Hydrogen Peroxide, an Endogenous Endothelium-Derived Hyperpolarizing Factor, Plays an Important Role in Coronary Autoregulation In Vivo

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Background—Recent studies in vitro have demonstrated that endothelium-derived hydrogen peroxide (H2O2) is an endothelium-derived hyperpolarizing factor (EDHF) in animals and humans. The aim of this study was to evaluate our hypothesis that endothelium-derived H2O2 is an EDHF in vivo and plays an important role in coronary autoregulation.

Methods and Results—To test this hypothesis, we evaluated vasodilator responses of canine (n=41) subepicardial small coronary arteries (≥100 μm) and arterioles (<100 μm) with an intravital microscope in response to acetylcholine and to a stepwise reduction in coronary perfusion pressure (from 100 to 30 mm Hg) before and after inhibition of NO synthesis with N’-monomethyl-L-arginine (L-NMMA). After L-NMMA, the coronary vasodilator responses were attenuated primarily in small arteries, whereas combined infusion of L-NMMA plus catalase (an enzyme that selectively dismutates H2O2 into water and oxygen) or tetraethylammonium (TEA, an inhibitor of large-conductance KCa channels) attenuated the vasodilator responses of coronary arteries of both sizes. Residual arteriolar dilation after L-NMMA plus catalase or TEA was largely attenuated by 8-sulfophenyltheophylline, an adenosine receptor inhibitor.

Conclusions—These results suggest that H2O2 is an endogenous EDHF in vivo and plays an important role in coronary autoregulation in cooperation with NO and adenosine. (Circulation. 2003;107:1040-1045.)

Key Words: endothelium-derived factors ■ microcirculation ■ ischemia ■ nitric oxide ■ adenosine

Coronary autoregulation is an important physiological compensatory mechanism that permits the myocardium to relax when coronary perfusion pressure is decreased.1 Nitric oxide (NO) and adenosine are known to be involved in ischemic autoregulation during low perfusion pressure.1,2 Vascular endothelial cells play an important role in modulating vascular tone by releasing at least 3 vasodilator factors, including NO, prostacyclin (PGI2), and endothelium-derived hyperpolarizing factor (EDHF).3,4 NO synthase inhibitors largely suppress acetylcholine (ACH)-induced dilation of human5 and canine6 epicardial coronary arterioles in vivo, indicating that EDHF may not be involved in the ACh-induced vasodilation of large coronary arteries in vivo. Conversely, in the coronary microcirculation, inhibition of NO synthase does not abolish ACh-induced vasodilation.7 Residual dilation to ACh after inhibition of NO synthase and cyclooxygenase has been attributed to EDHF. However, the nature of EDHF has been controversial since the first report on its existence.3,4 The natures of EDHF that have been proposed include cytochrome P-450 metabolites,8,9 endothelium-derived K+10 and electrical communications through gap junctions between endothelial cells and vascular smooth muscle cells.11 Recently, Matoba et al12,13 established that endothelium-derived hydrogen peroxide (H2O2) is a primary EDHF in mice and humans. Indeed, H2O2 is known to activate calcium-activated K+ channels (KCa)14 and to cause hyperpolarizations of vascular smooth muscle cells. Blockade of KCa channels inhibits the adenosine-induced coronary vasodilation.15 However, it remains to be examined whether H2O2 is an EDHF in the coronary circulation in vivo, and if so, whether H2O2 contributes to autoregulation as a compensatory mechanism for NO and adenosine. The aim of the present study was to elucidate those important issues in dogs. The results demonstrated that H2O2 is a primary EDHF in vivo and plays an important role in coronary autoregulation.

Methods

Animal Preparation
This study conformed to the Guideline on Animal Experiments of Kawasaki Medical School and the Guide for the Care and Use of Laboratory Animals of the United States Department of Health and Human Services. The use of animals was in accordance with the Guideline on Animal Experiments of the Ministry of Education, Science, Sports and Culture of Japan.

