoensymes, whereas the present study aimed to investigate the role of nNOS in the human microvasculature in vivo. Furthermore, SMTC is known to be capable of inhibiting eNOS, albeit at much higher concentrations than those used in the present study. 10 It was therefore important to undertake additional studies to confirm the nNOS specificity of SMTC. To exclude the possibility that SMTC may be acting via inhibition of eNOS, we compared the effects of SMTC and L-NMMA on acetylcholine-induced increases in blood flow. Doses of SMTC that reduced basal blood flow had no significant effect on the vasodilator response to acetylcholine, whereas L-NMMA markedly reduced the increase in flow induced by acetylcholine, consistent with numerous previous reports. 11 The lack of effect of SMTC on acetylcholine-induced vasodilation is consistent with previous animal data in which a similar in vivo concentration of SMTC did not affect the acetylcholine response but reduced basal arterial flow. 12 Although the vasodilator response to acetylcholine can be complex and can include both NO-dependent and -independent components, a major NO dependence is well
established both in healthy animals and in humans.\textsuperscript{1} The inhibition of the acetylcholine response by L-NMMA in the absence of any significant inhibition by SMTC thus provides compelling evidence that the latter does not have an impact on eNOS-mediated effects in the forearm at the doses studied. The vasodilator response to the NO donor SNP was unaffected by SMTC, as would be expected.

Taken together, the above results challenge the conventionally accepted notion that basal vascular NO generation is derived from eNOS. Instead, the present data suggest that basal forearm blood flow in humans is regulated mainly via local nNOS, whereas increases in flow stimulated by acetylcholine may be eNOS-mediated. Because L-NMMA is non-selective for nNOS and eNOS, it is able to reduce both basal flow and the stimulated response to acetylcholine, whereas SMTC only affects the former. The potentially independent regulation of basal versus stimulated endothelium-mediated increases in blood flow may explain the poor correlation between impairment of these 2 aspects of flow regulation in many disease settings.\textsuperscript{2} Furthermore, it may also explain why conditions such as hypercholesterolemia and hyperhomo-
CLINICAL PERSPECTIVE

Nitric oxide (NO) generated by NO synthases (NOS) has a pivotal role in regulating blood flow. In most vascular beds, continuous NO generation reduces basal tone and increases blood flow. Seminal studies that used local forearm infusion of a nonselective NOS inhibitor, L-NMMA (N\textsuperscript{G}-monomethyl-L-arginine), confirmed that this basal vasodilator effect of NO exists in humans. These effects have been attributed to local release of NO by endothelial NOS (eNOS). eNOS-derived NO also mediates increases in blood flow elicited by agonists such as acetylcholine, and impairment of these responses (known as “endothelial dysfunction”) is a precursor to atherosclerosis. More recently, it has been appreciated that a second NOS isoform, neuronal NOS (nNOS), may also be involved in vascular regulation. This in vivo study investigated for the first time in humans the contribution of nNOS to the regulation of microvascular tone and blood flow. With local infusion of a selective nNOS inhibitor, S-methyl-L-thiocitrulline, basal blood flow in the normal forearm was found to be dependent on tonic nNOS activity, whereas increases in blood flow stimulated by acetylcholine were dependent on eNOS. These findings indicate that nNOS and eNOS make distinct contributions to the physiological regulation of human vascular tone. Tonic NO generation by nNOS is important for regulation of basal vasomotor tone and may therefore influence blood pressure, whereas eNOS-generated NO facilitates dynamic alterations in blood flow distribution and has antiatherosclerotic effects at the level of the endothelium. Elucidation of the relative roles of these 2 NOS isoforms in disease settings requires further investigation.
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