Effects of Neuronal Nitric Oxide Synthase on Human Coronary Artery Diameter and Blood Flow In Vivo

Michael Seddon, MD, MRCP; Narbeh Melikian, MRCP; Rafal Dworakowski, MD, PhD; Husain Shabeeh, MRCP; Benyu Jiang, PhD; Jonathan Byrne, PhD, MRCP; Barbara Casadei, MD, DPhil; Philip Chowienczyk, FRCP; Ajay M. Shah, MD, FMedSci

Background—Nitric oxide (NO)–mediated local regulation of vascular tone is considered to involve endothelial NO synthase (eNOS). However, we recently reported that human forearm basal microvascular tone in vivo is tonically regulated by neuronal NO synthase (nNOS), in contrast to an acetylcholine-stimulated reduction in tone, which is eNOS dependent. Here, we investigated the in vivo effects of an nNOS-selective inhibitor, S-methyl-L-thiocitrulline (SMTC), on the human coronary circulation and on flow-mediated dilatation in the forearm.

Methods and Results—In patients with angiographically normal coronary arteries, intracoronary infusion of SMTC (0.625 μmol/min) reduced basal coronary blood flow by 34.1±5.2% (n=10; P<0.01) and epicardial coronary diameter by 3.6±1.2% (P=0.02) but had no effect on increases in flow evoked by intracoronary substance P (20 pmol/min). The nonselective NOS inhibitor Nω-monomethyl-L-arginine (25 μmol/min) also reduced basal coronary flow (by 22.3±5.3%; n=8; P<0.01) but, in contrast to SMTC, inhibited substance P–induced increases in flow (P<0.01). In healthy volunteers, local infusion of SMTC (0.2 μmol/min) reduced radial artery blood flow by 36.0±6.4% (n=10; P=0.03) but did not affect flow-mediated dilatation (P=0.55). In contrast, Nω-monomethyl-L-arginine (2 μmol/min) infusion reduced radial blood flow to a similar degree (by 39.7±11.8%; P=0.02) but also inhibited flow-mediated dilatation by ≈80% (P<0.01).

Conclusions—These data indicate that local nNOS-derived NO regulates basal blood flow in the human coronary vascular bed, whereas substance P–stimulated vasodilatation is eNOS mediated. Thus, nNOS and eNOS have distinct local roles in the physiological regulation of human coronary vascular tone in vivo. (Circulation. 2009;119:2656-2662.)

Key Words: blood flow ■ microcirculation ■ nitric oxide ■ vasculature ■ vasodilation

Nitrergic NOS (NO) generated from L-arginine by NO synthase (NOS) has a pivotal role in regulating blood flow.1 In most vascular beds, the continuous generation of NO reduces basal vasomotor tone and increases blood flow. As such, local NOS inhibition, eg, with the non–isoform-selective inhibitor Nω-monomethyl-L-arginine (L-NMMA), reduces resting blood flow both in animal studies and in humans.1,2 These effects have generally been attributed to NO generated by endothelial NOS (eNOS) expressed in the endothelium of blood vessels. NO derived from eNOS also mediates increases in blood flow elicited by agonists such as acetylcholine and substance P, and the impairment of such responses (known as endothelial dysfunction) predicts the development of atherosclerosis.3,4

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Recent data from animal studies indicate that NO generated by local neuronal NOS (nNOS) can influence vascular tone, raising the possibility that different NOS isoforms may subserve distinct effects on the regulation of blood flow.5 We recently reported the first investigation of the potential effects of local nNOS on microvascular tone in humans in a study in which the effects of local brachial arterial infusion of an nNOS-selective inhibitor, S-methyl-L-thiocitrulline (SMTC), were assessed. This study showed that SMTC caused a dose-dependent reduction in basal forearm blood flow in healthy volunteers without affecting acetylcholine-induced eNOS-mediated vasodilatation, whereas the nonselective NOS inhibitor L-NMMA inhibited acetylcholine-induced vasodilatation and reduced basal blood flow.6 These results indicated that basal forearm microvascular tone in healthy humans in vivo is regulated by local nNOS-derived NO, whereas acetylcholine-stimulated vasodilatation is eNOS mediated.

