Roles of Nitric Oxide and Adenosine in the Regulation of Coronary Conductance in the Basal State and During Reactive Hyperemia

Jun Otomo, MD; Naoki Nozaki, MD; Hitonobu Tomoike, MD

Nitric oxide (NO) and adenosine are important mediators in the regulation of coronary vascular tone and are released into the interstitium from the vascular endothelium and myocardium, respectively. The roles of these autacoids in the regulation of coronary flow in the basal and reactive hyperemic states were examined in Langendorff rabbit hearts perfused with oxygenated Krebs-Henseleit solution at 37 °C and 110 mmHg pressure. Instantaneous perfusion pressure-flow relationships were analyzed to derive coronary conductance both in the basal state and during the early phase of reperfusion (hyperemic state). N\textsuperscript{\textsteriskleft} -nitro-L-arginine methyl ester (L-NAME) at increasing concentrations (10\textsuperscript{-6} to 10\textsuperscript{-4} mol/L) (n = 7) and 8-phenyltheophylline (8-PT) at increasing concentrations (10\textsuperscript{-9} to 10\textsuperscript{-6} mol/L) (n = 7) were applied to assess the role of NO and adenosine, respectively. L-NAME dose-dependently reduced the coronary conductance in both the basal and early hyperemic states, while 8-PT dose-dependently reduced conductance only in the hyperemic state. Changes in conductance during the early hyperemic phase correlated well with changes in the debt repayment ratio for either L-NAME (r = 0.94) or 8-PT (r = 0.99). These data suggest that a flow-related NO release mechanism regulates the coronary conductance in both the basal and hyperemic states while the metabolic regulation of adenosine release plays a role in the presence of ischemia.

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Key Words: Coronary conductance; Nitric oxide; Adenosine; Reactive hyperemia; Pressure-flow relationship

Temporary interruption of arterial inflow to organs and tissues results in vasodilation with subsequent reactive hyperemic response. Reactive hyperemia of the heart is mediated by vasodilator metabolites and/or myogenic responses. Two well-known vasodilatory metabolites are adenosine in myocardium and nitric oxide (NO) in vascular endothelium. The role of adenosine in the metabolic control of coronary blood flow is well accepted. A type of hyperemia seen after temporary coronary occlusion, namely reactive hyperemia, has been shown to be mediated by adenosine and by NO. However, how and when these mediators affect coronary conductance in the basal and hyperemic states remain undetermined. In the present study, we evaluated the role of NO and adenosine in the temporal regulation of coronary blood flow in the basal and reactive hyperemic states by examining the inhibitory effects of N\textsuperscript{\textsteriskleft} -nitro-L-arginine methyl ester (L-NAME) on NO formation and of 8-phenyltheophylline (8-PT) on adenosine receptors. We analyzed beat-to-beat pressure-flow trajectories consecutively before, during, and after coronary occlusion and determined quantitatively conductances in the basal and hyperemic states.

Materials and Methods

Heart Perfusions

An isolated, perfused Langendorff rabbit heart preparation was used for these studies. The investigation conforms to the Guide for the Care and use of Laboratory Animals, published by the US National Institutes of Health (NIH publication No.85-23, revised 1985). Animal use was also in accordance with institutional guidelines. Japanese white rabbits weighing 2.0–2.5 kg were heparinized (100 U/kg, 2-2-2 Iida-Nishi, Yamagata 990-23, Japan)
intravenously) to prevent the formation of intracorony microthrombi\textsuperscript{16} and were anesthetized with pentobarbital sodium (50 mg/kg iv). The heart was rapidly excised and the aorta was dissected free and cannulated. The heart was immediately perfused at a constant pressure of 110 mmHg with Krebs-Henseleit
Table 1  Extent of Reactive Hyperemia (n=6)

