Upregulation of Vascular Extracellular Superoxide Dismutase in Patients With Acute Coronary Syndromes

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Objective—We examined the vascular expression levels of extracellular superoxide dismutase (EC-SOD), a major antioxidant enzyme in the cardiovascular system, in patients with acute coronary syndromes.

Methods and Results—Twenty-one consecutive patients with acute myocardial infarction (AMI), 14 patients with unstable angina, 11 patients with stable angina, and 20 control subjects were studied. The levels of vascular EC-SOD expression were assessed by the difference in plasma EC-SOD concentrations before and after intravenous heparin injection. In the patients with AMI, vascular EC-SOD expression (ng/mL) was significantly higher on day 1 after the onset of AMI (148±10) as compared with the control subjects (116±6, P<0.05). The vascular EC-SOD expression returned to the normal range on day 7 (104±8), and that level persisted thereafter. The vascular EC-SOD expression was also significantly higher in the patients with unstable angina (160±13) than in those with stable angina (122±10) or in the controls (116±6, P<0.05 each). Moreover, in the patients with AMI, higher levels of vascular EC-SOD expression on day 1 were significantly associated with smaller myocardial infarct size (P<0.05).

Conclusions—This is the first clinical demonstration showing that vascular EC-SOD may be upregulated in acute coronary syndromes in humans in vivo. EC-SOD may play an important protective role against increased oxidative stress during acute ischemic coronary events. (Arterioscler Thromb Vasc Biol. 2004;24:106-111.)

Key Words: superoxide dismutase ■ acute coronary syndromes ■ myocardial infarction ■ angina pectoris ■ oxidative stress

Superoxide anion is thought to play an important role in the pathogenesis of atherosclerotic coronary artery diseases.1–3 This radically exerts multiple deleterious cardiovascular actions, such as the induction of proinflammatory genes, oxidation of low-density lipoprotein (LDL), stimulation of smooth muscle cell proliferation, or damage of endothelial cells and cardiomyocytes. Moreover, superoxide anion reacts with a potent vasodilator and important antiatherogenic factor, nitric oxide, causing loss of nitric oxide bioactivity and subsequent formation of the oxidant peroxynitrite (ONOO–). Superoxide anion is produced in a variety of cells, such as the induction of proinflammatory genes, oxidation of low-density lipoprotein (LDL), stimulation of smooth muscle cell proliferation, or damage of endothelial cells and cardiomyocytes. Moreover, superoxide anion reacts with a potent vasodilator and important antiatherogenic factor, nitric oxide, causing loss of nitric oxide bioactivity and subsequent formation of the oxidant peroxynitrite (ONOO–). Superoxide anion is produced in a variety of cells, such as the induction of proinflammatory genes, oxidation of low-density lipoprotein (LDL), stimulation of smooth muscle cell proliferation, or damage of endothelial cells and cardiomyocytes. Moreover, superoxide anion reacts with a potent vasodilator and important antiatherogenic factor, nitric oxide, causing loss of nitric oxide bioactivity and subsequent formation of the oxidant peroxynitrite (ONOO–).

Reduced production of superoxide anion has been shown under atherosclerotic/inflammatory vascular conditions and during reperfusion after myocardial ischemia, leading to the development of atherosclerosis, myocellular ischemia/reperfusion injury (myocardial dysfunction), and/or arrhythmias.1–3 Superoxide dismutase (SOD) is an enzyme responsible for the dismutation of superoxide anion to hydrogen peroxide and oxygen.4–6 Three different isoforms of SOD have been described. Copper/zinc SOD (Cu/Zn-SOD)7 and manganese SOD (Mn-SOD)8 are intracellularly present in the cytosol and mitochondria, respectively. By contrast, extracellular SOD (EC-SOD)9 localizes in extracellular space by binding to heparan sulfate proteoglycans in the interstitial matrix and on cell membranes. This could allow EC-SOD to efficiently scavenge superoxide anion in its specific location. EC-SOD is mainly synthesized and secreted from vascular smooth muscle cells and macrophages, and distributes throughout the vascular wall with high concentrations in endothelial cells and the intima.6,10 EC-SOD is the predominant arterial SOD isoform, as evidenced by the facts that EC-SOD activity is ∼100 times higher in human coronary artery and thoracic aorta than in other tissues (skeletal muscle or fat tissue),6,11 and that 70% of total SOD activity is derived from EC-SOD in human aorta.12 Thus, EC-SOD plays a central role in cardiovascular antioxidant mechanisms.4–6

It has been shown that vascular overexpression of EC-SOD improves endothelial dysfunction in rats13 and reduces myocardial and cerebral infarct size in rabbits14...
and mice,\textsuperscript{15} respectively, and that vascular deficiency of EC-SOD impairs vasorelaxation\textsuperscript{16} and enlarges the infarct size\textsuperscript{17} in mice. These results indicate that the expression levels of EC-SOD in the vasculature are critically important for the regulation of vasomotor function and the development of vascular disorders. It has recently been reported that vascular EC-SOD is downregulated in patients with stable exertional angina pectoris, suggesting the pathogenetic role of EC-SOD in chronic coronary artery disease.\textsuperscript{18} However, the role of EC-SOD in acute coronary syndromes remains to be clarified.

