Oxygen-derived Free Radicals Related Injury in the Heart During Ischemia and Reperfusion

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It has been suggested recently that oxygen-derived free radicals may play an important role in the genesis of reperfusion injury and arrhythmias. Free radicals have a very short half-life (ranging from mili- to microseconds), hence almost all the reports supporting the free radical hypothesis of reperfusion cell injury have been indirect. We have applied electrone spin resonance spectrometry to measure directly the amount of free radicals generated during ischemia and reperfusion. The concentration of free radicals in mitochondria increased significantly during ischemia (for 20 and 40 min). The concentration of free radicals after reperfusion was higher than that during ischemia, and a large amount of free radical generation occurred within the first 60 sec of reperfusion and returned to the level of prereperfusion at 5 min after reperfusion. The concentration of free radicals in the reperfusion-induced ventricular fibrillation group was significantly higher than that in the non-occurrence group. The administration of liposomal superoxide dismutase reduced the incidence of reperfusion-induced ventricular fibrillation and that prevented the free radical generation during reperfusion. This study showed that enhanced generation of free radicals occurred at the onset of ventricular fibrillation and that free radical scavenger prevented the development of arrhythmias and free radical generation during reperfusion.

We have obtained more circumstantial evidence for an involvement of free radicals in the genesis of reperfusion injury and arrhythmias.

THERE is little doubt that the net effect of early reperfusion is beneficial on myocardial infarction. Although the restoration of blood flow arrests the progression of necrosis,[1] paradoxically it is accompanied by functional derangements including a prolonged contractile impairment and various ventricular arrhythmias.[2,3] Reperfusion of the ischemic myocardium promotes the generation of oxygen-derived free radicals,[4] which have been implicated in the genesis of reperfusion-induced arrhythmias and depression of contractility.[5]

Free radicals are molecules or fragments of molecules containing an unpaired electron in their outer orbitals. Unpaired electron tends to acquire a pair, therefore, free radicals attract electrons from other molecules and can lead to a chain reaction as new free radicals are produced. During reperfusion there is a dramatic increase in free radical generation. On the other hand, the accumulation of free radicals in tissue is limited by a family of radical scavengers which include superoxide dismutase, catalase and glutathione peroxidase. These scavengers serve as intracellular defense mechanisms against the overproduction of free radicals, and tissue can be

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damaged if the formation of free radical exceeds the capacity of these protective mechanism. The potential role of the oxygen-derived free radicals system in myocardial injury has been the subject of many recent investigators. It has been recently suggested that oxygen-derived free radicals may play an important role in the genesis of reperfusion-induced arrhythmias[6-9] In support of this, it was found that the addition of either superoxide dismutase (SOD) or catalase to the isolated, perfused rat heart reduced the incidence of reperfusion-induced arrhythmias[6,8] This evidence, however, could not show a definitive association between the generation of free radical and the genesis of reperfusion-induced arrhythmias, hence radical scavengers may be beneficial by other mechanisms unrelated to the inhibition of free radical production. It is necessary to measure directly the generation of free radical during reperfusion and to investigate the relation with the occurrence of reperfusion-induced arrhythmias. Unfortunately, almost all the reports supporting the free radical hypothesis of reperfusion-induced arrhythmias and cell injury did not measure directly the free radicals in the heart. Since free radicals have a short half-life, generation of free radicals during ischemia and reperfusion has not been directly measured because of the instability of these products[6-9]

Electrone spin resonance spectrometry has been widely used to identify and characterize free radicals in simple chemical systems. We have applied electrone spin resonance spectrometry to measure directly free radicals in the heart mitochondria during ischemia and reperfusion, and then have further investigated the possible involvement of free radicals in the occurrence of reperfusion-induced arrhythmias. Also, the effect of liposomal superoxide dismutase (L-SOD) on reperfusion-induced ventricular arrhythmias was investigated in this study.

