Role of Oxygen-Derived Free Radicals in Myocardial Edema and Ischemia in Coronary Microvascular Embolization

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Background. Oxygen-derived free radicals are thought to injure the ischemic heart during coronary microvascular embolization.

Methods and Results. To test this idea, microspheres (15 μm in diameter) were repetitively administered into the left anterior descending coronary artery to cause microvascular embolization in dogs. Myocardial contractile and metabolic dysfunctions were significantly attenuated after treatments with recombinant human superoxide dismutase, an acyl derivative of ascorbic acid (CV3611, 2-O-octadecylascorbic acid), and xanthine oxidase inhibitor (allopurinol). The free radical scavengers and inhibitor enhanced the coronary hyperemic flow response during embolization, and the total number of microspheres causing maximal embolization was increased by these drugs. When 8-phenyltheophylline was additionally administered with superoxide dismutase, these beneficial effects were abolished, indicating that coronary effects of these drugs may be due to increased release of adenosine during coronary microvascular embolization.

Conclusions. We conclude that oxygen radicals worsen the ischemic injury in coronary microembolization. (Circulation 1991;84:828–840)

Although reperfusion after prolonged coronary occlusion results in partial salvage of the ischemic myocardium, maximal salvage is limited by the no-reflow phenomenon or perfusion impairment observed during reperfusion.1-2 The perfusion deficit during reflow may be caused by blood cell plugging in capillaries,3,4 compression of capillaries by swollen myocardial cells,5 and luminal obstruction by endothelial cellular swelling.6-8 Engler et al4 demonstrated that leukocyte plugging plays an important role in perfusion deficit after reperfusion. If patchy myocardial ischemia due to microvascular obstructions induces subsequent cellular swelling, this process may cause further perfusion deficits and aggravate myocardial ischemia. Although recent studies suggest that myocardial and vascular endothelial cellular swellings due to ischemia are caused, in part, by oxygen-derived free radicals,9 the role of free radicals in coronary microvascular obstructions has not been studied. Previous studies from our laboratory demonstrated that myocardial ischemia induced by coronary microvascular obstructions is characterized by the sustained release of adenosine, which causes hyperemic flow in the microcirculation surrounding the patchy ischemic lesion.10-12 In these lesions, oxygen-derived free radicals may be continuously generated. Because free radicals may play an important role for an increase in vascular permeability during ischemia13 and cellular swelling during reflow,14 radical scavengers may be effective in attenuating the severity of cellular swelling and, hence, the extent of myocardial necrosis15 after coronary microvascular obstruction. We have reported that adenosine released from the ischemic myocardium criti-
cally determines the contractile and metabolic dysfunction.10–12 Coronary blood flow responses may be improved by free radical scavengers if free radical generation alters adenosine metabolism. Thus, the present study was designed to determine 1) whether coronary microvascular embolization results in myocardial cell swelling, 2) whether free radical scavengers improve myocardial contractile and metabolic dysfunction and prevent myocardial edema (we have examined the effects of free radical scavengers, i.e., superoxide dismutase [SOD, Ube Industries, Tokyo], CV36116 [2-O-octadecylascorbic acid, Takeda Chemical Industries, Osaka, Japan], and the xanthine oxidase inhibitor, allopurinol, on ventricular and coronary dysfunction, lactate production, and myocardial edema after coronary microvascular embolization in dogs), and 3) whether adenosine plays a central role in the beneficial effects of radical scavengers.

Methods

Instrumentation

Eighty-five mongrel dogs weighing 12–30 kg were anesthetized with pentobarbital sodium (30 mg/kg i.v.) and mechanically ventilated through a cuffed endotracheal tube. 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 extracorporeal bypass, containing an electromagnetic flow probe (FF-050T, Nihon Kohden, Tokyo). Coronary perfusion pressure was determined through the bypass tube proximal to the cannula (14-15G). A collecting tube (1 mm in diameter and 8 cm in length) was inserted into a small coronary vein near the center of the perfused area with an extracorporeal bypass for coronary venous blood sampling. The drained venous blood was collected in the reservoir placed at the level of the right atrium and was returned to the jugular vein. A miniature pressure transducer (P-7, Königsberg Instruments, Inc., Pasadena, Calif.) was inserted into the left ventricular (LV) cavity through a stab incision at the ventricular apex. In 41 dogs (protocol 1), a pair of ultrasonic crystals (5-MHz, Schuessler, Cardiff By The Sea, Calif.) were implanted into the endocardial one third of the perfusion area for measurements of myocardial segment length. LV pressure and its first derivative (dP/dt) were measured. End-diastolic length was determined at the R wave on the electrocardiogram, and end-systolic length was determined at the minimal dP/dt. Fractional shortening was calculated as follows: (end-diastolic length minus end-systolic length) divided by end-diastolic length as an index of myocardial contractility of the perfused area. Heart rate averaged 126 beats/min and was not changed during each study.

