Superoxide Dismutase Enhances Ischemia-Induced Reactive Hyperemic Flow and Adenosine Release in Dogs
A Role of 5'-Nucleotidase Activity

Masafumi Kitakaze, Masatsugu Hori, Seiji Takashima, Kunimitsu Iwai, Hiroshi Sato, Michitoshi Inoue, Akira Kitabatake, and Takenobu Kamada

To test the hypothesis that 5'-nucleotidase activity during ischemia is attenuated by oxygen-derived free radicals, we measured ischemia-induced reactive hyperemic flow, adenosine release, and 5'-nucleotidase activity in dogs (n=62). A 1-minute occlusion of the coronary artery caused reactive hyperemic flow (307±5 versus 92±1 ml·100 g⁻¹·min⁻¹ at baseline) with increased release of adenosine (14.4±1.4 versus 0.4±0.1 nmol·100 g⁻¹·min⁻¹ at baseline). Superoxide dismutase augmented (p<0.001) both peak coronary blood flow (333±6 ml·100 g⁻¹·min⁻¹) and repayment (436±12 versus 320±7 ml/100 g in the untreated group). Adenosine release during reperfusion was augmented (22.7±1.9 nmol·100 g⁻¹·min⁻¹, p<0.001), and 8-phenyltheophylline completely abolished the enhanced reactive hyperemia. Enzymatic assay of 5'-nucleotidase activity revealed that the administration of superoxide dismutase increases ecto-5'-nucleotidase activity in ischemic myocardium. When an inhibitor of ecto-5'-nucleotidase, αβ-methyleneadenosine 5'-diphosphate, was administered, the effects of superoxide dismutase were completely abolished. Thus, we conclude that 1) the augmentation of reactive hyperemic flow caused by superoxide dismutase is attributed to the enhanced release of adenosine and 2) the enhanced release of adenosine over the untreated controls is attributed to the protection of ecto-5'-nucleotidase activity during ischemia. (Circulation Research 1992;71:558–566)

KEY WORDS • myocardium • ischemia • reperfusion • oxygen-derived free radicals • coronary blood flow

Myocardial ischemia and reperfusion are believed to generate oxygen-derived free radicals, which may cause coronary vascular and myocardial injuries.1-4 Oxygen-derived free radicals may directly affect coronary vasomotor tone.5-10 Furthermore, oxygen-derived free radicals inactivate and degrade several enzymes and proteins that may play important roles in regulation of myocardial function and coronary vasomotor tone.5-10 Oxygen-derived free radicals degrade endothelium-derived relaxing factors11 and Na⁺,K⁺-ATPase activity.10 This suggests that they have the ability to inactivate crucial chemical mediators and enzymes involved in the regulation of coronary blood flow. We have previously reported that recombinant human superoxide dismutase (SOD) enhances hyperemic coronary flow that is due to microembolization.12 Because this coronary hyperemic flow is reported to be mainly attributed to released adenosine,13 oxygen-derived free radicals may affect adenosine metabolism in the ischemic and reperfused myocardium. Both membrane-bound and cytosolic 5'-nucleotidase activities are responsible for adenosine production during ischemia.14-18 If oxygen-derived free radicals attenuate 5'-nucleotidase activity, SOD may restore the 5'-nucleotidase activity and enhance the release of adenosine from the ischemic myocardium.19

To test this idea, we measured reactive hyperemic flow response and adenosine release after a 1-minute coronary occlusion with and without the administration of SOD. Furthermore, to examine the possibility that the deactivation of 5'-nucleotidase activity is caused by oxygen-derived free radicals and that SOD may restore 5'-nucleotidase activity, we assessed 5'-nucleotidase activity of the ischemic myocardium by enzymatic assay with and without SOD treatment.

Materials and Methods

Instrumentation

Sixty-two mongrel dogs weighing 14–22 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 oxygen (100% O₂, 3–5 l/min). The chest was opened through the left fifth intercostal space, and the heart was suspended in a pericardial cradle. The left anterior descending coronary artery was cannulated and perfused with blood via the left carotid artery through an extracorporeal bypass tube. Coronary blood flow (CBF) of the perfused area was measured with an electromagnetic flow probe attached at the bypass tube, and coronary perfusion pressure (CPP) was monitored at the tip of the coronary artery cannula. A small short collecting tube (1 mm in diameter and 7 cm in length) was cannulated into a small coronary vein near the center of the perfused area to sample coronary venous blood. The drained venous blood was collected in a reservoir placed at the level of the left atrium and was returned to the jugular vein. High fidelity left ventricular pressure was measured by a micromanometer (model P-5, Konigsberg Instruments, Inc., Pasadena, Calif.) placed in the left ventricular cavity through the apex. A pair of ultrasonic crystals was placed in the inner one third of the myocardium approximately 1 cm apart to measure myocardial segment length with an ultrasonic dimension gauge (5 MHz, Schuessler, Cardiff by the Sea, Calif.). Heart rate averaged 138±2 beats per minute during the control conditions and did not change throughout the study.

