Coronary Nitric Oxide Production in Response to Exercise and Endothelium-Dependent Agonists

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Background—Endothelium-derived nitric oxide (NO) contributes to epicardial coronary artery vasodilation during exercise. However, blockade of NO production does not impair the increase in coronary blood flow (CBF) during exercise, suggesting that NO is not obligatory for exercise-induced coronary resistance vessel dilation. In contrast, the increases in CBF produced by endothelium-dependent agonists are decreased after NO blockade. Consequently, this study was performed to determine whether the increase in coronary NO production in response to agonists is greater than that which occurs during exercise.

Methods and Results—We measured the oxidation products of NO (nitrate+nitrite=NOx) in aortic and coronary sinus plasma using chemiluminescence to assess NOx production across the coronary circulation in chronically instrumented dogs during a 3-stage treadmill exercise protocol and in response to intracoronary administration of the endothelium-dependent agonists acetylcholine (37.5 μg/min) and bradykinin (3.0 μg/min). No coronary NOx production could be detected at rest or during the first 2 stages of exercise; only at the highest level of exercise was a small increase in coronary NOx production measured. In contrast, coronary production of NOx was significantly increased in response to endothelium-dependent agonists.

Conclusions—Coronary NO production in response to endothelium-dependent agonists is greater than in response to the increase in shear stress associated with exercise. These findings support previous studies suggesting that NO is not required for the coronary vasodilation that occurs in the normal heart during exercise. (Circulation. 2000;101:2526-2531.)

Key Words: nitric oxide • endothelium • exercise • blood flow

Nitric oxide (NO) production by vascular endothelium increases in response to stimuli that cause activation of constitutive endothelial cell NO synthase (eNOS). Thus, agonists such as acetylcholine (ACh) and bradykinin (BK)1,2 increase coronary blood flow (CBF) by engaging specific endothelial cell receptors that stimulate production of NO and other vasodilator substances.3,4 NO production by the vascular endothelium is also responsive to mechanical stimulation produced by increases in shear stress,5 pulsatility,6 and axial strain.7 These mechanical effects contribute to the epicardial coronary artery dilation that occurs in response to increases in blood flow during reactive hyperemia or exercise.

Blockade of NO production with analogues of L-arginine markedly blunts the increase in CBF produced by endothelium-dependent vasodilators. In contrast, inhibition of NO production did not impair the normal increase in coronary flow during treadmill exercise in dogs.8 This disparity in the effect of NO synthesis blockade could be explained by differences in the potency of exercise and endothelium-dependent agonists to stimulate NO production. Only 1 previous study9 reported a small increase in coronary NO production during exercise. Although NO production has been detected in response to endothelium-dependent agonists in isolated hearts, no studies have been performed in vivo to compare its production with that by exercise in the same subjects. Consequently, the present study was performed to directly measure coronary NO production during increases of coronary flow produced by endothelium-dependent agonists and by exercise.

Methods

Studies were carried out in 17 adult mongrel dogs weighing 25 to 30 kg, trained to run on a motor-driven treadmill. All studies were performed in accordance with the “Position of the American Heart Association on Research Animal Use” adopted in November 1984 and were approved by the Animal Care Committee of the University of Minnesota.

Surgical Instrumentation

Animals were premedicated with acepromazine (10 mg IM), anesthetized with sodium pentobarbital (30 mg/kg IV), intubated, and
ventilated with room air supplemented with oxygen. A left thoracotomy was performed in the fifth intercostal space. A heparin-filled polyvinyl chloride catheter, 3.0-mm OD, was introduced into the internal thoracic artery and advanced into the ascending aorta. Similar catheters were placed in the left atrium and the LV. A solid-state micromanometer (Koningsberg Instruments Inc, model P5) was also introduced into the LV at the apex. A fourth heparin-filled catheter was introduced into the coronary sinus through the right atrial appendage and advanced to within 1 cm of the anterior interventricular vein to allow selective sampling of the venous effluent from myocardium perfused by the LAD. Approximately 1.5 cm of the proximal LAD was dissected free, and a Doppler velocity probe (Craig Hartley, 2.5- to 3.5-mm ID) was placed around the vessel. A heparin-filled silicone rubber catheter (0.3-mm ID) was introduced into the LAD distal to the flow probe for intracoronary administration of agonists. The pericardium was loosely closed, the catheters were tunneled subcutaneously to exit at the base of the neck, and the thoracotomy was closed in layers. Catheters were protected with a nylon vest and flushed daily with heparinized saline.

