HUMAN SUPEROXIDE DISMUTASE FAILED TO LIMIT THE SIZE OF MYOCARDIAL INFARCT AFTER 20-, 30-, OR 60-MINUTE ISCHEMIA AND 72-HOUR REPERFUSION IN THE RABBIT

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The effect of superoxide dismutase (SOD) on the size of the myocardial infarct resulting from various durations of ischemia and a 72-hour reperfusion was examined in the rabbit. A coronary branch of the circumflex artery was occluded for 20, 30, or 60 min and then reperfused. Seventy-two hours after the coronary occlusion, the infarct size and the size of the area at risk (vascular bed of occluded coronary artery) were determined by histology (hematoxylin-eosin and Mallory's staining) and by fluorescent particles, respectively. Human SOD (45,000 units/kg) was injected intravenously as a bolus in SOD-treated rabbits, while only saline was administered to control rabbits. The percentage of the area at risk which actually infarcted (%I/AAR) was 25.5 ± 12.9% (mean ± S.D.) in the 20-min ischemia control group (n = 9), 19.7 ± 10.2% in the 20-min ischemia SOD group (n = 9), 44.8 ± 9.0% in the 30-min ischemia control group (n = 9), 41.0 ± 6.3% in the 30-min ischemia SOD group (n = 9), 74.2 ± 13.8% in the 60-min ischemia control group (n = 9), and 76.6 ± 8.2% in the 60-min ischemia SOD group (n = 7). The %I/AAR was not significantly different between the control and SOD groups for any duration of ischemia. Heart rate, blood pressure, and the size of area at risk were comparable in all six groups. These findings suggested that oxygen-free radicals produced during initial moments of reperfusion were unlikely to contribute to myocardial necrosis regardless of the duration of ischemia in the rabbit.

THE role of oxygen-free radicals has received considerable interest as a possible mechanism for injury in ischemia-reperfusion of the heart. Superoxide dismutase (SOD) was previously found to reduce reperfusion arrhythmia and improve the functional recovery of myocardium following ischemia and reperfusion, which indirectly suggested the existence of injurious oxygen-free radicals in the myocardium at reperfusion. However, whether the oxygen-free radicals upon reperfusion cause myocardial necrosis is still very controversial. Several studies have reported that SOD plus catalase or SOD alone limits the myocardial infarct size. On the other hand, other studies have not observed such protective effects for SOD. Several explanations have been proposed for

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study was designed to examine 1) the role of free radicals, produced upon reperfusion and accessible to intravascular SOD, in myocardial necrosis, and 2) the dependence of such free radical injury, if present, on ischemia duration.

**MATERIALS AND METHODS**

**Surgical Preparation and SOD Regimen:**

The surgical preparation was essentially the same as in our previous study. Sixty-three male Japanese White Rabbits weighing 2.5 to 3.0 kg, were anesthetized with intravenous sodium pentobarbital (40 mg/kg). The rabbit was intubated through a cervical tracheostomy and mechanically ventilated with room air and supplemented oxygen. Catheters (PE 90) were placed in the right carotid for blood gas and blood pressure monitoring, and in the right jugular vein for SOD or saline injection. The respirator and oxygen supplement was adjusted to control arterial blood gas and pH within the physiological range. Precordial electrocardiography (ECG) was monitored using bipolar leads across the chest. The chest was opened via a left thoracotomy, the pericardium was opened, and the heart was exposed. Silk thread (4–0) was passed around a branch of the left circumflex artery with a taper needle, and the ends of the tie were threaded through a small vinyl tube. Heparin (1,000 units) was administered intravenously, and 3 min later, the coronary branch was occluded by pulling the snare, which was then fixed by clamping the tube with a mosquito clamp. Myocardial ischemia was confirmed by ST segment elevation in the ECG and regional cyanosis of the myocardial surface.