**Experimental Protocols**

**Protocol 1: Effects of recombinant human SOD on reactive hyperemia.** We investigated the effects of SOD (human recombinant, 5,340 IU/mg, >99% purity) on the coronary hyperemic flow response (peak CBF and the flow debt/repayment ratio) after a 1-minute coronary occlusion. Twenty-one dogs were used in this protocol. Both CPP and CBF were continuously measured. Coronary arterial and venous blood were sampled for blood gas analysis and the determination of adenosine concentrations in six dogs. After the hemodynamic stabilization, a 1-minute occlusion of the bypass tube to the left anterior descending coronary artery was followed by complete reperfusion. After the first occlusion and reperfusion, we waited 15 minutes for hemodynamic and metabolic stabilization and then started the administration of SOD. Ten minutes after the onset of continuous intracoronary infusion of SOD (25 μg·kg⁻¹·min⁻¹), a 1-minute coronary occlusion followed by reflow was again performed. SOD was infused continuously throughout the protocol except during the coronary occlusion. For assessment of adenosine release during reactive hyperemia, coronary venous blood from the perfused area was sampled continuously until CBF returned to the baseline level. Each 1-ml sample of blood was collected in a separate sampling tube, and each interval of time sampling (I) was measured. Adenosine release (AdR) during the ith sampling interval (AdRᵢ) was calculated as follows: [mean CBF (ml/sec) during interval]×1/1(i) (seconds)×[coronary arteriovenous differences of adenosine (mol/ml) in the ith tube]. Thus, adenosine release during hyperemia (AdRh) was obtained by the summation of AdRᵢ, and the average rate of AdR (mol·100 g⁻¹·min⁻¹) was calculated as follows: AdRh×[60/duration of hyperemia (seconds)]×[100/myocardial weight of perfused area (g)]. The average number of samplings during the hyperemic response was 14.3.

To test the effects of SOD on the severity of the ischemia, hemodynamic parameters (i.e., left ventricular pressure, dP/dt, and segment length of the perfused area) were measured in five dogs. In these dogs, 1 minute of coronary occlusion followed by reperfusion was performed with and without SOD administration. End-diastolic length (EDL) was determined at the R wave of the electrocardiogram, and end-systolic length (ESL) was determined at the minimal dP/dt. Fractional shortening was calculated by (EDL−ESL)/EDL as an index of myocardial contractility of the perfused area. To assess the myocardial metabolic states during ischemia in these dogs, we also measured the lactate concentrations in the coronary arterial and venous blood 5, 10, 15, 30, 60, and 120 seconds after the onset of reperfusion with and without SOD administration.

To test the reproducibility of the coronary reactive hyperemic response, we performed three consecutive 1-minute coronary occlusions and subsequent reperfusion in the untreated state with a time interval of 25 minutes in five dogs. Before each coronary occlusion and after the third coronary occlusion/reperfusion, adenosine (2 μg·kg⁻¹·min⁻¹) was infused into the perfused area to test whether coronary vascular responses to exogenous adenosine is altered after 1 minute of coronary occlusion.

Furthermore, we tested the effects of administration of SOD on reactive hyperemia after 15, 30, and 45 seconds and 1 minute of coronary occlusion in five dogs.

**Protocol 2: Effects of 8-phenyltheophylline on the enhancement of reactive hyperemic flow responses caused by SOD.** Reactive hyperemic flow response is reported to be partially attributed to released adenosine during reperfusion. To test the hypothesis that enhancement of reactive hyperemic flow response caused by SOD is attributed to the enhanced release of adenosine, we examined the effects of SOD under treatment with 8-phenyltheophylline on reactive hyperemia in five dogs. Ten minutes after the onset of the continuous intracoronary infusion of 8-phenyltheophylline (30 μg·kg⁻¹·min⁻¹), SOD was additionally administered for 10 minutes. We confirmed that this dose of 8-phenyltheophylline completely abolishes the vasodilatory effect of adenosine (4 μg·kg⁻¹·min⁻¹ i.c.). The coronary artery was occluded for 1 minute; this occlusion was followed by complete reperfusion. Administration of 8-phenyltheophylline and SOD was continued throughout the study except during the coronary occlusion. Both CPP and CBF were measured in this protocol.