Acute coronary syndromes result from a reduction in coronary blood flow caused by changes in vascular tone, platelet aggregation, and/or platelet-fibrin thrombus formation at the site of fissured or ruptured atherosclerotic plaques.\textsuperscript{19} Increased oxidative stress has been evidenced in patients suffering from acute myocardial infarction and unstable angina pectoris.\textsuperscript{20} Although the functional significance of EC-SOD under such oxidative conditions is of biological interest, no study has ever addressed the possible involvement of this major antioxidant enzyme. Thus, the present study was designed to examine the alterations of vascular EC-SOD expression in patients with acute coronary syndromes.

### Materials and Methods

#### Study Subjects

Twenty-one consecutive patients with acute myocardial infarction (AMI) who were admitted to our hospital within 6 hours after the onset of chest pain, 14 patients with unstable angina pectoris, 11 patients with stable angina pectoris, and 20 age- and sex-matched control subjects were prospectively studied. The characteristics of the patients are shown in Table 1. All patients gave written informed consent. The present study was reviewed and approved by the Human Research Committee at the University of Occupational and Environmental Health, School of Medicine, Japan, and was performed according to the Institutional Guidelines. The diagnosis of AMI was made by a history of typical chest pain, characteristic ECG alterations including ST-segment elevation and appearance of abnormal Q waves, and an increase in serum cardiac enzymes.\textsuperscript{19} The diagnosis of unstable angina pectoris was made when a more severe, prolonged, or frequent angina, superimposed on a preexisting pattern of stable exertion-related angina pectoris or of angina pectoris at rest, was associated with reversible ischemic ST-segment changes.\textsuperscript{19} The diagnosis of stable angina pectoris was made by a typical chest pain with ischemic ST-segment depression on exertion or at rest.\textsuperscript{19} All patients had at least one significant organic stenosis (>75% stenosis of the luminal diameter by the AHA classification) or significant coronary artery spasm after intracoronary injection of acetylcholine (>75% contraction of the luminal diameter with ischemic ST-segment changes and/or chest pain) proved by coronary arteriography. Age- and sex-matched healthy volunteers without any symptoms or any ECG abnormality were selected as control subjects. At the time of the study, there was no patient receiving warfarin or heparan therapy that may affect the measurement of plasma EC-SOD concentrations. Patients with renal failure, malignancy, profound anemia, inflammatory diseases, and disorders of the blood coagulation system were excluded from the present study.

In the patients with AMI on day 1 after the onset and with unstable angina, the time from when the cardiovascular drugs were last taken to when the blood sampling was performed (hours) was as follows: aspirin, 9.5±1.8 (n = 16) and 5.1±0.6 (n = 10); nitrates, 2.4±0.8 (n = 13) and 5.2±0.9 (n = 13); calcium channel blockers, 8.1±2.7 (n = 13) and 5.7±0.7 (n = 13); angiotensin-converting enzyme inhibitors, 5.2±3.7 (n = 2) and 4.5±1.2 (n = 2); angiotensin II type 1 receptor blockers, 10.0 (n = 1) and 4.5±1.7 (n = 2); beta blockers, (n = 0) and 3.0±1.5 (n = 2), respectively.

#### Evaluation of Vascular EC-SOD Expression Levels

EC-SOD anchors to heparan sulfate proteoglycans on the surface of the arterial wall, and is released into the blood by heparan admin-

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**Table 1. Clinical Characteristics of the Study Patients**