Whether nNOS-derived NO also influences conduit artery tone and blood flow in other vascular beds such as the coronary circulation remains to be investigated. Previous studies using intracoronary infusion of L-NMMA have...
shown that NO is important in the basal regulation of human coronary microvascular and conduit artery tone.7–11 However, the relative contribution of nNOS and eNOS to these effects is unknown because selective NOS inhibitors have not previously been studied in humans. The aims of the present study were to study the effects of nNOS on human forearm conduit arteries in vivo and to investigate the effects of nNOS-derived NO in the human coronary circulation in vivo.

Methods

The studies were approved by the local Research Ethics Committee, and all study participants provided written informed consent. SMTC at a purity >99% was obtained from Calbiochem, UK (Beeston, Nottingham, UK) and prepared to standards suitable for human use in a nationally accredited pharmaceutical manufacturing facility as previously described.4 L-NMMA and substance P were purchased from Chinalfa (Lautelfingen, Switzerland), and isosorbide dinitrate (ISDN) was obtained from Schwarz Pharma (Leavesden Park, Watford, Hertfordshire, UK). The use of all investigational agents (L-NMMA, SMTC, and substance P) for mechanistic human studies was approved by an independent Research Ethics Committee in accordance with national regulations and included review of an independent expert evaluation of the potential toxicity of SMTC.

Forearm Conduit Artery Studies

Ten healthy, normotensive, normocholesterolemic male volunteers (age, 26.8±3.5 years) were not taking any regular medications were studied. Subjects abstained from caffeine for at least 12 hours before the studies. Eight subjects participated in 2 studies, 1 each with SMTC or L-NMMA, performed in random order 1 week apart. Two subjects participated in the SMTC study in the first 2 subjects, we infused SMTC containing drinks, and cigarettes for at least 24 hours and from food for at least 6 hours before cardiac catheterization.

Cardiac catheterization was performed via the right femoral approach in a quiet, temperature-controlled cardiac catheterization laboratory with digital cineangiography (Philips, Best, The Netherlands). After diagnostic coronary angiography, a 0.014-in wire equipped with a Doppler crystal at its tip (FloWire, Volcano Therapeutics Inc, Rancho Cordova, Calif) was advanced through a 6F coronary guiding catheter into a proximal coronary artery segment that was straight and free of any branches within 1 cm of the tip. The Doppler wire was positioned in the left anterior descending artery in 15 subjects, in the circumflex artery in 3 subjects, and in the right coronary artery in 2 subjects. The Doppler wire was interfaced with a real-time spectral analysis system (ComboMap Pressure and Flow system, Volcano Therapeutics, Inc) to enable recording of the average peak velocity (APV) of blood flow. All drugs were infused directly into the coronary artery via the guiding catheter at infusion rates of 2 mL/min. APV recordings were taken during each step and before angiography to avoid the effects of contrast. Coronary angiography was performed with nonionic contrast medium (Omnipaque GE Healthcare, Little Chalfont, UK) with the study artery positioned near the isocenter; the angle of projection was set at the beginning of the protocol and was not altered during the rest of the study. After angiography, we waited for the APV to return to its precontrast value before progressing to the next step in the protocol. Aortic pressure and ECG were recorded throughout the protocol. Digital analysis was performed offline with an automated quantitative coronary angiography edge-detection system (Philips). Diameter measurements were made in a 2.5- to 5-mm-long segment near the distal tip of the Doppler FloWire in 2 subjects, in 3 different operators using side branches and the FloWire tip as anatomic landmarks. Coronary blood flow (in mL/min) was derived from the APV and arterial diameter according to a validated formula.12

Preliminary Safety Protocol

Because SMTC had not previously been administered in the human coronary circulation in vivo, in the first 2 subjects, we infused SMTC for 10 minutes at a 100-fold lower dose (0.00625 µmol/min) than the planned dose for the main protocol (0.625 µmol/min) and then for another 10 minutes at a 10-fold lower dose (0.0625 µmol/min) and then washed it out for an additional 10 minutes. Aortic pressure and the 12-lead ECG were continuously recorded. Our previous study in the forearm had demonstrated that the effects of SMTC were reversible by L-arginine and saline washout.6

