Systemic Inhibition of Nitric Oxide Synthase Unmasks Neural Constraint of Maximal Myocardial Blood Flow in Humans

Philipp A. Kaufmann, MD; Ornella Rimoldi, MD; Tomaso Gnecchi-Ruscone, MD; Robert S. Bonser, MD, FRCS, FRCP; Thomas F. Lüscher, MD, FESC, FRCP; Paolo G. Camici, MD, FESC, FRCP

Background—Nitric oxide (NO) is an endothelial mediator that regulates vascular smooth muscle tone, but it may exert its cardiovascular action also by modulating the autonomic control of vasomotor tone. We assessed the effect of simultaneous inhibition of both endothelial (eNOS) and neuronal (nNOS) NO synthase isoforms on myocardial blood flow (MBF) and coronary flow reserve (CFR) in volunteers and in (denervated) transplant recipients.

Methods and Results—MBF (mL · min⁻¹ · g⁻¹) was measured at rest and during adenosine-induced hyperemia with positron emission tomography and ¹⁵O-labeled water. CFR was calculated as adenosine/resting MBF. Measurements were repeated during one of the following intravenous infusions: group 1 (n=12), saline; group 2 (n=9), 3 mg/kg N⁶-monomethyl-L-arginine (L-NMMA), which crosses the blood-brain barrier and inhibits both eNOS and nNOS; group 3 (n=13), 10 mg/kg L-NMMA; group 4 (n=8), phenylephrine titrated to simulate the hemodynamic changes in group 3; and group 5 (n=6), 10 mg/kg L-NMMA infused into the heart transplant recipients. After intervention, hyperemic MBF and CFR were unchanged in groups 1, 2, and 4. By contrast, both hyperemic MBF (+53%, P<0.0001 versus baseline) and CFR (+52%, P<0.0001 versus baseline) increased in group 3, whereas they remained unchanged in group 5, which suggests that an intact cardiac innervation was required for the increase in MBF and CFR observed in group 3.

Conclusions—The results of the present study suggest that maximal adenosine-induced hyperemia and CFR in humans are constrained by neurally mediated vasodilation, which can be relieved by systemic NOS inhibition with L-NMMA. (Circulation. 2004;110:1431-1436.)

Key Words: nitric oxide ■ nitric oxide synthase ■ blood flow ■ imaging

Nitric oxide (NO) has been primarily recognized as an endothelial mediator that directly regulates vascular smooth muscle tone. Accordingly, it has been shown that intracoronary administration of the NO synthase (NOS) inhibitor N⁶-monomethyl-L-arginine (L-NMMA) reduces epicardial coronary artery diameter and blood flow through inhibition of endothelial NOS (eNOS).

Growing evidence, however, suggests that NO may also exert its action on the coronary circulation indirectly by modulating the autonomic control of vasomotor tone. A recent noninvasive study in humans has demonstrated that an intact cardiac innervation is required for the physiological vasodilator response of the coronary circulation during cold pressor test.

Although the exact role of NO in this context remains to be defined, previous in vivo and ex vivo studies in animals have supported the concept that NO derived from neuronal NO synthase (nNOS) contributes to the regulation of vasomotor tone and blood pressure. It has also been demonstrated that NO produced by neurons in the central nervous system acts as a neurotransmitter, the primary effect of which is to constrain sympathetic excitability. Intravenous infusion of the NOS inhibitor L-NMMA in both animals and humans has been shown to increase overall efferent sympathetic activity, although the associated rise in blood pressure would be expected to induce a baroreflex-mediated decrease in sympathetic tone. This effect of intravenous L-NMMA infusion on efferent sympathetic activity has been attributed to its central inhibition of nNOS because L-NMMA crosses the blood-brain barrier. The inhibition of central nNOS, in turn, would lessen the physiological constraint exerted by NO on sympathetic output.

In the present study, we aimed to assess the net effect of simultaneous inhibition of both NOS isoforms on myocardial blood flow (MBF) and coronary flow reserve (CFR) measured noninvasively by positron emission tomography (PET).
We hypothesized that systemic L-NMMA infusion would inhibit central nNOS, thus disinhibiting efferent sympathetic activity, which in turn would increase CFR in healthy human volunteers but not in heart transplant recipients because of their cardiac denervation.