<table>
<thead>
<tr>
<th></th>
<th>AMI (n = 21)</th>
<th>Unstable Angina (n = 14)</th>
<th>Stable Angina (n = 11)</th>
<th>Control (n = 20)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>67.9±1.9 [51–81]</td>
<td>70.6±1.9 [55–83]</td>
<td>65.7±3.6 [42–80]</td>
<td>66.5±1.7 [43–76]</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (male/female, n)</td>
<td>16/5</td>
<td>6/8</td>
<td>8/3</td>
<td>15/5</td>
<td>NS</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>65.9±5.5</td>
<td>55.9±3.5</td>
<td>62.4±3.2</td>
<td>58.9±1.8</td>
<td>NS</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>157.7±4.7</td>
<td>155±4.3</td>
<td>158±2.2</td>
<td>158±1.5</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23.1±0.6</td>
<td>23.0±0.8</td>
<td>24.5±0.8</td>
<td>23.3±0.6</td>
<td>NS</td>
</tr>
<tr>
<td>Blood pressure (mm Hg, systolic/diastolic)</td>
<td>131±4/73±3</td>
<td>143±5/76±4</td>
<td>136±4/75±3</td>
<td>140±5/81±3</td>
<td>NS</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>87.3±3.6*</td>
<td>78.4±4.4</td>
<td>69.0±2.8</td>
<td>66.3±2.1</td>
<td>*P&lt;0.05</td>
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<tr>
<td>Blood biochemical data</td>
<td></td>
<td></td>
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<tr>
<td>Fasting blood sugar (mg/dL)</td>
<td>117.4±5.8</td>
<td>108.1±6.2</td>
<td>104.9±5.8</td>
<td>101.9±3.0</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>192.8±7.1</td>
<td>194.7±6.3</td>
<td>196.1±13.8</td>
<td>172.3±6.1</td>
<td>NS</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>146.8±15.7</td>
<td>137.1±21.2</td>
<td>136.7±15.9</td>
<td>137.7±17.3</td>
<td>NS</td>
</tr>
<tr>
<td>Urea nitrogen (mg/dL)</td>
<td>16.9±1.2</td>
<td>18.5±1.8</td>
<td>15.6±1.2</td>
<td>14.4±1.1</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>1.3±0.4</td>
<td>0.9±0.1</td>
<td>0.8±0.7</td>
<td>0.9±0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Left ventricular ejection fraction (%)</td>
<td>53.4±2.9*</td>
<td>61.7±3.9</td>
<td>64.4±2.1</td>
<td>69.0±4.7</td>
<td>*P&lt;0.05</td>
</tr>
<tr>
<td>NYHA class</td>
<td>4.0±0.0*</td>
<td>2.4±0.2</td>
<td>1.6±0.2</td>
<td>1.1±0.1</td>
<td>*P&lt;0.05</td>
</tr>
<tr>
<td>Killip classification</td>
<td>2.1±0.3</td>
<td>1.0±0.0</td>
<td>1.0±0.0</td>
<td>1.5±0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Previous myocardial infarction, n (%)</td>
<td>2 (9)</td>
<td>3 (21)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>NS</td>
</tr>
<tr>
<td>Coronary risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>4 (18)</td>
<td>6 (43)</td>
<td>2 (18)</td>
<td>5 (25)</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>12 (54)</td>
<td>10 (71)</td>
<td>8 (73)</td>
<td>9 (45)</td>
<td>NS</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>5 (24)</td>
<td>4 (29)</td>
<td>6 (55)</td>
<td>4 (20)</td>
<td>NS</td>
</tr>
<tr>
<td>Current smoking, n (%)</td>
<td>9 (41)</td>
<td>5 (36)</td>
<td>6 (55)</td>
<td>7 (35)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM. n indicates patient number; NS, not significant. *P<0.05 vs control subjects.
tation competitively. On the basis of these properties, the levels of vascular EC-SOD expression were evaluated by the difference in plasma EC-SOD concentrations before and 10 minutes after intravenous bolus injection of 5000 U heparan (heparan-releasable EC-SOD), as previously reported. In the present study, after heparan injection, plasma EC-SOD concentrations acutely increased at 5 minutes, reached the maximum level at 10 to 15 minutes, and then returned to the baseline level at 6 hours. The time course of plasma EC-SOD concentrations after heparan injection was almost identical among the 4 groups studied (data not shown).

In most cases, venous blood sampling was performed in the morning under fasting condition. The blood samples of the patients with AMI on day 1 or of those with unstable angina pectoris were taken immediately after admission to our hospital or during anginal attacks, respectively. In the patients with AMI on day 1, the levels of vascular EC-SOD expression were obtained at 3.0±0.4 (mean±SEM) hours after the onset of AMI, and maximum values of serum creatine kinase and lactate dehydrogenase concentrations (the highest of a series of measurements post AMI) were obtained at 20.1±1.3 and 31.8±2.6 hours after the onset of AMI, respectively.