Effect of SMTC or L-NMMMA on Basal Coronary Flow and Stimulated Increases in Flow

The planned study dose of SMTC (0.625 µmol/min) was chosen on the basis of our previous forearm studies to achieve a local coronary artery concentration of ~5 µmol/L and to be devoid of eNOS-inhibitory effects. The dose of L-NMMA (25 µmol/min) was chosen on the basis of prior studies of the effects of intracoronary L-NMMA in humans8,10,11 and the fact that L-NMMA is significantly (at least 10-fold) less potent than SMTC at inhibiting nNOS.

After 7 minutes of intracoronary saline infusion (2 mL/min), baseline parameters were recorded. Substance P (20 pmol/min) was then coinfused (2 µL/min) for 2 minutes to induce endothelium-dependent vasodilatation. After return of the APV to baseline, endothelium-independent function was assessed as the response to ISDN (1-mg bolus). Once parameters had again returned to baseline, either SMTC (0.625 µmol/min; n = 10) or L-NMMA (25 µmol/min; n = 8) was infused for 7 minutes. This infusion was then continued, and substance P (20 pmol/min) was coinfused (2 µL/min) for 2
minutes to assess the effect of SMTC or L-NMMA on endothelium-dependent vasodilatation. Finally, while SMTC or L-NMMA was continued, the response to ISDN was reassessed (Figure 1). We chose to examine the response to substance P rather than acetylcholine to avoid the possibility of vasoconstriction in patients with coronary risk factors.

Statistical Analyses

Data are shown as mean±SEM. Vasoconstrictor responses to SMTC and L-NMMA were calculated as percentage decrease in basal blood flow. Coronary vasodilator responses to substance P and ISDN were calculated as increases in coronary blood flow above the immediately preceding baseline (in mL/min). Prespecified comparisons of responses to flow (FMD) or drugs were made relative to the immediately preceding baseline by paired t test using Bonferroni correction for multiple comparisons within the same experiment. This test also was used to compare responses in the absence and presence of the individual inhibitors.

In the forearm studies, in which the same subjects received both L-NMMA and SMTC, effects of SMTC on FMD were compared with those of L-NMMA using repeated-measures ANOVA and testing for an interaction between type of inhibitor (ie, L-NMMA or SMTC) and change in FMD in the presence and absence of inhibitor. In the coronary studies, in which L-NMMA and SMTC were given to different groups of subjects, a 2-way ANOVA was used to test for differences in inhibition between the groups.

In cases when lack of inhibition by SMTC was an important negative finding, 95% confidence intervals were calculated for the degree of inhibition caused by SMTC. Differences were considered significant if \( P<0.05 \).

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

Forearm Studies

SMTC (0.2 \( \mu \text{mol/min} \)) reduced basal radial artery blood flow by 36.0±6.4% (from 25.4±7.8 mL/min during saline infusion to 17.4±6.9 mL/min; \( P=0.03 \); Figure 2A) but did not affect basal radial artery diameter (2.59±0.1 mm before versus 2.56±0.1 mm after SMTC; \( P=0.22 \); Figure 2B). The magnitude of FMD was similar in the absence and presence of SMTC (6.0±0.6% versus 6.4±0.5%, respectively; \( P=0.55 \); Figure 2C).