MATERIALS AND METHODS

1. Generation of free radical in ischemic and reperfused myocardial mitochondria

Forty-two mongrel dogs of either sex, weighing 9–15 kg, were anesthetized with sodium pentobarbital (30 mg/kg body weight i.v.). After endotracheal intubation the dogs were ventilated with room air using a Harvard respirator. Thoracotomy was performed on the 4th intercostal space and the pericardium was opened. Nineteen dogs were used as experimental animals of ischemia and were divided into three groups. One group was the sham-operated control (n = 9), and both the other two groups were ischemic groups (n = 5, n = 5). The left anterior descending coronary artery was dissected free from surrounding tissue just distal to origin of the first diagonal branch. In the ischemic group the left anterior descending coronary artery (LAD) was occluded with an intracranial arterial clamp for 20 and 40 min, respectively. Soon after the onset of LAD occlusion, the ischemic area was clearly apparent as a sharply delineated cyanotic area over anterior of left ventricle. If a dog did not demonstrate the appearance of epicardial cyanosis over the left ventricle, that dog was excluded from this study. Twenty-three dogs were used as experimental animals of reperfusion and they were divided into two groups; non-reperfused (n = 6) and reperfused groups (n = 17). The reperfused group was further divided into three groups; 30 sec of reperfusion (n = 6), 60 sec of reperfusion (n = 4), and 5 min of reperfusion (n = 7), respectively. The LAD was occluded as mentioned above for 15 min and then followed by each duration of reperfusion. If a dog developed ventricular fibrillation during ischemia and reperfusion, it was excluded from the measurement of free radical, because the duration of ischemia and/or reperfusion is variable in these dogs.

2. Relation between the occurrence of reperfusion-induced ventricular fibrillation and the generation of free radical

Nineteen mongrel dogs were used. The LAD was occluded for 15 min and then followed by 5 min reperfusion. An electrocardiogram (limb lead II) was monitored continuously throughout the experiment for analysis of the occurrence of reperfusion-induced ventricular fibrillation. If the dog developed ventricular fibrillation during reperfusion, the heart was immediately excised and mitochondrial fractions of non-reperfused and reperfused areas were prepared. The dogs that developed ventricular fibrillation during ischemia were excluded from this study. If the dog did not develop ventricular fibrillation during ischemia and reperfusion, the heart was excised at 5 min of reperfusion and then mitochondrial fractions of non-reperfused and reperfused areas were prepared.