Dogs used in this study were maintained in accordance with the guidelines of the American Physiological Society. Only animals that were lawfully ac-

quired were used in this study, and their retention and use were in compliance with regulations of our university and in accordance with the NIH Guide (US Department of Health, Education, and Welfare publication No. [NIH] 80-23, Washington, DC, US Government Printing Office, 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. Changes in coronary blood flow, lactate production, and fractional shortening after repetitive injections of 15-μm microspheres. Forty-one dogs with instrumentation were used to elucidate the relation between the extent of embolization and ischemic changes and to define the maximal dose of microspheres that can obstruct the functional coronary vascular beds. After hemodynamic stabilization, coronary perfusion pressure, LV pressure, myocardial segment length, and mean regional coronary blood flow were determined. On completion of these control studies, 15-μm microspheres (5.0×10⁴ microspheres/ml baseline coronary blood flow) were injected into the LAD through the bypass tube. Because the quantitative relation between the number of microspheres causing microvascular embolization and the functional and metabolic changes of myocardium is critically important in this study, we used microspheres that were 15 μm in diameter (15±1 μm, 3M Co., St. Paul, Minn.) because the loss of 15 μm microspheres through shunting to the coronary vein is negligible (<3% of the total dose).16–18 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 by a vortex mixer at least 1 minute before injection. After stabilization, usually within a few minutes, hemodynamic studies (LV pressure, coronary blood flow, and coronary perfusion pressure), blood sampling, and recording of segment length were performed, and another dose (5.0×10⁴ microspheres/ml coronary blood flow) of microspheres was administered. These procedures were repeated until coronary blood flow was reduced to less than 8 ml/min. The number of microspheres required for embolization to reduce coronary blood flow to almost zero (<8 ml/min) was also recorded. Arterial blood was sampled simultaneously with coronary venous blood for analysis of lactate concentration and oxygen content. Lactate concentration was assessed by enzymatic assay, and the lactate extraction rate was calculated as follows: (coronary arteriovenous difference in lactate concentration multiplied by 100) divided by the arterial lactate concentration. Coronary arterial venous oxygen difference (AVO₂D) was calculated as follows: coronary arterial oxygen content minus venous oxygen content. Myocardial oxygen extraction was calculated as follows: AVO₂D divided by the arterial oxygen content. MVO₂ (ml/100 g/min) was calculated
as follows: coronary blood flow (ml/100 g/min) multiplied by AVO2D (ml/dl).

Protocol 2. Changes in tissue water content and histological changes after a single injection of 15-μm microspheres. We investigated the effects of the free radical scavengers, human recombinant SOD and CV3611, and the xanthine oxidase inhibitor allopurinol, on myocardial edema after coronary microvascular embolization. In 44 dogs, 15-μm microspheres (2.8±0.2×10⁶ microspheres/g myocardium), which were well stirred as in protocol 1, were administered into the LAD by a single injection. Heart rate, LV pressure, and coronary perfusion pressure were measured before and 10 and 60 minutes after the resulting embolization. Arterial and coronary venous blood samples were also obtained for analysis of lactate and adenosine concentrations at these study intervals. At the end of the experiment, the heart was excised, and transmural myocardial samples were taken from 34 dogs after 60 minutes of ischemia. In 10 other dogs, the protocol was completed 10 minutes after embolization, and myocardial tissue samples were obtained in the same manner.

In both protocols, the perfusion area was determined by injection of Evans’ blue dye into the bypass tube. The mean tissue weight of the perfused area in protocols 1 and 2 were 37±3 and 34±5 g, respectively.

Drug Administration

Eighty-five dogs (41 in protocol 1, 44 in protocol 2) were divided into the following six groups: group A, control; group B, SOD (2.5 mg/kg) was administered by bolus injection 10 minutes before embolization and by continuous infusion (2.5 mg/kg/hr i.v.) throughout the experiments; group C, CV3611 (10 mg/kg) was orally administered 2 hours before embolization; group D, allopurinol (50 mg/kg i.v.) was administered by injection 2 hours before embolization and by continuous infusion (30 mg/kg/hr i.v.) throughout the experiments; group E, 8-phenyltheophylline (30 μg/kg/min i.c.) was administered during treatment with SOD as in group A; and group F, only 8-phenyltheophylline (30 μg/kg/min i.c.) was infused as the control for group E. Ten dogs in protocol 2, killed 10 minutes after embolization for analysis of early myocardial edema formation, were randomly divided into group A (control) and group B (SOD treatment). Administrations of these drugs elicited no significant changes in basal hemodynamic parameters (Tables 1 and 2).

Histological Examinations

Several transmural samples (0.3–0.7 g each) were obtained both from the center of the embolized area (LAD area) and from the center of the control nonembolized area (area perfused by the left circumflex coronary artery, LCx). Tissue water content was obtained from more than six samples of each area. These samples were weighed and then dried overnight at 90°C. The dry samples were weighed again, and the tissue water contents were calculated as follows: (wet weight minus dry weight) divided by dry weight.

Remaining myocardial tissue samples were subjected to histological examination. Samples were immediately layered with glutaraldehyde (2.5% glutaraldehyde in a phosphate buffer of 350 mosmol/l at pH 7.4) for electron microscopic study. Sections were then cut into approximately 1–2-μm cubes that were placed in the same mixture of glutaraldehyde. Blocks of myocardial tissue underwent further fixation in glutaraldehyde at 4°C overnight. After postfixation in OsO₄, dehydration was accomplished in a graded series of ethanol solutions, and the tissue was em-
Figure 2. Schematic of experimental protocol 2. In 34 dogs, microspheres (15 μm in diameter, 3.0 ± 0.1 × 10^6 microspheres/coronary blood flow [milliliter per minute]) were injected into the left anterior descending coronary artery and coronary hemodynamics and metabolic changes (see text) were measured 60 minutes after embolization with and without pharmacological interventions. Group A: Saline (20 ml/hr i.v.) was continuously infused during the experiment (control). Group B: Recombinant human superoxide dismutase (SOD, 2.5 mg/kg/hr i.v.) was given 10 minutes before embolization and continuously infused (2.5 mg/kg/hr i.v.) throughout the experiment. Group C: CV3611 (10 mg/kg) was orally given 120 minutes before embolization, and saline (20 ml/hr i.v.) was continuously infused during the experiment. Group D: Allopurinol (50 mg/kg i.v.) was administered 120 minutes before embolization and continuously infused (30 mg/kg/hr i.v.) during the experiment. Group E: 8-Phenyltheophylline (8-PT, 30 μg/kg/min i.c.) was administered under the treatment with SOD as in group B. Group F: 8-Phenyltheophylline (8-PT) was administered without the SOD treatment. After completion of experiments, tissue water content was measured, and histological examinations were performed in excised hearts. In 10 other dogs, identical studies were done 10 minutes after embolization with and without SOD treatment.