**NO Production During Exercise**

Studies of coronary NO production during exercise (n=12) and in response to endothelium-dependent agonists (n=10) were performed 1 to 3 weeks after surgery. Interventions were performed in random order on separate days. Aortic and LV pressures were measured with transducers at mid-chest level (Spectramed Inc, model TNF-R). The fluid-filled catheter in the LV was used to calibrate the Koningsberg micromanometer. LAD blood flow was measured with the Doppler velocity probe. Data were recorded on an 8-channel direct-writing recorder (Coulbourne Instruments Inc). After all recording instruments were connected, the dog was placed on the treadmill. Fifteen minutes later, resting hemodynamics were recorded, and 3 mL of blood was withdrawn from the aortic and coronary venous catheters and placed on ice for measurement of NOx and for blood gas analysis. A 3-stage graded treadmill exercise protocol was then performed as follows: 3.2 km/h at 0% grade (stage 1), 6.4 km/h at 0% grade (stage 2), and 6.4 km/h at 10% grade (stage 3). Three minutes into each exercise stage, aortic and coronary venous blood samples were withdrawn for NOx and blood gas measurements. After completion of exercise, the blood samples were centrifuged at 2500 rpm for 15 minutes at 4°C to remove the formed elements and were stored at −70°C.

**NO Production in Response to Endothelium-Dependent Agonists**

On a separate day, the dogs (n=10) were placed in a sling, and catheters were reconnected as described above. Approximately 30 minutes later, resting hemodynamics were recorded, and 3 mL of aortic and coronary vein blood was withdrawn for measurement of NOx. The coronary flow response to infusion of ACh in doses of 3.75, 7.5, 15, 37.5, and 75 μg/min at rates of 0.15 to 3.0 mL/min was measured in the initial 4 dogs. Because infusion of ACh in a dose of 37.5 μg/min produced an increase in coronary flow similar to that during exercise, this dose was used to compare with NO production during exercise. ACh was infused through the coronary catheter at a rate of 37.5 μg/min while hemodynamics and the LAD Doppler signal were monitored. When the increase in coronary flow reached steady state, blood was withdrawn from the aortic and coronary venous catheters for measurement of NOx, and the infusion was discontinued. Thirty minutes later, the coronary NOx production and flow responses to BK were determined. A dose-response curve to BK dissolved in normal saline was performed at doses of 0.3, 0.6, 1.5, 3, and 4 μg/min at flow rates between 0.15 and 3.0 mL/min in 4 dogs. Because BK in a dose of 3 μg/min caused an increase in coronary flow similar to that during exercise stage 3 in our preliminary studies, this dose was infused into the LAD for determination of NOx production. After the increase in coronary flow during BK infusion had reached steady state, aortic and coronary venous blood was withdrawn for measurement of NOx.

To assess NOx production in response to an endothelium-independent vasodilator, SNP dissolved in normal saline and protected from exposure to light was infused into the LAD catheter at rates of 0.3 to 3.0 μg·kg⁻¹·min⁻¹ in 5 dogs. Aortic and coronary venous blood samples were collected for measurement of NOx production during infusion of SNP at a dose of 1.5 μg·kg⁻¹·min⁻¹.

**NO Production After Inhibition of NOS With L-NNA**

To demonstrate that the increase in NOx in response to endothelium-dependent agonists was mediated through NOS, 4 additional dogs were studied before and after inhibition of NOS with L-NNA. With the dogs standing quietly in a sling, arterial and coronary venous blood samples were collected during baseline conditions and during the infusion of ACh (37.5 μg/min) and BK (3 μg/min). The dogs then underwent the graded treadmill exercise protocol. One hour later, L-NNA (10 mg/kg) was infused through the coronary catheter over 30 minutes. Thirty minutes later, blood sampling was performed during infusion of the endothelium-dependent agonists and exercise in the same order.