3. Effects of liposomal superoxide dismutase on

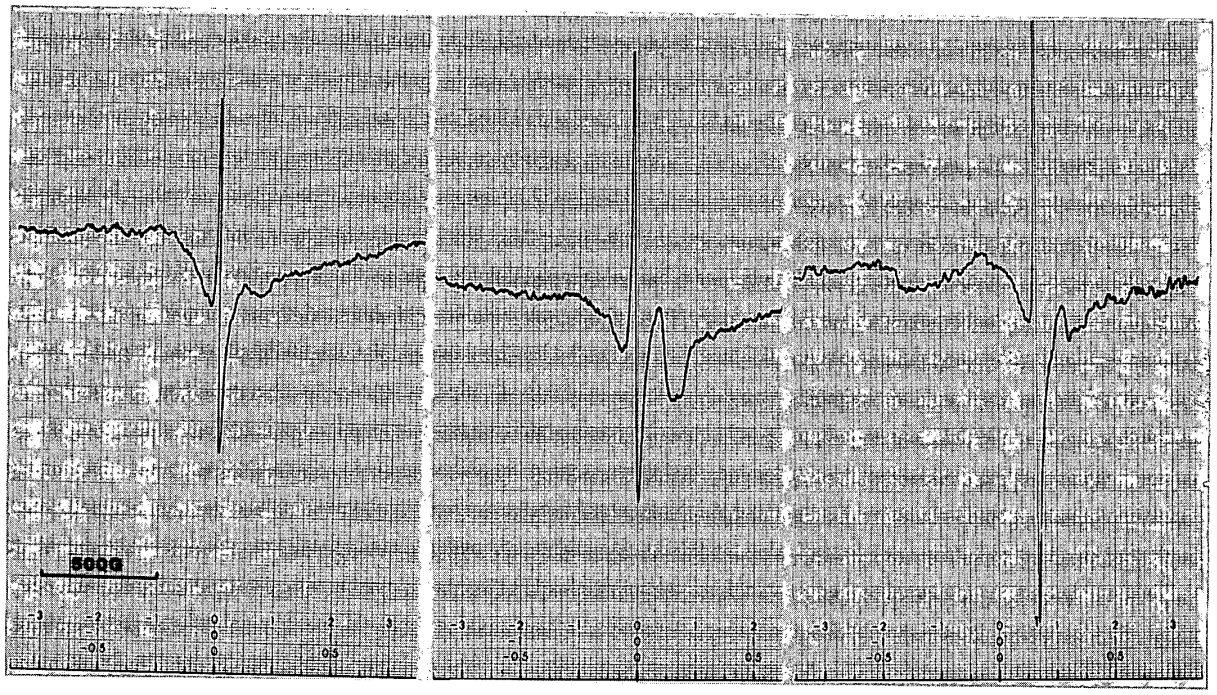

Fig.1. The typical ESR spectra of control, ischemic, and postischemic reperfusion heart mitochondrial preparations.
Left: control, Middle: 15 min ischemia, Right: 15 min ischemia and followed by 30 sec of reperfusion

reperfusion-induced ventricular fibrillation and the generation of free radical

Thirty-one mongrel dogs were used as experimental animals for investigation of reperfusion-induced arrhythmias, and 12 dogs were used for investigation of free radical generation during reperfusion. Liposomal superoxide dismutase was kindly provided by Toyo Soda Manufacturing Co., Ltd, Tokyo in Japan. In the L-SOD-treated group, L-SOD (3×10^4 U/kg/h) was administrated directly into the left atrium starting 15 min before LAD occlusion and followed by the same dose infusion throughout the experiment. In the non-treated control group, the same volume of physical saline solution was directly administered into the left atrium using the same method as for the L-SOD-treated group. An electrocardiogram was recorded as already mentioned for analysis of the occurrence of arrhythmias. The LAD was occluded for 15 min and then reperfusion was performed for 5 min, and the occurrence of reperfusion-induced ventricular fibrillation was compared with L-SOD-treated group (n = 12) and the non-treated group (n = 16). The dogs that developed ventricular fibrillation during reperfusion were immediately excised and then mitochondrial fractions of non-reperfused and reperfused areas were prepared. In addition, for the investigation of the effect of L-SOD on the generation of free radical, the LAD was occluded for 15 min and then followed by 30 sec (n = 6) and 60 sec (n = 6) of reperfusion, respectively. L-SOD was administrated using the same method as mentioned above. The concentration of mitochondrial free radicals in the L-SOD-treated group was compared with that in the non-treated group for each duration of the reperfusion.

4. Preparation of mitochondria

Hearts were rapidly excised and mitochondrial fractions were prepared by the modified method of Chance and Hagihara[10] from the non-ischemic, ischemic, non-reperfused, and reperfused areas, respectively. The heart was minced in ice-cooled 0.25 M sucrose and suspended in an five-fold ice-cooled isolation medium containing 0.25 M sucrose, 1 M Tris/HCl buffer (pH 7.4). The mixture was filtered through cheesecloth and 2 ml of 0.25 M sucrose (pH 7.4) and 2 mg bacterial alkaline protease (nagarse) were added to 1 g of sediment and then left to stand at 0°C for 20 min. The mixture was gently homogenized at 800 rpm with a teflon homogenizer and the suspension was centrifuged at 1000 x g at 4°C for 10 min. The sediment was suspended in a small amount of isolation medium contain-