**Methods**

**Study Population**

We studied 42 healthy male volunteers (age range 35 to 60 years). None of them had a history of cardiovascular disease, smoking, or any other cardiovascular risk factor. Accordingly, none of the volunteers was receiving any form of treatment. Enrollment criteria included normal heart rate, blood pressure, ECG, and 2D echocardiogram and low clinical probability of coronary artery disease. In all volunteers, CFR measured by PET was within normal limits in all left ventricular segments, which made the presence of relevant obstructive coronary artery disease or coronary microvascular dysfunction very unlikely.

In addition, we studied 6 heart transplant recipients (age range 52 to 60 years; 2 with dilated cardiomyopathy and 4 with heart failure secondary to ischemic heart disease). They were studied 6.5 ± 2 months after orthotopic transplantation, when the heart is known to be still denervated. They were recruited from the Transplant Unit, Queen Elizabeth Medical Centre, Birmingham, United Kingdom. All transplant recipients had a good ejection fraction, measured by echocardiography within 3 months of PET scanning. In addition, all recipients were free from rejection at the time of scanning and had no rejection history between measurement of ejection fraction and PET scanning. Similar to the volunteers, all patients with uncontrolled cardiovascular risk factors and current smokers were excluded. The lipid profile was assessed in all volunteers and patients, and those with total cholesterol higher than 6.4 mmol/L (250 mg/100 mL) were excluded from the study. In addition, all subjects were carefully instructed to refrain from intake of caffeine-containing beverages or food during the 24 hours preceding the study. A screening test for caffeine was performed in a blood sample taken immediately before the PET scan from each subject. Caffeine was not detectable in any of the blood samples.

**Measurement of MBF**

MBF was measured with 15O-labeled water (H215O) and an ECAT 931-08/12 15-slice PET scanner (CTI/Siemens). H215O (700 to 900 MBq) was injected as an intravenous bolus over 20 seconds at an infusion rate of 10 mL/min, and dynamic scanning was acquired over a period of 5.5 minutes.

The sinograms obtained were corrected for attenuation and reconstructed on a MicroVax II computer (Digital Equipment Corp) with dedicated array processors and standard reconstruction algorithms. On factor images, generated by iterative reconstruction, regions of interest were drawn within the left atrium and ventricular myocardium on consecutive image planes. These were projected onto the dynamic H215O images to generate blood and tissue time activity curves, which were fitted to a single tissue compartment tracer kinetic model to give values of MBF (mL · min⁻¹ · g⁻¹).

**Study Protocol**

Under baseline conditions, MBF was measured both at rest and during pharmacologically induced hyperemia (IV adenosine 140 µg · kg⁻¹ · min⁻¹; Figure 1). Arterial blood pressure was recorded by automatic cuff sphygmomanometer at 1-minute intervals, and the ECG was monitored continuously throughout the procedure. A 12-lead ECG was recorded at baseline and every minute during adenosine administration. Thereafter, repeat resting and hyperemic MBF measurements were performed after one of the following 30-minute intravenous infusions (Figure 1): group 1 (n = 12), isotonic saline; group 2 (n = 9), 3 mg/kg L-NMMA (Clinalfa) dissolved in 50 mL of isotonic saline; group 3 (n = 13), 10 mg/kg L-NMMA dissolved in 50 mL of isotonic saline; group 4 (n = 8), phenylephrine (1 mg in 100 mL) infused intravenously to achieve an increase in arterial blood pressure similar to that observed in group 3; and group 5 (n = 6), 10 mg/kg L-NMMA dissolved in 50 mL of isotonic saline infused in the heart transplant recipients. The 5 study groups did not differ with regard to gender, body mass index, and lipid profile, although transplanted patients were significantly older (Table 1).

**CFR and Coronary Resistance**

CFR was calculated as the ratio of adenosine to resting MBF. To account for the variability of coronary driving pressure, resting and minimal (ie, during adenosine infusion) coronary resistance (mm Hg · mL⁻¹ · min⁻¹ · g⁻¹) was also calculated as the ratio of mean arterial pressure to MBF.

