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INHIBITION OF POSTISCHEMIC REPERFUSION ARRHYTHMIAS BY AN SOD DERIVATIVE THAT CIRCULATES BOUND TO ALBUMIN WITH PROLONGED *IN VIVO* HALF-LIFE

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Abstract—Intravenously administered superoxide dismutase (SOD) rapidly disappeared from the circulation and often failed to prevent oxidative tissue injury. Thus, although superoxide radicals have been postulated to play an important role in the pathogenesis of postischemic reflow-induced tissue injury, conclusive evidence supporting this concept is lacking. We have synthesized an SOD derivative (SM-SOD) that circulated bound to albumin and accumulated in injured tissues whose local pH was decreased. Transient occlusion followed by reperfusion of the coronary artery elicited severe ventricular arrhythmias in rats. Intravenous administration of SM-SOD markedly inhibited the reflow-induced arrhythmias. SOD did not show such inhibitory effect. Kinetic analysis revealed that SM-SOD accumulated in the acidic lesion of the injured heart soon after reflow and returned to the circulation thereafter. These and other results suggest that superoxide radical and/or its metabolites would play a critical role in the pathogenesis of reperfusion arrhythmias and that SM-SOD may be useful for protecting acute myocardial injury induced by such hazardous oxygen metabolites.

Arrhythmias sometimes occur as the consequence of spontaneous relief of coronary spasm in variant angina [1] or reperfusion of the ischemic region with thrombolysis during the early stage of acute myocardial infarction [2]. Although superoxide radical and its metabolites formed during reperfusion of an ischemic heart have been postulated to play an important role in the pathogenesis of electrophysiological derangements that trigger serious ventricular arrhythmias [3, 4], conclusive evidence supporting this concept is lacking. If superoxide radical played a critical role in the reperfusion injury, selective dismutation of this chemical species by superoxide dismutase (SOD)† would inhibit the occurrence of reperfusion arrhythmias and protect the myocardial tissue. However, in some cases, particularly in *in vivo* experiments, SOD failed to block reperfusion arrhythmias presumably because of its short half-life in the circulation ($T_{1/2} = 4$ min). We have synthesized an SOD derivative (SM-SOD) by covalently linking poly (styrene co-maleic acid) butyl esters (SM), a hydrophobic organic anion, which circulated bound to albumin with a half-life of 6 hr [5] and accumulated in an injured site of a tissue whose local pH was decreased [6]. The present work shows the inhibitory effect of SM-SOD on postischemic reflow-induced arrhythmias *in vitro* and *in vivo*. Dynamic changes in myocardial pH during ischemia and reflow are also demonstrated.

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† Abbreviations: SOD, superoxide dismutase; SM, poly (styrene co-maleic acid) butyl ester; SM-SOD, SM-linked SOD; LAD, left anterior descending artery; PVC, premature ventricular tachycardia; VT, ventricular tachycardia; Vf, ventricular fibrillation.

MATERIALS AND METHODS

Materials. Xanthine, xanthine oxidase, cytochrome *c* and bovine erythrocyte-type SOD were purchased from Sigma Chemical Co. (St Louis, MO). SOD was purified from human erythrocytes by the method of Gärtner *et al.* [7]. SM-SOD was synthesized from SOD by linking 2 moles of SM to the lysyl amino groups of SOD as described previously [5, 8]. Radioactive samples of SOD and SM-SOD were prepared by using ^{125}I-labeled Bolton–Hunter reagent (New England Nuclear Co., Boston, MA) as described in Ref. 9. Specific radioactivity of enzyme samples was 2,200 cpm per μg protein. Protein concentration was determined by the method of Lowry *et al.* [10] using bovine SOD as the standard.

Animals. Male Wistar rats, 230–280 g, were fed laboratory chow and water *ad lib.* and used for all studies after 16 hr of fasting.

