Superoxide Dismutase Restores Contractile and Metabolic Dysfunction Through Augmentation of Adenosine Release in Coronary Microembolization

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Background. This study was undertaken to test the hypothesis that administration of superoxide dismutase (SOD) restores the contractile and metabolic dysfunction in coronary microembolization and that these beneficial effects of SOD are attributable to the restoration of 5'-nucleotidase activity and subsequent augmentation of adenosine release.

Methods and Results. In 78 dogs before and after an injection of microspheres (15 μm in diameter) into the left anterior descending coronary artery, regional coronary blood flow (CBF), fractional shortening (FS), and lactate extraction ratio (LER) were measured with and without administration of recombinant human SOD (50 μg/kg/min i.c.). In the untreated dogs (n=6), both FS and LER decreased after coronary microembolization (2.0×10^5 microspheres per ml CBF [ml/min]). FS and LER decreased from 24.2±1.3% to 5.1±1.2% and from 23.0±1.1% to −10.5±2.9%, respectively. These ischemic changes were associated with coronary hyperemic flow (141±8 versus 92±1 ml/100 g/min) and adenosine release (5.8±0.5 versus 0.4±0.1 nmol/100 g/min). Pretreatment with SOD augmented the hyperemic flow to 164±4 ml/100 g/min and enhanced the release of adenosine (9.6±0.6 nmol/100 g/min) associated with improvement of functional and metabolic dysfunction (FS, 14.8±2.3%; LER, 15.1±3.1%). Administration of SOD at 10 minutes (n=5) and 30 minutes (n=5) after coronary embolization restored the contractile function and lactate metabolism (at 10 minutes: FS, 16.7±2.2% and LER, 16.7±3.9%; at 30 minutes: FS, 11.1±1.3% and LER, 7.2±3.1%). However, administration of SOD 60 minutes after coronary embolization (n=6) did not restore the contractile and metabolic dysfunction. The restoration of the contractile and metabolic dysfunction by SOD treatment was blunted by adenosine receptor blockade with 8-phenylthioprylline (n=5). Myocardial 5'-nucleotidase activity at 2 hours after embolization was restored with SOD treatment at 10 minutes (n=5) and 30 minutes (n=5) after embolization. However, SOD treatment at 60 minutes after embolization (n=6) did not restore 5'-nucleotidase activity compared with the SOD pretreatment group. Furthermore, coronary submaximal vasodilation induced by papaverine (n=5) and adenosine (n=5) abolished the beneficial effects of SOD.

Conclusions. We conclude that 1) in sustained myocardial ischemia, SOD treatment attenuates ischemic injury caused by coronary microembolization by restoration of 5'-nucleotidase activity and augmentation of adenosine release; 2) this beneficial effect of SOD is observed even after coronary microembolization; and 3) the beneficial effects of SOD are attributable to coronary vasodilation produced by augmented adenosine release. (Circulation 1993;87:982–995)

Key Words: ischemia • microembolization • free radicals • superoxide dismutase • adenosine • vasodilation • 5'-nucleotidase

Reperfusion after prolonged coronary occlusion restores contractile and metabolic dysfunction of the ischemic myocardium.1 However, the complete restoration is substantially limited by blood cell plugging of small coronary vessels, cellular membrane damage by free radicals, and calcium overload of myocardium.2-7 Aggregated platelets and activated leukocytes during ischemia and reperfusion are also thought to plug small coronary arteries, which may initiate and sustain myocardial ischemia.8,9 In small ischemic lesions surrounded by the hyperemic area,10,11 persistent oxygen supply to the ischemic lesions may continuously generate oxygen-derived free radicals.12 Indeed, several lines of evidence show that oxygen-derived free radicals generated during ischemia and reperfusion injure the myocardium and that administration of superoxide dismutase (SOD) attenuates the myocardial injury.13,14 A recent study in our laboratory and Culture of Japan and by a grant from the Uehara Memorial Foundation, Japan.

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demonstrated that myocardial injury induced by coronary microembolization is markedly attenuated by SOD, suggesting that coronary microembolization results in the sustained production of oxygen-derived free radicals. Since oxygen-derived free radicals are reported to cause an increase in vascular permeability, cellular swelling, and enzyme inactivation, sustained free radical generation may accelerate the contractile and metabolic dysfunction in coronary microembolization. Our study also reveals that enhanced release of adenosine caused by SOD treatment in coronary microembolization causes coronary hyperemic flow and improves myocardial metabolic and contractile function. Adenosine in the ischemic myocardium is thought to be formed mainly by dephosphorylation of 5'-AMP by 5'-nucleotidase. There are two types of 5'-nucleotidase in the myocardium: ecto- and cytosolic 5'-nucleotidase. Both enzymes are reported to be responsible for adenosine production. Because the active site of the ecto-5'-nucleotidase faces the extracellular space, this enzyme is susceptible to extracellular free radicals. This evidence may lead us to support the view that SOD administration may preserve ecto-5'-nucleotidase activity and increase adenosine production in coronary microembolization.