**Protocol 3: Effects of α,β-methyleneadenosine 5'-diphosphate on the enhanced reactive hyperemic flow and adenosine release caused by SOD.** Oxygen-derived free radicals may attenuate 5'-nucleotidase activity, which is thought to regulate adenosine production during ischemia. We hypothesized that oxygen-derived free radicals attenuate 5'-nucleotidase activity and that administration of SOD protects this enzyme activity and thereby enhances release of adenosine. If blockade of ecto-5'-nucleotidase treated with α,β-methyleneadenosine 5'-diphosphate (AOPCP) abolishes the effects of SOD, we can argue that the effects of SOD on enhancements of reactive hyperemic flow and adenosine release
are tightly linked to the alteration of ecto-5′-nucleotidase activity during ischemia and reperfusion. Conversely, if administration of AOPCP does not abolish the effects of SOD on reactive hyperemia and adenosine release, we can conclude that the effects of SOD on reactive hyperemia and adenosine release are not related to 5′-nucleotidase. To test this idea, we examined the changes in reactive hyperemic flow induced by a 1-minute coronary occlusion with and without administration of SOD under treatment with AOPCP (n=6). Ten minutes after the onset of the continuous intracoronary infusion of AOPCP (80 μg·kg⁻¹·min⁻¹), intracoronary infusion of SOD was administered continuously for 10 minutes. The coronary artery was occluded for 1 minute; this occlusion was followed by reperfusion. Administration of AOPCP and SOD was continued throughout the study except during coronary occlusion. Both CPP and CBF were measured, and coronary arterial venous blood was sampled for blood gas analysis and adenosine measurement in this protocol.

Furthermore, to confirm that treatment with SOD preserves the 5′-nucleotidase activity during 1 minute of coronary occlusion, we measured 5′-nucleotidase activity of the myocardium with and without SOD treatment in 34 dogs. The myocardium was sampled from the endocardium at 1 minute of ischemia (n=7 in each) and at 3 minutes (n=5 in each) and 5 minutes (n=5 in each) of reperfusion with and without SOD treatment. The activity of 5′-nucleotidase was measured by the enzymatic assay.

### Hemodynamic Measurements

Before and during reactive hyperemia after 15, 30, 45, and 60 seconds of coronary occlusion, coronary hemodynamic parameters were measured. The duration of reactive hyperemia was defined as the time between the release of coronary occlusion and the return of CBF to preoclusion flow. Flow debt was defined as preocclusion baseline flow rate times the duration of occlusion, and flow repayment was defined as the area under the flow versus the time curve during reactive hyperemia minus the preocclusion baseline flow times the duration of reactive hyperemia. The area was computed by planimetry.

### Chemical Analysis

Coronary arterial and venous blood oxygen difference (AVO₂D) was assessed by the difference between coronary arterial and venous oxygen contents. Myocardial oxygen consumption (MVO₂, ml·100 g⁻¹·min⁻¹) was calculated as CBF (ml·100 g⁻¹·min⁻¹)×AVO₂D (ml/dl). Lactate was assessed by the enzymatic assay, and the lactate extraction ratio was obtained by coronary arteriovenous differences in lactate concentration multiplied by 100 and divided by arterial lactate concentration.

### Adenosine measurement

The methods of adenosine measurements have been reported previously. Briefly, 1 ml blood was drawn into a syringe containing 0.5 ml dipyridamole (0.02%) and 100 μl of 2-deoxyglucose (0.1 mg/ml) with EDTA (500 mM) to block both uptake of adenosine by red blood cells and degradation of adenosine. After centrifugation, the supernatant was obtained, and the adenosine content was determined by radioimmunoassay. Adenosine in the plasma (100 μl) was succinylated by 100 μl dioxane containing succinic acid anhydride and triethylamine. After a 20-minute incubation, the mixture was diluted with 100 μl adenosine 2′,3′-O-disuccinyl-3-[¹²⁵I]iodotyrosine methyl ester (0.5 pmol) and 100 μl diluted anti-adenosine serum. The mixture was kept in a cold water (4°C) bath for 18 hours, and second antibody solution (500 μl goat anti-rabbit immunoglobulin G antiserum) 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 degradation during this blood sampling procedure has been reported to be negligible.