Reperfusion arrhythmias in isolated perfused heart. Under light ether anesthesia, animals were intravenously injected with 0.2 ml of heparin solution (1000 units/kg). After 30 sec, the heart was excised and immediately placed in an ice-cold perfusion medium until contraction ceased (approximately for 15 sec). Then, the ascending aorta was cannulated and the heart was perfused retrogradely by the method of Langendorff [11] at a constant perfusion pressure of 110 cm H_2O. Under these conditions, coronary flow was approximately 15 ml/min per g wet weight. Modified Krebs–Henseleit bicarbonate buffer [12] was used as the standard perfusion medium, which was equilibrated with 95% oxygen plus 5% carbon dioxide. The isolated heart was perfused for an initial 20 min with the standard buffer at 37° and the left anterior descending artery (LAD) was occluded for 10 min by mild suction with a small

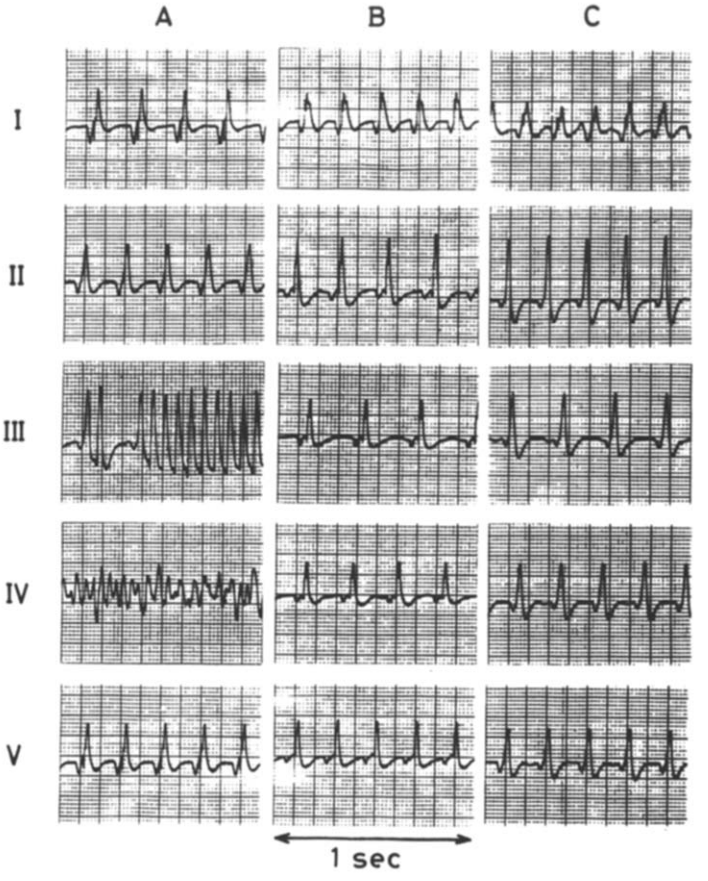

Fig. 1. Effect of SOD and SM-SOD on reperfusion arrhythmias in the isolated perfused heart. LAD of the isolated perfused heart was occluded for 10 min followed by reperfusion with the standard buffer solution (A) at 37° as described in the text. In some cases, the perfusion medium was added with either 25 units/ml of SOD (B) or SM-SOD (C) 15 min before occlusion. During the experiments, the pericardial ECG was recorded. The data represent a typical result taken from 30 similar experimental runs. I, before occlusion; II, 10 min after occlusion; III, 20 sec after reperfusion; IV, 50 sec after reperfusion; V, 3 min after reperfusion.

plastic tube as described in Ref. 13. Then, the heart was reperfused by releasing the occlusion. In most cases, this treatment elicited a marked ventricular arrhythmias which disappeared within 3 min after reperfusion. After 15 min of reperfusion, the perfusion medium was added with 25 units/ml of either SOD or SM-SOD. After 15 min, LAD was again occluded for 10 min at the same site as that for the first occlusion. An epicardial ECG recording was obtained via two silver electrodes attached to the heart and the ECG trace was continuously recorded throughout the experiments. Coronary flow was measured at 5 min intervals.

Postischemic reflow arrhythmias in vivo. Under pentobarbital anesthesia (50 mg/kg), the trachea was cannulated and artificial respiration was started with room air using a volume of 1.5 ml/100 g body weight and a rate of 55 strokes/min to maintain normal pO_2, pCO_2 and pH of the blood. Systemic blood pressure was monitored from the femoral artery by a transducer and a standard lead I ECG was continuously recorded. The chest was opened by thoracotomy and the pericardium was incised. Then, LAD was occluded for 15 min without resuscitating the animals. Then, blood was reflowed by releasing the occlusion. In most cases, postischemic reflow arrhythmias were elicited by this treatment, which disappeared within 3 min of reperfusion. Animals that showed unusual reactions under such conditions were not used for further experiments. At 15 min after the first reperfusion, either 5 mg/kg of SOD, SM-SOD or 0.2 ml of saline was injected into the tail vein. After 15 min, LAD was again occluded for 15 min at the same site as that for the first occlusion.