Determination of Adenosine Concentration

The method of adenosine concentration was described in detail elsewhere. In brief, 2 ml blood was withdrawn into a syringe that contained 1 ml dipyridamole (0.01%) and 0.2 ml MnCl₂ (10 mM) to block the uptake of adenosine by the red blood cells and to prevent adenosine degradation. Blood samples were quickly put into iced water to prevent release of adenosine from red blood cells. After centrifugation, the supernatant was mixed with an equal volume of 10% trichloroacetic acid to remove the coagulated protein. Residual trichloroacetic acid was removed by water-saturated ether from the extraction of the supernatant; thereby, the sensitive radioimmunoassay method for analyzing adenosine was used. Adenosine in the plasma (100 μl) was succinylated by 100 μl dioxane, containing succinic acid anhydride and triethylamine. After a 10-minute incubation, the mixture was diluted with 800 μl of 0.3 M imidazole buffer (pH 6.5). The assay mixture contained 100 μl sample, 100 μl succinyl hydrogen-3-labeled adenosine (25,000 cpm in an amount of 1 pmol), and 100 μl diluted antiadenosine serum. After the mixture was kept in an ice-cold water bath for 24 hours, a cool suspension of dextran-coated charcoal (500 μl) was added. The charcoal was spun down, and 0.5 ml supernatant was counted for radioactivity in a liquid scintillation counter. The amount of adenosine degradation during

bedded in an epoxy resin. Semithin (0.5–1.0 μm) sections were cut and stained with toluidine blue for light microscopic examination to study the distribution of myocardial and interstitial edema. Ultrathin sections were produced and stained with uranyl acetate and lead citrate for examination with an electron microscope (100CX, Hitachi, Tokyo).
the sampling procedure and the degradation rate of adenosine are negligible.\textsuperscript{10,11}

**Statistical Analysis**

Statistical analysis was performed with paired and unpaired $t$ tests on all data. The repeated measures analysis of variance was also used to test the differences of responses of each parameter against the extent of microvascular embolization. All values were expressed as mean±SEM, and $p<0.05$ was considered significant.

**Results**

**Effects of SOD, CV3611, and Allopurinol on Myocardial Ischemia and the Total Number of Microspheres for Maximal Embolization**

Tables 1 and 2 depict the basal hemodynamic data and myocardial oxygen consumption before embolization in the six study groups. The basal hemodynamic parameters were comparable among the groups, and drug administrations per se did not significantly alter these parameters.