The research ethics committees of Hammersmith Hospital and Queen Elizabeth Medical Centre approved the study protocol. Radiation exposure was licensed by the UK Administration of Radioactive Substances Advisory Committee (ARSAC). All patients gave informed and written consent before the study.

**Statistical Analysis**

Data are reported as mean ± SD. Statistical comparisons of hemodynamic data, MBF, CFR, and coronary resistance during the different study conditions were performed by ANOVA for repeated measure-

<table>
<thead>
<tr>
<th>TABLE 1. Subject Characteristics</th>
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<tbody>
<tr>
<td>Group</td>
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<tr>
<td>-------</td>
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<tr>
<td>1</td>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
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<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
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</tbody>
</table>
results the common side effects caused by adenosine, such as flushing and chest tightness. None of the subjects experienced any symptoms or ECG changes after infusion of L-NMMA or phenylephrine.

Hemodynamics
At baseline, heart rate and mean arterial blood pressure at rest and during adenosine were higher in group 5. Hemodynamic parameters remained unchanged in groups 1 and 2 after the infusions. By contrast, mean arterial pressure was significantly increased in groups 3 (+10±1%; P<0.05), 4 (+11±1%; P<0.05), and 5 (+11±1%; P<0.05) compared with baseline and compared with groups 1 and 2. This was accompanied by a decrease in heart rate in groups 3 (−13±1%; P<0.05) and 4 (−13±1%; P<0.05) but not in group 5 (+2±3%; P=NS; Table 2).

MBF, CFR, and Coronary Resistance
MBF and CFR data for each study group are presented in Table 3. In patients who had received transplants (group 5), resting MBF was significantly higher than in groups 1 through 4 both at baseline and after infusion of L-NMMA. In groups 1, 2, 4, and 5, resting and hyperemic MBF and CFR after infusions were comparable to their respective baseline values. Similar to the other groups, resting MBF was unaffected by the infusion of L-NMMA in group 3. By contrast, hyperemic MBF and CFR increased significantly after the infusion of the high dose (10 mg/kg) of L-NMMA compared with the baseline value and with the values in groups 1, 2, 4, and 5 after infusions. However, when the same high dose of L-NMMA was administered to the patients who had received transplants, no changes in MBF and CFR were observed compared with baseline (Figure 2).

At baseline, coronary resistance was comparable in the 5 groups both at rest and during adenosine infusion (data not shown). The changes in minimal coronary resistance after the infusions are illustrated in Figure 3. After the infusion of 10 mg/kg L-NMMA, a further decrease (−35%, P<0.0005) was observed in group 3, whereas there was a nonsignificant trend toward an increase in group 5.

Discussion
The present study demonstrates that intravenous administration of L-NMMA at 10 mg/kg in healthy volunteers increases adenosine-induced hyperemia by ~50%. This effect appears to necessitate an intact cardiac innervation, because no increase was observed in transplant recipients (6.5±2 months after transplantation), whose hearts remain totally denervated for >1 year after the operation.16

Our findings, that L-NMMA did not decrease resting MBF while increasing hyperemic MBF and CFR, are only appar-

### TABLE 2. Hemodynamics

<table>
<thead>
<tr>
<th>GR</th>
<th>MAP (mm Hg)</th>
<th>HR (bpm)</th>
<th>RPP</th>
<th>MAP (mm Hg)</th>
<th>HR (bpm)</th>
<th>RPP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Adenosine</td>
<td></td>
<td>Baseline</td>
<td>Adenosine</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>89±12</td>
<td>62±6</td>
<td>7615±1559</td>
<td>86±12</td>
<td>93±15</td>
<td>11 584±2842</td>
</tr>
<tr>
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<td>86±9</td>
<td>57±9</td>
<td>6566±1062</td>
<td>86±10</td>
<td>88±15</td>
<td>10 471±2181</td>
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<td>3</td>
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<td>60±9</td>
<td>6930±1218</td>
<td>90±9</td>
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<td>11 741±1968</td>
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<tr>
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<td>60±11</td>
<td>7278±1797</td>
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<td>84±16</td>
<td>10 164±2587</td>
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<tr>
<td>5</td>
<td>104±10</td>
<td>92±14</td>
<td>12 576±1597</td>
<td>98±10</td>
<td>95±13</td>
<td>12 566±1735</td>
</tr>
</tbody>
</table>

GR indicates group; HR, heart rate (bpm); MAP, mean arterial pressure (mm Hg); and RPP, rate pressure product (systolic blood pressure x HR).