<table>
<thead>
<tr>
<th>Duration of coronary occlusion(sec)</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal flow (ml/min)</td>
<td>22±2</td>
<td>22±3</td>
<td>23±3</td>
<td>24±3</td>
<td>23±2</td>
<td>24±2</td>
</tr>
<tr>
<td>Peak hyperemic flow (ml/min)</td>
<td>38±3</td>
<td>43±1</td>
<td>51±2**</td>
<td>63±2**</td>
<td>73±1**</td>
<td>80±2**</td>
</tr>
<tr>
<td>Debt repayment ratio</td>
<td>0.47±0.11</td>
<td>0.51±0.13</td>
<td>0.69±0.14*</td>
<td>0.79±0.17*</td>
<td>0.84±0.17*</td>
<td>0.78±0.12*</td>
</tr>
</tbody>
</table>

Values are means±SE. *, p<0.05; **, p<0.01 vs 5 sec occlusion.

Table 2  Time Course of Reactive Hyperemia for 30 sec of Coronary Occlusion (n=4)

<table>
<thead>
<tr>
<th>Trial times</th>
<th>1–2</th>
<th>3–4</th>
<th>5–6</th>
<th>7–8</th>
<th>9–10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (per min)</td>
<td>187±16</td>
<td>183±13</td>
<td>179±19</td>
<td>174±19*</td>
<td>170±21*</td>
</tr>
<tr>
<td>Basal flow (ml/min)</td>
<td>33±3</td>
<td>31±3</td>
<td>30±3</td>
<td>31±2</td>
<td>32±2</td>
</tr>
<tr>
<td>Perfusion pressure (mmHg)</td>
<td>103±1</td>
<td>104±1</td>
<td>104±1</td>
<td>104±1</td>
<td>103±1</td>
</tr>
<tr>
<td>Peak hyperemic flow (ml/min)</td>
<td>66±3</td>
<td>65±3</td>
<td>65±3</td>
<td>65±4</td>
<td>65±3</td>
</tr>
<tr>
<td>Debt repayment ratio</td>
<td>0.62±0.08</td>
<td>0.77±0.14</td>
<td>0.68±0.15</td>
<td>0.70±0.14</td>
<td>0.72±0.11</td>
</tr>
</tbody>
</table>

Coronary conductance (ml/min per mmHg)

- Basal: 0.55±0.06, 0.55±0.04, 0.54±0.04, 0.55±0.05, 0.56±0.05
- Early hyperemia: 1.05±0.04, 1.09±0.06, 1.11±0.06, 1.11±0.07, 1.13±0.07

Values are means±SE. *, p<0.05; **, p<0.01 vs trial 1–2.

Table 3  Effect of Nω-Nitro-L-Arginine Methyl Ester on Reactive Hyperemia (n=7)

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>10⁻⁶</th>
<th>3×10⁻⁶</th>
<th>10⁻⁵</th>
<th>10⁻⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (l/min)</td>
<td>181±11</td>
<td>181±12</td>
<td>178±14</td>
<td>158±14**</td>
<td>144±16**</td>
</tr>
<tr>
<td>Basal flow (ml/min)</td>
<td>28±4</td>
<td>28±3</td>
<td>24±3**</td>
<td>19±3**</td>
<td>18±3**</td>
</tr>
<tr>
<td>Perfusion pressure (mmHg)</td>
<td>108±1</td>
<td>107±1</td>
<td>109±1*</td>
<td>110±1*</td>
<td>110±1*</td>
</tr>
<tr>
<td>Peak hyperemic flow (ml/min)</td>
<td>69±4</td>
<td>70±3</td>
<td>56±5**</td>
<td>43±3**</td>
<td>39±4**</td>
</tr>
<tr>
<td>Peak flow ratio</td>
<td>2.84±0.24</td>
<td>2.40±0.22</td>
<td>2.50±0.24</td>
<td>2.54±0.27</td>
<td>2.89±0.44</td>
</tr>
<tr>
<td>Debt repayment ratio</td>
<td>0.73±0.13</td>
<td>0.71±0.11</td>
<td>0.51±0.02</td>
<td>0.53±0.06*</td>
<td>0.35±0.06**</td>
</tr>
</tbody>
</table>

Values are means±SE. Reactive hyperemia was analyzed after 30 sec coronary occlusion.