Definition of arrhythmias and statistical analysis. Arrhythmias occurring within 3 min after reperfusion were classified as premature ventricular contractions (PVCs), ventricular tachycardia defined as five or more consecutive PVC (VT) and ventricular fibrillation (Vf). The number of PVC and the duration of VT and Vf were expressed as mean ± SE. A one-way analysis of variance was first carried out to test for any differences between the mean values of all groups. If a difference was established, each of the treated group was compared with the control group using the unpaired *t*-test. An analogous procedure

was followed for distributions of discrete variables (e.g., VT). An overall χ^2 test for a $2 \times N$ table was constructed, followed by a sequence of 2×2 χ^2 tests using the Yates correction, in order to compare individual groups.

Accumulation of SM-SOD in the injured heart. Under pentobarbital anesthesia, animals were intravenously injected with 50 μg of radioactive SOD or SM-SOD (11,000 cpm/rat). After 15 min, LAD was occluded for 15 min followed by reflow as described above. At the indicated times, the heart was excised and immediately placed in 20 mM phosphate buffer, pH 6.0, containing 0.15 M NaCl (PBS) and allowed to beat in this buffer at 37° for 1 min to eliminate the blood remaining within the atriums and ventricles. Then, the heart was cannulated via the aorta and slowly perfused with 1 ml ice-cold PBS over a period of 5 sec to remove the blood remaining within the vascular bed. The perfusate and the heart thus prepared were determined for radioactivity.

Determination of myocardial pH. Under pentobarbital anesthesia and artificial respiration, a needle-type pH electrode (Microelectrode Inc., MI-402) was inserted into the left ventricular wall 3 mm distal to the site for occlusion of LAD; the tip of the electrode was localized at the center of the ischemic lesion. The electrode was sustained by a soft spring to minimize electrical noise caused by beating of the heart. The change in myocardial pH was monitored by a TOA pH meter Model HM-5B during the experiments.

RESULTS

Postischemic reperfusion arrhythmias in vitro

To study the mechanism for ischemia and reperfusion-induced tissue injury, LAD of the isolated perfused heart was transiently occluded and ECG was recorded throughout the experiments (Fig. 1). No significant arrhythmias occurred during occlusion of LAD. However, reperfusion of the ischemic heart with the standard buffer elicited severe arrhythmias within 5–20 sec. In most cases, both VT and Vf were observed for 40–70 sec after reperfusion, which disappeared thereafter under the experimental conditions (Fig. 1A). The occurrence of both VT and Vf was markedly inhibited by adding either SOD or SM-SOD to the perfusion medium (Fig. 1B and C). Under identical conditions, equimolar amounts of heat-denatured enzyme preparations and SM failed to inhibit the occurrence of arrhythmias (data not shown). Table 1 summarizes the quantitative aspects of the inhibitory effect of SOD and SM-SOD on arrhythmias induced *in vitro*.

Postischemic reperfusion arrhythmias in vivo

To test whether similar inhibition of postischemic reperfusion arrhythmias by these enzymes also occurs *in vivo*, the effect of intravenously injected SOD and SM-SOD was observed in the rat. As described for the experiments with isolated perfused heart, marked arrhythmias including VT and Vf were also elicited by a transient occlusion followed by reflow of LAD (Fig. 2A). Interestingly, intravenously injected SOD failed to inhibit the occurrence of VT and Vf (Fig. 2B). The inhibitory effect

Table 1. Effect of SM-SOD on reperfusion arrhythmias in the isolated perfused heart

	First reperfusion					Second reperfusion				
		Incidence (%)		Duration (sec)			Incidence (%)		Duration (sec)	
Treatment	PVCs (/3 min)	VT	Vf	VT	Vf	PVCs (/3 min)	VT	Vf	VT	Vf
Control	56 ± 7	100	85.7	34.0 ± 5.7	22.6 ± 5.5	29 ± 7	100	71.4	46.0 ± 9.1	9.4 ± 2.6
SOD	37 ± 13	100	85.7	30.4 ± 8.9	21.7 ± 8.1	25 ± 8	71.4	14.2	3.7 ± 1.5†	0.6 ± 0.6*
SM-SOD	37 ± 7	100	71.4	30.0 ± 5.0	22.7 ± 5.4	30 ± 8	85.7	28.5	6.5 ± 1.6†	0.8 ± 0.6*

