Role of Nitric Oxide in Reactive Hyperemia of the Guinea Pig Heart

Miloslav M. Kostic and Jürgen Schrader

To evaluate the role of nitric oxide (NO) in the flow response after brief coronary arterial occlusion, NO formation by the isolated guinea pig heart was assessed by a specific difference spectrophotometric assay. Release of NO under basal conditions was 121.8±10.5 pmol/min and increased to 211.1±16.8 pmol/min after 60 seconds of coronary occlusion. Simultaneously, release of cGMP and adenosine increased by 87% and 65%, respectively. The kinetics of NO release paralleled the reactive hyperemic flow response. Inhibition of NO synthesis with nitro-L-arginine methyl ester (L-NAME, 30 μM) significantly reduced basal flow and attenuated reactive hyperemia, flow repayment, and repayment ratio. L-NAME decreased release of cGMP but significantly increased adenosine release under basal conditions and during reactive hyperemia. Oxyhemoglobin (5 μM) potentiated the effects of L-NAME. The stereoisomer nitro-D-arginine methyl ester was ineffective. Our results suggest 1) NO is an important regulator of coronary flow during reactive hyperemia as well as under basal flow conditions and 2) the significance of the increased adenosine release when NO synthesis is inhibited remains to be determined. (Circulation Research 1992;70:208-212)

Brief periods of coronary occlusion are followed by a prolonged overshoot of coronary blood flow known as reactive hyperemia. Coronary arterial occlusions as brief as 200 msec in duration can elicit coronary reactive hyperemia, and the magnitude of this response appears to be primarily related to myocardial metabolic activity.

The metabolites that are involved in mediating reactive hyperemia have not been fully defined but may involve changes in interstitial potassium, hydrogen ions, osmolarity, carbon dioxide, catecholamines, prostaglandins, and adenosine. Among them, adenosine was shown to play an important role. However, it appears that factors other than adenosine are collectively more important.

Nitric oxide (NO) formation by the vascular endothelium was recently shown to play a role in the regulation of blood pressure and to critically influence basal coronary vascular tone. The biosynthesis and physiology of NO has been recently reviewed. No information is available, however, about whether an accelerated cardiac NO formation may play a role during metabolic vasodilation. This study was therefore undertaken to explore the contribution of NO to myocardial reactive hyperemia. This involved measurements of NO release from the heart and use of the NO synthesis blocker nitro-L-arginine methyl ester (L-NAME). Furthermore, the role of adenosine relative to NO was investigated.

Materials and Methods

Hearts, isolated from guinea pigs with a body mass of 250 g, were perfused according to the Langendorff technique, either at constant pressure (60 cm H2O) or constant flow (10 ml/min). Perfusion medium, equilibrated with 95% O2-5% CO2 at 37°C, contained (mM) NaCl 140, KCl 4, CaCl2 1.8, MgCl2 1.0, NaH2PO4 0.4, glucose 5, pyruvate 2, HEPES 10, indomethacin 0.01, and ethyleno-9-(2-hydroxynon-3-yl) adenine (EHNA) 0.005, pH 7.4. Oxyhemoglobin (HbO2), prepared as described elsewhere, was added when the release of NO was measured. All hearts were electrically paced (300 beats per minute). Left ventricular systolic pressure and dP/dtmax was measured by means of a fluid-filled balloon inserted into the left ventricle through the cut mitral valve. Coronary flow was monitored with an electromagnetic flowmeter (Hellige, Freiburg, FRG). In constant-flow experiments, flow was maintained by means of a roller pump (Reichelt, Chemie Technik, Heidelberg, FRG) and coronary perfusion pressure was measured with a pressure transducer (Gould).

After the isolated heart perfusion was set up, 30 minutes was allowed for stabilization of the prepara-
tion. Reactive hyperemia was induced by occlusion of coronary inflow for 30 or 60 seconds. After each occlusion, 5–10 minutes was allowed to permit the heart to attain a new hemodynamic steady state.

For measurement of the cardiac release of cGMP and adenosine, samples of coronary effluent perfusate were collected for 1 minute before coronary occlusion and during the first 30 seconds of reactive hyperemia. This procedure was repeated two or three times, and the respective perfusates were combined to yield a total volume of about 15 ml, which was sufficient for subsequent analyses. After control samples for basal flow and reactive hyperemia were taken, the same protocol was followed after the medium was switched to one containing 30 μM nitro-arginine methyl ester (d-NAME), 30 μM L-NAME, 30 μM L-NAME plus 5 μM HbO₂. The hearts were equilibrated for 5 minutes with each medium before the first effluent perfusate samples were taken. In the case of hemoglobin, medium was supplemented with freshly prepared HbO₂, whereas L-NAME and d-NAME were infused directly into the perfusion cannula to which the heart was attached.

For analysis of metabolites released from guinea pig hearts, samples of effluent perfusate were processed as previously described. cGMP was determined with a commercially available radioimmunoassay (Amersham, Braunschweig, FRG). Adenosine was determined by radioimmunoassay.