**Measurement of NOx**

At physiological oxygen tensions, NO is rapidly oxidized,11 with an estimated half-life of 0.1 second in the coronary circulation.12 Although NO is virtually undetectable in plasma,13 the oxidation products nitrate (NOx) and nitrite (NO2−) are stable intermediates that can be accurately measured; the arteriovenous difference in NOx can then be used to estimate coronary NO production.9 Plasma samples for NOx determination were thawed and vigorously vortexed. The nitrite content of plasma was obtained by injecting 10-μL samples into the reaction chamber of the chemiluminescence analyzer (Sievers model 280) filled with 3 mL of 1N HCl, 3 mL of vanadium III chloride solution, and 0.6 mL of antifoaming agent (Dow Corning, FG-10) at 25°C. The reaction chamber was continuously bubbled with helium to strip NO into the gas phase. Nitrate content of the samples was determined in a similar manner after the reaction chamber had been heated to 90°C. At 90°C, both nitrite and nitrate are oxidized to NO; consequently, nitrate was subtracted from the total NOx content to obtain nitrate concentration. Standards were prepared by addition of known amounts of sodium nitrate and sodium nitrite to 1-mL aliquots of plasma; 10-μL samples were injected into the reaction chamber, and the output signal (mV) was used to construct standard curves for concentrations of nitrate [0 to 20 (μmol/L/L)] and nitrate [0 to 60 (μmol/L/L)].

**Data Analysis**

Heart rate and pressures were measured from the strip-chart recordings. LAD blood flow was calculated from the coronary Doppler shift by use of the equation q=2.5×(d'²×f)/(d), where q is coronary flow in mL/min, d is the ID of the vessel in mm, and f is the Doppler frequency shift in kHz.14 Because the artery is adherent to the flow probe in chronically instrumented animals, the coronary artery diameter is fixed at the site at which the velocity signal is obtained so that the OD of the artery is equal to the ID of the flow probe. On the basis of our previous experience, the ID of the artery was taken to be 80% of its OD.

Total plasma nitrate and nitrate concentration was expressed as NOx. The coronary arteriovenous difference of NOx (μmol/L) was obtained by subtracting coronary venous NOx from aortic NOx. The coronary production of NOx (nmol/min) was calculated by multiplying the arteriovenous difference by CBF. Data are expressed as mean±SEM. Data within groups were compared by ANOVA for repeated measures. A value of P<0.05 was considered significant. Individual comparisons were performed with the Wilcoxon signed-rank test.

**Results**

**Hemodynamic Response to Exercise**

The hemodynamic responses to exercise are shown in Table 1. Aortic pressure and heart rate increased progressively...
TABLE 1. Hemodynamics During Exercise (n=12)

<table>
<thead>
<tr>
<th>Mean Aortic Pressure, mm Hg</th>
<th>Heart Rate, bpm</th>
<th>LV Systolic Pressure, mm Hg</th>
<th>LV End-Diastolic Pressure, mm Hg</th>
<th>Rate-Pressure Product, mm Hg×bpm</th>
<th>CBF, mL/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>104±4</td>
<td>125±9</td>
<td>124±4</td>
<td>8±1</td>
<td>15 700±1800</td>
</tr>
<tr>
<td>Ex-1</td>
<td>114±4</td>
<td>167±7</td>
<td>145±4</td>
<td>11±1</td>
<td>24 400±1500</td>
</tr>
<tr>
<td>Ex-2</td>
<td>120±3</td>
<td>204±8</td>
<td>151±4</td>
<td>12±1</td>
<td>31 000±1800</td>
</tr>
<tr>
<td>Ex-3</td>
<td>120±3</td>
<td>222±12</td>
<td>159±3</td>
<td>12±1</td>
<td>35 400±2100</td>
</tr>
</tbody>
</table>