To test the hypothesis that SOD restores contractile and metabolic dysfunction after the onset of coronary microembolization, we administered SOD before and at 10, 30, 60, and 120 minutes after embolization of microspheres. We also determined whether the beneficial effects of SOD are attributable to coronary vasodilation induced by enhanced adenosine release. The effects of SOD on the activities of ecto- and cytosolic 5'-nucleotidase were also assessed to determine whether SOD preserves the enzyme activity induced in coronary microembolization.

Methods

Instrumentation

Seventy-eight mongrel dogs weighing 12–23 kg were anesthetized with sodium pentobarbital (30 mg/kg i.v.). The trachea was intubated, and the animal was ventilated with room air mixed with 100% oxygen (1–3 L/min). A left thoracotomy exposed the heart, which was supported in a pericardial cradle. The left anterior descending coronary artery (LAD) was cannulated and perfused with blood from the carotid artery through an extraanatomic bypass. Coronary perfusion pressure (CPP) was determined through the bypass tube proximal to the cannula (15G), and coronary blood flow (CBF) of the perfused area was measured with an electromagnetic flow probe (Nihon Kohden, FF-050T, Tokyo) at the bypass tube. Coronary venous blood was sampled from a collecting tube (1-mm diameter, 7-cm length) inserted into a small coronary vein near the center of the perfused area. The venous blood was collected in a reservoir placed at the level of the right atrium and returned to the jugular vein. A miniature pressure transducer (Konigsberg, P-5, Pasadena, Calif.) was inserted into the left ventricular (LV) cavity through the ventricular apex. In 48 dogs, a pair of ultrasonic crystals (Schuessler, 5 MHz, Cardiff by the Sea, Calif.) was implanted into the endocardial third of the perfusion area to measure myocardial segment length. LV pressure (LVP) and its first derivative (dP/dt) were measured. End-diastolic length (EDL) was determined at the R wave of the ECG, and end-systolic length (ESL) was determined at the minimum dP/dt. Fractional shortening (FS=EDL−ESL/EDL) was measured as an index of regional myocardial contractility in the perfused area. Heart rate averaged 142±2 min−1 and did not change during each study.

The animals used in this study were maintained in accordance with the guidelines of the American Physiological Society. Only animals that were lawfully acquired were used in this study; their retention and use complied with the regulations of our university and were in accordance with the NIH Guide (DHEW Publication No. [NIH] 80-12, revised 1978, reprinted 1980). Animals in the laboratory received every consideration for their comfort. Appropriate anesthetics were used, and muscle relaxants or paralytics were not used.

Experimental Protocols

Protocol 1: The effects of SOD administered before and at 10, 30, 60, and 120 minutes after a single injection of 15-μm microspheres on CBF, lactate production, and FS. Twenty-eight dogs were used in this protocol. After hemodynamic stabilization, LVP, CPP, CBF, and myocardial segment length were measured in each dog. The blood in the coronary artery and vein were sampled for measurements of lactate and adenosine concentrations and oxygen content. After measurement of hemodynamic parameters and blood sampling, 15-μm microspheres (2.0×103/1 mL of baseline CBF [mL/min]; 15±1 μm in diameter; 3M Co.) were injected into the LAD through the bypass tube. Microspheres suspended in 1 mL of 10% dextran containing 2 μL Tween 80 were agitated for at least 15 minutes in an ultrasonicator and stirred with a vortex mixer for at least 1 minute before the injection. In six dogs, human recombinant SOD (Ube Industries, Tokyo) was injected into the bypass tube (50 μg/kg/min) 10 minutes before microembolization and then was infused continuously for 130 minutes. Hemodynamic and metabolic parameters were measured before and at 10, 20, 30, 40, 50, 60, and 120 minutes after the injection of microspheres. In the other 16 dogs, SOD was infused into the bypass tube at 10 (n=5), 30 (n=5), or 60 minutes (n=6) after coronary microembolization. Again, hemodynamic and metabolic parameters were measured before and at 10, 20, 30, 40, 50, 60, and 120 minutes after the injection of microspheres. As control (n=6), a single injection of microspheres was done without SOD treatment.

Protocol 2: Effects of 8-phenyltheophylline on CBF, lactate production, and FS when SOD infusion is initiated 30 minutes after injection of 15-μm microspheres. We have previously reported that administration of SOD enhances both CBF and adenosine release during coronary microembolization. To determine whether the beneficial effect of SOD administered after coronary microembolization is due to its ability to enhance the release of adenosine and coronary hyperemic flow, SOD infusion (50 μg/kg/min i.c.) was initiated 30 minutes after microsphere embolization and was continued for 90 minutes while 8-phenyltheophylline was administered in five dogs. An intracoronary infusion of 8-phenyltheophylline (30 μg/kg/min i.c.) was initiated 10 minutes before microembolization and was continued for
130 minutes. Before and 10, 20, 30, 40, 50, 60, and 120 minutes after coronary microembolization, hemodynamic and metabolic parameters were measured.