### 5′-Nucleotidase measurement

Subendocardial myocardium in the left anterior descending coronary artery and left circumflex coronary artery areas were sampled
at 1 minute after the onset of ischemia and at 3 and 5 minutes of reperfusion with and without SOD treatment in 34 dogs. Myocardial tissue was frozen and stored under liquid nitrogen.

The myocardial tissue was homogenized using a Potter-Elvehjem homogenizer (30 strokes) for 5 minutes in 10 vol ice-cold 10 mM HEPES-KOH buffer (pH 7.4) containing 0.25 M sucrose, 1 mM MgCl₂, and 1 mM mercaptoethanol at 0°C. The crude homogenate was strained through a double-layered nylon sieve and again homogenized for 1 minute. For preparation of 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. For preparation of 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 fraction 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 divided into aliquots that were immediately frozen and stored at −80°C.

The activity of 5'-nucleotidase was assessed by the enzymatic assay technique²⁶ and was described in moles per gram wet weight per minute.

**Statistical Analysis**

Statistical analysis in Table 1 was performed by the paired t test.²⁷ The repeated-measures analysis of variance was also used to test the differences of responses of each parameter before, during, and after coronary occlusion (Tables 2–6 and Figures 2–4). All values were expressed as mean±SEM, and a value of p<0.05 was considered significant.

**Results**

Before and after the treatment with SOD, there were no significant changes in CPP, CBF, regional fractional shortening, and the metabolic parameters shown in Figures 1 and 2 and Table 1. Figure 1 depicts the

### Table 1. Coronary Hemodynamic and Metabolic Parameters Before and After the Pharmacological Interventions Before Ischemia

<table>
<thead>
<tr>
<th>Protocol</th>
<th>CPP (mm Hg)</th>
<th>CBF (ml/100 g·min⁻¹)</th>
<th>MVO₂ (ml/100 g·min⁻¹)</th>
<th>pH(a)</th>
<th>pH(v)</th>
<th>Ado(a) (pmol/ml)</th>
<th>Ado(v) (pmol/ml)</th>
<th>AdR(C) (nmol·g⁻¹·min⁻¹)</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>
<td>0.37±0.11</td>
</tr>
<tr>
<td>SOD</td>
<td>106±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>
<tr>
<td>8PT</td>
<td>104±9</td>
<td>91±2</td>
<td>7.00±0.10</td>
<td>7.40±0.01</td>
<td>7.39±0.01</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>8PT+SOD</td>
<td>105±10</td>
<td>93±2</td>
<td>7.20±0.20</td>
<td>7.40±0.01</td>
<td>7.38±0.01</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>8PT+SOD</td>
<td>104±10</td>
<td>92±1</td>
<td>6.89±0.47</td>
<td>7.40±0.01</td>
<td>7.38±0.01</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>8PT+SOD</td>
<td>104±10</td>
<td>92±1</td>
<td>6.89±0.47</td>
<td>7.40±0.01</td>
<td>7.38±0.01</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>8PT+SOD</td>
<td>104±10</td>
<td>92±1</td>
<td>6.89±0.47</td>
<td>7.40±0.01</td>
<td>7.38±0.01</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

CPP, coronary perfusion pressure; CBF, coronary blood flow; MVO₂, myocardial oxygen consumption; pH(a) and pH(v), pH in coronary arterial and venous blood, respectively; Ado(a) and Ado(v), adenosine concentrations in coronary arterial and venous blood, respectively; AdR(C), adenosine release; SOD, administration of superoxide dismutase; 8PT, administration of 8-phenylthophysphine; AOPCP, administration of α,β-methyleneadenosine 5'-diphosphate. Values are mean±SEM.
TABLE 2. Serial Changes in Fractional Shortening During and After Myocardial Ischemia With and Without the Administration of Superoxide Dismutase

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>SOD</th>
<th>Time after the onset of coronary occlusion (seconds)</th>
<th>Time after the onset of reperfusion (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>Untreated</td>
<td>24.5±1.2</td>
<td>...</td>
<td>5.3±2.4</td>
<td>2.2±2.4</td>
</tr>
<tr>
<td>SOD</td>
<td>23.7±1.4</td>
<td>24.1±1.5</td>
<td>6.1±2.0</td>
<td>3.7±1.9</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
There are no significant differences of fractional shortening for any time between untreated and superoxide dismutase (SOD)-treated groups.