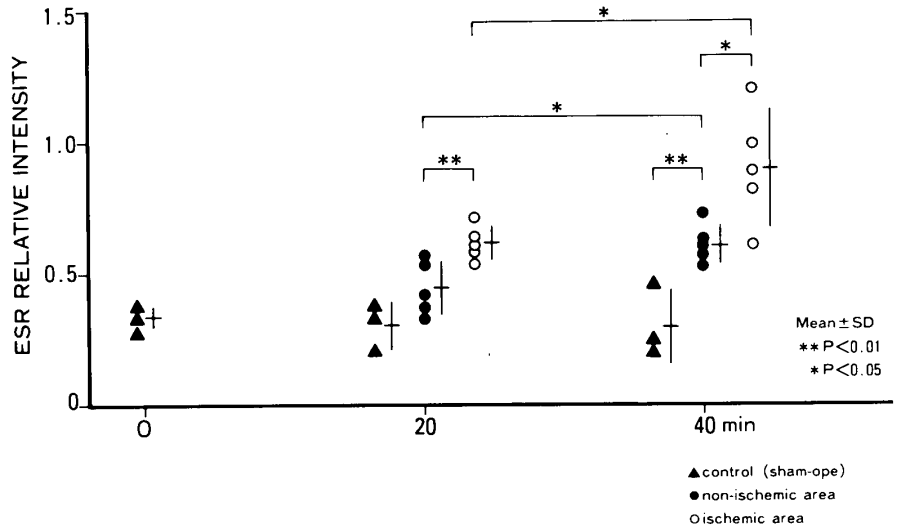

Fig.2. The changes in free radical concentrations during ischemia. The numbers on the ordinate indicate the ESR relative intensity, and time is plotted on the abscissa. Triangles indicate the sham-operated control group values. Closed circles indicate the non-ischemic areas and open circles indicate the ischemic areas.

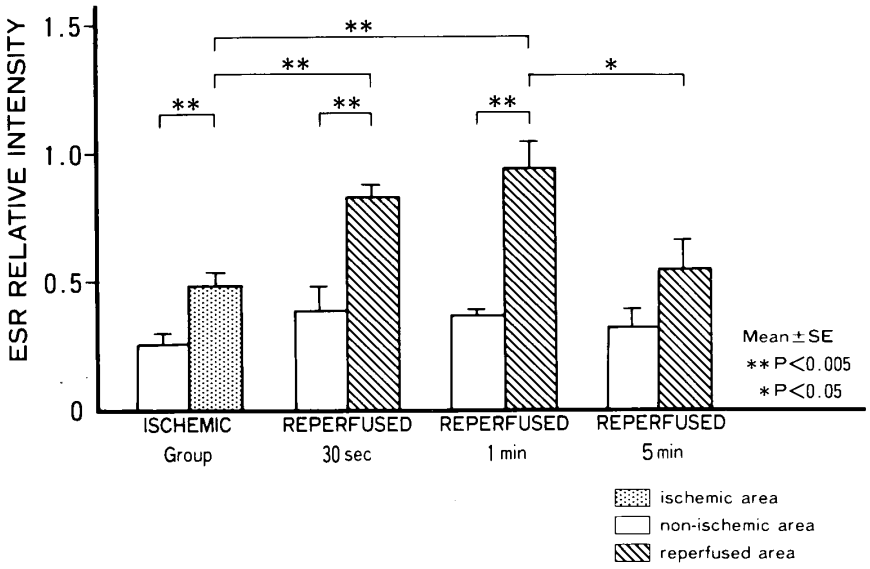

Fig.3. The changes in free redical concentrations during reperfusion. In ischemic group LAD was occluded for 15 min. In reperfused group LAD occluded for 15 min and followed by each duration of reperfusion. Open column indicates the non-ischemic-reperfused area and oblique line column indicates the reperfused area.

ing of 0.25 M sucrose and 0.01 M Tris/HCl buffer (pH 7.4) at 4°C. The suspension was centrifuged at 17000 rpm at 4°C for 10 min. The mitocondrial suspension (about 10 mg protein/ml) was frozen in liquid nitrogen.

5. Measurement of free radicals

Free radicals in myocardial mitochondria were measured using an electrone spin resonance spectrometry (JEOL JES-FE2XG, Japan) equipped with a variable temperature controller. Manganese chloride was used as the standard substance. Conditions of electrone spin resonance signal observation were as follows: Microwave power 8 mW, Sweep with 3300 ± 100 Gauss, Sweep time 4 min, Modulation 100 KHz 6.3 Gauss, Response time 1 sec, Temperature −150°C. The gravity value was determined by using manganese as a reference. The electron spin resonance (ESR) intensity was defined