The isolated heart was perfused for an initial 20 min with the standard buffer. Then, LAD was occluded for 10 min followed by reperfusion. Fifteen min after reperfusion, the perfusion medium was added with 25 units/ml of either SOD or SM-SOD. After 15 min of perfusion with SOD or SM-SOD, LAD was again occluded for 10 min followed by reperfusion. The number of PVCs and duration of VT and Vf are expressed as the means ± SE. PVCs, premature ventricular complexes; VT, ventricular tachycardia; Vf, ventricular fibrillation. N = 7 in each group, *$P < 0.05$, †$P < 0.01$.

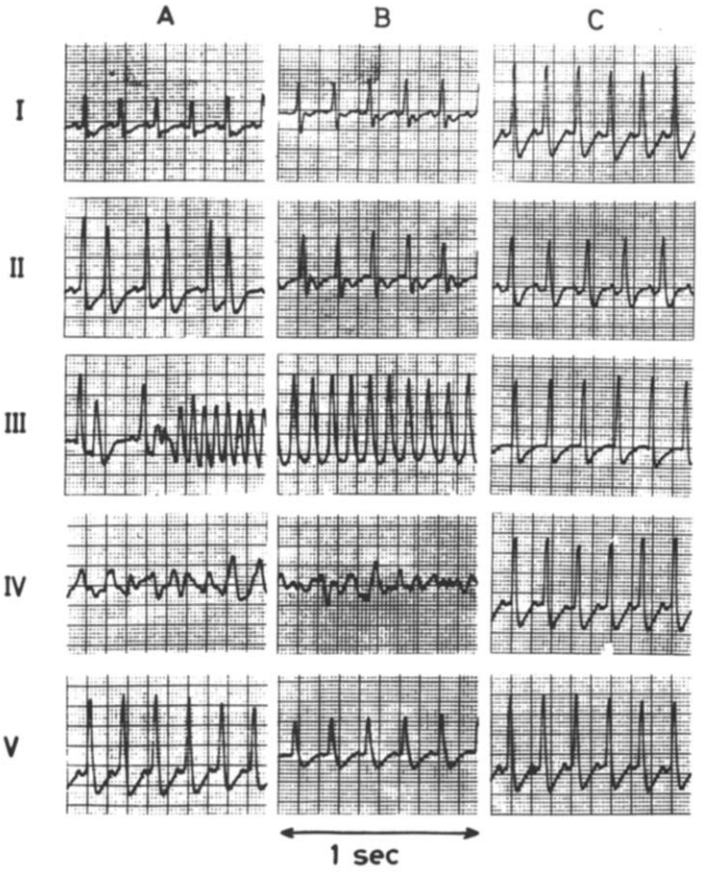

Fig. 2. Effect of SOD and SM-SOD on reflow arrhythmias *in vivo*. Under pentobarbital anesthesia and artificial respiration, animals were injected with either 0.2 ml of saline (A), 5 mg/kg of SOD (B), or 5 mg/kg of SM-SOD (C) into the tail vein. After 15 min, LAD was occluded for 15 min followed by reflow. Standard lead I ECG was recorded through the experiments. The data represent a typical result taken from 30 similar experimental runs. I, before ischemia; II, 15 min after occlusion of LAD; III, 10 sec after reflow; IV, 20 sec after reflow; V, 3 min after reflow.

was not seen even when a five-times higher dose of SOD than that used for the experiments shown in Fig. 2B was given to the animals (data not shown). In contrast, a single dose of SM-SOD almost completely inhibited the occurrence of both VT and Vf (Fig. 2C). Under identical conditions, equimolar amounts of SM and heat-denatured SM-SOD failed to inhibit the occurrence of arrhythmias (data not shown). Table 2 summarizes the effect of SOD and SM-SOD on arrhythmias *in vivo*.

Transient accumulation of SM-SOD in the reflowed heart

To elucidate the mechanism by which SM-SOD effectively inhibited postischemic reflow arrhythmias both *in vitro* and *in vivo* while SOD showed the inhibitory effect only in isolated perfused heart, radioactive SOD and SM-SOD were intravenously injected to rats and determined their *in vivo* fates. Consistent with the previous observations in intact rats [14], SOD rapidly disappeared from the circulation with a half-life of 4 min and appeared in urine, while SM-SOD circulated with a half-life of 6 hr in animals whose LAD was transiently occluded (Fig. 3).