representative recordings of changes in CPP and CBF before, during, and after a 1-minute coronary occlusion. Administration of SOD significantly augmented peak CBF and the repayment during reperfusion, although there were no significant changes in CPP and CBF at baseline. Figure 2 shows the summarized data of CBF and adenosine release during reperfusion after a 1-minute coronary occlusion with and without the SOD treatment. Although CPP was not different in the control and SOD-treated groups (106±5 versus 106±5 mm Hg before ischemia), both peak CBF (Figure 2A) and repayment (Figure 2B) during reperfusion were significantly augmented in the SOD-treated group compared with the untreated group. The percent increases in peak CBF and the debt/repayment ratio in the untreated group were 337±8% and 350±8%, respectively, and these two parameters in the SOD treatment were significantly (p<0.001) increased to 368±11% and 482±15%, respectively. The duration of reactive hyperemia was not different between the untreated and SOD-treated groups (126±4 and 132±4 seconds). Adenosine release during reperfusion after a 1-minute occlusion of the perfused area was significantly augmented in the SOD-treated group compared with the untreated group (Figure 2C). Table 2 shows the changes in fractional shortening before, during, and after a 1-minute coronary occlusion. There were no significant changes in the time courses of decreases in fractional shortening during ischemia between the untreated and SOD-treated groups. The extent of decreases in the lactate extraction ratio during reperfusion was also comparable between the untreated and SOD-treated groups (Table 3). These results indicate that SOD treatment did not alter the functional and metabolic severity of myocardial ischemia. Table 4 confirms that reactive hyperemia was highly reproducible in three consecutive coronary occlusion and reperfusion cycles with 25-minute intervals in five dogs. Increases in CBF caused by exogenous adenosine administration (2 μg · kg⁻¹ · min⁻¹) before the first, second, and third coronary occlusions and after the third coronary occlusion were 213±11, 225±9, 219±10, and 223±8 ml · 100 g⁻¹ · min⁻¹, respectively. This result indicates that the extent of increases in CBF in response to exogenous adenosine administration was not altered by 1 minute of coronary occlusion. Table 5 shows the effects of changes in the duration of coronary occlusion on the SOD-induced augmentation of reactive hyperemia. There was no augmentation of reactive hyperemia after 15 and 30 seconds of coronary occlusion even in the SOD-treated group. In 45 seconds of ischemia, SOD slightly augmented reactive hyperemia during reperfusion, indicating that 60 seconds of ischemia is necessary to generate enough oxygen-derived free radicals to attenuate adenosine production. These data indicate that SOD treatment significantly augments reactive hyperemic flow after at least 1 minute of coronary occlusion and suggest that this augmentation of CBF may be due to increased release of adenosine during ischemia.

We then antagonized the adenosine receptors by the administration of 8-phenyltheophylline to test whether the SOD-induced augmentation of adenosine release during reperfusion is in part responsible for the augmentation of reactive hyperemic flow. Administration of 8-phenyltheophylline did not change the hemodynamic and metabolic parameters in the untreated and SOD-treated groups (Table 1 and Figure 3). However, it attenuated both the peak CBF and repayment after 1 minute of coronary occlusion (Figure 3), confirming that reactive hyperemia after ischemia is in part mediated through released adenosine.18,20,22,23 Administration of SOD under treatment with 8-phenyltheophylline augmented neither peak CBF nor repayment during reperfusion. These results indicate that SOD-induced enhancements of peak CBF and repayment during reperfusion are mediated through enhanced adenosine release.

TABLE 3. Serial Changes in the Lactate Extraction Ratio After Myocardial Ischemia With and Without the Administration of Superoxide Dismutase