Hemodynamic Responses to Pharmacological Vasodilators

With the dogs standing in a sling, mean aortic pressure was 100±4 mm Hg, heart rate was 120±5 bpm, and CBF was 42±5 mm Hg (Table 2). Intracoronary infusion of ACh caused no significant change in aortic pressure, left ventricular (LV) end-diastolic pressure, or heart rate, whereas coronary flow increased to 108±6 mL/min at an intracoronary dose of 37.5 μg/min (P < 0.01). Infusion of BK (3 μg/min) resulted in no significant change in systemic hemodynamics, whereas coronary flow increased to 113±9 mL/min (P < 0.01). Infusion of sodium nitroprusside (SNP) at a dose of 37.5 μg/min in 5 dogs increased coronary flow to 110±12 mL/min (P < 0.05), with no significant change in systemic hemodynamics.

NO<sub>x</sub> Production in Response to Pharmacological Vasodilators

The baseline aortic concentration of NO<sub>x</sub> was 10.2±1.7 μmol/L and was not significantly different from the coronary venous concentration of 11.7±2.2 μmol/L (Figures 2 and 4); calculated coronary NO<sub>x</sub> production was 57±43 nmol/min (not significantly different from zero). During intracoronary infusion of ACh, NO<sub>x</sub> production increased to 108±76 nmol/min (P < 0.01 versus zero). During intracoronary BK, NO<sub>x</sub> production increased to 304±162 nmol/min (P < 0.01 versus zero). Infusion of the endothelium-independent vasodilator SNP resulted in a small but significant increase in transcoronary NO<sub>x</sub> production to 78±17 nmol/min (P < 0.05 versus zero).

NO<sub>x</sub> Production After L-NNA

Administration of N<sup>ω</sup>-nitro-l-arginine (L-NNA) increased resting mean arterial pressure from 105±10 to 123±7 mm Hg and decreased heart rate from 117±9 to 94±10 bpm. Resting CBF was not significantly changed after L-NNA (33±3 versus 40±8 mL/min), but the increases in CBF to ACh (76±11 versus 47±17 mL/min) and to BK (61±6 versus 50±10 mL/min) were significantly reduced. NO<sub>x</sub> production in response to ACh was reduced from 129±46 to −51±43 nmol/min after L-NNA, and in response to BK, it was reduced from 99±91 to −100±120 nmol/min. Similarly, after L-NNA, coronary NO<sub>x</sub> production during exercise stage 3 was −34±68 nmol/min. These findings indicate that the increase in NO<sub>x</sub> observed in response to endothelium-dependent agonists and exercise was due to activation of NOS.

Discussion

NO produced by the endothelium can diffuse into the lumen, where it is quickly oxidized to nitrate by Hb, with the formation of nitrite. Nitrite is then rapidly oxidized to nitrate by Hb, with the formation of nitrate. Nitrate is a stable product of NO metabolism and is not rapidly oxidized. Nitrite is also rapidly oxidized to nitrate in vivo, as indicated by the finding that the plasma nitrite concentration during resting conditions was <5% of the nitrate level. Therefore, levels of oxidation products of NO (nitrate + nitrite = NO<sub>x</sub>) essentially reflect the plasma concentration of nitrate. During resting conditions, aortic plasma NO<sub>x</sub> was 13.8±1.9 μmol/L, and coronary venous NO<sub>x</sub> was 14.1±2.1 μmol/L (Figure 1). These values were not significantly different and indicated no measurable NO production across the coronary circulation. Furthermore, in 5 of 12 animals, the gradients were negative, with aortic NO<sub>x</sub> levels higher than coronary venous levels (Figure 2). During the first 2 levels of exercise, no measurable production of NO<sub>x</sub> was observed, and =50% of the gradients were negative. During the highest level of exercise, the concentration of NO<sub>x</sub> was 15.4±2.6 μmol/L in aortic blood and 16.36±2.6 μmol/L in coronary venous blood, with a significant transcoronary gradient of NO<sub>x</sub> (Figures 1 and 3). Thus, NO<sub>x</sub> production across the coronary circulation (P < 0.05 versus zero) was detectable only during the heaviest level of exercise.
of equimolar amounts of methemoglobin (MetHb). NO can also form nitrite, which is the predominant oxidative product of NO metabolism in aqueous solutions, or peroxynitrite by reacting with superoxide anion. NO can also directly bind to cysteine groups on Hb to form S-nitrosohemoglobin or can nitrosylate plasma proteins such as albumin. These reactions are so efficient that the half-life of NO in the coronary circulation has been estimated to be ~100 ms, making measurements of true NO essentially impossible in in vivo studies. To circumvent this difficulty, the arteriovenous difference in the stable oxidative products of NO (nitrate and nitrite) across the coronary circulation can be used as an index of NO production.