In the same subjects studied on a separate occasion, L-NMMA (2 \( \mu \text{mol/min} \)) reduced basal radial blood flow to a similar extent (by 39.7±11.8%, from 30.9±7.8 mL/min during saline infusion to 18.8±6.7 mL/min; \( P<0.05 \); Figure 2A) and had no effect on basal radial artery diameter (2.71±0.1 mm before versus 2.67±0.2 after L-NMMA; \( P=0.39 \); Figure 2B). However, in contrast to SMTC, FMD was significantly inhibited by L-NMMA (6.5±0.2% before versus 1.0±0.3% after L-NMMA; \( P<0.01 \); Figure 2D). The mean change in FMD after SMTC was 0.4%, with 95% confidence intervals of from 1.0 to 1.8; ie, the upper limit of the potential reduction in FMD with SMTC was only 1.0% (compared with that induced by L-NMMA [ie, 5.5%]). The difference in inhibition of FMD by SMTC and L-NMMA was significant by repeated-measures ANOVA (\( P<0.01 \)). In the control group, FMD responses measured 25 minutes apart
Table. Clinical Characteristics of Study Participants in the Coronary Studies

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- BP indicates blood pressure; LV, left ventricular; EF, ejection fraction; LAD, left anterior descending artery; LCx, left circumflex artery; and RCA, right coronary artery.

were unchanged (FMD, 7.29±0.93% versus 7.44±0.82%; P=0.79).

Coronary Studies

All the drugs were well tolerated, and no subject had any symptoms or ECG signs of ischemia at any stage. Neither intracoronary SMTC nor L-NMMA caused any change in heart rate or systemic blood pressure. SMTC had no effect on coronary blood flow or epicardial diameter at the 100-fold and 10-fold lower doses used in the safety protocol (data not shown). The clinical characteristics of the patients included in the study protocol are shown in the Table.

Effect of SMTC or L-NMMA on Basal Coronary Flow and Epicardial Artery Diameter

Intracoronary SMTC infusion (0.625 μmol/min) reduced basal coronary flow by 34.1±5.2% (n=10; P<0.01; Figure 3A). SMTC also caused a small but significant reduction in epicardial artery diameter (−3.6±1.2%; P<0.05; Figure 3B). As expected, intracoronary L-NMMA infusion (25 μmol/min) reduced coronary flow by 22.3±5.3% (n=8; P<0.01; Figure 3A) and caused a small but significant reduction in epicardial artery diameter (−2.5±0.6%; P<0.05), similar in magnitude to that observed with SMTC (Figure 3B). Representative coronary Doppler traces demonstrating the effect of SMTC and L-NMMA on the baseline APV are shown in Figure 4.

Effect of SMTC or L-NMMA on the Responses to Substance P and ISDN

Despite causing a significant reduction in basal coronary blood flow, SMTC had no significant effect on the increase in flow evoked by intracoronary substance P infusion (25.7±5.9 mL/min before versus 25.4±4.5 mL/min after SMTC; P=0.92; Figure 5A) or on the epicardial artery dilatation caused by substance P (5.9±1.2% before versus 6.7±1.6% after SMTC; P=0.57; Figure 5B). In contrast, L-NMMA significantly inhibited the flow response to substance P (23.6±3.8 mL/min before versus 9.9±1.7 mL/min after L-NMMA; n=8; P<0.01; Figure 5C) and tended to attenuate the epicardial artery dilatation (5.6±1.3% before versus 3.0±0.8% after L-NMMA; P=0.16; Figure 5D). The mean effect of SMTC on the coronary flow response to substance P was 0.41 mL/min, with 95% confidence intervals of −5.6 to 6.12; ie, the upper limit of the potential reduction in flow response to substance P was 5.6 mL/min (compared with the effect of L-NMMA [ie, 14.7 mL/min]). The difference in inhibition of the flow response to substance P by SMTC and L-NMMA was significant by 2-way ANOVA (P<0.01).

SMTC had no effect on the increase in blood flow induced by ISDN (63.5±6.9 mL/min before versus 65.1±8.9 after SMTC; P=0.88) or on its vasodilator effect on epicardial diameter (6.9±1.2% before versus 5.4±1.2% after SMTC; P=0.12). Similarly, L-NMMA also had no effect on ISDN-induced increases in blood flow (47.0±6.6 mL/min before versus 49.4±9.0 mL/min after L-NMMA; P=0.79) or epicardial diameter (4.6±1.5% before versus 4.3±0.6% after L-NMMA; P=0.77).