Protocol 3: Effects of SOD treatment on 5'-nucleotidase activity 2 hours after a single injection of microspheres. To elucidate the mechanism by which SOD treatment augments adenosine release, we tested the hypothesis that the administration of SOD restores the activity of 5'-nucleotidase after coronary microembolization. The subendocardial tissues were sampled from the LAD and left circumflex coronary artery (LCx) areas 2 hours after a single injection of microspheres (2.0×10⁸/1 mL of CBF [mL/min]). SOD (50 μg/kg/min) was administered 10 minutes before (n=6) and at 10 (n=6), 30 (n=6), and 60 minutes (n=6) after microembolization. As control (n=6), a single injection of microspheres was done without SOD administration. Myocardial tissue (1.0−1.5 g) from the endocardial layer was quickly sampled and stored in liquid nitrogen.

Protocol 4: Effects of coronary vasodilation during SOD administration on lactate production and FS after a single injection of 15-μm microspheres. If the beneficial effects of SOD on myocardial contractile and metabolic dysfunction are attributable to coronary vasodilation caused by enhancement of adenosine release, the beneficial effects of SOD may be blunted when the coronary arteries are submaximally dilated. To test this idea, papaverine (20 μg/kg i.c., n=5) and adenosine (4 μg/kg/min i.c., n=5) were administered 10 minutes before coronary microembolization (2.0×10⁸/1 mL of baseline CBF) and then were infused continuously for 130 minutes. Furthermore, to test the effect of SOD under papaverine administration on the myocardial ischemia of comparable severity with protocol 1, the identical protocol was also performed before and after coronary embolization of a large dose of microspheres (3.0×10⁸/1 mL of baseline CBF) in five dogs. In protocols 1−4, the perfusion area was determined by injecting Evans blue dye into the bypass tube. The mean weight of the perfused tissue in protocols 1−4 were 36±3 g, 32±3 g, 36±4 g, and 38±4 g, respectively.

Chemical Analysis

Lactate concentration was assessed enzymatically, and lactate extraction ratio (LER) was obtained by determining the coronary arteriovenous difference in lactate concentration times 100 and divided by the arterial lactate concentration. Coronary arterial venous oxygen difference (ΔVO₂D) was calculated as the difference between coronary arterial and venous oxygen contents and MV₀₂ (mL/100 g/min) was calculated as the CBF (mL/100 g/min)×ΔVO₂D (mL/DL).

The method of adenosine measurement has been reported previously.⁹,°²²−²⁴ Briefly, 1 mL of blood was drawn into a syringe containing 0.5 mL dipyridamole (0.02%) and 100 μL of 2'-deoxycofroycin (0.1 mg/mL) with EDTA (500 mM) to block both the uptake of adenosine by red blood cells and the degradation of adenosine. After centrifugation, the supernatant was determined by radioimmunoassay. The adenosine in the plasma (100 μL) was succinylated with 100 μL of dioxane containing succinic acid anhydride and trimethylamine. After a 20-minute incubation, the mixture was diluted with 100 μL of dioxane 2',3'-O-dissucinyl-3-[¹⁸¹I]-iodotyrosine methyl ether (0.5 pmol) and 100 μL of diluted antiadenosine serum. The mixture was kept in a cold-water (0°C) bath for 18 hours, and a second antibody solution (goat anti-rabbit IgG serum, 500 μL) was added. After incubation at 4°C for 1 hour, unreacted materials were removed by centrifugation at 3,000 rpm at 4°C for 20 minutes. The radioactivity remaining in the tube was counted by a gamma counter. The amount of adenosine degraded during this blood sampling procedure is known to be negligible.¹⁰,²⁷

Measurement of 5'-Nucleotidase Activity

We measured the ecto (membrane bound) and endo (cytosolic)-5'-nucleotidase activities separately. The myocardial tissue was homogenized for 5 minutes in 10 volumes of ice-cold 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid potassium hydroxide (HEPES-KOH) buffer (pH 7.4) containing 0.25 M sucrose, 1 mM MgCl₂, and 1 mM mercaptoethanol. The crude homogenate was strained through a double-layered nylon sieve and was homogenized further for 1 minute. To prepare a crude membrane fraction, part of the homogenate was centrifuged at 1,000g for 10 minutes. The resultant pellet was washed three times and finally resuspended in the HEPES-KOH buffer. To prepare the cytosolic fraction, the remaining part of the homogenate was first centrifuged at 3,000g for 10 minutes, and the supernatant was centrifuged again at 200,000g for 1 hour. The membrane and cytosolic fractions were dialyzed at 4°C for 4 hours against 10 mM HEPES-KOH (pH 7.4) containing 1 mM MgCl₂, 1 mM mercaptoethanol, and 0.01% activated charcoal and then was divided into aliquots that were frozen immediately and stored at −80°C.

The activity of 5'-nucleotidase was assessed by an enzymatic assay technique²⁸ and was described as units of mol/g wet wt/min.

Statistical Analysis

Paired t tests were performed to test the differences for the data in Table 2.²⁹ ANOVA followed by Bonferroni's multiple comparison test was used for comparisons at each time point of the hemodynamic and metabolic parameters in "Results," all figures, and Tables 1 and 3.²⁹ All values are expressed as mean±SEM, and a value of p<0.05 was considered significant.