Coronary microcirculation was visualized using an intravital microscope as described previously.3-5 Briefly, the coronary microcirculation of dogs (n=12) was observed in response to acetylcholine (10-8 to 10-5 M) and a stepwise reduction in coronary perfusion pressure (from 100 to 30 mm Hg) before and after inhibition of NO synthesis with L-NMMA (5 mg/kg, i.v., intravenous infusion). Seven animals were also subjected to combined infusion of L-NMMA and catalase (600 U/kg, i.v., intravenous infusion). Residual arteriolar dilation to acetylcholine after L-NMMA was largely attenuated by 8-sulfophenyltheophylline (1 mg/kg, i.v., intravenous infusion), an adenosine receptor inhibitor. ACh (10-6 M) elicited a significant dilation in the coronary microcirculation, and the dilation was attenuated by L-NMMA, catalase, and 8-sulfophenyltheophylline.

The dilator responses of canine coronary arterioles to acetylcholine (10-8 to 10-5 M) were assessed before and after inhibition of NO synthesis with L-NMMA (5 mg/kg, i.v., intravenous infusion). The baseline arteriolar diameter was measured at rest and after a 10-min period of low perfusion pressure (100 mm Hg). L-NMMA attenuated ACh-induced arteriolar dilation, and this attenuation was largely reversed by catalase or TEA.

Received September 10, 2002; revision received November 5, 2002; accepted November 6, 2002.

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Circulation is available at http://www.circulationaha.org

DOI: 10.1161/01.CIR.0000050145.25589.65

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Laboratory Animals published by the US National Institutes of Health.

Mongrel dogs (n=41, 10 to 25 kg) of either sex were anesthetized with morphine (3 mg/kg IM) and sodium pentobarbital (25 mg/kg IV). After intubation, each animal was ventilated with a high-frequency jet ventilator (model VS600, IDC) with room air supplemented by 100% oxygen. Aortic pressure and left ventricular pressure were measured with an 8F pigtail double manometer catheter (SPC-784A, Millar). Heart rate was kept constant at 100 bpm by right ventricular pacing after atrioventricular node blocking by 40% formaldehyde.16

Needle-Probe Intravital Microscope

Briefly, the needle probe (4.5 mm in diameter) contains a gradient index lens surrounded by light guide fibers and a double-lumen sheath. A doughnut-shaped balloon on the tip avoids direct compression of the vessels by the needle tip.16

Measurements of Arteriolar Diameters

We placed the needle probe gently on the subepicardial microvessel. When a clear vascular image was obtained, the end-diastolic vascular images were taken at a rate of 30 pictures per second.16

Coronary Sinus Cannulation

A Sones catheter was inserted into the right external jugular vein and advanced into the coronary sinus. Blood samples drawn from the coronary sinus catheter were analyzed for plasma adenosine concentration.17

Lactate Measurements

Arterial and coronary venous lactate samples were drawn into syringes. Lactate concentration was measured with a YSI 2300 Stat Plus model lactate analyzer.17 Myocardial percent lactate extraction was calculated as (arterial−venous)/arterial values×100 (%).

Plasma Adenosine Measurements

The samples were then concentrated by evaporation and resuspended in 50 μL of high-performance liquid chromatography buffer.18 The adenosine in each sample was separated on a Shimadzu LC10 high-performance liquid chromatograph with a C-18 column using an ion-pairing buffer solution of tetrabutylammonium hydrogen sulfate and potassium phosphate with an acetonitrile gradient.19

Application of System for Controlled Perfusion of Coronary Arteries

To manipulate coronary arterial pressure, the heart was perfused with blood from the left femoral artery.19 An in-line flow probe (Transonic 4-Fr, connected to a T206 Transonic flowmeter) just proximal to the Gregg cannula in the left main coronary artery was used to measure phasic coronary blood flow. Coronary perfusion pressure was measured in the first diagonal branch of the left anterior descending coronary artery.

Experimental Protocols

After the surgical procedure and instrumentation, at least 30 minutes were allowed for stabilization while hemodynamic variables were monitored. The following protocols were examined.