For the quantitation of NO release, isolated hearts were perfused with constant flow (10.23±0.44 ml×min⁻¹, n=10) with medium containing 5 μM HbO₂. NO concentration in the effluent perfusate was continuously measured by a specific difference spectrophotometric assay based on the rapid NO-induced oxidation of HbO₂ to methemoglobin. Because HbO₂ traps the entire amount of NO released in less than 100 msec, the measurement of the extinction difference between the absorption maximum and isobestic point of HbO₂ versus methemoglobin (α₁, 401 nm; α₂, 411 nm) in a flow-through cell with a double wavelength spectrophotometer (Hitachi 557, Perkin-Elmer, FRG) permitted the continuous assay of released NO. The NO concentration was calculated from the extinction coefficient estimated to 38 mM⁻¹×cm⁻¹ under our experimental conditions. Amounts of NO released under basal conditions and during reactive hyperemia were calculated according to the criteria applied for quantitative flow debt and repayment estimations.

Quantitative analysis of features characterizing the reactive hyperemic flow response were performed according to criteria described elsewhere.

L-NAME and d-NAME were obtained from Serva, Heidelberg, FRG; indomethacin and bovine hemoglobin from Sigma, Munich, FRG; and EHNA from Burroughs Wellcome, Beckenham, UK.

Statistical significance of differences was estimated using paired Student’s t test.

### Results

In a first experimental series the influence of NO synthesis inhibitor L-NAME on reactive hyperemic flow response of the isolated guinea pig heart was investigated. As shown in the representative recording given in Figure 1, L-NAME reduced basal coronary flow and greatly attenuated reactive hyperemia. From the data compiled in Table 1 it can be seen that L-NAME but not the stereoisomer D-NAME reduced basal coronary flow by 16% and attenuated the duration of reactive hyperemia and

![Figure 1. Representative recordings of the coronary effects of nitro-l-arginine methyl ester (L-NAME) and oxyhemoglobin (HbO₂) plus L-NAME. Reactive hyperemia followed a coronary occlusion period of 30 seconds. CF, coronary flow.](image)

### Table 1. Effects of Nitro-l-arginine Methyl Ester, Nitro-l-arginine Methyl Ester, and Oxyhemoglobin on Basal Flow and Reactive Hyperemia After 30 Seconds of Coronary Occlusion in the Isolated Guinea Pig Heart

<table>
<thead>
<tr>
<th>Condition</th>
<th>Basal flow (ml×min⁻¹)</th>
<th>Duration (seconds)</th>
<th>Maximal flow (ml×min⁻¹)</th>
<th>Flow repayment (ml)</th>
<th>Repayment ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.72±0.54</td>
<td>61.60±3.99</td>
<td>14.58±0.94</td>
<td>4.65±0.36</td>
<td>1.57±0.09</td>
</tr>
<tr>
<td>D-NAME (30 μM)</td>
<td>5.50±0.47</td>
<td>68.00±2.94</td>
<td>14.64±1.19</td>
<td>4.59±0.41</td>
<td>1.68±0.06</td>
</tr>
<tr>
<td>L-NAME (30 μM)</td>
<td>4.79±0.45†</td>
<td>45.40±1.84‡</td>
<td>14.12±1.19</td>
<td>3.43±0.35‡</td>
<td>1.45±0.09§</td>
</tr>
<tr>
<td>HbO₂ (5 μM)+L-NAME (30 μM)</td>
<td>4.57±0.38</td>
<td>36.82±2.31‖</td>
<td>13.08±0.87‖</td>
<td>2.36±0.18‖</td>
<td>1.07±0.10‖</td>
</tr>
</tbody>
</table>

All values represent mean±SEM of six paired experiments. D-NAME, nitro-d-arginine methyl ester; L-NAME, nitro-l-arginine methyl ester; HbO₂, oxyhemoglobin.

*Repayment ratio=flow repayment/flow debt.
†p<0.005, ‡p<0.005, §p<0.05 vs. D-NAME.
‖p<0.05 vs. L-NAME.
The kinetics of coronary venous NO concentration closely paralleled the changes in coronary resistance. L-NAME (30 μM) reduced basal and postischemic rate of NO release by 55% and 33%, respectively (Figure 3). Again the kinetics of coronary venous NO concentration in the presence of L-NAME closely paralleled the respective changes in coronary resistance (Figure 2). D-NAME (30 μM) influenced neither the time course of NO release (Figure 2) nor the basal and postischemic rates of NO liberation (Figure 3).

Release of cGMP into the effluent perfusate of isolated hearts was recently shown by us to reflect the formation of NO by coronary endothelial cells.9 Similarly, in the present study L-NAME not only diminished NO release but also reduced basal cGMP release (274±54 fmol×min⁻¹) by 34%. During reactive hyperemia cGMP release was enhanced and L-NAME again significantly attenuated this effect by 35% (Figure 4). Simultaneous presence of L-NAME and 5 μM HbO₂ further reduced the release of cGMP under basal conditions and during reactive hyperemia (Figure 4).