Results

Effects of SOD on Myocardial Contractile and Metabolic Dysfunction in Coronary Microembolization (Protocol 1)

Heart rate, mean atrial pressure, and LV dP/dt were comparable in the untreated and SOD−, 8-phenylthio-1,2-diphenylhydrazine−, adenosine−, and papaverine-treated groups and were unchanged throughout protocols (Table 1). An intracoronary infusion of these chemicals had no significant effect on these hemodynamic parameters.

Figure 1 shows CPP (panel A) and CBF (panel B) before and after the injection of microspheres. Coronary perfusion pressure was not changed in SOD treatment groups. In the untreated group, CBF increased from 91±2 to 141±8 mL/100 g/min, and a high level of CBF was maintained for 120 minutes after microsphere injection; SOD treatment significantly increased the hyperemic flow (176±16 mL/100 g/min at 120 minutes of microembolization, p<0.001 versus the untreated
**Table 1.** Systemic Hemodynamic Parameters Before and During Coronary Microembolization

<table>
<thead>
<tr>
<th>Heart rate (bpm)</th>
<th>n</th>
<th>Baseline</th>
<th>Pharmacological intervention</th>
<th>Time after onset of microembolization (min)</th>
<th>10</th>
<th>30</th>
<th>60</th>
<th>120</th>
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<tr>
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<td>...</td>
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<tr>
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<td>144±11</td>
<td>143±7</td>
<td>140±5</td>
<td>139±7</td>
<td>138±12</td>
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</tr>
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<td>145±8</td>
<td>...</td>
<td>139±10</td>
<td>146±5</td>
<td>144±7</td>
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<td>130±9</td>
<td>149±8</td>
<td>144±9</td>
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<td>148±3</td>
<td>143±7</td>
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<td>144±11</td>
<td>147±9</td>
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<td>151±12</td>
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<tr>
<td>Peak positive LV dp/dt (mm Hg/sec)</td>
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<td>...</td>
<td>2,199±290</td>
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<tr>
<td>SOD 10 minutes</td>
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<td>...</td>
<td>2,200±198</td>
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<td>...</td>
<td>2,239±231</td>
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<td>2,200±312</td>
<td>2,019±201</td>
<td>2,011±223</td>
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<td>2,069±301</td>
<td>2,342±218</td>
<td>2,215±311</td>
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</table>

Values are mean±SEM. bpm, Beats per minute; SOD, superoxide dismutase; LV, left ventricular.

group by ANOVA), whereas it did not change CBF and any metabolic parameters at the baseline condition (Table 2). When SOD was administered 10 minutes after microsphere injection, CBF began to increase 10–20 minutes later and remained high (177±27 mL/100 g/min at 120 minutes of microembolization, p<0.05 versus the untreated group by ANOVA). When SOD was administered 30 minutes after microembolization, CBF increased gradually to 166±9 mL/100 g/min at 120 minutes of microembolization (p<0.001 versus the untreated group by ANOVA). However, when SOD was administered 60 minutes after microembolization, it did not further increase CBF. These results indicate that SOD further increases CBF even after the onset of ischemia induced by coronary embolization. Figure 2 shows the changes in FS after coronary microembolization. An injection of microspheres decreased FS from 24.2±1.3% to 5.1±1.2%, and this reduction in FS was sustained for 120 minutes (5.5±2.7%). In contrast, when SOD was administered continuously before embolization, the decreases in FS after coronary microembolization were smaller than the untreated group (14.8±2.3% at 120 minutes of microembolization, p<0.001 versus the untreated group by ANOVA). When SOD treatment was initiated 10 minutes after microembolization, the decreased FS was restored to

**Table 2.** Coronary Hemodynamic and Metabolic Parameters Before and After Administration of SOD Before Coronary Microembolization

<table>
<thead>
<tr>
<th>CPP (mm Hg)</th>
<th>CBF (mL/100 g/min)</th>
<th>MVO₂ (mL/100 g/min)</th>
<th>pH (a)</th>
<th>pH (v)</th>
<th>Ado (a) (pmol/mL)</th>
<th>Ado (v) (pmol/mL)</th>
<th>AdR (nmol/100 g/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>106±5</td>
<td>92±1</td>
<td>7.35±0.52</td>
<td>7.41±0.01</td>
<td>7.38±0.02</td>
<td>10.0±1.6</td>
<td>13.9±1.3</td>
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<tr>
<td>SOD 105±5</td>
<td>91±1</td>
<td>7.56±0.48</td>
<td>7.39±0.02</td>
<td>7.39±0.01</td>
<td>11.0±2.3</td>
<td>14.4±2.1</td>
<td>0.31±0.05</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Statistical analysis was performed by paired t test. CPP, coronary perfusion pressure; CBF, coronary blood flow; MVO₂, myocardial oxygen consumption; pH (a) and pH (v), pH in coronary arterial and venous blood; Ado (a) and Ado (v), adenosine concentrations in coronary arterial and venous blood; AdR, adenosine release; SOD, administration of superoxide dismutase.
the SOD pretreatment level at 120 minutes of microembolization (p<0.005 versus the untreated group by ANOVA). When SOD treatment was initiated 30 minutes after microembolization, the decreased FS was partially restored. SOD treatment at 60 minutes after the injection of microspheres did not restore the decrease in FS. These differences in the degree of restoration of FS were related to the increases in CBF (Figure 1), suggesting that the beneficial effect of SOD on contractile dysfunction is attributable to the augmentation of increases in CBF. The administration of SOD also improved the metabolic dysfunction caused by coronary microembolization. Figure 3 shows the changes in MVO_2 (panel A) and LER (panel B) caused by SOD treatment after coronary microembolization. SOD treatments before and after microembolization restored the decreases in LER. The improvement of anaerobic myocardial metabolism, as gauged by LER, was comparable with the recovery of the FS. MVO_2 did not change significantly after the treatment with SOD. Since we have previously reported that coronary hyperemia induced by microembolization is mainly due to