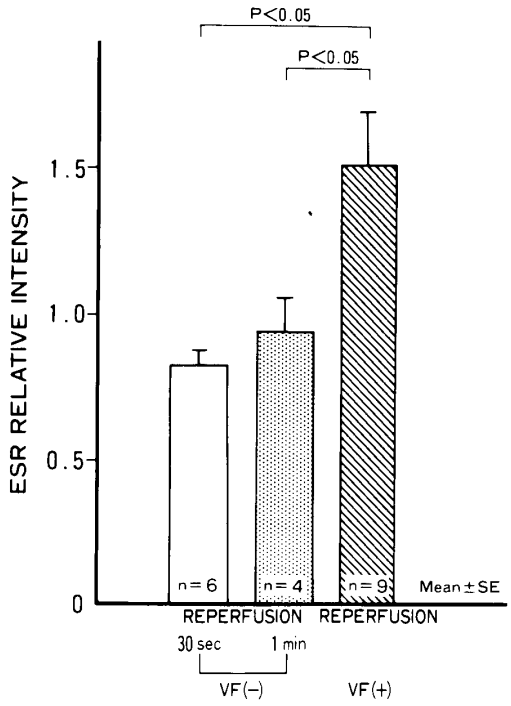

Fig.4. The relation between the occurrence of reperfusion-induced ventricular fibrillation and the generation of free radicals. Reperfusion-induced ventricular fibrillation occurred within the first 60 sec after reperfusion. This shows the concentration of free radical in reperfused area in group with ventricular fibrillation was significantly increased within the first 60 sec after reperfusion. VF(−) indicates group without ventricular fibrillation and VF(+) indicates group with ventricular fibrillation.

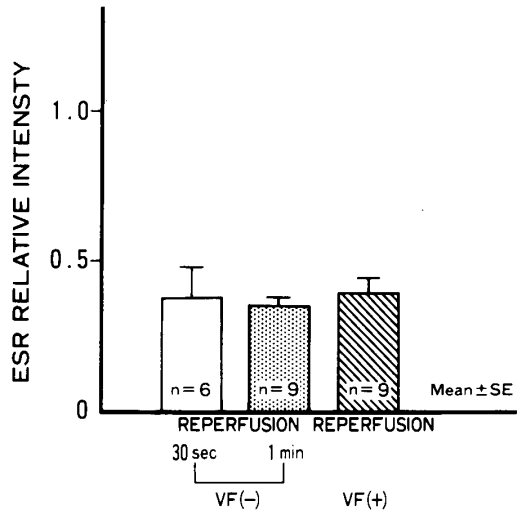

Fig.5. The concentration of free radical in non-ischemic-reperfused areas in groups with and without ventricular fibrillation. The generation of free radical in the non-reperfused area was not affected by occurrence of ventricular fibrillation. VF(−) indicates group without ventricular fibrillation and VF(+) indicates group with ventricular fibrillation.

as the ratio of the maximum deflexion of the ESR signals of samples to that of the standard signals of manganese chloride which inserted in the same resonance cavity of the ESR spectrometry. ESR relative intensity was expressed as intensity per miligram mitochondrial protein which was determined by the method of biuret reaction. Since the relative intensity of the ESR peaks observed is directly proportional to the free radical concentration of mitochondria, free radical concentrations of samples are shown as the ESR relative intensity in this study.

6. Statistical analysis

Statistical analysis was made by paired or non-paired Student's t test except for analysis of the effect of L-SOD on ventricular fibrillation. Analysis of the effect of L-SOD was done by chi-squared test. Differences for $P < 0.05$ were considered significant.

RESULTS

1. Free radical concentration in ischemia and reperfusion

Figure 1 shows typical ESR spectra from non-ischemic, ischemic, and reperfused mitochondrial preparations, respectively. The ESR signal was observed at g = 2.005 (g value) between the third and fourth signals of manganese chloride. Fig. 2 shows the changes in free radical concentrations during ischemia. There was no significant difference among the sham-operated control groups, 0.34 ± 0.04 (mean ± SD), 0.31 ± 0.14, and 0.30 ± 0.14, respectively. On the other hand, in a group in which the LAD was occluded for 20 min, the values of ESR relative intensity in ischemic and non-ischemic areas were 0.62 ± 0.06 (mean ± SD) and 0.45 ± 0.10. In a group in which the LAD was occluded for 40 min, the values of ESR relative intensity in ischemic and non-ischemic area were 0.90 ± 0.22 (mean ± SD) and 0.61 ± 0.07. The ischemic areas have significantly higher concentrations of free radicals than those in non-ischemic areas ($p < 0.01$, $p < 0.05$). Furthermore, the concentration of free radicals in ischemic areas increased significantly the longer the duration of ischemia. There was no significant difference in the free radicals of the non-ischemic area between the

TABLE I THE EFFECT OF L-SOD ON THE OCCURRENCE OF REPERFUSION-INDUCED VENTRICULAR FIBRILLATION

Group	*Incidence of VF*
Control	*9 / 16 (56%)*
Liposomal SOD (3×10^4 U/kg/h)	*1 / 12 (8%)**

**p < 0.05 compared with control*

sham-operated control and the 20 min ischemia. The value of the non-ischemic area in the 40 min ischemia was significantly higher than that in the sham-operated control. These data show that there is a significant generation of free radicals during ischemia.