Figure 3 shows the mean changes in fractional shortening in the embolized area (LAD area), lactate extraction rate, coronary $\text{AVO}_2\text{D}$, and coronary blood flow after repetitive injections of microspheres. In group A (control), coronary blood flow was increased at the low doses of microspheres (up to 2.0x10\textsuperscript{5} microspheres/g myocardium). However, at the higher doses, flow decreased to less than 8 ml/min as the number of microspheres was increased up to 5.2±0.4x10\textsuperscript{5} microspheres/g myocardium as observed in our previous studies.\textsuperscript{10-12} In contrast to the biphasic change in coronary blood flow, both fractional shortening and lactate extraction rate were almost linearly decreased when the extent of embolization was increased. After the injection of a small amount of microspheres (1.0x10\textsuperscript{5} microspheres/g myocardium), lactate production occurred, and after injection of a larger amount of microspheres (3.0x10\textsuperscript{5} microspheres/g myocardium), the negative values of fractional shortening indicate dyskinetic motion of the ventricular wall. With drug interventions (groups B, C, and D), the maximal coronary hyperemic response was observed at a larger dose of microspheres (2.0x10\textsuperscript{5} microspheres/g myocardium) than at the control dose (1.0x10\textsuperscript{5} microspheres/g myocardium), whereas the valve of the maximal flow was comparable in the control and the three treated groups (160±13, 170±20, 169±21, and 169±18 ml/100 g/min in groups A [control], B, C, and D, respectively). At microsphere doses higher than 2.0x10\textsuperscript{5} microspheres/g myocardium, coronary blood flow was decreased in a dose-dependent manner. The maximal number of microspheres required to decrease flow to 8 ml/min in the treated groups was significantly ($p<0.01$) larger than that in the control (5.2±0.4x10\textsuperscript{5} microspheres/g myocardium in group A, 9.1±0.7x10\textsuperscript{5} microspheres/g myocardium in group B, 7.8±0.5x10\textsuperscript{5} microspheres/g myocardium in group C, and 9.9±1.0x10\textsuperscript{5} microspheres/g myocardium in group D). Both coronary $\text{AVO}_2\text{D}$ (Figure 3) and oxygen extraction (control: 0.57±0.02 versus 0.33±0.02 at 2.0x10\textsuperscript{5} microspheres/g myocardium, in the untreated condition [group A]) were significantly ($p<0.001$) attenuated by treatment. The extents of decreases of coronary $\text{AVO}_2\text{D}$ in the treated conditions (groups B, C, and D) were larger ($p<0.001$) than those in group A up to 4.0x10\textsuperscript{5} microspheres/g myocardium, and the extents of oxygen extraction in the treated conditions (groups B, C, and D) were also larger ($p<0.001$) up to 4.0x10\textsuperscript{5} microspheres/g myocardium (0.42±0.02, 0.40±0.02, and 0.43±0.02 at 2.0x10\textsuperscript{5} microspheres/g myocardium in groups B, C, and D, respectively). In the treated groups (groups B, C, and D), decreases in both fractional shortening ($p<0.001$) and lactate extraction rate ($p<0.001$) after repeated emboli- lizations were less than those in the control group between the embolization ranges of 1.0x10\textsuperscript{5} and 4.0x10\textsuperscript{5} microspheres/g myocardium. In the treated groups, dyskinetic wall motion was observed at the dose of 4.0x10\textsuperscript{5} microspheres/g myocardium, and lactate production only occurred at the doses of more than 4.0x10\textsuperscript{5} microspheres/g myocardium. Although the maximal number of microspheres required for embo-
TABLE 2. Effects of 8-Phenyltheophylline on Improvements of Contractile and Metabolic Parameters Due to the Treatments With SOD (Groups E and F)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>8-PT</th>
<th>SOD</th>
<th>Extent of embolization (% maximal embolization)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0–10</td>
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<td>Coronary perfusion pressure (mm Hg)</td>
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<td></td>
<td></td>
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<tr>
<td>Without SOD</td>
<td>103±8</td>
<td>102±6</td>
<td>102±6</td>
<td>104±2</td>
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<tr>
<td>With SOD</td>
<td>98±6</td>
<td>97±7</td>
<td>97±5</td>
<td>100±5</td>
</tr>
<tr>
<td>Coronary blood flow (ml/100 g/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without SOD</td>
<td>92±2</td>
<td>92±3</td>
<td>92±3</td>
<td>108±5</td>
</tr>
<tr>
<td>With SOD</td>
<td>91±3</td>
<td>92±3</td>
<td>91±3</td>
<td>107±8</td>
</tr>
<tr>
<td>MVO2 (ml/100 g/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Without SOD</td>
<td>6.6±0.3</td>
<td>6.4±3</td>
<td>6.4±3</td>
<td>5.8±0.7</td>
</tr>
<tr>
<td>With SOD</td>
<td>6.4±0.4</td>
<td>6.5±3</td>
<td>6.3±3</td>
<td>5.2±0.5</td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Without SOD</td>
<td>24.7±1.1</td>
<td>24.7±2.0</td>
<td>24.7±2.0</td>
<td>12.5±1.3</td>
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<tr>
<td>With SOD</td>
<td>25.3±2.6</td>
<td>24.5±1.0</td>
<td>23.2±1.0</td>
<td>12.6±1.3</td>
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<tr>
<td>Lactate extraction rate (%)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Without SOD</td>
<td>28.4±4.0</td>
<td>27.9±3.9</td>
<td>27.9±3.9</td>
<td>-9.8±3.4</td>
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<td>With SOD</td>
<td>31.3±3.6</td>
<td>30.7±2.5</td>
<td>29.0±2.5</td>
<td>-8.6±2.9</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
Group E, with SOD (n=5); group F, without SOD (n=5). 8-PT, 8-phenyltheophylline; SOD, superoxide dismutase; MVO2, myocardial oxygen consumption.

There are no significant changes in each parameter between the SOD-treated and untreated conditions by repeated measures analysis of variance.

Effects of 8-Phenyltheophylline on the Beneficial Actions of SOD

We have previously reported that coronary hyperemic flow due to released adenosine is beneficial during coronary microvascular embolization.10–12 To test whether the beneficial effects of SOD are attrib-

![Bar graphs of changes in mean fractional shortening, lactate extraction rate, coronary arteriovenous oxygen difference, and coronary blood flow during repetitive injections of microspheres in untreated recombinant human superoxide dismutase (SOD), CV3611, and allopurinol groups (protocol 1). Density of microspheres causing embolization was calculated as the injected number of the microspheres per gram of myocardium. Up to 4.0×10^5 microspheres/g myocardium caused embolization; fractional shortening, lactate extraction rate, and coronary blood flow in SOD, CV3611, and allopurinol groups were significantly (p<0.001) larger than those in the control group; p values were obtained from the post hoc test appropriately corrected for multiple comparison.

Embolized microspheres (× 10^5/g·myocardium)
Effects of Histological Changes in the Basal Condition After Embolization in the Treated and Untreated Groups

The effects of SOD during treatment with 8-phenyltheophylline were examined in the baseline conditions before and after microvascular embolization. When 8-phenyltheophylline was administered before the microspheres, there were no significant changes in hemodynamic parameters (Table 2). As shown in Table 2, in the presence of the adenosine receptor antagonist, all beneficial effects of SOD during microvascular embolization were no longer seen. Furthermore, the increased number of microspheres required for maximal embolization during treatment with SOD was abolished when 8-phenyltheophylline was administered (5.4±0.3×10^5 microspheres/g myocardium in group E; 5.3±0.2×10^5 microspheres/g myocardium in group F; p=NS). These results indicate that the beneficial effect of the SOD treatment on microcirculation-induced ischemia is mainly attributed to the enhanced adenosine release; however, the increased number of microspheres required for maximal embolization with SOD treatment in group B may be due to the other factors as well.