Table 3. 5'-Nucleotidase Activities 2 Hours After a Single Injection of Microspheres With and Without SOD Treatment

<table>
<thead>
<tr>
<th>Enzyme Activity (nmol/g wet wt/min)</th>
<th>Pretreatment</th>
<th>10</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecto enzyme activity</td>
<td>9.1±0.3††</td>
<td>15.5±0.6*</td>
<td>12.7±0.4††</td>
<td>9.1±0.7††</td>
</tr>
<tr>
<td>LCx</td>
<td>6.5±0.4</td>
<td>6.6±0.5</td>
<td>7.1±0.5</td>
<td>6.9±0.8</td>
</tr>
<tr>
<td>Cytosolic enzyme activity</td>
<td>93.2±3.5</td>
<td>99.6±7.1</td>
<td>97.1±4.2</td>
<td>89.9±5.7</td>
</tr>
<tr>
<td>LCx</td>
<td>6.6±0.4</td>
<td>6.6±0.5</td>
<td>7.1±0.5</td>
<td>6.9±0.8</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Six dogs in each group were used. LAD and LCx, subendocardial myocardium in the left anterior descending and circumflex coronary arterial areas; SOD, superoxide dismutase.

*p<0.01 vs. no treatment; †p<0.05 vs. pretreatment; ‡p<0.05 vs. 10 minutes after microembolization by ANOVA followed by Bonferroni's multiple comparison test.

Figure 1. Plots show coronary perfusion pressure (panel A) and coronary blood flow (panel B) before and after coronary microembolization. C1 and C2 indicate control conditions, i.e., 10 minutes and immediately before embolization, respectively. Pretreatment of superoxide dismutase (SOD) and treatments 10 and 30 minutes after embolization augmented the hyperemic flow response, whereas SOD administration 60 minutes after embolization failed to increase the coronary blood flow. Values are mean±SEM. *p<0.05, **p<0.01, +p<0.005, ++p<0.001 vs. the no treatment group by ANOVA followed by the Bonferroni test.
endogenous adenosine in ischemic myocardium, we also measured adenosine release. As was expected, pretreatment with SOD further increased \((p<0.005\) versus the untreated group by ANOVA) adenosine release in the embolized myocardium at 120 minutes of microembolization (Figure 4). When SOD was administered at 10 or 30 minutes after the onset of coronary microembolization, adenosine release was further increased. However, at 60 minutes after microembolization, SOD did not enhance adenosine release. These results suggest that the increased CBF is mediated through the enhancement of adenosine release.

**Effects of 8-Phenyltheophylline on CBF, Lactate Production, and FS When SOD Infusion is Initiated 30 minutes After an Injection of 15-µm Microspheres (Protocol 2)**

If there is a cause–effect relation between the functional recovery and enhanced adenosine release after SOD treatment, the beneficial effects of SOD administered before and after the injection of microspheres should be blunted by treatment with an adenosine antagonist, 8-phenyltheophylline. Treatment with 8-phenyltheophylline alone changed neither CBF (Figure 5A) nor FS \((22.5\pm0.0\%\) versus \(22.4\pm2.1\%\) in the control, Figure 5B). During treatment with 8-phenyltheophylline, an injection of microspheres did not increase CBF and markedly reduced FS. When SOD was administered 30 minutes after microsphere injection, neither CBF nor FS was increased, indicating that the beneficial effects of SOD in coronary microembolization are mediated through the enhancement of adenosine release.

**Effects of SOD Treatment on 5′-Nucleotidase Activity 2 Hours After a Single Injection of Microspheres (Protocol 3)**

To determine cellular mechanisms by which SOD enhances the release of adenosine, 5′-nucleotidase activity of the myocardium was measured at 2 hours after the injection of microspheres with and without SOD treatment (Table 3). With SOD pretreatment, ecto-5′-nucleotidase activity was augmented \((p<0.001\) versus the untreated group by ANOVA). SOD administration initiated 30 minutes after the onset of coronary microembolization also increased ecto-5′-nucleotidase activity \((p<0.01\) versus the untreated group by ANOVA) but did not reach the levels in SOD pretreatment group. However, when SOD was administered 60 minutes after the onset of coronary microembolization, ecto-5′-nucleotidase activity was not augmented.