The changes in free radical concentration during reperfusion are presented in Fig. 3. There were time-dependent changes in free radical generation during reperfusion.

The value of ESR relative intensity in ischemic area of pre-reperfusion (baseline) was 0.48 ± 0.05 (mean ± SE). On the other hand, the values of ESR relative intensity in ischemic areas of 30 sec, 60 sec, and 5 min after reperfusion were 0.82 ± 0.05 (mean ± SE), 0.94 ± 0.11, and 0.54 ± 0.12, respectively. The values of ESR relative intensity in ischemic areas of 30 sec, and 60 sec after reperfusion were significantly higher than that of baseline. The value of 5 min after reperfusion, however, was not significantly different from that of pre-reperfusion and returned to the baseline value. This study showed that a significant increase in free radical generation occurred within the first 60 sec after reperfusion and then returned to baseline by 5 min after reperfusion. On the other hand, there was no significant difference between the values of ESR relative intensity in non-ischemic-reperfused areas among the reperfusion groups.

2. Relation between the occurrence of reperfusion-induced ventricular fibrillation and the generation of free radicals

Nine out of 19 dogs (47%) developed ventricular fibrillation during reperfusion, and all of them occurred within the first 60 sec after reperfusion. We used, therefore, the values of ESR relative intensity in ischemic area of 30 and 60 sec after reperfusion as control groups.

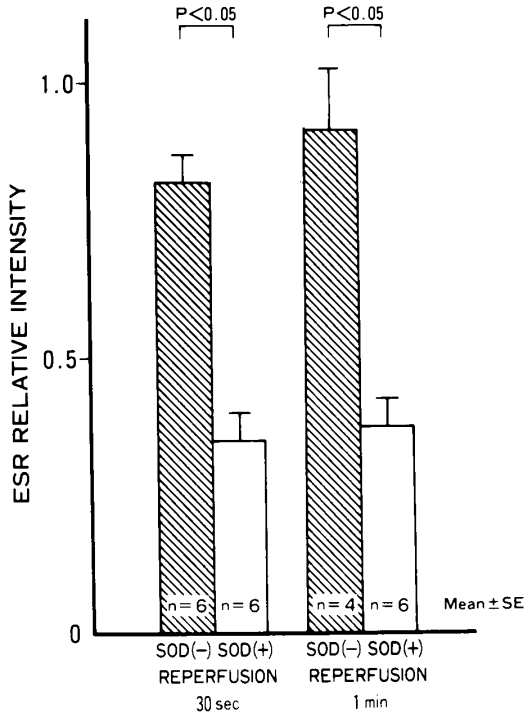

Fig.6. The effect of L-SOD on the generation of free radicals. The oblique line column indicates the non-treated control group and the open column indicates the L-SOD-treated group. The LAD was occluded for 15 min and followed by 30 sec and 60 sec of reperfusion, respectively.

Fig. 4 shows the concentrations of reperfused mitochondrial free radicals in a group with ventricular fibrillation and control groups. The ESR relative intensity in reperfused area with ventricular fibrillation was 1.50 ± 0.18 (mean ± SE). On the other hand, the values of ESR relative intensity in the control groups without ventricular fibrillation were 0.89 ± 0.05 (mean ± SE) and 0.94 ± 0.11, at 30 and 60 sec after reperfusion, respectively. The group with ventricular fibrillation had a significantly higher concentration of free radicals in the reperfused area than those of the groups without ventricular fibrillation ($p < 0.05$). Fig. 5 shows the concentrations of free radicals in the non-ischemic-reperfused areas in the groups with and without ventricular fibrillation. There was no significant differences among those groups. The generation of free radicals in the non-ischemic-reperfused area was not affected by ventricular fibrillation.