The coronary artery was occluded for 20, 30, or 60 min and then reperfused by releasing the snare. After 15 min of ischemia, the rabbit was randomly placed into control or SOD-treated groups. In the SOD-treated groups, human recombinant Cu-Zn SOD, 45,000 units/kg dissolved in saline (71,360 units/ml) was intravenously injected 5 min before reperfusion. The human SOD was obtained courtesy of Pharmacia AB, Uppsala, Sweden. The control animals received only saline. Reperfusion was confirmed by hyperemia over the ventricular surface. The vinyl tube was removed, and the ends of the silk thread were tied together to make a loop and then left in the thorax. The chest was closed in layers, and the tracheostomy repaired with 4–0 silk. These surgical procedures were performed...
### TABLE I SUMMARY OF HEMODYNAMIC DATA

<table>
<thead>
<tr>
<th>GROUP</th>
<th>N</th>
<th>PRE-OCLUSION</th>
<th></th>
<th>OCCLUSION</th>
<th></th>
<th>REPERFUSION</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HP bpm</td>
<td>BP mmHg</td>
<td>HR bpm</td>
<td>BP mmHg</td>
<td>HR bpm</td>
<td>BP mmHg</td>
</tr>
<tr>
<td>Group 20C</td>
<td>9</td>
<td>263 ± 12</td>
<td>108 ± 24 / 86 ± 15</td>
<td>259 ± 12</td>
<td>111 ± 21 / 89 ± 15</td>
<td>257 ± 18</td>
<td>107 ± 21 / 86 ± 18</td>
</tr>
<tr>
<td>Group 20S</td>
<td>9</td>
<td>263 ± 30</td>
<td>114 ± 24 / 88 ± 9</td>
<td>257 ± 33</td>
<td>111 ± 15 / 86 ± 9</td>
<td>258 ± 27</td>
<td>109 ± 12 / 86 ± 9</td>
</tr>
<tr>
<td>Group 30S</td>
<td>9</td>
<td>248 ± 33</td>
<td>117 ± 9 / 93 ± 6</td>
<td>248 ± 33</td>
<td>116 ± 9 / 92 ± 6</td>
<td>240 ± 24</td>
<td>113 ± 12 / 88 ± 9</td>
</tr>
<tr>
<td>Group 60C</td>
<td>9</td>
<td>249 ± 33</td>
<td>109 ± 18 / 89 ± 15</td>
<td>250 ± 33</td>
<td>108 ± 15 / 89 ± 15</td>
<td>239 ± 33</td>
<td>102 ± 18 / 84 ± 15</td>
</tr>
<tr>
<td>Group 60S</td>
<td>7</td>
<td>276 ± 40</td>
<td>119 ± 19 / 96 ± 16</td>
<td>275 ± 42</td>
<td>118 ± 19 / 97 ± 13</td>
<td>258 ± 45</td>
<td>109 ± 21 / 86 ± 13</td>
</tr>
</tbody>
</table>

The mean ± standard deviation. Heart rate (HR) and blood pressure (BP) were measured 2 min before coronary occlusion (PRE-OCLUSION), 2 min after coronary occlusion (OCCLUSION), and 2 min after reperfusion (REPERFUSION).

### TABLE II SUMMARY OF HEART WEIGHT, SIZE OF AREA AT RISK, AND INFARCT SIZE

<table>
<thead>
<tr>
<th>GROUP</th>
<th>N</th>
<th>ISCHEMIA min</th>
<th>H-WT g</th>
<th>AAR cm³</th>
<th>INFARCT cm³</th>
<th>INFARCT SIZE % OF AAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 20C</td>
<td>9</td>
<td>20</td>
<td>7.4 ± 0.9</td>
<td>0.74 ± 0.30</td>
<td>0.20 ± 0.12</td>
<td>25.5 ± 12.9</td>
</tr>
<tr>
<td>Group 20S</td>
<td>9</td>
<td>20</td>
<td>7.9 ± 0.9</td>
<td>0.62 ± 0.18</td>
<td>0.13 ± 0.09</td>
<td>19.7 ± 10.2</td>
</tr>
<tr>
<td>Group 30C</td>
<td>9</td>
<td>30</td>
<td>7.7 ± 0.6</td>
<td>0.80 ± 0.33</td>
<td>0.37 ± 0.18</td>
<td>44.8 ± 9.0ab</td>
</tr>
<tr>
<td>Group 30S</td>
<td>9</td>
<td>30</td>
<td>7.8 ± 1.2</td>
<td>0.64 ± 0.36</td>
<td>0.27 ± 0.18</td>
<td>41.0 ± 6.3ab</td>
</tr>
<tr>
<td>Group 60C</td>
<td>9</td>
<td>60</td>
<td>7.8 ± 0.9</td>
<td>0.85 ± 0.36</td>
<td>0.66 ± 0.36ab</td>
<td>74.2 ± 13.8abcd</td>
</tr>
<tr>
<td>Group 60S</td>
<td>7</td>
<td>60</td>
<td>8.2 ± 0.8</td>
<td>1.04 ± 0.32</td>
<td>0.79 ± 0.24abcd</td>
<td>76.6 ± 8.2abcd</td>
</tr>
</tbody>
</table>