### TABLE 3. MBF and CFR

<table>
<thead>
<tr>
<th>GR</th>
<th>n</th>
<th>Baseline</th>
<th>Intervention*</th>
<th>Baseline</th>
<th>Intervention*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MBF, mL·min⁻¹·g⁻¹</td>
<td></td>
<td>MBF, mL·min⁻¹·g⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>0.89±0.11</td>
<td>3.26±0.62</td>
<td>0.99±0.11</td>
<td>3.65±0.38</td>
<td>2.79±0.76</td>
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<tr>
<td>2</td>
<td>9</td>
<td>0.78±0.12</td>
<td>3.42±0.74</td>
<td>0.81±0.06</td>
<td>3.51±0.64</td>
<td>2.91±0.77</td>
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<tr>
<td>3</td>
<td>13</td>
<td>0.88±0.13</td>
<td>3.40±0.94</td>
<td>0.90±0.17</td>
<td>5.23±0.64</td>
<td>2.65±0.67</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>0.81±0.08</td>
<td>3.31±0.87</td>
<td>0.81±0.17</td>
<td>3.65±0.92</td>
<td>2.93±0.79</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>1.31±0.19</td>
<td>3.08±0.28</td>
<td>1.33±0.22</td>
<td>2.76±0.65</td>
<td>2.43±0.56</td>
</tr>
</tbody>
</table>

**GR indicates group.**

*Intervention: infusion according to protocol.

†P<0.05, ‡P=0.0001 vs baseline; §P<0.05, ||P<0.005 vs all other groups.
ently in contradiction with a number of previous studies showing a decrease in MBF after administration of L-NMMA.2,29,30 In fact, in those studies, L-NMMA was infused directly into the coronary circulation at doses that block coronary eNOS but without any significant systemic hemodynamic effect.

The vasodilator effect of adenosine was originally thought to be based solely on the direct stimulation of A2 adenosine receptors on vascular smooth muscle cells, which mediate an increase in the second-messenger cAMP by stimulating adenylyl cyclase. Therefore, this agent has been used frequently in animal and human studies to evaluate endothelium-independent vasodilation.31 However, in the past decade, it has been appreciated that adenosine also acts, at least in part, as an endothelium-dependent vasodilator,32,33 both via flow-mediated dilation34 and via direct stimulation of endothelial cells.35

We infused L-NMMA intravenously at a dose that has been shown to increase blood pressure and activate the baroreflex and to effectively block eNOS and nNOS in experimental animals36 and humans.14,37 Given that the coronary circulation extracts ~5% of cardiac output, with our systemic doses of L-NMMA we would expect to achieve coronary concentrations of ~50 μmol (group 2) and 164 μmol (groups 3 and 5), respectively, which compare well with the figures of 32,30 125,2 and 320 μmol3 shown to block coronary eNOS when administered via an intracoronary route. Thus, it is reasonable to assume that with our high-dose L-NMMA infusion, we have successfully blocked a substantial amount of both eNOS and nNOS. Although we cannot distinguish the isolated contribution of each NOS isoform inhibition, we can assume that the difference between local versus systemic infusion of L-NMMA must be due to the additional nNOS inhibition, resulting in a net increase in flow.

We did not observe any change in resting MBF after intravenous infusion of 3 or 10 mg/kg L-NMMA. This is in apparent contradiction with previous reports, which found a decrease in cross-sectional area and coronary flow after intracoronary L-NMMA administration.2,3,30 However, there is no study in the literature in which resting MBF was measured before and after intravenous L-NMMA infusion. Buus et al33 found a decrease in adenosine-induced hyperemia after intravenous infusion of the NOS inhibitor N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME), which does not cross the blood-brain barrier. Resting MBF was not assessed. In all other studies in which a decrease in resting MBF after L-NMMA administration was observed, L-NMMA was injected via intracoronary routes. The only case in which a dose of L-NMMA similar to the one used by us was injected systemically is the study by Owyla et al,14 in which MBF was not measured. They found, however, an increase in effenter muscle sympathetic nerve activity that supports our interpretation of the data of the present investigation.