**Effect of Coronary Vasodilation During SOD Administration on Lactate Production and FS After a Single Injection of 15-µm Microspheres (Protocol 4)**

If the beneficial effects of SOD are attributed to increased CBF, these effects may be blunted when the coronary arteries are submaximally dilated with vasodilators. To test this idea, we examined the effects of SOD
on coronary microembolization during infusions of papaverine and adenosine. Figure 6 shows the changes in CBF that occurred with adenosine and papaverine treatments in coronary microembolization. When papaverine and adenosine were administered into the LAD, CBF increased to 257±12 and 238±4 mL/100 g/min, respectively. After the injection of microspheres, CBF decreased and remained at low levels. In contrast to the increase in CBF after SOD treatment (30 minutes after microembolization) in protocol 1, treatments with these vasodilators abolished the ability of SOD to increase CBF after microembolization. During coronary vasodilation with papaverine and adenosine, SOD affected neither the functional nor the metabolic deterioration of the myocardium (Figures 7 and 8). However, when coronary vessels are submaximally dilated by papaverine and adenosine, FS and LER decreased less than those in protocol 1 without vasodilators although the same numbers of microspheres were embolized (2.0×10⁵ spheres/1 mL of CBF [mL/min]). Because we observed that the 15-μm-diameter microspheres do not pass through the small coronary arteries,¹⁰ the improvement of myocardial perfusion caused by papaverine and adenosine may attenuate the extent of decreases in FS and LER in 2.0×10⁵ spheres embolization. Therefore, we embolized a larger dose of microspheres (3.0×10⁵/1 mL of CBF [mL/min]) to decrease both FS and LER to the comparable extents observed in protocol 1 (Figures 7 and 8). In this condition, the administration of SOD did not restore either contractile or metabolic dysfunction. These results indicate that the beneficial effect of SOD is attributable to coronary vasodilation caused by enhanced adenosine release.

**Discussion**

The present study demonstrates that 1) administration of SOD restores the contractile and metabolic dysfunction in coronary microembolization; 2) the beneficial effects of SOD are mediated through enhanced release of adenosine, which augments coronary vasodilation; and 3) the enhancement of adenosine release by SOD is attributable to restoration of 5’-nucleotidase activity.

**Cardiovascular Actions of SOD in Coronary Microembolization**

We have previously reported the pathophysiology of acute coronary embolization and found that endoge-
nous adenosine plays beneficial roles in myocardial cellular injury in coronary microembolization. In this study, we showed that in embolization with 15-μm microspheres, maximal CBF (167±17 from 97±4 mL/100 g/min) was obtained in 37±5% of the total embolization (5.0±0.5x10^5/g myocardium). In the more extensive embolization, resting coronary flow decreased almost linearly toward zero. Therefore, we embolized 2.0x10^5/g microspheres that may cause maximal hyperemic flow in the present study. We also revealed that this size of microsphere causes a 100–200-μm ischemic area but does not cause myocardial necrosis. In these circumstances, the coronary hyperemic flow response in the intact surrounding area is caused by endogenous adenosine released from the ischemic myocardium, suggesting that oxygen may be continuously supplied to the ischemic foci, and oxygen-derived free radicals may be continuously generated. Oxygen-derived free radicals are also derived from activated neutrophils through NADPH oxidase and through arachidonic acid metabolism and a variety of cytotoxic products, e.g., neutral protease and leukotrienes. These factors may contribute to continuous generation of oxygen-derived free radicals in coronary microembolization.

We need to consider the multiple cardiovascular effects of oxygen-derived free radicals in coronary microembolization. First of all, oxygen-derived free radicals are reported to accelerate the formation of myocardial edema which may worsen the severity of perfusion deficits and aggravate myocardial ischemia. If oxygen-derived free radicals injure the myocardium directly, adenosine release from the ischemic myocardium may be increased. Thus, SOD may decrease adenosine release by attenuation of ischemic changes, since adenosine is released in response to the severity of the ischemic injury. In the present study, however, adenosine release was enhanced by SOD (Figure 4). This observation suggests that the beneficial effect of SOD is not attributed to the attenuation of myocardial edema and injury caused by oxygen-derived free radicals in coronary microembolization. Oxygen-derived free radicals may also cause coronary endothelial edema. SOD may attenuate the endothelial edema and improve myocardial perfusion in coronary microembolization, resulting in an increase of the washout rate of adenosine. This hypothesis is consonant with the results of our present study. However, if this is the case, SOD must be effective even under the condition of 8-phenyltheophylline treatment, which does not interfere with endothelial cellular function. In the present study, the beneficial effects of SOD were abolished by 8-phenyltheophylline (Figure 5), indicating that attenuation of endothelial edema does not play a major role in the beneficial effects of SOD. It is also reported that SOD inactivates the degradation of endothelium-derived relaxing factor (EDRF), which may enhance coronary vasodilation during ischemia and reperfusion. SOD-induced attenuation of the inactivation of EDRF may
not be directly related to our observation because 8-phenyltheophylline abolished the beneficial effects of SOD (Figure 5). However, because adenosine may increase EDRF release, we cannot deny the interaction between SOD and EDRF through the effects of adenosine in endothelial cells. EDRF derived from stimulation of adenosine receptors in SOD treatment may synergistically contribute to enhancement of coronary hyperemic flow. Oxygen-derived free radicals may directly affect the coronary arteries, and SOD-induced changes in coronary arterial tone may enhance coronary hyperemia and attenuate myocardial contractile and metabolic dysfunction. If this is the case, the release of adenosine may not be increased by SOD treatment, because decreases in coronary arterial tone are thought to decrease the adenosine concentration in coronary venous blood. In the present study, the release of adenosine was augmented by SOD treatment in coronary microembolization, indicating that SOD directly affects adenosine metabolism. We have presented further evidence that the activity of 5'-nucleotidase, which is responsible for adenosine production during ischemia, is preserved by SOD. Taken together, the present results show that SOD increases coronary reactive hyperemia mainly by enhancing adenosine release and that the other direct coronary and myocardial effects of SOD are unlikely to be involved in enhancement of reactive hyperemia in coronary microembolization.