**Measurement of Plasma EC-SOD Concentrations**

The blood was collected in vacuum tubes containing sodium EDTA. The blood samples were centrifuged at 3000 rpm, 4°C, for 15 minutes, and the supernatants were stored at –80°C. Plasma EC-SOD concentrations were measured by a two-step ELISA, as we previously reported. The lower limit of detection was 50 pg/mL and the working range was up to 50 ng/mL. This ELISA system showed no cross-reactivity with other SOD isoforms.

**Statistical Analysis**

Results are expressed as the mean value±SEM. Statistical analysis was performed by an analysis of variance, a χ² test, an unpaired t test, or regression analysis (least squares linear estimation) where appropriate. If a significant F value was found in an analysis of variance, the Scheffe’s post-hoc test for multiple comparisons was used to identify the differences among groups. The values were considered to be statistically significant when P<0.05.

**Results**

**Patient Characteristics**

Clinical characteristics, including age, sex, body weight, height, body mass index, blood pressure, blood biochemical data, Killip classification, a history of old myocardial infarction, and the presence of coronary risk factors were comparable among the patients with AMI, unstable angina, stable angina, and the control subjects (Table 1). On the other hand, heart rate and class of the New York Heart Association (NYHA) were significantly higher in the patients with AMI than in the control subjects, and left ventricular ejection fraction evaluated by cardiac echocardiography was significantly lower in the patients with AMI than in the controls (Table 1). However, no significant correlation was noted between these factors and the levels of vascular EC-SOD expression (correlation coefficient: -0.06 on heart rate, 0.19 on class of NYHA, and 0.01 on left ventricular ejection fraction, all n=66).

**Levels of Vascular EC-SOD Expression in Patients With Acute Coronary Syndromes**

In the patients with AMI, the expression of vascular EC-SOD was significantly higher on day 1 after the onset of AMI (148±10 ng/mL, n=21) as compared with the control subjects (116±6 ng/mL, n=20, P<0.05, Figure 1). The vascular EC-SOD expression significantly decreased and returned to the normal range on day 7, and that level persisted until day 21. The error bars indicate SEM. *P<0.05 vs control subjects

![Figure 1](image1)

**Figure 1.** The levels of vascular EC-SOD expression in the patients with AMI. The vascular EC-SOD expression was significantly higher on day 1 after the onset of AMI (closed circle) as compared with the control subjects (open circle, P<0.05). The vascular EC-SOD expression was significantly decreased and returned to the normal range on day 7, and that level persisted until day 21. The error bars indicate SEM. *P<0.05 vs control subjects

The vascular EC-SOD expression was comparable between the patients with stable angina (122±10 ng/mL, n=11) and the control subjects (116±6 ng/mL, n=20, Figure 2). By contrast, the vascular EC-SOD expression was significantly higher in the patients with unstable angina (160±13 ng/mL, n=14) than in those with stable angina or in the controls (P<0.05 each, Figure 2).

Plasma EC-SOD concentrations before heparan administration were not significantly different among the patients with AMI, unstable angina, stable angina, or the control subjects (data not shown).

**Effects of Oxygen Inhalation or Medication on Vascular EC-SOD Expression Levels**

To investigate whether or not the increase in vascular EC-SOD expression observed in this study may have been
because of oxygen administration in the emergency room or the regular use of cardiovascular drugs, the effects of oxygen inhalation or medication on vascular EC-SOD expression were examined in the patients with AMI, or in those with unstable angina pectoris. Oxygen inhalation or medication with aspirin, nitrates, calcium channel blockers, angiotensin-converting enzyme inhibitors, angiotensin II type 1 receptor blockers, or beta blockers did not significantly affect the levels of vascular EC-SOD expression in the patients with AMI on day 1, or in those with unstable angina pectoris (Table 2). The medication also did not significantly change the levels of vascular EC-SOD expression in the patients with AMI on day 7, 14, or 21, or in those with stable angina pectoris (data not shown). In addition, although all of the patients with AMI on days 7, 14, and 21 were treated with aspirin and nitrates, there was no significant difference in the levels of vascular EC-SOD expression between these patients and the control subjects (Figure 1). On the other hand, the medication with angiotensin II type 1 receptor blockers tended to increase the levels of vascular EC-SOD expression in a small number of the patients with AMI on day 1 or unstable angina pectoris (Table 2). However, even when the patients taking this drug were excluded, identical statistical results of significantly higher vascular EC-SOD expression in those patients were still noted (P<0.05 each). No patients received antioxidant drugs such as vitamin C, vitamin E, or probucol.