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>SOD</th>
<th>Time after the onset of reperfusion (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Untreated</td>
<td>26.5±3.9</td>
<td>...</td>
<td>-42.1±6.5</td>
</tr>
<tr>
<td>SOD</td>
<td>25.7±2.9</td>
<td>26.7±3.0</td>
<td>-41.0±8.2</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
There are no significant differences in the lactate extraction ratio for any time between untreated and superoxide dismutase (SOD)-treated groups.
We hypothesized that oxygen-derived free radicals attenuate 5'-nucleotidase activity and that treatment with SOD restores or increases this enzyme activity. To test this hypothesis, we first examined the effects of AOPCP, an inhibitor of ecto-5'-nucleotidase, on the enhanced release of adenosine and reactive hyperemic flow induced by SOD treatment. Administration of AOPCP did not change the coronary hemodynamic and metabolic parameters. However, AOPCP significantly attenuated adenosine release and reactive hyperemia during reperfusion, which also supports the role of adenosine in reactive hyperemia (Figure 4). Under treatment with AOPCP, the administration of SOD did not increase the reactive hyperemia and adenosine release (Figure 4). This observation indicates that 1) enhanced adenosine release during reperfusion under SOD treatment is attributed to ecto-5'-nucleotidase activity and 2) one of the target sites of oxygen-derived free radicals during ischemia and reperfusion is ecto-5'-nucleotidase. Table 6 strengthens this observation. Enzymatic assay for ecto-5'-nucleotidase and cytosolic 5'-nucleotidase activities before, during, and after ischemia were measured in the sampled myocardial tissues. In the untreated state, ecto-5'-nucleotidase activity in the ischemic area was significantly (p<0.05) increased compared with the nonischemic left circumflex coronary artery area. However, under SOD treatment this enzyme activity was further increased (9.1±0.4 versus 7.4±0.5 μmol·g wet wt⁻¹·min⁻¹, p<0.05), suggesting that oxygen-derived free radicals generated during ischemia blunt myocardial ability to increase ecto-5'-nucleotidase activity during ischemia and that SOD overcomes this effect. Intriguingly, 5 minutes after the onset of coronary reperfusion, ecto-5'-nucleotidase activity returned to the control level in the untreated and SOD-treated groups, and there were no significant differences between these two values. Cytosolic 5'-nucleotidase did not change during ischemia and reperfusion with or without SOD treatment.

**Discussion**

In the present study, we conclude that 1) the augmentation of reactive hyperemic flow caused by administration of SOD is attributed to the enhanced release of adenosine and 2) the enhanced release of adenosine is attributed to the increase in ecto-5'-nucleotidase activity during ischemia. Before reaching these conclusions, we should consider the possibilities that several other coronary vasomotor and myocardial effects of SOD may phenomenologically explain these observations during ischemia and reperfusion.

**Possible Mechanisms for the Effects of SOD on Enhanced Reactive Hyperemia**

Oxygen-derived free radicals are produced mainly during reperfusion after myocardial ischemia. Furthermore, several lines of evidence reveal that oxygen-derived free radicals are also generated during myocardial ischemia.28-4 In this case, oxygen-derived free radicals accelerate the formation of myocardial edema and may increase the severity of ischemia, which is thought to enhance adenosine release during the following reperfusion. However, in the present study, the time course of decreases in fractional shortening and lactate production during ischemia and reperfusion were identical with and without SOD treatment (Tables 2 and 3), indicating that the extent of myocardial ischemia in the untreated and SOD-treated groups is comparable. It is unlikely that 1 minute of ischemia produces significant edema in the myocardium. SOD is

**TABLE 4. Reproducibility of Reactive Hyperemia After Three Subsequent 1-Minute Coronary Occlusions**

<table>
<thead>
<tr>
<th></th>
<th>CPP (mm Hg)</th>
<th>Baseline CBF (ml·100 g⁻¹·min⁻¹)</th>
<th>Peak CBF (ml·100 g⁻¹·min⁻¹)</th>
<th>Repayment (ml/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First ischemia</td>
<td>108±4</td>
<td>93±2</td>
<td>312±7</td>
<td>342±8</td>
</tr>
<tr>
<td>Second ischemia</td>
<td>107±3</td>
<td>92±2</td>
<td>320±8</td>
<td>356±9</td>
</tr>
<tr>
<td>Third ischemia</td>
<td>107±5</td>
<td>92±3</td>
<td>319±7</td>
<td>351±7</td>
</tr>
</tbody>
</table>

CPP, coronary perfusion pressure; CBF, coronary blood flow. Values are mean±SEM.

There are no significant differences for each parameter between groups treated with and without SOD.