Relation Between SOD and 5'-Nucleotidase Activity in Coronary Microembolization

The other intriguing finding in the present study is that administration of SOD restores the myocardial contractile and metabolic function even after coronary microembolization. This observation suggests that oxygen-derived free radicals are continuously generated during coronary microembolization and attenuate 5'-nucleotidase activity. At 30 minutes after the onset of coronary microembolization, the administration of SOD partially restored the ecto-5'-nucleotidase activity. However, at 60 minutes after an injection of microspheres, SOD did not restore the activity of ecto-5'-nucleotidase. These results indicate that ecto-5'-nucleotidase activity may be irreversibly inactivated by a sustained exposure to oxygen-derived free radicals of more than 60 minutes and that SOD administered within this period can restore the enzyme activity in coronary microembolization.
Our results indicate that ecto-5′-nucleotidase activity is increased by myocardial ischemia in coronary microembolization and that SOD can further increase this enzyme activity. We did not clarify the mechanism by which myocardial ischemia increases 5′-nucleotidase activity. Because we measured this enzyme activity in vitro, high-energy phosphates, proton ions, or divalent cations seem unlikely to be the candidate for increasing this enzyme activity, although these factors may affect 5′-nucleotidase activity and adenosine production in vivo. In our previous study, activation of protein kinase C enhanced 5′-nucleotidase activity in rat myocytes, indicating that protein phosphorylation may contribute to the increased 5′-nucleotidase activity. The preliminary study of protein kinase C and 5′-nucleotidase suggests that catecholamine release in the ischemic heart may increase 5′-nucleotidase activity in coronary microembolization.

What happens to 5′-nucleotidase in coronary microembolization? Two phenomena appear to be overlapped to alter 5′-nucleotidase activity: One is the increased 5′-nucleotidase activity by ischemia itself, and the other is the decreased 5′-nucleotidase activity by oxygen-derived free radicals. With SOD treatment, the activation of the enzyme caused by ischemia can override the enzyme inactivation caused by oxygen-derived free radicals, leading to the increases in 5′-nucleotidase activity in the ischemic area (Table 3). In the present study, SOD increased ecto-5′-nucleotidase activity more than the untreated condition because SOD eliminates oxygen-derived free radicals and thereby attenuates the potency to decrease in nucleotidase activity. In addition, SOD may induce new enzyme activity on the cell surface and contribute to improved 5′-nucleotidase activity.

In the present study, we could not elucidate the mechanism by which oxygen-derived free radicals influence the activity of ecto-5′-nucleotidase. One plausible possibility is that oxygen-derived free radicals may damage the cellular membrane around ecto-5′-nucleotidase and temporarily promote internalization of this enzyme. 5′-Nucleotidase, located either at the cellular surface or in the cytosol, is reported to contribute to adenosine release during ischemia.23-25,39,49-51 The allosteric changes of 5′-nucleotidase may be responsible in part for its activation,19,20 and oxygen-derived free radicals may modify its allosteric modulation. Another possibility is that oxygen-derived free radicals degrade the
protein structure and thereby abolish its activity. The activity of 5'-nucleotidase would be reversibly inhibited by an allosteric mechanism and irreversibly inhibited if destroyed. Oxygen-derived free radicals may affect 5'-nucleotidase by either mechanism.