Effects of SOD, CV3611, and Allopurinol on Tissue Water Content and Metabolic and Histological Changes

Table 3 depicts the coronary hemodynamic parameters and metabolic changes before and after administration of a single dose of the microspheres (3.0±0.1×10^5 microspheres/g myocardium) that caused embolization. Systemic and coronary hemodynamic parameters (i.e., heart rate, coronary perfusion pressure, and coronary blood flow) and metabolic parameters (i.e., coronary AVo_2,D, lactate extraction rate, MVo_2, and adenosine release) at the basal condition before embolization were comparable. Administration of drugs per se did not alter the hemodynamic and metabolic conditions (Table 3). After embolization in the LAD area, coronary perfusion pressure remained unchanged in all groups. In group A (control), coronary blood flow slightly decreased after embolization and was associated with a decrease in coronary AVo_2,D. In other groups (groups B, C, and D), however, coronary blood flow was significantly (p<0.001) increased with a decrease in the arteriovenous oxygen difference compared with the changes in these parameters in the untreated condition. This hyperemic flow was sustained 60 minutes after embolization, and there was no significant difference among the treated groups. Lactate production in the treated groups was significantly (p<0.001) less than that in the control group, indicating that the ischemic changes were attenuated by the treatment with SOD, CV3611, or allopurinol. MVo_2 in the embolized area was also significantly (p<0.001) less in the control group (Table 3), although heart rate and coronary perfusion pressure were not significantly different from those in the treated groups. Adenosine was massively released in all groups after embolization (Table 3), and the changes in the extent of released adenosine under SOD, CV3611, and allopurinol conditions during 60 minutes are significantly (p<0.05) larger than those under the untreated condition.

Figure 4 shows the tissue water content of the embolized area (LAD area) and the nonembolized area (LCx area) 10 and 60 minutes after embolization with or without SOD treatment. There were no significant differences in the tissue water content in the nonischemic area between the control and SOD-treated groups. SOD treatment significantly attenuated tissue water gain in ischemic areas both 10 (9±2%) and 60 minutes (19±3%) after embolization. Figure 5 depicts the tissue water content 60 minutes after embolization in the treated groups (SOD, CV3611, and allopurinol). There were no significant differences in the tissue water content in the nonischemic area. In the LAD area, tissue water gains 60 minutes after embolization in the treated groups were significantly less than those in the control group, but they were not significantly different among the three treated groups.

When 8-phenyltheophylline was given, the beneficial effects of SOD for the reduction of edema formation were abolished; 60 minutes after embolization, tissue water content became 4.9±0.6 g H_2O/g dry wt, and SOD under the treatment of SOD did not change the tissue water content 60 minutes after embolization (5.0±0.4 g H_2O/g dry wt).

Microscopic examination 60 minutes after embolization in the control group showed a marked myocardial edema in the demarcated patchy lesions composed of 10–15 injured myocytes (86–166 μm in

![Graph](http://circ.ahajournals.org/https://circ.ahajournals.org/)  
**Figure 4.** Bar graphs of myocardial tissue water content in the embolized (LAD area) and the nonembolized area (LCx area) 10 and 60 minutes after embolization with (B) or without (A) SOD treatment. Tissue water content was obtained as (wet weight minus dry weight) divided by dry weight. *p<0.01 vs. control group A assessed by the post-hoc test appropriately corrected for multiple comparison. Increases in tissue water content in LAD area compared with LCx area is significantly (p<0.001) attenuated in the SOD group compared with the control group 10 and 60 minutes after embolization. LAD, left anterior descending coronary artery; LCx, left circumflex coronary artery; SOD, recombinant human superoxide dismutase.
diameter, Figure 6). Electron microscopy showed the marked intracellular edema in the control group (Figure 7). Although mild alterations in mitochondrial structures and subsarcolemmal vesicle formation were observed, neither the amorphous dense bodies nor sarcolemmal disruption was observed. In the interstitial space, extracellular edema was also observed. However, endothelial cell swelling was not often observed, even in the control (untreated) group. Although granulocytes were occasionally observed in the capillary lumens, adherence to the endothelium was seldom observed. In the treated groups, both interstitial and intracellular edema was much less than that in the control group, which is compatible with the results in the tissue water content. These histological changes were comparable among the treated groups. The density of the ischemic lesions and both interstitial and intracellular edema could not be quantified in the present study because the tissue samples for electron microscopic examination were too small.

**Discussion**

In the present study, we found in coronary microvascular embolization that 1) sustained patchy myocardial ischemia produced marked myocardial edema, 2) oxygen-derived free radicals played an important role in precipitation of myocardial ischemia and edema formation, and 3) some of the beneficial effects of radical scavengers and allopurinol were mediated by enhanced adenosine release.
Coronary Microvascular Embolization Model and the Role of Adenosine

We have reported that a large amount of adenosine is released after coronary microvascular embolization with microspheres, causing hyperemic coronary flow.\(^\text{10}\) Histological examination of the embolized myocardium showed patchy ischemic foci distributed throughout the ventricular wall of the embolized area; each ischemic focus ranged between 86 and 166 \(\mu\)m in a tangential section. Thus, it is likely that adenosine released by the ischemic myocytes is washed out through the local coronary vessels in the nonischemic areas distributed between the ischemic foci. Therefore, the measured adenosine in the coronary vein may be proportional to the total amount of adenosine production at the ischemic myocytes although the washout efficacy may also influence the released amount. Accordingly, in our ischemic model, the amount of adenosine release reflects an extent of myocardial ischemia caused by the microvascular blockade. Adenosine is a potent coronary vasodilator and may enhance the extent of washout of ischemic metabolites, including adenosine. The exposure to adenosine augments the oxygen delivery through coronary vasodilation. It is also known that adenosine attenuates leukocyte activation and adhesion and inhibits free radical formation by leukocytes. Negative inotropic effects of adenosine may also elicit protective effects against ischemic injury in concert with other beneficial actions. Redistribution of coronary blood flow by adenosine may be disadvantageous for the ischemic myocardium if the steal phenomenon is induced. Therefore, the role of adenosine may be complex in the pathophysiology of microvascular embolization, and thus, the results obtained in the study should be carefully interpreted.