The mean ± standard deviation. ISCHEMIA = duration of ischemia; H-WT = heart weight; AAR = area at risk. (a) p < 0.05 vs group 20C; (b) p < 0.05 vs group 20S; (c) p < 0.05 vs group 30C; (d) p < 0.05 vs group 30S.
under sterile conditions, and a combination of 50 mg ampicillin and 50 mg cloxacillin was injected intramuscularly for prophylaxis of infection. The rabbit was returned to its cage for recovery.

Seventy-two hours after the surgery, the rabbit was heparinized with 2,000 units of intravenous heparin and then sacrificed by a pentobarbital overdose. The heart was removed and mounted on a Langendorff apparatus. The coronary arteries were perfused with saline at 80 mmHg to wash out the remaining blood. In the reperfused animals, the coronary branch was re-occluded by ligating the remaining tie left around the branch. Fluorescent particles (3–30 μ in diameter, Duke Scientific Co.) were injected into the perfusion line to mark the zone which was not supplied by the occluded coronary branch (i.e., non-ischemic zone; Fig. 1). The heart was then taken off of the Langendorff apparatus, and the atria were removed, weighed and fixed in 20% buffered formalin for a day. The heart was then stored in 10% buffered formalin.

Measurement of Infarct and Area at Risk:

The heart was sectioned in 3 mm slices from the apex to the base, using a tissue slicer, and each slice was embedded in paraffin. The slice containing the coronary tie was excluded from the analysis because it had localized necrosis of the myocardium which was obviously related to the ligature. Two thin sections were cut from each paraffin block using a microtome. One was stained with hematoxylin-eosin (HE) and the other with Mallory's connective tissue stain modified by Heidenhain. The field of the occluded coronary artery, i.e., area at risk, was observed by illuminating the stained slide preparation with ultraviolet light and was traced on the slide itself. The slide preparation was mounted on a projector and enlarged 7 times. The infarct and area at risk were traced on papers. The traced areas were determined by a cut-and-weigh technique. As described in the previous study, heart slices shrank during the process of fixation and embedding in paraffin. To calculate the original area at risk and infarct volumes, we divided the areas which were obtained by tracing by 0.72413 and then multiplied by the sample thickness (3 mm).

Statistics:

All results are expressed as mean ± standard deviation. Multiple comparisons of the groups were performed by one-way analysis of variance with the Bonferroni test. Linear regression was obtained by the least square method, and the
coronary occlusion, but before randomization. One in the 20-min ischemia control group and another in the 20-min ischemia SOD-treated group were found dead in the cage on the second post-operative day. None of the rabbits were killed by arrhythmia during early reperfusion. Four rabbits were excluded because the area at risk was ambiguous. The remaining 52 rabbits contributed to the following analysis.

Table I summarises the hemodynamic data for all six groups: 20-min ischemia control group (group 20C), 20-min ischemia SOD group (group 20S), 30-min ischemia control group (group 30C), 30-min ischemia SOD group (group 30S), 60-min control group (group 60C), and 60-min SOD group (group 60S). There were no significant differences among any of the six groups in heart rate, and systolic and diastolic blood pressures two min before and after coronary occlusion, and two min after reperfusion. The heart weight, the mass of area at risk and the infarct size are summarized in Table II. Heart weight and size of the area at risk were also comparable among the groups.