3. Effects of L-SOD on reperfusion-induced ventricular fibrillation and the generation of free radical

Table I shows the effect of L-SOD on the occurrence of reperfusion-induced ventricular fibrillation. In the non-treated group 19 out of 16 dogs (56%) developed ventricular fibrillation during reperfusion. Only one out of 12 dogs (8%), however, developed ventricular fibrillation during reperfusion in the L-SOD-treated group. The occurrence of reperfusion-induced ventricular fibrillation was significantly lowered by the administration of L-SOD ($p < 0.05$). Fig. 6 shows the effect of L-SOD on the generation of free radical during reperfusion. As already mentioned, a large quantity of free radical generation occurred within the first 60 sec after reperfusion with a peak at 30 sec and then returned to the level of baseline by 5 min after reperfusion. We investigated, therefore, the effects of L-SOD at 30 and 60 sec after reperfusion. The ESR relative intensity of the L-SOD-treated group was significantly lower than those of the non-treated groups at 30 and 60 sec after reperfusion (0.35 ± 0.05 vs 0.82 ± 0.05, $p < 0.05$; 0.38 ± 0.05 vs 0.94 ± 0.11, $p < 0.05$). The administration of L-SOD reduced significantly the occurrence of reperfusion-induced ventricular fibrillation and the generation of free radicals in the reperfused mitochondria.

DISCUSSION

There is much convincing evidence that oxygen-derived free radicals may play an important role in cellular injury during myocardial ischemia and reperfusion. It has been suggested recently that oxygen-derived free radicals may play an important role in the genesis of reperfusion-induced arrhythmias.[6-9] In support of this, it has been found that the addition of either SOD or catalase in the isolated, perfused rat heart reduced the incidence of reperfusion-induced ventricular fibrillation.[6,8] Similar results have been obtained by others.[11] However, since generation of free radicals at the onset of reperfusion-induced arrhythmias has not been directly measured, almost all the evidence supporting the free radical hypothesis of ischemic-reperfusion cell damage including reperfusion-induced arrhythmias have been indirect. Two fundamental questions, then, arise. Namely, is there generation of free radicals at reperfusion? And is there a true relation between the generation of free radicals and the occurrence of reperfusion-induced arrhythmias? We have applied ESR spectrometry to measure directly free radical generation for investigation of the arrhythmogenesis of free radicals during reperfusion. ESR spectrometry allows the direct measurement of chemical species with unpaired electrone. It is possible to measure the amount of the generation of free radicals during ischemia[12] and reperfusion[13] by using ESR spectrometry. Superoxide radicals are continuously formed in vivo, the major source being mitochondrial respiration. We therefore used mitochondria of heart as the material to measure free radical generation. Duration of ischemia and reperfusion was chosen according to the study, in which reperfusion after a 10- to 15-minute period of ischemia resulted in the highest incidence of reperfusion-induced ventricular fibrillation, and that occurred within the first few minutes of reperfusion.[14,30]

This study showed that the concentration of free radicals was significantly increased during ischemia, and that the increase was more in proportion to the length of the ischemic duration. The concentration of free radicals after reperfusion was significantly higher than that during ischemia, and a large amount of free radical generation occurred within the first 60 sec after reperfusion and returned to the pre-reperfusion level by 5 min after reperfusion. Zweier and co-woerkers have also shown that during ischemia there is a significant increase in the concentration of oxygen radicals, and that a dramatic burst in free radicals occurred within 10 seconds of reperfusion.[15] If the ischemia was complete, and the tissue was completely anoxic, no free radicals could be generated under these conditions. The region of total ischemia, however, may be very unusual in clinical myocardial ischemia due to the collateral circulation. Therefore, despite coronary occlusion, some oxygen will reach the ischemic tissue, and it has been calculated that oxygen tensions which were reduced by approximately 90% could still maintain oxygen-derived free radical formation at levels that were 83% of normal.[16,17] Varying degrees of partial ischemia and hypoxia may be a commonly encountered situation in the human heart. Our results are considered to reflect these clinical situations and consequently have shown that free radicals could be generated under ischemic conditions as well as during reperfusion. In addition, free radical concentration in the non-ischemic area was significantly increased as

compared with that in the sham-operated control. There are several potential causes of increased free radical generation during ischemia and reperfusion. They include dissociation of the intramitochondrial electron transport system;[18] the depletion of the protective scavenging mechanisms;[19] accumulation of neutrophils;[20] hypoxanthine-xanthine oxidase system;[21] enhanced arachidonic acid metabolism;[22] and the auto-oxidation of catecholamine;[23] respectively. We assume that the stimulation of catecholamine which was released as the result of ischemia has an effect on the non-ischemic myocardial free radical generation as well as on the ischemic area.