Discussion

We recently performed the first human studies of the effects of a selective nNOS inhibitor, SMTC, on the local regulation of blood flow and found that basal microvascular tone in the forearm is under the tonic control of nNOS-derived NO.6 SMTC caused a dose-dependent reduction in basal forearm blood flow via stereospecific inhibition of the L-arginine/NO pathway without affecting acetylcholine-induced vasodilatation, consistent with an nNOS-selective action. However, the possible role of local nNOS in blood flow or in conduit artery tone in other human vascular beds was not addressed. In this regard, the coronary circulation is of particular importance, being a major factor influencing heart function in health and disease. Whereas changes in endothelial NO-dependent vasomotor function in the human forearm and brachial artery often parallel changes in the coronary circulation,8 it is well recognized that these 2 vascular beds differ significantly in

Figure 3. Effects of SMTC and L-NMMA on basal coronary blood flow and epicardial artery diameter. A, SMTC (0.625 μmol/min) and L-NMMA (25 μmol/min) significantly reduced basal coronary artery blood flow (P<0.05). B, SMTC and L-NMMA both caused a small but significant reduction in conduit artery diameter (P<0.05).
Intracoronary SMTC infusion at a dose of 0.625 μmol/min resulted in a concentration of 5 μmol/L, approximately half the concentration studied in the forearm. This concentration caused a significant reduction in basal coronary blood flow, comparable to that seen in previous studies with L-NMMA. However, a substantially higher dose of L-NMMA (40-fold greater) was necessary to achieve a similar reduction in flow, consistent with in vitro data showing comparable nNOS inhibition with lower concentrations of SMTC than L-NMMA. In vivo animal studies also demonstrated that SMTC was more selective for nNOS over eNOS, inhibiting nNOS-mediated responses without affecting eNOS-mediated responses to acetylcholine. These results suggest that the reduction in basal coronary flow with either drug was the result of local nNOS inhibition.

Local substance P infusion was used to stimulate eNOS activity and induce coronary vasodilatation. SMTC had no effect on the increases in blood flow evoked by intracoronary substance P, whereas L-NMMA caused a significant inhibition of the substance P-induced increase in coronary flow. These results are consistent with the notion that SMTC, at the doses used in the present study, is relatively selective for nNOS over eNOS.

**Figure 4.** Representative coronary Doppler spectral traces. A and B, Effect of SMTC and L-NMMA on basal APV.

**Figure 5.** Effect of intracoronary SMTC or L-NMMA on the increases in blood flow and epicardial artery diameter induced by substance P. A and B, SMTC did not affect the flow response or increase in epicardial artery diameter after substance P. C and D, L-NMMA caused a significant inhibition of the substance P-induced increase in coronary flow (*P<0.05) and tended to attenuate substance P-induced epicardial diameter dilatation.
study, is selective for nNOS, whereas L-NMMA inhibits both local nNOS and eNOS. They are also in keeping with the lack of effect of SMTC on acetylcholine-induced increases in forearm blood flow in our previous study, which in contrast were inhibited by L-NMMA. As expected, neither SMTC nor L-NMMA had any significant effect on the increase in coronary flow evoked by ISDN.

The data discussed so far pertain to microvascular tone and the regulation of blood flow. We also investigated the effects of SMTC on conduit artery tone. In the coronary circulation, SMTC caused a small but significant reduction in epicardial artery diameter similar in magnitude to that observed with a 40-fold higher dose of L-NMMA, suggesting that nNOS also may have a role in the regulation of basal tone at the level of the conduit coronary arteries. In the forearm, however, we found that local infusion of SMTC had no effect on radial artery diameter despite reducing basal blood flow to an extent similar to the coronary circulation. Interestingly, local infusion of L-NMMA also had no effect on radial artery diameter, consistent with previous results. The lack of effect of either NO inhibitor on radial artery diameter may suggest that the basal release of NO from either eNOS or nNOS is minimal at the level of this peripheral conduit artery, in contrast to epicardial coronary arteries.