**Effects of Hypercholesterolemia and Aging on Vascular EC-SOD Expression Levels**

We next examined the mechanisms by which the vascular EC-SOD expression is upregulated. Because it was conceivable that increased oxidative stress may lead to compensatory upregulation of vascular EC-SOD expression in patients with acute coronary syndromes, the influence of well-characterized oxidative conditions such as hypercholesterolemia or aging on vascular EC-SOD expression was studied. When the data of the 4 patient groups were analyzed all together (n=66), the levels of vascular EC-SOD expression were positively correlated with serum concentrations of total cholesterol (r=0.28, P<0.05, Figure 3A) and with age (r=0.31, P<0.05, Figure 3B).

**Relationship Between Vascular EC-SOD Expression Levels and Myocardial Infarct Size**

To clarify the role of EC-SOD in acute coronary syndromes, the correlation between the extent of vascular EC-SOD expression and myocardial infarct size was examined. As the levels of vascular EC-SOD expression on day 1 after the onset of AMI became higher, maximum values of serum concentrations of creatine kinase (n=21, r=0.44, P<0.05, Figure 4A) and lactate dehydrogenase (n=21, r=0.52,
Markers of myocardial infarct size, with an onset after intravenous bolus injection of 5000-U heparan, to avoid the occurrence of its adverse effects, including bleeding complications. However, the heparan-releasable EC-SOD with this amount has been shown to reflect the levels of total vascular EC-SOD expression in vivo, and has been widely used as a useful marker for evaluating human vascular EC-SOD expression in a number of clinical studies. 

The amount of heparan might need to be adjusted by body weight. However, because it was difficult to measure the body weight of the patients, or for us to ask the patients their precise body weight during acute coronary events, we did not adjust the amount of heparan by body weight. To consider the factor of body weight, we compared it among the patients with AMI, unstable angina pectoris, stable angina pectoris, and the control subjects, and found that there was no significant difference in average body weight among them. A previous study reported that the amount of vascular EC-SOD released into the blood increases linearly in proportion to the amount of heparan administration per body weight (up to 150 U/kg). This amount of heparan releases only a limited part of vascular EC-SOD, which has been estimated to be 3% of total vascular EC-SOD. For ethical reasons, we did not use doses of >5000-U heparan, to avoid the occurrence of its adverse effects, including bleeding complications. However, the heparan-releasable EC-SOD with this amount has been shown to reflect the levels of total vascular EC-SOD expression in vivo, and has been widely used as a useful marker for evaluating human vascular EC-SOD expression in a number of clinical studies.

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The amount of heparan might need to be adjusted by body weight. However, because it was difficult to measure the body weight of the patients, or for us to ask the patients their precise body weight during acute coronary events, we did not adjust the amount of heparan by body weight. To consider the factor of body weight, we compared it among the patients with AMI, unstable angina pectoris, stable angina pectoris, and the control subjects, and found that there was no significant difference in average body weight among them. A previous study reported that the amount of vascular EC-SOD released into the blood increases linearly in proportion to the amount of heparan administration per body weight (up to 150 U/kg). This amount of heparan releases only a limited part of vascular EC-SOD, which has been estimated to be 3% of total vascular EC-SOD. For ethical reasons, we did not use doses of >5000-U heparan, to avoid the occurrence of its adverse effects, including bleeding complications. However, the heparan-releasable EC-SOD with this amount has been shown to reflect the levels of total vascular EC-SOD expression in vivo, and has been widely used as a useful marker for evaluating human vascular EC-SOD expression in a number of clinical studies.
Protective Role of Vascular EC-SOD

To clarify the role of EC-SOD in acute coronary syndromes, we investigated the relationship between the extent of vascular EC-SOD expression and myocardial infarct size. As the levels of vascular EC-SOD expression on day 1 after the onset of AMI became higher, maximum values of serum concentrations of creatine kinase and lactate dehydrogenase, markers of myocardial infarct size, became lower. These results are consistent with previous reports that vascular overexpression of EC-SOD by administration of recombinant enzyme or by gene transfer protects the heart against irreversible ischemia/reperfusion injury, limiting the size of myocardial infarction by ≈50% in rats ex vivo or rabbits in vivo, respectively. Thus, it is conceivable that vascular EC-SOD may play an important cardioprotective role in reducing myocellular injury. The vasculoprotective effect of vascular EC-SOD overexpression on neointimal formation has also been reported.27

Conclusions

The present findings provide the first clinical evidence for an increase in vascular EC-SOD expression in patients having acute myocardial ischemic episodes. Upregulation of vascular EC-SOD may thus play an important compensatory role in the presence of increased oxidative stress to maintain the balance of cardiovascular redox status in humans in vivo.

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