A large quantity of free radical generation occurred within the first 60 sec of reperfusion, and returned to the level of baseline by 5 min after reperfusion. This phenomenon follows the same time course as the appearance of reperfusion-induced ventricular fibrillation.[14,24,30] In fact, all reperfusion-induced ventricular fibrillation occurred within the first one minute of reperfusion in this study. Moreover, the concentration of free radicals in the reperfused area of the group with ventricular fibrillation was significantly higher than that of the group without ventricular fibrillation. These observations are consistent with the hypothesis that the major part of reperfusion injury and arrhythmias occur within the first few minutes after reperfusion and that enhanced generation of free radicals occurs at the onset of ventricular fibrillation.

In the next place, we investigated whether the free radical scavenger could reduce the generation of free radicals and consequently prevent the occurrence of reperfusion-induced ventricular fibrillation or not. The native enzymes have circulating half-lives of only 6–9 min but L-SOD have circulating half-lives of 2–5 hours. It can transfer across cell membranes by endocytosis or fusion and can cause up to a 100-fold increase in cellular specific activity.[25] Dose and duration of administration of L-SOD was based on other reports.[26,27] This study showed that the administration of L-SOD reduced the incidence of reperfusion-induced ventricular fibrillation and the generation of free radicals during reperfusion. Only one dog developed ventricular fibrillation in spite of the administration of L-SOD. This dog had the same concentration of free radicals as the dogs that developed ventricular fibrillation without treatment of L-SOD. The oxygen metabolites potentially responsible for tissue damage are superoxide anion, hydrogen peroxide, and hydroxyl radical. Recent data suggest that superoxide and hydrogen peroxide may themselves be less toxic, but they may result in the generation of the more reactive hydroxyl radical via an iron-catalyzed Harber-Weiss reaction or Fenton reaction.[28] It has to be pointed out that these reactions require iron or another oxidized metal ion as a catalyst, as well as the simultaneous presence of both hydrogen peroxide and superoxide anion.[29] Therefore, effective scavenging action of L-SOD alone might be sufficient to prevent generation of hydroxyl radicals and, hence, tissue damage and reperfusion-induced ventricular fibrillation. These results showed that the free radical scavenger prevented the development of ventricular arrhythmias, and that the inhibition or removal of the source of free radical production decreased the severity of rhythm disturbances. This study yields the evidence supporting the hypothesis that oxygen-derived free radicals may play a pivotal role in the genesis of reperfusion-induced ventricular fibrillation.

The mechanisms of reperfusion-induced ventricular fibrillation are very complicated.[30] As has often been speculated that ischemia-induced intracellular potassium loss, extracellular potassium accumulation and a general loss of transmembrane ionic balance, including potassium, calcium and magnesium, would be particularly good candidates for a common final mechanism.[31] Such ionic perturbations could be triggered by membrane injury. The plasma membrane is also a critical site of the free radical reactions. The unsaturated fatty acids present in membranes and the transmembrane proteins containing oxidizable amion acids are susceptible to free radical damage. Also, increased membrane permeability caused by lipid peroxidation or oxidation of structurally important proteins, can cause a breakdown to transmembrane ion gradients and inhibition of integrated cellular metabolic processes.[32–34] Thus, the anti-arrhythmic effects of anti-free radical interventions might be explained by the protection of membrane injury resulting from lipid peroxidation arising from the production of oxygen-derived free radicals.

In conclusion, by using ESR spectrometry, we have demonstrated that free radicals were generated in significant numbers during ischemia and reperfusion, and there was good correlation

between free radical generation and the occurrence of reperfusion-induced ventricular fibrillation. We showed the evidence supporting the hypothesis that oxygen-derived free radicals might play an important role in the genesis of reperfusion-induced ventricular arrhythmias. Consequently, a drug such as L-SOD may be a useful adjunct to thrombolytic therapy, angioplasty, or surgical revascularization to attenuate the deleterious consequence of restoring oxygen to ischemic tissue.

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