1) ACh (1.0 μg/kg IC for 2 minutes)-induced, EDHF-mediated coronary vasodilation was evaluated before and after inhibition of NO synthase (Nω-monomethyl-L-arginine [L-NMMA], 2 μmol/min for 20 minutes)20 with cyclooxygenase blockade (ibuprofen, 12.5 mg/kg IV, an inhibitor of the synthesis of vasodilator prostaglandins) to evaluate the role of EDHF and NO alone without PGI2 or catalase (40 000 U/kg IV and 240 000 U · kg−1 · min−1 IC for 10 minutes, an enzyme that selectively dismutates H2O2 into water and oxygen)21 or tetraethylammonium (TEA, 10 μg · kg−1 · min−1 IC for 10 minutes, an inhibitor of large-conductance Kc channels).22

2) Coronary perfusion pressure was changed in a stepwise manner from 100 to 30 mm Hg before and after L-NMMA plus ibuprofen. Coronary perfusion pressure was slowly reduced (~1 minute) to the next lower level.

3) To determine the nature of EDHF and the mechanism of EDHF-mediated vasodilation, additional experiments were performed in coronary subepicardial arterioles with a CCD intravital microscope. The inhibitors TEA and catalase were used. In additional experiments, feedback vasodilator responses during coronary autoregulation were examined before and after catalase, and after catalase followed by L-NMMA compared with L-NMMA followed by catalase.

4) To evaluate the compensatory effects of adenosine, coronary venous blood samples were drawn. To evaluate the interaction among EDHF, NO, and adenosine, we also evaluated vasodilator responses after TEA or catalase with L-NMMA followed by adenosine receptor blockade (8-sulfophenyltheophylline, 8-SPT, 25 μg · kg−1 · min−1 IC for 5 minutes).22 All drugs were obtained from Sigma Chemical Co.

Statistical Analysis

Results are expressed as mean±SEM. Vascular and coronary blood flow responses were analyzed by 2-way ANOVA followed by Scheffe’s post hoc test. Student’s t test was used for both paired and unpaired comparisons. The criterion for statistical significance was a value of P<0.05.

Results

Role of EDHF in Endothelium-Dependent Vasodilation of Coronary Microvessels In Vivo

The vasodilator responses of small coronary arteries (~100 μm) to ACh were significantly attenuated by L-NMMA (in the presence of ibuprofen) and were further attenuated by catalase (Figure 1, left) or TEA. By contrast, the vasodilator responses of arterioles (~100 μm) were relatively resistant to L-NMMA (in the presence of ibuprofen) but were markedly attenuated by catalase (Figure 1, right) or TEA. We confirmed that the inhibitory effects of catalase were comparable to those of TEA (data not shown).

Hemodynamics and Blood Gases During Decreasing Coronary Perfusion Pressure

In each experimental condition, mean aortic pressure at baseline was constant and comparable (control, 72±7 mm Hg; L-NMMA, 74±8 mm Hg; and L-NMMA plus catalase, 59±6 mm Hg). Coronary arterial pressure was significantly attenuated by L-NMMA (10±1 mm Hg) and catalase (5±1 mm Hg) in the control condition. Coronary arterial pressures in the presence of ibuprofen were not significantly different from those of the control condition (Figure 2). Coronary arterial pressures were significantly lower in the absence of ibuprofen in the presence of L-NMMA compared with those of control and L-NMMA in the presence of ibuprofen, consistent with the release of EDHF. Coronary arterial pressures were lower in the presence of L-NMMA compared with those of control, L-NMMA, and L-NMMA plus catalase. The decrease in coronary arterial pressures in the presence of catalase was not statistically significant compared with those of control, L-NMMA, and L-NMMA plus ibuprofen.
followed by catalase, 72±6 mm Hg). P O 2, P CO 2, and pH were maintained within the physiological range (pH, 7.35 to 7.45; P CO 2, 25 to 40 mm Hg; P O 2, >70 mm Hg) throughout the experiments. Transmural coronary blood flow at 70 mm Hg of perfusion pressure was comparable among the 3 conditions (Figure 2). However, the flow was significantly reduced to a comparable extent at 50 and 30 mm Hg of perfusion pressure in all 3 conditions (Figure 2).