**TABLE 5. Reactive Hyperemia After 15, 30, and 45 Seconds of Ischemia With and Without Superoxide Dismutase Treatment**

<table>
<thead>
<tr>
<th></th>
<th>CPP (mm Hg)</th>
<th>Baseline CBF (ml·100 g⁻¹·min⁻¹)</th>
<th>Peak CBF (ml·100 g⁻¹·min⁻¹)</th>
<th>Repayment (ml/100 g)</th>
</tr>
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<tbody>
<tr>
<td>15-Second ischemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without SOD</td>
<td>106±5</td>
<td>90±1</td>
<td>229±13</td>
<td>234±15</td>
</tr>
<tr>
<td>With SOD</td>
<td>106±5</td>
<td>90±2</td>
<td>222±17</td>
<td>235±16</td>
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<tr>
<td>30-Second ischemia</td>
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<tr>
<td>Without SOD</td>
<td>106±3</td>
<td>89±2</td>
<td>301±13</td>
<td>306±11</td>
</tr>
<tr>
<td>With SOD</td>
<td>108±5</td>
<td>89±1</td>
<td>312±8</td>
<td>314±9</td>
</tr>
<tr>
<td>45-Second ischemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without SOD</td>
<td>106±4</td>
<td>90±1</td>
<td>332±6</td>
<td>340±11</td>
</tr>
<tr>
<td>With SOD</td>
<td>107±6</td>
<td>91±2</td>
<td>371±23</td>
<td>390±17</td>
</tr>
</tbody>
</table>

CPP, coronary perfusion pressure; CBF, coronary blood flow; SOD, superoxide dismutase. Values are mean±SEM.

There are no significant differences for each parameter between groups treated with and without SOD.
reported to inactivate the degradation of endothelium-derived relaxing factor, which may enhance the coronary vasodilation during ischemia and reperfusion. Indeed, endothelium-derived relaxing factor may be released in the hypoxic myocardium. However, SOD-induced enhancement of endothelium-derived relaxing factor release may not be involved in the present observation because the enhanced reactive hyperemia is completely abolished by treatment with 8-phenyltheophylline (Figure 3).

Oxygen-derived free radicals may also directly affect the coronary arteries and SOD-induced changes in coronary arteries may be responsible for the enhancement of reactive hyperemia. However, if this were the case, the release of adenosine might not be increased, because a decrease in coronary tone aside from adenosine-induced vasodilation is thought to decrease the adenosine concentration in coronary venous blood. In the present study, the release of adenosine was augmented by the SOD treatment, indicating that SOD directly affects the adenosine metabolism. Furthermore, the adenosine production with AOPCP also blocked the augmented hyperemic flow. This would not be expected if the hyperemic flow was mediated by the action of the free radicals directly on the coronary arteries. Administration of SOD and subsequent enhancement of adenosine release may inhibit platelet aggregation and leukocyte adhesion during ischemia and reperfusion, causing enhancement of reactive hyperemic flow. However, if platelet aggregation and leukocyte adhesion are involved during 1 minute of coronary occlusion and subsequent reperfusion, CBF, MVo2, adenosine concentration of the coronary vein, and the lactate extraction ratio could not return to the control levels. However, this is not the case (Tables 1, 3, and 4), indicating that the enhancement of reactive hyperemia and adenosine release due to SOD treatment is not attributed to the inhibition of platelet aggregation and leukocyte adhesion. Taken together, we believe that SOD increases reactive hyperemic flow mainly because of enhanced adenosine release and that the other coronary and myocardial effects of SOD are not significantly involved in the SOD-induced enhancement of reactive hyperemia.

Effects of SOD on the Production of Adenosine During Ischemia and Reperfusion

In the present study, we have shown that the administration of SOD increased the adenosine concentration detected in the coronary venous blood during reperfu-
sion. Several possibilities need to be considered to explain this observation. Oxygen-derived free radicals may directly degrade adenosine in the coronary vessels and decrease the adenosine concentration. However, this seems unlikely because administration of AOPCP abolished the effects of SOD. SOD may also increase the myocardial AMP content of ischemic myocardium, a substrate for the production of adenosine through ecto-5'-nucleotidase. Another possibility is that oxygen-derived free radicals may desensitize adenosine receptors in the coronary vessels. However, this idea is denied by the observation in the present study that increases in CBF in response to exogenous adenosine are not reduced after 1 minute of coronary occlusion (see "Results"). Laxson et al.\(^3\) also reported that three 10-minute coronary artery occlusions separated by 30-minute reflow periods do not alter maximal responses of adenosine in the increases in coronary flow. On the other hand, we observed that the administration of AOPCP abolished the effects of SOD on adenosine production, supporting the idea that enhanced adenosine release during reperfusion is due to the preservation of ecto-5'-nucleotidase activity. Several lines of evidence\(^8\)–\(^10\) support the idea that oxygen-derived free radicals inactivate several enzyme activities. Ecto-5'-nucleotidase that is bound to the myocardial and endothelial cellular surface is thought to be a target of the injury caused by oxygen-derived free radicals. The attenuation of 5'-nucleotidase activity caused by oxygen-derived free radicals may be transient and return to the baseline level within a few minutes, since multiple occlusions within a relatively short period of time produced the reproducible and similar alterations in reactive flow responses (Table 4).