To get further insight into the behavior of SM-SOD in the microcirculation of the injured heart, ^{125}I-labeled SM-SOD was injected intravenously and determined for radioactivity accumulated in the myocardial tissue. Radioactivity associated with the heart of the control animals was fairly low. Occlusion of LAD did not appreciably affect the levels of radioactivity associated with the ischemic heart. However, immediately after reperfusion, SM-SOD accumulated transiently in the heart. It reached a maximum within 20–30 min after reperfusion and decreased thereafter; about 0.4% of the injected dose accumulated in the reflowed heart. These observations suggested that SM-SOD, but not SOD, would circulate in the vascular bed of the reflowed myocardium with high concentrations and effectively dismutate superoxide radicals both in the circulation and in the injured heart.

Changes in myocardial pH during ischemia and reflow

Previous studies in this laboratory [6] revealed that SM-SOD accumulated in tissues whose local pH was decreased. To test whether transient accumulation of SM-SOD to the reflowed heart might reflect the

Table 2. Effect of SM-SOD on reflow-induced arrhythmias *in vivo*

Treatment	First reflow					Second reflow				
	PVCs (/3 min)	Incidence (%)		Duration (sec)		PVCs (/3 min)	Incidence (%)		Duration (sec)	
		VT	Vf	VT	Vf		VT	Vf	VT	Vf
Control	33 ± 10	100	16.7	24.3 ± 3.1	1.7 ± 1.3	14 ± 3	83.3	16.7	7.8 ± 1.7	0.9 ± 0.6
SOD	16 ± 4	100	33.3	22.7 ± 3.8	1.7 ± 0.8	16 ± 5	75.0	25.0	9.2 ± 2.4	0.6 ± 0.3
SM-SOD	20 ± 3	100	25.0	24.0 ± 6.1	1.0 ± 0.5	7 ± 2	16.7*	8.3	0.7 ± 0.5*	0.2 ± 0.2

After stabilization of the heart rate in all animals, LAD was occluded for 15 min followed by reflow of the blood. At 15 min after the first reperfusion, either 1 mg of SOD, SM-SOD or 0.2 ml of saline was injected into the tail vein. After 15 min, LAD was again occluded for 15 min followed by reflow of the blood. The number of PVCs and duration of VT and Vf are expressed as the means ± SE. PVCs, premature ventricular complexes; VT, ventricular tachycardia; Vf, ventricular fibrillation. N = 12 in each group, *P < 0.01.

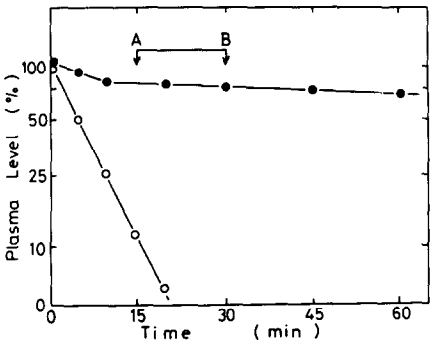

Fig. 3. Fate of SOD and SM-SOD in the circulation. Under pentobarbital anesthesia, heparinized animals were intravenously injected with radioactive enzyme samples (100,000 cpm/rat). At indicated times, 0.1 ml of blood samples were corrected from the left femoral vein and determined for radioactivity. Transient occlusion followed by reflow of LAD was performed (from A to B) as described for Fig. 2. Other conditions were the same as in Fig. 2. Open circles, SOD, closed circles, SM-SOD.

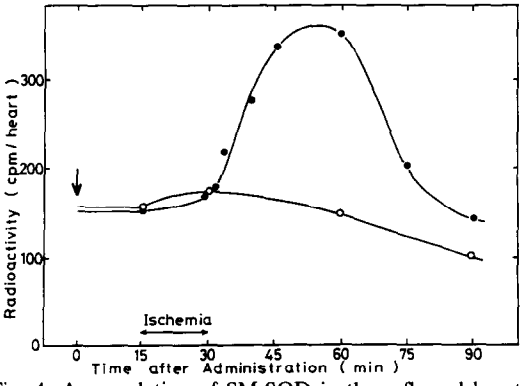

Fig. 4. Accumulation of SM-SOD in the reflowed heart. Under pentobarbital anesthesia and artificial respiration, 50 μg of radioactive SM-SOD (110,000 cpm/rat) was injected from the tail vein. After 15 min, LAD was occluded for 15 min followed by reflow. At the indicated times, the heart was excised and determined for the tissue-associated radioactivity as described in the text. Open circles, control groups; closed circles, occluded and reflowed groups.

decrease in cardiac pH, changes in the local pH in the myocardium were monitored by a needle-type electrode. As shown in Fig. 5, the local pH of the ischemic myocardium rapidly decreased after occlusion of LAD from 7.4 to 6.5. The lowered pH rapidly increased to about 7.1 within 3 min after reflow. However, it required about 30 min to return to the normal range of myocardial pH.