Our 2-step dose-finding study revealed a different effect of the low (3 mg/kg) and high (10 mg/kg) doses of L-NMMA on adenosine-induced hyperemia. The reasons for the lack of any response at the low L-NMMA dose are not clear, although a different ED\textsubscript{50} for L-NMMA-induced blockade of eNOS and nNOS has been suggested.14 Alternatively, inhibition of the
tonic central neuronal constraint on sympathetic outflow may be too limited at low L-NMMA doses to counterbalance the local effect of eNOS inhibition, whereas this balance might be shifted at high L-NMMA doses. It is also possible that the lower dose simply might have been too low to induce any effect.

In contrast to the lower L-NMMA dose, there was a significant increase in blood pressure after infusion of the high dose. This was paralleled by a baroreflex-mediated decrease in heart rate in healthy volunteers but not in transplanted subjects, which is a further confirmation that their hearts were still denervated. The increase in perfusion pressure cannot explain the observed increase in hyperemic MBF and CFR as in group 4, where phenylephrine was titrated to achieve an increase in blood pressure similar to that obtained in group 3; in fact, MBF and CFR were unaffected by this change in pressure. This is in line with previous results with intracoronary Doppler catheter measurements18 that showed no change in baseline and hyperemic MBF assessed during intravenous phenylephrine infusion (targeting a 30-mm Hg blood pressure increase).

It has been suggested that NO may, in addition to its direct vasodilator action, exert its cardiovascular actions by direct chronotropic effects9 and by modulating sympathetic vasoconstrictor tone.40–42 It remains controversial whether inhibition of nNOS elicits sympathoexcitatory effects or not, because it may exert opposing effects at different sites.43 The present study is the first to report a net increase in adenosine-induced hyperemic MBF in response to L-NMMA. Our results suggest that this is mainly due to central nNOS inhibition, which leads to increased sympathoexcitatory effectors that override local eNOS blockade.

The finding that pharmacologically induced vasodilation may lead to submaximal MBF is consistent with our previous observation that dipyridamole-induced hyperemia can increase by ~40% after administration of a selective α1-adrenoceptor blocker.38 We hypothesize that the reflex sympathetic activation elicited after the systemic administration of vasodilators such as adenosine and dipyridamole would result in a further fall in minimal coronary resistance, which, however, is blunted by an NO-modulated suppression of sympathetic outflow in the central nervous system. This is in line with our previous study,26 which showed that an increase in the standard adenosine rate of 140 μg · kg−1 · min−1 does not further increase maximal MBF. The present results suggest that when the vasodilator is administered during systemic inhibition of NO, this constraint is removed, and the overall effect is a further dilatation and a higher hyperemic flow. The further fall in minimal resistance could be due to activation of β2-adrenoceptors on coronary arterioles, as recently reported by Sun and coworkers.44 The findings by Buus et al33 showing that inhibition of NO by L-NAME elicits α-adrenergic–mediated vasoconstriction, which limits adenosine-induced hyperemia, are only in apparent contradiction with the present data. It is worth pointing out that L-NAME does not cross the blood-brain barrier. Therefore, the experimental conditions are equivalent to the transplant recipients in the present study, in whom we observed an analogous trend toward reduced CFR after NO inhibition.

Although in 30% of transplant recipients an upregulation of inducible NOS has been found to be associated with decreased CFR (<2),45 we believe that this has little impact on the present results because, first, L-NMMA blocks all NOS isoforms, and second, only 1 transplant recipient had a CFR below 2.

In conclusion, the results of the present study suggest that maximum adenosine-induced hyperemic MBF and CFR in humans are constrained by neurally mediated vasoconstriction. The latter can be relieved by systemic NOS inhibition with L-NMMA.

Acknowledgments

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References


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