An important physiological response at the level of both conduit and microvascular arteries is FMD, an arterial response stimulated by increased local blood flow and shear stress. FMD, which has been demonstrated to be endothelium dependent and NO mediated, is widely used as an index of endothelial function in humans and is impaired under conditions that predispose to atherosclerosis. We found that SMTC had no effect on FMD in the radial artery, whereas L-NMMA inhibited the response, as previously demonstrated. These results are consistent with the accepted notion that FMD is an eNOS-mediated response and indicate that, despite the lack of evidence for basal release of NO (presumably from nNOS), radial arteries are capable of stimulated eNOS-mediated responses. We did not investigate FMD in the coronary circulation, but it is likely that the increase in epicardial diameter observed after substance P, which was inhibited by L-NMMA but not SMTC, reflects the combination of a direct effect on the epicardial artery and the result of FMD. Indeed, FMD is considered an important regulatory mechanism in the coronary circulation in humans, and when induced by exercise or atrial pacing, it has been shown to be inhibited by intracoronary L-NMMA.

The results of the present study suggest that local nNOS-derived NO is a key regulator of basal blood flow not only in the human forearm but also in the coronary vascular bed, whereas agonist (substance P)–induced increases in flow are eNOS mediated. Shear stress–dependent increases in conduit artery tone (ie, FMD) also appear to be eNOS rather than nNOS dependent. Taken together, our results indicate that nNOS and eNOS may have distinct local roles in the physiological regulation of human coronary and forearm vascular tone in vivo. The precise site of local nNOS-derived NO release could not be defined from the present study but may include perivascular nerves and/or cells within the vessel wall, both of which have been reported to express nNOS protein. Recently, it has been suggested that red blood cell–derived NO could influence vascular function; the possible contribution of such a source also needs to be considered, although the NO isosform in red cells was found to be eNOS. Future studies need to address the question of the precise sites of local NO generation by different isoforms and evaluate how the relative roles of eNOS and nNOS may be altered in disease settings.

Study Limitations
We used a single dose of SMTC or L-NMMA to limit the duration of the study and total dose of NO inhibitor. However, these doses produced comparable inhibition of basal flow. Furthermore, the conclusions of the present study are significantly strengthened by our previous study in the forearm circulation in which we undertook dose-response studies. We studied a single dose of substance P, whereas the data could be strengthened by the determination of dose-response curves to substance P in the presence of NO inhibitors. The present protocol was designed to minimize the impact of dye boluses on coronary flow and diameter, but the use of an infusion catheter inside the guiding catheter could have minimized this potential problem. It should also be noted that we could not undertake coronary studies in healthy volunteers and that most subjects with atypical chest pain had at least 1 risk factor for coronary disease, which could potentially have influenced the results. Finally, demonstration of similar results with a different nNOS inhibitor would strongly support the present data, but unfortunately, no other nNOS inhibitors are currently available and approved for human use.

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Disclosures
None.

References
Changes in microvascular tone regulate blood flow and its distribution in different vascular beds. Nitric oxide (NO) is known to be a key paracrine factor that regulates vessel tone, both under resting conditions and in settings where blood flow increases, e.g., in response to agonist stimulation. It has been thought that this NO derives from the endothelial isoform of NO synthase (eNOS), expressed in the endothelium. However, in a recent first-in-humans study with an inhibitor of neuronal NO synthase (nNOS) called S-methyl-l-thiocitrulline, we reported that basal microvascular tone and blood flow in the forearm were under the control of nNOS-derived NO rather than eNOS, whereas increases in flow stimulated by acetylcholine involved eNOS. In the present study, we investigated whether nNOS affects the in vivo response of large arteries to increased flow (flow-mediated dilatation) and whether nNOS-derived NO plays a role in the human coronary circulation. Our results suggest that basal coronary flow in humans is regulated by nNOS-derived NO, whereas increases in flow stimulated by substance P involve eNOS-derived NO. We also found that flow-mediated dilatation in the forearm involves eNOS-derived NO and not nNOS. These results indicate that nNOS and eNOS have distinct local roles in the physiological regulation of human coronary vascular tone in vivo. It will be important in future studies to determine the relative contribution of eNOS and nNOS to blood flow regulation in disease settings and to assess whether they should be individually targeted.