DISCUSSION

The present work demonstrates that SM-SOD efficiently inhibited postischemic reflow-induced arrythmias both *in vitro* and *in vivo*, which indicated

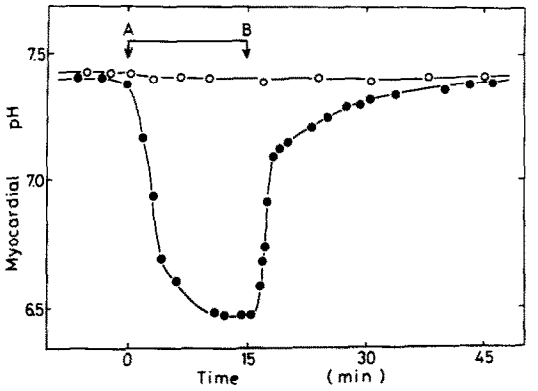

Fig. 5. Changes in myocardial pH during ischemia and reflow. Under pentbarbital anesthesia and artificial respiration, a needle pH electrode was inserted into the left ventricular wall. The local pH in the myocardium was monitored during the experiments. LAD was occluded for 15 min followed by reflow. Other conditions were the same as in Fig. 2A. Open circles, intact heart; closed circles, transiently occluded heart; (A) start of occlusion; (B) start of reflow.

that reactive oxygen species play a critical role in the pathogenesis of postischemic reflow arrhythmias. Since SM-SOD circulated with a half life of 6 hr and efficiently accumulated in the injured heart, significant dismutation of superoxide radicals would have occurred both in the circulation and in the reflowed myocardium. Ischemia increases the vascular permeability of a tissue [15]. Thus, transient accumulation of SM-SOD in the reflowed heart would partly be due to the increase in the vascular permeability. Since SM is a hydrophobic organic anion with 7 carboxyl groups (pK_a about 6.5), lipophilic nature of SM-SOD markedly increased when the anionic groups of the SM moiety were protonated [16]. Thus, SM-SOD transiently accumulates in tissues and binds to cell surface membranes when its environmental pH is decreased [6]. The local pH of an ischemic tissue has been shown to decrease predominantly due to enhanced anaerobic glycolysis [17, 18]. In fact, the present experiments revealed that the local pH of the myocardium decreased rapidly after occlusion of LAD. Although the lowered pH reversibly increased to about 7.1 within 3 min after reflow, it took about 30 min before the myocardial pH returned to its normal range. Thus, the decrease in pH of the ischemic myocardium may also participate, at least in part, in the accumulation of SM-SOD in the reflowed heart. The transient nature of accumulation might suggest that SM-SOD would have localized in an extracellular space of myocardium which was readily accessible to the microcirculation rather than in intracellular compartments. Thus, reactive oxygen metabolites, such as superoxide radicals, occurring in an extracellular space of the ischemic myocardium might be of critical importance in the pathogenesis of postischemic reflow arrhythmias. Although reflow-induced arrhythmias are common and consistent in such animal models, their occurrence in patients is much less frequent and their exact relationship to reflow is subject to debate. Thus, possible involvement of reactive oxygen species in the pathogenesis of ischemia/reflow-induced arrhythmias in human subjects should be studied further.

Preliminary experiments in this laboratory revealed that, when LAD was occluded permanently, about 70% of the rats died within 30 min predominantly due to irreversible ventricular fibrillation. To our surprise, the mortality of the animals was decreased to about 15% by a single dose of SM-SOD (10 mg/kg body wt). This might suggest that superoxide radical and/or its metabolite(s) also play a critical role in determining the prognosis of acute myocardial infarction. Mechanisms by which reactive oxygen species were generated in the ischemic myocardium and electrophysiological disturbance of the heart was elicited are under our current investigation.

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