Figure 2 illustrates the percentage of area at risk which actually infarcted (%I/AAR) in all six experimental groups. The %I/AAR was significantly enlarged when the ischemic duration was longer, reaching the following percentage; group 20C, 25.5 ± 12.9%; group 30C, 44.8 ± 9.0%; and group 60C, 74.2 ± 13.8% (p < 0.05 in any comparison). However, there were no significant differences detected between any SOD-treated group and its control group in duration of ischemia. The relationship between the size of the area at risk and infarct size is illustrated in Fig. 3. Thus, a significant linear correlation was observed in all six groups. Group 20C, Y = 0.33X – 0.05, r = 0.07; group 20S, Y = 0.47X – 0.16, r = 0.88; group 30C, Y = 0.54X – 0.07, r = 0.93; group 30S, Y = 0.48X – 0.04, r = 0.98; group 60C, Y = 0.97X – 0.16, r = 0.97; group 60S, Y = 0.73X + 0.03, r = 0.95 (all p < 0.05). The regression was again not different between any SOD-treated group and its control, suggesting that SOD had no protective effect regardless of the size of the area at risk.

RESULTS

Seven of the 63 rabbits operated on died of ventricular fibrillation during coronary occlusion. Five rabbits died of ventricular fibrillation during

**DISCUSSION**

In the present study, SOD failed to limit the size of myocardial infarct after 20, 30 or 60 min of ischemia followed by a 72-hour reperfusion. This finding suggested that oxygen-free radicals
generated during initial moments of reperfusion do not contribute to myocardial necrosis in the rabbit heart, and that the "window of ischemia duration", within which oxygen-free radicals play as significant a role as ischemia per se, is unlikely to occur.

Recently, free radical generation in the heart was demonstrated by electron spin resonance (ESR) spectroscopy with or without spin-trapping agents. Free radical burst signals, which peaked after about 5 min of reperfusion were demonstrated during early reperfusion following ischemia in vitro and in vivo. Furthermore, these free radical signals were suppressed by intravenously administered SOD suggesting that the radicals are accessible to intravascular SOD. The SOD regimen of the present study (45,000 u/kg of SOD as a bolus 5 min prior to reperfusion) was designed to scavenge for such free radicals released soon after reperfusion. Figure 4 illustrates the calculated time-course of plasma SOD levels in the present and previously reported studies. Such calculation was based on a plasma half-life for SOD of 6 min in rabbits (personal communication from A-C Bylund-Fellenius) and 22 min in dogs and a plasma volume of 4% of body weight. Since SOD, a protein with a molecular weight of 32,000 daltons, is not expected to distribute readily across the capillary walls, its distribution volume was assumed to be equal to the plasma volume. As shown in Fig. 4, the SOD regimen used in the present study should achieve SOD plasma level higher than those obtained in the previous studies after 5–10 min of reperfusion, at a time when according to the ESR studies, the free radical burst is expected to peak. The present study did not support the hypothesis that this burst of free radicals during the initial moments of reperfusion causes significant myocardial necrosis in vivo.

The failure of SOD to limit myocardial infarct size made quite a contrast to the protective effects of SOD and other free radical scavengers against reperfusion arrhythmias. However, a recent study by Hearse and Tosaki provided data regarding the mechanism of the anti-free radical interventions, possibly explaining the negative results in infarct size studies and positive results in reperfusion arrhythmia studies. In bicarbonate buffer-perfused isolated rat heart, PBN (N-ter-butyl-alpha-phenylnitrone), a spin-trap agent which was administered prior to reperfusion, did not reduce the absolute incidence of reperfusion-induced ventricular fibrillation, but did shift the relationship between the duration of ischemia and the incidence of ventricular fibrillation. Following 10 min of ischemia, ventricular fibrillation occurred following reperfusion in 100% of control hearts but in only 50% of
those treated with PBN. However, the incidence of ventricular fibrillation was also 100% in the PBN-treated group when the ischemic duration was 30 min. These findings indicated that PBN’s protective effect was a delaying action, and that PBN removed the free radicals so that the duration of ischemia that could be tolerated before the heart became vulnerable to reperfusion arrhythmia was extended by 20 min (i.e., from 10 to 30 min).2 However, this delaying effect might in fact be much shorter (i.e., less than 20 min) in the in vivo situation, in which metabolic changes caused by ischemia progress much faster than in vitro.28,29 If a similar delaying effect is assumed for the mechanism of SOD’s cardioprotective effect following reperfusion, it might explain the present finding that the duration of the ischemia–infarct size relationship was not significantly shifted in vivo (Fig. 2). Other explanations are also possible. Firstly, free radicals generated in the heart upon reperfusion might not be toxic enough to kill the myocytes, although they could still show arrhythmogenicity. Secondly, the radicals might be diminished by scavengers in the blood.30