Edema Formation in Coronary Microvascular Embolization

It was recently reported that thromboembolism in small coronary vessels is not infrequent, especially after coronary reperfusion.\(^\text{20}\) Platelet aggregation and leukocyte plugging in intramural small vessels may cause or at least augment the no-reflow phenomenon after recanalization of the occluded coronary arteries either spontaneously or by thrombolytic therapy. To mimic coronary microcirculatory abnormalities, we administered 15-\(\mu\)m microspheres in the coronary arteries to induce embolization, although in our model, myocardial tissue is not ischemic before microvascular embolization. As previously reported,\(^\text{10,12}\) coronary microvascular embolization induced hyperemic flow that was associated with a massive release of adenosine, whereas myocardial ischemic injury, assessed by both fractional shortening and lactate extraction rate, was progressive in a dose-dependent manner (Figure 3). In our experimental model, we also observed a marked increase in tissue water content by 32\% 1 hour after embolization. Light and electron microscopic examinations revealed a well-demarcated ischemic area associated with marked intracellular and extracellular edema (Figure 6). In the surrounding area adjacent to the ischemic area, no edematous changes were observed. Because the total tissue water content determined in

![Image](http://circ.ahajournals.org/)

**FIGURE 7.** Electron microscopic section of myocardium 60 minutes after embolization from control (group A). Prominent intracellular edema is observed with marked separation of organelles. In subsarcolemmal space, vesicle formation (V) is observed. Interstitial space is widened and contains edema fluid (E). Note that this injured cell is adjacent to the intact cell (IC). Original magnification, \(\times3,780\).
TABLE 3. Coronary Hemodynamic and Metabolic Parameters Before and After Microvascular Embolization (Protocol 2)

<table>
<thead>
<tr>
<th>Heart rate (beats/min)</th>
<th>Before embolization</th>
<th>After embolization</th>
<th>10 Minutes</th>
<th>60 Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Untreated (n=6)</td>
<td>123±6</td>
<td>...</td>
<td>115±6</td>
<td>118±5</td>
</tr>
<tr>
<td>B. SOD (n=6)</td>
<td>125±7</td>
<td>123±6</td>
<td>118±8</td>
<td>123±4</td>
</tr>
<tr>
<td>C. CV3611 (n=6)</td>
<td>121±6</td>
<td>120±7</td>
<td>118±5</td>
<td>120±6</td>
</tr>
<tr>
<td>D. Allopurinol (n=6)</td>
<td>118±8</td>
<td>120±5</td>
<td>123±8</td>
<td>123±6</td>
</tr>
</tbody>
</table>

Coronary perfusion pressure (mm Hg)

| A. Untreated (n=6)     | 89±7                | ...               | 90±6       | 92±4       |
| B. SOD (n=6)           | 90±6                | 88±5              | 86±4       | 88±4       |
| C. CV3611 (n=6)        | 88±5                | 90±6              | 91±5       | 86±3       |
| D. Allopurinol (n=6)   | 92±6                | 90±6              | 88±5       | 86±6       |

Coronary blood flow (ml/100 g/min)

| A. Untreated (n=6)     | 90±2                | ...               | 85±7       | 80±7       |
| B. SOD (n=6)           | 90±5                | 95±5              | 141±19*    | 138±15†    |
| C. CV3611 (n=6)        | 91±3                | 95±2              | 132±17*    | 138±10†    |
| D. Allopurinol (n=6)   | 92±6                | 91±3              | 140±12†    | 140±18*    |

Coronary vascular resistance (mm Hg/ml/100 g/min)

| A. Untreated (n=6)     | 1.00±0.03           | ...               | 1.05±0.03  | 1.13±0.05  |
| B. SOD (n=6)           | 1.01±0.04           | 0.99±0.05         | 0.61±0.07† | 0.65±0.08† |
| C. CV3611 (n=6)        | 0.99±0.05           | 0.98±0.05         | 0.63±0.08† | 0.66±0.07† |
| D. Allopurinol (n=6)   | 1.02±0.04           | 1.01±0.04         | 0.62±0.07† | 0.62±0.09* |

AVO2D (ml/dl)

| A. Untreated (n=6)     | 7.0±0.3             | ...               | 5.0±0.3    | 5.3±0.6    |
| B. SOD (n=6)           | 7.3±0.8             | 7.1±0.7           | 4.6±0.6    | 4.2±0.4    |
| C. CV3611 (n=6)        | 6.4±0.6             | 7.0±0.6           | 5.0±0.5    | 5.1±0.8    |
| D. Allopurinol (n=6)   | 6.8±0.6             | 6.8±0.7           | 4.8±0.6    | 5.2±0.8    |

MVO2 (ml/100 g/min)

| A. Untreated (n=6)     | 6.4±0.4             | ...               | 4.5±0.5    | 4.3±0.9    |
| B. SOD (n=6)           | 6.6±0.7             | 6.7±0.6           | 6.4±1.1*   | 5.9±0.9*   |
| C. CV3611 (n=6)        | 6.0±0.7             | 6.7±0.7           | 6.6±1.0*   | 5.9±0.7*   |
| D. Allopurinol (n=6)   | 6.3±0.8             | 6.2±0.5           | 6.4±1.2*   | 6.6±0.8*   |