*, p<0.05; **, p<0.01 vs before L-NAME.

solution at 37℃. The composition of the Krebs-Henseleit solution was as follows (in mmol/L): NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.18, NaHCO₃ 24.9, KH₂PO₄ 1.18, and glucose 5.6. The solution was bubbled continuously with 95% oxygen and 5% carbon dioxide and the pH was maintained at 7.4. The perfusion apparatus was an all-glass water-jacketed system. A screw-type occluder, in-line electromagnetic flow probe (Nihon-Kohden, FF-050T and MFV 3100, Tokyo) and 2 small side arms were attached between the perfusion apparatus and the cannula. Perfusion pressure was monitored at the side branch using a fluid-filled system. Drugs were infused at a constant speed (1/60 of the basal coronary flow) into the aortic root from the other side-arm of the perfusion system. Heart wet weight was 5.2±0.1 g.

PO₂ and PCO₂ were periodically analyzed during experiments and were more than 600 mmHg and 40±3 mmHg, respectively. The left ventricle was continuously vented through a cannula inserted into the left ventricular cavity via the mitral valve with negative pressure to reduce the metabolic stimulus to coronary vasomotion and to keep the metabolic component of coronary tone constant. Hearts were allowed to stabilize before starting the experimental protocol.

Experimental Protocols

Experiment 1. Measurements were made under basal conditions and during and after abrupt coronary occlusions of 5, 10, 20, 30, 45, and 60 sec duration in 6 rabbits. An occlusion for 30 sec was repeated every 10 min for 120 min in another 4 rabbits.
Fig 2. Typical tracings of pressure-flow trajectories before and after L-NAME. The relationship between pressure and flow shifted rightward and the area circumscribed by trajectories became smaller after L-NAME owing to decreases in slope at B and E. An inflection between D and E (D-E: see Fig 1 legend) became unclear after L-NAME.

Fig 3. Dose-related effects of L-NAME on slope 1 (○) and slope 2 (○). *p<0.05 and **p<0.01 vs before L-NAME (C).

Experiment 2. Thirty seconds of coronary occlusion was repeated before and 10 min after continuous infusion of L-NAME in 7 rabbits. Doses of L-NAME increased stepwise from $10^{-6}$ mol/L to $3 \times 10^{-6}$, $10^{-5}$ and $10^{-4}$ mol/L.

Experiment 3. Thirty seconds of coronary occlusion was repeated before and 15 min after continuous infusion of 8-PT in 7 rabbits. Doses of 8-PT increased stepwise from $10^{-9}$ mol/L to $10^{-8}$, $10^{-7}$, and $10^{-6}$ mol/L.

Data Analysis
Data were recorded on a pen recorder (Nihon-Denki Sanei, Japan) and were simultaneously digitized using an 8-channel multipurpose data acquisition system (MacLab, Analog Digital Instruments, NSW, Australia) connected to a Macintosh computer (IICi, Apple computer, Cupertino, CA).

Flow debt, reactive hyperemic flow, and repayment of flow debt were calculated as described by Coffman and Gregg. Areas of flow debt and reactive hyperemic flow were measured on slow-speed analog tracings by a planimeter. The debt repayment ratio was calculated as the ratio of reactive hyperemic flow to flow debt. To analyze coronary conductance in the basal and hyperemic states, pressure-flow trajectories were displayed using digitized data (Fig 1a, top). Stroke coronary flow and perfusion pressure in each cardiac cycle were calculated from the digitized data and were plotted as shown in Fig 1a, bottom. From beat-to-beat pressure-flow relationships, conductances in the basal (B) and early hyperemic (E) states were assessed on slope 1 and 2 (Fig 1b), respectively. From this, the reactive hyperemia was divided into early and late phases, which were quantified by the conductance and debt repayment ratio, respectively.
Table 4 Effect of 8-Phenyltheophylline on Reactive Hyperemia (n=7)