**Basal Production of NO and Regulation of Resting Myocardial Blood Flow**

Chu et al demonstrated that inhibition of NO synthesis with N\(^\text{G}\)-monomethyl-L-arginine (L-NMMA) resulted in a decrease in epicardial coronary artery diameter without a significant change in CBF in awake dogs. Similar findings were reported by Parent et al in response to N\(^\text{G}\)-nitro-L-arginine methyl ester (L-NAME). Jones et al, using intravital microscopy in open-chest dogs, showed that L-NAME caused constriction of small arteries but compensatory vasodilation of arterioles so that CBF was unchanged. Thus, NO is not critical for the maintenance of resting blood flow in the normal heart.

Previous investigators have reported either no measurable basal NO production in the coronary circulation or a small positive transcoronary gradient of NO\(_x\). We failed to find significant coronary NO\(_x\) production during resting conditions. Although true NO production has not been directly measured in the intact animal because of its rapid degradation, Kelm and Schrader examined basal NO release in isolated perfused guinea pig hearts by measuring the conversion of oxyhemoglobin to MetHb by NO. They observed that resting NO production was very low, at 161 ± 11 pmol/min, a value that may in part explain our failure to detect coronary NO production during resting conditions. Because circulating plasma levels of nitrate are much higher (micromolar), picomolar increases in NO\(_x\) across the heart may be too small to be detected during basal conditions. In contrast to previous reports, the plasma samples in our study were not deproteinated, so that nitrosylated proteins as well as nitrate and nitrite were reduced to NO. Despite this modification, we failed to find significant coronary NO production during resting conditions.

**NO Production During Exercise**

Blockade of NO production impairs epicardial artery dilation during exercise but does not decrease CBF, indicating that NO production is not required for coronary resistance vessel dilation during exercise. Although it is likely that NO is produced continuously in the coronary circulation and increases with increasing levels of exercise (shear stress), only at the highest level of exercise in the present study was sufficient NO produced to be detected. To the best of our knowledge, only 1 previous study has measured coronary NO production during exercise. Bernstein et al found coronary NO production during 3 levels of treadmill exercise, but only at the second stage of exercise was NO production significantly greater than during resting conditions. Their method of measuring NO\(_x\) differed from ours, in that they converted deproteinated plasma nitrate to nitrite by use of Aspergillus reductase, which was then converted to NO with HCl and injected into a chemiluminescence analyzer. We injected plasma directly into a reactor containing vanadium III and HCl at 90°C to convert all nitrate, nitrite, and nitrosylated proteins to NO. Nevertheless, basal levels of NO produced by the coronary circulation were too small to be detected, in part because of the relatively high background levels of nitrate in blood.

**Figure 2.** A, Individual data points of coronary arteriovenous NO\(_x\) difference measured in 12 dogs during rest and 3 levels of treadmill exercise. Only at highest level of exercise (EX-3) was NO\(_x\) gradient positive. B, Coronary arteriovenous NO\(_x\) difference during rest and in response to endothelium-dependent agonists ACh and BK (n=10) and endothelium-independent agonist SNP (n=5).