Lactate extraction rate (%)

| A. Untreated (n=6)     | 19.8±3.9            | ...               | -17.5±3.4  | -18.0±3.2  |
| B. SOD (n=6)           | 17.3±5.3            | 18.1±2.0          | -1.0±3.1*  | -1.0±4.0*  |
| C. CV3611 (n=6)        | 20.1±4.4            | 20.0±3.8          | -2.4±3.0*  | -1.0±2.8*  |
| D. Allopurinol (n=6)   | 19.7±4.2            | 19.8±4.8          | -0.2±3.4*  | -1.8±3.0*  |

AdR (nmol/100 g/min)

| A. Untreated (n=4)     | 0.4±0.8             | ...               | 5.6±1.8    | 5.8±1.7    |
| B. SOD (n=4)           | 0.3±1.0             | 0.1±0.8           | 9.8±2.2    | 10.1±2.4   |
| C. CV3611 (n=4)        | 0.5±0.9             | 0.5±1.1           | 8.8±3.0    | 9.4±2.9    |
| D. Allopurinol (n=4)   | 0.8±1.5             | 0.4±1.1           | 10.1±2.9   | 10.2±3.0   |

Values are mean±SEM.

AVO2D, coronary arteriovenous oxygen difference; MVO2, myocardial oxygen consumption; AdR, adenosine release into coronary vein.

*p<0.05 and tp<0.01 (unpaired t test) vs. the untreated condition. Significant differences in the responses of coronary blood flow (p<0.001), coronary vascular resistance (p<0.001), AVO2D (p<0.05), MVO2 (p<0.001), lactate extraction rate (p<0.001), and AdR (p<0.05) to microvascular embolization were observed between A vs. B, C, and D. These p values are obtained from the post hoc test appropriately corrected from multiple comparison.
the present study reflects the water content of both ischemic and nonischemic areas, the actual increase in tissue water content in the ischemic region should be more than the observed value. Previous studies reported tissue water gains of 10–20% during postischemic reperfusion or persistent low perfusion. Thus, in our opinion, edema in our embolized model is more prominent than in previously reported ischemic models.

It is known that with coronary reperfusion after ischemia, a cellular swelling occurs, and “explosive” swelling is observed in irreversibly injured cells. During postischemic reflow, an increment of the tissue water content can be detected as early as 30 seconds after reperfusion. However, reflow is not essential for cellular swelling in the ischemic heart because myocardial edema was also observed in the ischemic model of diminished coronary perfusion without abrupt reflow. In the latter model, cellular water content was increased by 10.9% during 45 minutes of low perfusion, whereas the extracellular volume was unchanged. Even during coronary occlusion without reflow, intracellular swelling can occur accompanying a decrease in the extracellular space with total tissue water content unchanged. In the present histological study, we fixed the tissue with high osmolar glutaraldehyde buffer (350 mosmol/l) to avoid artificial increases in extracellular tissue water content in the ischemic myocardium. Thus, existence of edema both in extracellular and intracellular spaces in our model (Figure 6) indicates that the water supply from the nonischemic surrounding area augments the edema formation both in the extracellular and intracellular spaces.

The Role of Free Radicals in Myocardial Ischemia and Edema Formation

During ischemia, ATP is broken down to AMP, which is ultimately metabolized to hypoxanthine. When molecular oxygen is reintroduced to cells containing high concentrations of hypoxanthine, xanthine oxidase converted from xanthine hydrogenase during ischemia produces superoxide anions. In ischemia due to large coronary artery occlusion, oxygen restored during reperfusion acts as the electron acceptor, and superoxide produced as hypoxanthine is metabolized to uric acid. In our ischemic model, adenosine, a precursor of hypoxanthine, was released, providing the substrate for oxygen-derived free radical formation. Oxygen diffuses to the ischemic foci from the interspersed hyperemic myocardium, and thus, production of free radicals may be most prominent at the periphery of the ischemic region. In this microvascular ischemic model, the ischemic area was not reperfused. Ischemic regions are surrounded by hyperemic perfusion as is evidenced by a significant increase in total coronary blood flow associated with a decrease in AV02D (Figure 3). Thus, ischemic areas with patchy distribution may be supplied with water and oxygen from the surrounding hyperemic areas. Because superoxide anion (O2−) produced at the cellular membranes can move to the adjacent cells within a distance of 100 μm when SOD is absent, the entire portion of the ischemic region that is 86–166 μm in diameter may become a target of free radicals. Another possible source of oxygen radicals is activated neutrophils that produce oxygen-derived free radicals through NADPH oxidase and through arachidonic acid metabolism and release a variety of other cytotoxic products, including lysosomal hydrolases, neutral proteases, eicosatetraenoic acids, and leukotrienes. However, leukocyte accumulation was not prominent upon histological examination in this model.