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>10^{-9}</th>
<th>10^{-8}</th>
<th>10^{-7}</th>
<th>10^{-6}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (l/min)</td>
<td>174±5</td>
<td>168±7</td>
<td>162±7*</td>
<td>158±7**</td>
<td>153±7**</td>
</tr>
<tr>
<td>Basal flow (ml/min)</td>
<td>26±3</td>
<td>27±3</td>
<td>26±3</td>
<td>20±4</td>
<td>21±3</td>
</tr>
<tr>
<td>Perfusion pressure (mmHg)</td>
<td>105±1</td>
<td>105±1</td>
<td>105±1</td>
<td>106±1</td>
<td>107±1*</td>
</tr>
<tr>
<td>Peak hyperemic flow (ml/min)</td>
<td>64±4</td>
<td>63±4</td>
<td>60±4*</td>
<td>56±4**</td>
<td>47±4**</td>
</tr>
<tr>
<td>Peak flow ratio</td>
<td>2.63±0.32</td>
<td>2.46±0.22</td>
<td>2.41±0.23</td>
<td>2.45±0.23</td>
<td>2.34±0.19</td>
</tr>
<tr>
<td>Debt repayment ratio</td>
<td>0.65±0.07</td>
<td>0.58±0.08</td>
<td>0.49±0.08</td>
<td>0.41±0.09*</td>
<td>0.37±0.08**</td>
</tr>
</tbody>
</table>

Values are means±SE. Reactive hyperemia was analyzed after 30 sec coronary occlusion. *p < 0.05; **p < 0.01 vs before 8-PT.

Fig 4. Typical tracings of pressure-flow trajectories before and after 8-phenyltheophylline. The area circumscribed by trajectories became smaller after 8-PT owing to a significant decrease in slope 2. An inflection between D and E (D-E: see Fig 1 legend) became unclear after 8-PT.

Statistics

All values are expressed as means±SEM. In all protocols, 1-way analysis of variance (ANOVA) was used to test the treatment effect. When ANOVA demonstrated a statistically significant result, Fisher’s protected least significant difference was used to identify the subgroup difference. Statistical differences between 2 means were estimated by Student’s t test for paired observations. The perfusion pressure and coronary flow at slope 1 and 2 were fitted to a linear equation of the form y=a+b*x. The variables y and x are flow and perfusion pressure, respectively, and a and b are the coefficients of the equation. Correlation coefficients (r²) for the least-squares fit through the data points were determined. A p value less than 0.05 was considered to be significant.

Results

The peak level of hyperemic flow increased depending on occlusion time, however the debt repayment ratio remained constant for occlusion durations of 20—60 sec (Table 1). Repeated trials of 30 sec coronary occlusion and subsequent hyperemia showed that the level of peak hyperemic flow, the debt repayment ratio and basal coronary conductance remained constant for 10 occlusions for 120 min (Table 2). The early hyperemic conductance increased, but the increase was not statistically significant (Table 2).

Table 3 summarizes the effects of L-NAME on reactive hyperemia. The basal coronary flow and conductance were 28±4 ml/min and 0.48±0.05 ml/min per mmHg, respectively, and decreased dose-dependently after pretreatment with L-NAME. The basal flow and coronary conductance at the maximum dose of L-
NAME (10^{-4} \text{ mol/L}) were 18 \pm 3 \text{ ml/min and 0.28} \pm 0.03 \text{ ml/min per mmHg (p<0.01 vs before L-NNAME)}. The peak level of hyperemia and the debt repayment ratio, but not the peak flow ratio, decreased simultaneously and dose-dependently after L-NNAME. Fig 2 shows instantaneous pressure-flow relationships before and after pretreatment with L-NNAME. The trajectory of pressure-flow relationships before, during, and after coronary occlusion shifts rightward and downward and the area circumscribed by the envelope decreased after L-NNAME (Fig 2, right). Both slopes 1 (closed circles; basal conductance) and 2 (open circles; early hyperemic conductance) decreased dose-dependently (Fig 3).