**Mechanisms by Which SOD and Adenosine are Beneficial in Coronary Microembolization**

Several lines of evidence indicate that free radical scavengers do not attenuate ischemia and reperfusion injury. In the present study, however, SOD strikingly improved the myocardial contractile and metabolic dysfunction in coronary microembolization. We have previously reported that a large amount of adenosine is released after coronary embolization with microspheres and thereby increases CBF. As we discussed earlier, the histological study revealed small foci of ischemia distributed throughout the ventricular wall in coronary microembolization. Furthermore, we observed that the coronary hyperemia caused by released adenosine is sustained at least for several hours. Thus, it is likely that SOD easily reaches the site where oxygen-derived free radicals are generated. Adenosine release enhanced by SOD treatment may directly relax the coronary vessels and increase local oxygen delivery to the ischemic myocardium, leading to improvements of myocardial contractile and metabolic dysfunctions in coronary microembolization. In contrast, in the coronary occlusion and reperfusion model, SOD infused into a systemic vein may not reach the ischemic area during the occlusion. The other factor to blunt the beneficial effects of SOD is the degree of myocardial injury. Even if SOD reaches the affected myocardium, severe tissue damage caused by ischemia may blunt the potential benefit of SOD. Przyklenk and Kloner reported that SOD increases regional myocardial blood flow during reperfusion after 2 hours of ischemia, which may be consonant with our observation; however, administration of SOD reduced neither myocardial infarct size nor the severity of contractile function. This difference in effect of SOD may be attributed to the experimental models: Since our microembolization procedure may cause less severe reversible ischemic injury compared with the 2 hours of ischemia and reperfusion model, SOD may have the ability to improve contractile function in the present study.

We showed that SOD increases adenosine production during coronary microembolization; however, because adenosine has multiple effects on the cardiovascular system, the question arises as to which effects of adenosine are beneficial in coronary microembolization. Indeed, adenosine increases CBF, attenuates β-adrenoceptor-mediated increases in myocardial contractility, and inhibits platelet aggregation and the activation of leukocytes. All or any of these factors may improve myocardial contractile and metabolic dysfunction in coronary microembolization. In the present

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**FIGURE 7.** Plot of fractional shortening before and after coronary microembolization when the coronary arteries are submaximally dilated with adenosine or papaverine. No increase in fractional shortening was observed during superoxide dismutase (SOD) treatment. Closed circles are derived from the protocol in which a larger dose of microspheres (3.0×10^3/1 mL of baseline coronary blood flow [mL/min]) was embolized to induce severe ischemia. Values are mean±SEM.
study, the beneficial effects of adenosine are attributable to its coronary vasodilatory effect. This conclusion is derived from the fact that submaximal coronary vasodilation caused by exogenous adenosine and papaverine administration abrogates the beneficial effects of SOD and is compatible with our previous studies showing that both $\alpha_1$- and $\alpha_2$-adrenoceptor stimulation improve the ischemic changes through adenosine-mediated coronary vasodilation.\textsuperscript{30,61,62} Carty et al\textsuperscript{63} also observed a comparable phenomenon: Administration of an adenosine agonist during severe coronary constriction further improves myocardial metabolic function by increasing coronary flow. The increased contractile function may be attributable to an increase in coronary hyperemic flow caused by SOD treatment known as Gregg's phenomenon.\textsuperscript{64} However, if the increased myocardial function is simply induced by Gregg's phenomenon, the severity of ischemia should have been worsened because Gregg's phenomenon increases myocardial oxygen consumption. In the present study, LER was increased during SOD treatment, indicating that the severity of ischemia was also improved. Therefore, Gregg's phenomenon is thought to minimally modify the beneficial effect of SOD in the present study.

**Clinical Relevance**

Although early reperfusion is most important to attenuate ischemic injury in acute myocardial infarction, reperfusion cannot fully recover the reversible ischemic changes that occur partly through the non- or low-reflow phenomenon of blood cell plugging\textsuperscript{23} and luminal obstruction by endothelium\textsuperscript{40,65,66} or myocardial cellular swelling.\textsuperscript{67} Coronary microembolization in the present study may mimic this microvascular obstruction in the heart; however, we need to consider the differences in the situations between the realistic clinical settings and the present study. In the clinical settings, coronary microvascular obstruction overlaps the ischemic and reperfused myocardial damage, and coronary microembolization in the present study occurred in the intact myocardium. The other clinical relevance of coronary microembolization is the pathophysiology of unstable angina. The histological examination of patients with unstable angina revealed that the episodic

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**FIGURE 8.** Plots show myocardial oxygen consumption (panel A) and lactate extraction ratio (panel B) before and after coronary microembolization when the coronary arteries are submaximally dilated with adenosine and papaverine. No significant changes in myocardial oxygen consumption and lactate extraction ratio were observed during superoxide dismutase (SOD) treatment. Values are mean±SEM.
growth of the thrombus was accompanied by intermittent fragmentation of thrombus and that this microembolization causes microembolic occlusion of small intramyocardial arteries. These findings suggest that microembolization in the nondamaged myocardium may also clinically occur. In these microvascular injuries, adenosine, which is augmented by SOD administration, may play a key role in attenuation of microvascular injury. Indeed, it is reported that adenosine attenuates the microvascular obstruction after reperfusion and attenuates contractile dysfunction mainly because of suppression of the platelet and neutrophil activations. Recently, a potentiatior of adenosine release, AICA-ribose, has been effective in attenuation of the ischemic reperfusion injury. This evidence strongly suggests that endogenous adenosine is not sufficient to attenuate reperfusion injury and that additional exogenous administration or potentiation of endogenous adenosine can further reduce the ischemic injury. Our results imply that SOD is another potentiator of adenosine in the ischemic heart and deserves further studies to approve its beneficial effects.

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