**Pathophysiological and Clinical Relevance**

The mechanism of reactive hyperemia after myocardial ischemia is important because it is used experimentally and clinically for the assessment of coronary flow reserve. Adenosine has been reported to be partially responsible for reactive hyperemia.\(^18\)–\(^22\) Saito et al.\(^23\) reported that 30% of hyperemic flow after a brief period of coronary occlusion is attributed to adenosine release. Our findings agree well with this hypothesis. Our study further elucidated one of the mechanisms for reactive hyperemia, i.e., oxygen-derived free radicals. More intriguingly, the role of oxygen-derived free radicals in reactive hyperemia seems solely attributed to the attenuation of 5'-nucleotidase activity, which results in reduced adenosine production. It should be noted that the effects of SOD become prominent after at least 60 seconds of ischemia (Table 4). This result suggests that it may take at least 60 seconds of ischemia to generate enough free radicals to attenuate 5'-nucleotidase. On the other hand, if a longer period of ischemia is produced, the activity of this enzyme may be irreversibly attenuated.

However, when we apply the present results to coronary physiology, we need to consider the substantial differences between the anesthetized and conscious animals. Hintze and Vatner\(^31\) reported that the extent of reactive hyperemic flow was increased in conscious dogs compared with anesthetized dogs; CBF increased to 355±30% after 30 seconds of coronary occlusion. However, in the anesthetized dogs in the present study, CBF increased to 322±18% (Table 5). Furthermore, Olsson et al.\(^32\) reported that coronary vasodilatory response to adenosine was twice as large in conscious dogs as in anesthetized dogs. These results suggest that reactive hyperemic responses may be attenuated in the present study and that the increases in coronary flow responded to the release of adenosine during reactive hyperemia with and without administration of SOD. These differences in the coronary circulation between conscious and anesthetized dogs may be related to the changes in the sympathetic tones in the coronary vessels and myocardium that are due to general anesthesia and acute thoracotomy and may also be related to the changes in coronary vascular tones that are due to coronary cannulation and the bypass tube.

The present study indicates the tight linkage between SOD and adenosine production through the preservation of 5'-nucleotidase activity, suggesting that the beneficial effects of SOD on ischemia and reperfusion injury may be at least partially attributed to the enhanced release of adenosine. This hypothesis agrees with our previous observation that SOD administration attenuates the severity of the myocardial contractile and metabolic dysfunction in coronary microembolization and that these beneficial effects are abolished by treatment with 8-phenyltheophylline.\(^12\) Although further efforts are necessary before our results can be applied to

<table>
<thead>
<tr>
<th>Ecto-5'-nucleotidase activity (nmol · g wet wt (^{-1}) · min (^{-1}))</th>
<th>Without SOD</th>
<th>With SOD</th>
<th>Without SOD</th>
<th>With SOD</th>
<th>Without SOD</th>
<th>With SOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Minute of ischemia</td>
<td>7.4±0.5</td>
<td>9.1±0.4*</td>
<td>83.5±5.2</td>
<td>91.5±5.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Minutes of reperfusion</td>
<td>7.0±0.2</td>
<td>7.0±0.4</td>
<td>78.3±0.6</td>
<td>80.3±5.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 Minutes of reperfusion</td>
<td>6.8±0.5</td>
<td>6.9±0.5</td>
<td>81.0±8.4</td>
<td>81.5±2.5</td>
<td></td>
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</tr>
</tbody>
</table>

**TABLE 6. Ecto-5'-Nucleotidase and Cytosolic 5'-Nucleotidase Activities in the Ischemic and Reperfused Myocardium With and Without Superoxide Dismutase Treatment**

LAD, myocardium in the area of the left anterior descending coronary artery; LCx, myocardium in the area of the left circumflex coronary artery; SOD, superoxide dismutase. Values are mean±SEM. *p<0.05 vs. the corresponding value without SOD.
566 Circulation Research Vol 71, No 3 September 1992

clinical fields, our results hint that oxygen-derived free radicals may play an important role in attenuation of adenosine production during ischemia and that the administration of SOD may be beneficial for preserving adenosine production.

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