It is well established that adenosine is formed during reactive hyperemia at an accelerated rate.4 However, it was quite surprising to find that in the presence of the NO synthesis inhibitor, adenosine release was inversely related to NO and cGMP release (Figure 4). Basal release of adenosine was significantly higher in the presence of L-NAME (23±5 versus 41±7 pmol×min⁻¹; n = 5). During reactive hyperemia, L-NAME but not D-NAME further increased cardiac adenosine release (177±61 versus 246±94 pmol×min⁻¹). Again, HbO₂ potentiated the effects of L-NAME on cardiac adenosine release (Figure 4).

**Discussion**

This study provides two new insights into the regulation of coronary flow during reactive hyperemia: 1) nitric oxide is involved in mediating the reactive hyperemic flow response in the isolated perfused guinea pig heart; and 2) when NO synthesis is inhibited adenosine is formed at an accelerated rate, which may compensate for the loss of NO-mediated vasorelaxation.

Several lines of evidence presented in this study suggest that NO is an important causal factor during reactive hyperemia. 1) The kinetics of NO release parallels the changes in coronary resistance (Figure 2). 2) More cGMP is released during reactive hyperemia (Figure 4), which is most likely a consequence of the NO-induced stimulation of soluble guanylate cyclase activity and subsequent washout.9,16 3) The NO synthesis inhibitor L-NAME, but not its D-isomer, attenuates reactive hyperemia (Figure 1, Figure 2, Figure 3, Figure 4).
and this effect is associated with a diminished NO formation (Figures 2 and 3) and cGMP release (Figure 4). 4 HbO₂, a rapidly acting scavenger of NO,₁₄,₁₇ potentiates the coronary effects of L-NAME (Table 1, Figures 1 and 4) as well as that of N⁰-monomethyl L-arginine. It finally should be noted that the half-life of NO in the coronary circulation was recently reported by us to be only 0.1 second.⁹ Such a short half-life of biological activity is an important prerequisite to explain the rapid and transient changes in coronary resistance during reactive hyperemia to be elicited by locally formed NO.

The quantities of NO formed by the unstressed heart are sufficient to control basal coronary vascular tone.⁹ In support of this idea is the present finding that L-NAME significantly reduced basal coronary flow and diminished basal NO and cGMP release. The absolute extent to which NO controls coronary vascular tone, however, is difficult to assess at present. It is possible that the increased formation of adenosine from the normoxic heart in the presence of L-NAME may prevent the full vasoconstriction to develop when NO synthesis is blocked. Similar consideration also applies to reactive hyperemia.

Changes in the supply/demand ratio for oxygen decrease high energy phosphates¹⁸ and constitute the most potent stimulus for cardiac adenosine formation.¹⁹,²⁰ Reduction of coronary flow by inhibition of NO synthesis most likely decreased local tissue Po₂, thereby triggering the formation of adenosine. This sequence would imply that the relation between adenosine and NO is of an indirect nature. It should be noted, however, that hypoxia has been demonstrated to stimulate the formation of endothelium-derived relaxing factor/NO in conduit vessels²¹ but was ineffective in the isolated heart in liberating NO.²²

Using adenosine deaminase to degrade adenosine into vasoactive inosine, Saito et al.⁶ have shown that repayment of flow debt after 30 seconds of coronary occlusion was reduced by 33%. In the presence of L-NAME and L-NAME plus HbO₂, we found that under otherwise similar conditions repayment of flow debt was reduced by 26% and 50%, respectively. It therefore appears that NO and adenosine together play a major role in the vascular resistance changes during reactive hyperemia. Both vasodilatory metabolites are likely to interfere with each other so that whenever NO formation is reduced, adenosine may compensate for the loss of this vasodilator activity. The interplay between NO and adenosine may be another example of the generally held opinion that an important vascular system such as the coronary circulation is controlled by several metabolites interacting with each other in a complex way.²³

The mechanism(s) by which cardiac NO formation is accelerated in the phase of reactive hyperemia is presently not fully understood. Synthesis of NO from l-arginine is an oxygen-requiring process and it is unlikely that ischemia as such stimulates NO synthesis. It is conceivable, however, that initiation of flow after the ischemic period can elicit an endothelium-dependent flow-induced release of NO,²⁴ which then potentiates and perpetuates hyperemia initially caused by other metabolic factors. Still another plausible explanation is that cardiac NO synthesis may be stimulated by extracellular ATP.

We have recently shown that ATP is released during reactive hyperemia from the isolated guinea pig heart in quantities that are equipotent to that of adenosine.²⁵ Theophylline, which blocks the coronary vascular actions of adenosine and ATP equally well,²⁶ attenuates not only the reactive hyperemic flow re-
sponse but also the coronary release of NO and cGMP (authors' unpublished observation, 1991). It is thus likely that two purines may be involved in setting the tone during coronary reactive hyperemia, although different mechanisms are involved: adenosine acts through activation of coronary A2-receptors and extracellular ATP acts by well-characterized endothelial mechanisms through liberation of NO.

References

KEY WORDS • reactive hyperemia • nitric oxide synthesis • adenosine • cGMP • nitro-1-arginine methyl ester
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