In this microvascular ischemic model, oxygen-derived free radicals play an important role in enhancing myocardial patchy ischemia and edema formation because SOD, allopurinol (a xanthine oxidase inhibitor), and CV3611 markedly reduced both ischemic injury and myocardial edema. CV3611 is reported to scavenge superoxide anions and hydroxyl radicals and lipid peroxides. Of interest, the beneficial effects of the radical scavengers and allopurinol may be mediated by enhanced release of adenosine (Table 3). This increased release of adenosine may be attributed to the improved washout of adenosine by augmenting flow and attenuation of the interstitial edema. However, not only was the concentration of adenosine in blood from the coronary vein enhanced, but also the total amount of adenosine release was augmented by all three treatments. Another possibility is that free radical production may limit adenosine release by limiting flow through embolized vessels without affecting adenosine production; in untreated animals, adenosine produced by ischemic myocytes may be degraded before it can diffuse out of the ischemic zone, whereas the free radical scavengers may increase flow slightly and allow more adenosine to exit the ischemic zone before it is degraded. Although this hypothesis could not be excluded, it is more likely that the release of adenosine may improve myocardial ischemia and attenuate myocardial edema because the beneficial effect of SOD was negated by inhibiting the effect of adenosine with 8-phenyltheophylline treatment. This hypothesis is also supported by our previous studies; treatment with prazosin worsens myocardial ischemia by inhibiting release of adenosine, and treatment with clonidine ameliorates ischemic changes by enhancing the release of adenosine.

The mechanisms of augmented adenosine release by the three agents are unknown. Free radicals generated in the ischemic myocardium produced by coronary microvascular embolization may inhibit the release of adenosine and, hence, exacerbate the myocardial ischemia. Reduction in free radicals by the three agents may, thus, cause adenosine release. Alternatively, CV3611 or allopurinol may directly cause purine catabolic pathways to increase the relative amount of adenosine.
It is reported that superoxide anions and hydroxyl radicals increase the vascular permeability in skeletal muscle, brain, and lung. Thus, oxygen-derived free radicals generated in the microvascular embolization model may enhance the interstitial edema. If the effect of SOD observed in the present study is primarily attributed to prevention of increased vascular permeability induced by free radicals, then SOD treatment should have been effective in the absence of adenosine effect. In contrast, SOD was ineffective for the prevention of myocardial edema when adenosine receptors were blocked by 8-phenyltheophylline. Several possible mechanisms can be considered. It is reported that SOD inhibits the degradation of endothelium-derived relaxing factor, which may improve the microvascular perfusion and enhance the release of adenosine. Also, SOD may reduce edema formation because of its colloid osmotic effect and improve the washout of adenosine. However, this hypothesis is not plausible in this case because the total dose of SOD (5 mg protein/kg body wt) was too small to exert the osmotic effect, and both CV3611 and allopurinol have only minor effects on osmolarity. The second possibility is that oxygen-derived free radicals may cause the formation of lipid peroxides and hydroperoxides by reacting with polyunsaturated fatty acids, which, in turn, inactivate or degrade the membrane enzymes, for example, 5'-nucleotidase, which is an important enzyme in the production of adenosine in the ischemic myocardium. The third possibility is that oxygen-derived free radicals may directly destroy the adenosine molecule. Although the underlying mechanisms of augmented adenosine release by reducing free radicals are not clarified in the present study, the interaction between adenosine production and free radicals may play a central role both in the patchy myocardial ischemia and edema formation in acute microvascular embolization.

Effects of Radical Scavengers and Inhibitor on the Number of Microspheres for Maximal Microvascular Embolization

Another finding in the microvascular embolization model is that the maximal number of microspheres required to abolish flow was increased by allopurinol (9.9 ± 1.0 x 10^5 microspheres/g myocardium), which was almost twofold over control (5.2 ± 0.4 x 10^5 microspheres/g myocardium). Although the beneficial effects of the scavengers and inhibitor on myocardial edema and function may be mediated by enhanced adenosine production, the marked increase in the number of microspheres for embolization could not be explained by an increase in adenosine release because in previous studies prazosin (which inhibits the release of adenosine), clonidine (which enhances the vasodilatory effect of adenosine), and theophylline (which blocks the adenosine receptors) did not alter the maximal number of microspheres required for embolization. These lines of evidence imply that factors other than enhanced release of adenosine may determine the maximal number of microspheres required for embolization. Vascular compression by edema may augment the coronary perfusion deficit, and reduction of myocardial edema by the radical scavengers and inhibitor may prevent this and, thus, increase the number of microspheres for total obstruction of the vascular bed. Another plausible mechanism is that in control experiments microspheres are tightly trapped in the vascular lumen either by the endothelial cell swelling or vasoconstriction induced by free radicals, whereas the radical scavengers and inhibitor prevent the endothelial cellular swelling or vasoconstriction, allowing only a partial obstruction of microspheres. This hypothesis may be supported by the recent report that SOD and catalase given at the time of reperfusion attenuate both endothelial vascular injury and the "low reflow" phenomenon. In the present study, we carefully examined endothelial cell swelling in the electron microscopic study; however, endothelial cell swelling was not frequently observed, and the effect of the scavengers and inhibitor could not be clearly observed. In the microvascular embolization model, ischemic lesions are patchily distributed, and technical difficulties in differentiation of vasculatures in ischemic area versus nonischemic area may limit histological study. Whatever the mechanism, in the sustained patchy myocardial ischemia induced by coronary microvascular embolization, oxygen-derived free radicals may play a major role in the formation of myocardial ischemia and edema probably through the effects on adenosine release and microvascular endothelial cell injury.

However, it should be noted that in the heart with thromboembolism of small vessels during reperfusion after an ischemic episode, the tissue is already ischemic, and the effect of the thromboemboli in the small vessel is to make reflow heterogeneous and to prolong ischemia. In the present microvascular embolization model, the myocardium is normal before microvascular embolization. The exact clinical relevance of the microvascular embolization model to atherosclerotic coronary artery disease remains to be determined. Nonetheless, these studies suggest an important relationship among reduction of free radicals, adenosine, and ischemic damage.

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