**Figure 3.** NO\(_x\) production measured as coronary venous minus aortic NO\(_x\) concentration multiplied by CBF during 3 levels of treadmill exercise and in response to endothelium-dependent agonists and SNP. *P<0.05 vs zero.
NO Production in Response to Endothelium-Dependent Agonists

In contrast to the modest response to exercise, we observed significant increases in coronary NO production during vasodilation produced by the endothelium-dependent agonists ACh and BK. Furthermore, this increase in NO was mediated by activation of NOS, because the increase in NOx was blocked by L-NNA. Although the average increase in CBF was greater in response to agonists than to exercise, NOx in flow can be blocked with L-NNA. This is supported by findings in isolated rabbit hearts, in which NO production in coronary venous blood was significantly greater than aortic NOx from the blood, it is unreasonable to assume that the heart is able to accumulate nitrite or nitrate over a substantial period of time. It is more likely that the negative gradient implies a shift in the partitioning of NOx between the plasma and the red blood cells (for which NOx was not analyzed). Such a mechanism could occur because changes in the ambient oxygen tension from aorta to coronary sinus can cause changes in the binding of NO to Hb. The decrease in PO2 across the coronary circulation might result in accumulation of a greater fraction of NOx metabolites in the erythrocytes, with a resultant decrease in the plasma NOx content. For example, formation of HbNO occurs preferentially at low PO2 values, such as exist in coronary venous blood. Thus, Wennmalm et al. observed that when nitrite was incubated with whole blood ex vivo, a significant fraction was converted to HbNO in venous blood (O2 saturation=61%) but not in arterial blood. Because the coronary venous PO2 during rest and exercise was lower than during infusion of agonists, we cannot exclude greater partitioning of NO into the erythrocytes during these interventions. Because products derived from NO contained within the erythrocytes could not be determined, a significant portion of the byproducts of NO metabolism may be unaccounted for when the plasma was analyzed.

The chemiluminescent method for measuring NOx in the present study was highly reproducible. All specimens were measured in duplicate, with an average difference of <0.5 μmol/L. The method used was more direct than that previously reported, in which plasma nitrate was first reduced to nitrite with Aspergillus reductase and then acidified to liberate NO into the headspace gas, which was then injected into the chemiluminescent analyzer. Although the present technique eliminated several steps in the analytic procedure that could potentially introduce some degree of error, and despite performing the analysis on plasma to include NO in the form of nitrosylated proteins, we nevertheless failed to detect coronary NO production during resting conditions or at low levels of exercise. Only during administration of endothelium-dependent agonists or during heavy exercise was coronary NO production detected. Although it is likely that NO is produced continuously in small quantities by the coronary endothelium, the net NO production appears to be too small to detect in the presence of the relatively high background levels of plasma nitrate that exist in the intact animal during basal conditions.

**Table 2. Hemodynamics During Infusions of Agonists**

<table>
<thead>
<tr>
<th></th>
<th>Mean Aortic Pressure, mm Hg</th>
<th>Heart Rate, bpm</th>
<th>LV Systolic Pressure, mm Hg</th>
<th>LV End-Diastolic Pressure, mm Hg</th>
<th>CBF, mL/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (n=10)</td>
<td>100±4</td>
<td>120±6</td>
<td>122±6</td>
<td>9±2</td>
<td>42±5</td>
</tr>
<tr>
<td>ACh (n=10) (37.5 μg/min)</td>
<td>102±5</td>
<td>131±8</td>
<td>125±5</td>
<td>11±2</td>
<td>108±6</td>
</tr>
<tr>
<td>BK (n=10) (3 μg/min)</td>
<td>107±6</td>
<td>133±7</td>
<td>132±6</td>
<td>10±2</td>
<td>113±9</td>
</tr>
<tr>
<td>SNP (n=5) (37.5 μg/min)</td>
<td>95±89</td>
<td>132±9</td>
<td>116±9</td>
<td>10±3</td>
<td>110±12</td>
</tr>
</tbody>
</table>

**Figure 4.** Aortic and coronary venous NOx (μmol/L) during rest and in response to intracoronary infusion of ACh, BK, and SNP. Coronary venous NOx was significantly greater than aortic NOx in response to each agonist. *P<0.05 vs aorta.

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