**Role of EDHF in Coronary Autoregulation**

Under control conditions, coronary autoregulatory vasodilator responses to decreasing perfusion pressure were noted in arterioles but not in small arteries (Figure 3, left). In coronary arterioles, the autoregulatory vasodilator responses were significantly attenuated by L-NMMA (in presence of ibuprofen) and were further attenuated by catalase (Figure 3, right). Compared with the responses after L-NMMA alone (Figure 4A), L-NMMA followed by TEA (Figure 4B) or catalase (Figure 4C) caused a comparable extent of inhibition on the responses of arterioles. The arteriolar responses were also comparable after L-NMMA followed by catalase (Figure 4C) and after catalase followed by L-NMMA (Figure 4D) irrespective of the order of drug administration. The arteriolar responses at 30 mm Hg of coronary perfusion pressure were significantly reduced after L-NMMA, catalase, or TEA alone and were further decreased after L-NMMA followed by catalase, catalase followed by L-NMMA, or TEA followed by L-NMMA with decreased myocardial lactate extraction (Figure 5 and the Table).

**Compensatory Effects of Adenosine in Coronary Autoregulation**

Coronary venous adenosine concentrations were increased in response to decreasing coronary perfusion pressure after L-NMMA alone (Figure 6A), L-NMMA followed by catalase (Figure 6A), catalase followed by L-NMMA (Figure 6B), and TEA followed by L-NMMA (Figure 6C) but were not further increased after catalase or TEA alone (Figure 6, B and C). The blockade of adenosine receptor with 8-SPT suppressed the residual EDHF-mediated arteriolar dilation after L-NMMA followed by catalase at 30 mm Hg of coronary perfusion pressure with decreased myocardial lactate extraction (Figure 7A, Table). Coronary venous adenosine concentrations were increased in response to decreasing perfusion pressure after L-NMMA but were not further increased after L-NMMA followed by catalase (Figure 7B or TEA. This was also the case with L-NMMA followed by TEA and 8-SPT (data not shown).

**Discussion**

The major findings of the present study are that (1) H 2 O 2 is a primary EDHF in the canine coronary circulation in vivo and (2) H 2 O 2 plays an important role in coronary autoregulation as a compensatory mechanism for NO and adenosine. To the best of our knowledge, this is the first report that demonstrates the importance of H 2 O 2 as an endogenous EDHF that plays an important role in coronary autoregulation in vivo.
Critique of Experimental Model and Methodology

On the basis of the previous reports, we chose adequate doses of L-NMMA, catalase, TEA, and 8-SPT to inhibit NO, H₂O₂, KᵥCa, and adenosine, respectively. The methodological validity of the present study was confirmed previously. 16

H₂O₂ as an Endogenous EDHF in the Coronary Circulation In Vivo

Recently, Matoba et al 12,13 showed that H₂O₂ is a primary EDHF in canine coronary microvessels in a cooperative manner. In the present study, the arteriolar vasodilation during coronary autoregulation was not completely inhibited by L-NMMA plus ibuprofen or by catalase, and the residual arteriolar dilation was further inhibited after administration of all 3 inhibitors. These results indicate the negative feedback interaction between NO and EDHF during coronary autoregulation in vivo. The compensatory effect of adenosine may also be important. Stepp et al 1 suggested that adenosine plays an important role in the transition to ischemia at a coronary perfusion pressure of 50 mm Hg after KᵥATP channel blockade with glibenclamide. In the present study, coronary venous adenosine concentrations were increased at a perfusion pressure of <50 mm Hg within the vasoactive range for endogenous adenosine, as previously reported. 29

Table 1. Myocardial Lactate Extraction Rate (%) at 30 mm Hg of Coronary Perfusion Pressure