Table 4 summarizes the effects of 8-PT on reactive hyperemia. The basal coronary flow and coronary conductance were 26 \pm 3 \text{ ml/min and 0.46} \pm 0.02 \text{ ml/min per mmHg, respectively, before 8-PT and were unchanged at 21} \pm 3 \text{ ml/min and 0.43} \pm 0.02 \text{ ml/min per mmHg at a dose of 10^{-6} \text{ mol/L (not significant vs the basal state, Table 4). The peak flow ratio also remained constant after 8-PT. The debt repayment ratio was 0.65} \pm 0.07 \text{ before 8-PT and decreased dose-dependently to 0.37} \pm 0.08 \text{ (p<0.01) at the maximum dose of 8-PT, 10^{-6} \text{ mol/L (Table 4). Fig 4 shows typical examples of instantaneous tracings of pressure-flow trajectories before and after pretreatment with 8-PT. The pressure-flow relationship in the hyperemic state (E) moves down and to the right after 8-PT. Dose-related changes in slopes 1 (closed circles) and 2 (open circles) are summarized in Fig 5. Slope 1 remained constant up to a dose of 10^{-6} \text{ mol/L, but slope 2 was reduced significantly and dose-dependently after 8-PT. Changes in conductance during the early phase of hyperemia (slope 2) induced by either L-NNAME or 8-PT correlated well with changes in the debt repayment ratio (Fig 6).}

**Discussion**

In the present study, we showed that adenosine and nitric oxide (NO) act differently as mediators of basal coronary tone and reactive hyperemia. The reactive hyperemic state was divided into the early and late phases, as assessed by coronary conductance and debt repayment ratio, respectively. Flow-induced vasodilatation via NO was clearly observed during the basal and hyperemic states. Adenosine, which is known to be released from ischemic myocardium, affected the initial dilatation phase as well as the late phase of repayment but not the basal conductance.

The debt repayment ratio is often used as an approximation of coronary reserve and is about 4 for hyperemia following occlusions of 10–15 sec. In the present study, the debt repayment ratio was below unity. The magnitude of hyperemia is determined by the level of the basal flow, the level of compression of the coronary microvessels, and the intactness of adrenergic receptors. As we used an oxygenated crystalloid solution for coronary perfusion, the absence of blood corpuscles as well as the low oxygen-carrying capacity of the perfusate increased basal flow. The basal flow in the present study was 22 \pm 2 \text{ ml/min, which was 5 times higher than during blood perfusion. The oxygen content of the crystalloid solution used was one-tenth of that of arterial blood and the oxygen-carrying capacity of the solution was roughly 50% of that of in situ blood. Nevertheless, pretreatment with 8-PT did not alter basal flow, which suggests that the perfusate used...
did not cause significant production of adenosine or any other metabolic marker of myocardial ischemia in the basal state.

The present nonworking heart model using continuous suction of the left ventricular cavity did not need excess oxygen owing to the lower level of pressure-work, as previously noted\(^\text{17}\) and may be tolerant of the low oxygen carrying-capacity of the perfusate. One of advantages of the present model was the ability to evaluate the mechanisms of coronary flow regulation from the standpoint of coronary vessels per se, without significant influence of either myocardial contraction or changes in myocardial oxygen consumption.

The slope of the pressure-flow relationship has been termed the instantaneous resistance\(^\text{21}\). To obtain correct values for instantaneous resistance, it is essential that measurements are taken over such a short time that metabolic or reflex adaptation cannot affect the results. In the experiment described here, reflex adaptation may not be less strong because of the condition of isolated heart. As conductance measurement was performed within 5 sec to minimize the effects of autoregulation on coronary conductance, the present protocol should meet the maximum allowable time of measurement\(^\text{22}\). It is also necessary to minimize the compliance effect\(^\text{23}\). Possible compliance effects during systole and diastole will, at least in part, cancel each other out as pressure and flow were beat averaged. Such analysis of beat-averaged pressure and flow minimized the effects of phase shifts in measurements of pressure and flow on the trajectories of these variables.