In canine models, the infarct size resulting from 60 or 90 min of ischemia was reportedly limited by SOD5–8,11,15 but the infarct size caused by 40 min or 3 hours of ischemia was not,12,14 suggesting the “window of ischemia duration” for free radical-mediated injury to be about 90 min. However, in a recent study by Richard et al.16 infarct size after 90 min of ischemia was not limited by SOD plus catalase in dogs. The present study also does not support the hypothesis of a “window of ischemia duration” for free radical-mediated reperfusion injury. As far as infarct size is concerned, 40-min and 90-min ischemias in the dog heart are equivalent to 20-min and 30-min ischemias in our rabbit model, respectively, and in this study the infarct size was not limited by SOD in either the 20- or 30-min ischemia rabbit groups (Fig. 2).

Figure 4 also reveals that failure of SOD to limit infarct size in Uraizee et al.’s study14 is unlikely to be due to an insufficient SOD dose, because the plasma level of SOD in their study was calculated to be similar to those in the studies by Jolly et al.5 and Werne et al.11 who reported a significant limitation of infarct size by SOD.

We can not exclude the possible presence of myocardial injury by free radicals generated during the late reperfusion period (i.e., free radicals produced after the initial burst of radicals occurring at the time of reperfusion). As discussed in our previous report,13 leukocytes infiltrating the damaged myocardium may injure the myocytes by producing free radicals over hours or days. A recent study by Tamura et al.31 reported that polyethelene glycol-conjugated SOD (PEG-SOD), which has plasma half-life of 30 hours, was observed to have a protective effect even 4 days after reperfusion. The myocardial infarct size resulting from 90 min of ischemia, expressed as a percentage of the area at risk (%I/AAR), was 29.2% in the PEG-SOD-treated dogs, as compared with 44.2% in PEG-albumin treated controls.31 A similar degree of infarct size limitation was reportedly found by neutrophil depletion in a previous study from the same laboratory; %I/AAR after 90 min ischemia was reduced from 47.1% to 27.0% by an anti-neutrophil serum. However, when the duration of ischemia was elongated to 4 hours, such a limitation could not be demonstrated.33 These findings suggest a narrow “window of ischemia duration” of around 90 min for neutrophil-mediated reperfusion injury.

The failure of SOD to limit the myocardial infarct size in the rabbit in vivo has some clinical implications since the rabbit heart and the human heart share important characteristics. Firstly, the rabbit heart has poor coronary collaterals, similar to the human’s.34,35 The collateral blood flow to the ischemic zone, which substantially influences the infarct size in the dog,36,37 is negligible in the rabbit.38 Secondly, the rabbit heart has an extremely low level of xanthine dehydrogenase, in contrast with the dog and rat hearts.39,40 Xanthine dehydrogenase is a possible source of free radicals during reperfusion, which may cause the myocardial necrosis in the dog.41,42 However, it was recently revealed that the level of the xanthine dehydrogenase is very low in the human heart.39,43 These factors suggest that the rabbit is a better model than the dog for investigation of free radical injury in the human heart. The pig is another species in which the heart lacks xanthine dehydrogenase,39 and similarly it has been shown that the infarct size of pig heart is not reduced by SOD.17 The failure of SOD to limit infarct size in the present rabbit model and the porcine heart17 indicate that administration of SOD prior to coronary reperfusion in patients with acute myocardial infarction in order to reduce myo-

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cardial necrosis, is not valid. However, extrapolation of the present results to a clinical situation obviously needs caution. The validity of SOD at the time of coronary thrombolysis or coronary angioplasty in humans remains unknown, requiring further investigations.

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