<table>
<thead>
<tr>
<th>Protocol</th>
<th>n</th>
<th>Control Response</th>
<th>First Inhibitor</th>
<th>Second Inhibitor</th>
<th>Third Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11</td>
<td>30 ± 4</td>
<td>L-NMMA 23 ± 4</td>
<td>L-NMMA + TEA 14 ± 4</td>
<td>...</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>36 ± 1</td>
<td>TEA 30 ± 1</td>
<td>TEA + L-NMMA 20 ± 4</td>
<td>...</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>33 ± 5</td>
<td>L-NMMA 21 ± 5</td>
<td>L-NMMA + catalase 12 ± 3</td>
<td>...</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>34 ± 1</td>
<td>Catalase 22 ± 1</td>
<td>Catalase + L-NMMA 15 ± 1</td>
<td>...</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>33 ± 1</td>
<td>L-NMMA 23 ± 1</td>
<td>L-NMMA + TEA 13 ± 1</td>
<td>L-NMMA + TEA + 8SPT 2 ± 7</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>31 ± 2</td>
<td>L-NMMA 17 ± 1</td>
<td>L-NMMA + catalase 12 ± 1</td>
<td>L-NMMA + catalase + 8SPT 2 ± 2</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM. 
*P < 0.05 vs control; †P < 0.05 vs L-NMMA; ‡P < 0.05 vs TEA; §P < 0.05 vs catalase; ||P < 0.05 vs L-NMMA plus TEA; ¶P < 0.05 vs L-NMMA plus catalase.
The residual arteriolar dilation (~5%) after combined administration of ibuprofen, L-NMMA, and TEA or catalase was completely blocked by 8-SPT, indicating that an increased concentration of adenosine compensated for the loss of action of NO and EDHF. It remains to be examined why the adenosine concentrations did not increase further after administration of L-NMMA followed by TEA compared with L-NMMA alone. Cabell et al. reported that TEA inhibited adenosine-induced vasodilatation of canine subepicardial coronary arteries in vitro. Furthermore, Gao and Vanhoutte reported that H2O2 relaxed canine bronchial smooth muscle and elevated cAMP concentrations. Chaytor et al. reported that EDHF-mediated relaxation to ACh was associated with an increase in smooth muscle cAMP concentrations. These findings suggest that a cAMP-mediated pathway is involved, at least in part, in the autoregulatory coronary vasodilatation through large-conductance K_{Ca} channels.

Role of EDHF in Myocardial Ischemia During Coronary Autoregulation

K_{Ca} channels contribute substantially to the coronary vasodilation in myocardial ischemia. However, it remains to be examined whether EDHF contributes to autoregulatory coronary vasodilation. The present results demonstrate that EDHF/H2O2 contributes substantially to the vasodilatation during coronary autoregulation in vivo. Regarding the mechanism involved in the K_{Ca} channel opening during coronary autoregulation, cellular acidosis and increase in intracellular Ca^{2+} concentration after ischemia have been postulated to open K_{Ca} channels as a compensatory mechanism after inhibition of NO synthesis. We have previously demonstrated that subendocardial arteriolar dilation during reactive hyperemia is more sensitive to L-NMMA than subepicardial arteriolar dilation. These findings indicate that the perfusion of the subendocardium is more dependent on NO than that of the subepicardium. EDHF/H2O2 may compensate coronary blood flow, especially in the endocardium, in myocardial ischemia during coronary autoregulation.

Clinical Implications and Conclusions

The synthesis and action of endothelium-derived NO are impaired under various pathological conditions, such as hypertension and hyperlipidemia. Previous studies suggested that hypertension causes a compensatory increase in the activity of potassium channels. Cosentino et al. reported that in a transgenic mouse model of hyperphenylalaninemia, reduction in arterial tetrahydrobiopterin, a cofactor of endothelial NO synthase, was accompanied by a decrease in endothelial NO production, whereas H2O2 production was increased. In the present study, NO and EDHF/H2O2 apparently compensated each other to cause coronary autoregulatory vasodilatation in vivo. These results may represent a negative feedback interaction between the 2 relaxing factors.

In conclusion, we were able to demonstrate that H2O2 is a primary EDHF in the canine coronary circulation in vivo and plays an important role in coronary autoregulation as a compensatory feedback mechanism for NO and adenosine. These findings may have important clinical implications, because hyperpolarizing mechanisms contribute substantially to endothelium-dependent vasodilatation in myocardial ischemia.

Acknowledgments

This work was supported in part by grants 12558114 and 14657178 from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, Tokyo, Japan.
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_Circulation_. 2003;107:1040-1045; originally published online February 3, 2003; doi: 10.1161/01.CIR.0000050145.25589.65

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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http://circ.ahajournals.org/content/107/7/1040

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