The present study unveiled the importance of the time-dependent profile of reactive hyperemia, by analyzing the trajectories of pressure-flow relationships. We were able to derive conductances quantitatively in the basal state and during the early phase of reactive hyperemia. The pressure-flow relations are also modified by an intracavitary pressure, a prominent factor in the waterfall mechanism\(^\text{24}\). To minimize cavity pressure, we vented the left ventricular cavity with negative pressure. Thus, the present pressure-flow relationships represent characteristics unique to change in coronary vascular conductance.

Endothelial cells of the internal vascular lining play an important role in flow-mediated regulation of vascular tone of the epicardial coronary artery\(^\text{25,26}\). Recently, the role of the endothelium in the development of reactive hyperemia was confirmed in canine coronary circulation using N\(^6\)-monomethyl-L-arginine (L-NMMA)\(^\text{10}\), a competitive inhibitor of the biosynthesis of nitric oxide\(^\text{12,13}\). L-NMMA suppressed the flow debt repayment after 10-, 20- and 60-sec coronary occlusions, which accords well with the findings of present study. Suppression of coronary vascular tone and autoregulation were also noted in isolated rabbit hearts in the presence of L-NMMA, L-NAME, N\(^\text{ω}\)-iminoethyl-L-ornithine or N\(^\text{ω}\)-nitro-L-arginine\(^\text{27–29}\).

Basal coronary flow was attenuated by L-NAME in our experimental Langendorff set-up, as previously reported for NO synthesis inhibition (L-NMMA) in the isolated buffer perfused rabbit heart\(^\text{6,20}\). Lefroy et al\(^\text{30}\) reported a reduction in distal coronary diameter and coronary flow after intracoronary infusion of L-NMMA in 12 normal subjects. However, the reduction in basal flow induced by the NO synthesis inhibitor was larger under buffered conditions than in normal subjects or in situ canine heart\(^\text{10}\). This discrepancy between isolated and in situ hearts may depend on the absence of hemoglobin, as hemoglobin is a potent NO inhibitor in perfused blood systems. Vascular endothelium produces endothelium-derived nitric oxide in the presence of hypoxia\(^\text{31}\), which also plays a role in increasing basal coronary flow in the Langendorff model. Increased flow velocity may also stimulate the release of NO via augmented shear stress on the endothelium\(^\text{26–30}\).

We invariably noted the presence of an inflection on pressure-flow trajectories immediately after reperfusion, and this inflection was obscured by pretreatment with L-NAME. This finding suggests that the onset of NO release precedes the onset of coronary vasodilation. In other words, flow-related increase in shear stress may stimulate instantaneous release of NO\(^\text{32,33}\).

The interval from the onset of reperfusion to the inflection point (1–2 sec) corresponded to the latent period of the response of coronary smooth muscle to exogenously applied NO as previously reported\(^\text{34}\).

The present study confirmed that 8-PT dose-dependently suppresses reactive hyperemia but not basal flow, which is consistent with previous observations\(^\text{10,35}\). However, we discovered that 8-PT slowed the flow increase during the early phase of reactive hyperemia. Ischemia caused release of adenosine, which promptly dilated resistance vessels. Thus, NO and adenosine seem to play an important role in the development of reactive hyperemia, and these mediators are released by different stimuli during the process of reactive hyperemia. In conclusion, our data demonstrate for the first time that reactive hyperemia can be analyzed from the trajectories of beat-to-beat pressure-flow relations. The present study provides a basis for further work including study of hypertension-, atherosclerosis- or diabetes-induced changes in the microcirculation.
Acknowledgment

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