Experimental Studies

Continuous Detection of Superoxide *In Situ* during Ischemia and Reperfusion in the Rabbit Heart

Shinichi USHIRODA, MD, Yukio MARUYAMA, MD,
and Minoru NAKANO,¹ MD

SUMMARY

In order to clarify the time course of superoxide generation *in situ* during ischemia and reperfusion in the rabbit heart, we used a method of enhanced chemiluminescence (CL) with 2-methyl-6-[p-methoxyphenyl]-3, 7-dihydroimidazo [1, 2- α]pyrazin-3-one (MCLA) as a specific probe for detecting superoxide radicals. The surface of the rabbit heart was exposed to a photomultiplier tube in a light-proof box. We introduced a reversible snare occluder into the box to continuously observe the light emission. An ischemia-reperfusion group (I/R, $n = 7$) was subjected to 30 mins of coronary occlusion, followed by 90 mins of reperfusion. We performed the same procedure (except for coronary occlusion) in the sham-operated group ($n = 4$). Another group of rabbits ($n = 4$) subjected to I/R received superoxide dismutase (SOD: 20 mg/kg, iv) during reperfusion to observe the CL response.

In the I/R-group, the increase in CL began at 13 ± 2 (mean $\pm$ SEM) mins and peaked at 52 ± 12 mins of reperfusion. CL in the I/R-group gradually increased from 818 ± 350 counts/10 secs in the preischemic period to 1077 ± 401 counts/10 secs during reperfusion ($p < 0.01$). In contrast, there was no increase in CL in the sham-operated group. The administration of SOD briefly attenuated CL by $24.1 \pm 6.8\%$ for a period of 24.3 ± 6.8 mins.

The superoxide generation *in situ* in the ischemic rabbit heart appears to increase gradually and persists for a period following reperfusion. (Jpn Heart J 1997; 38: 91-105)

Key words: Free radicals, Superoxide, Reperfusion, Chemiluminescence, Myocardial infarction

CORONARY artery reperfusion by means of thrombolytic therapy, percutaneous transluminal angioplasty, or bypass surgery is rapidly emerging as the fundamental strategy in the management of acute myocardial infarction. However, it has more recently been suggested that restoration of

From the First Department of Internal Medicine, Fukushima Medical College, Fukushima, Japan and ¹Department of Photon and Free Radical Research, Immunoresearch Laboratories Co., Ltd., Takasaki, Japan.

Address for correspondence: Yukio Maruyama, MD, Professor and Chairman, First Department of Internal Medicine, Fukushima Medical College, 1 Hikarigaoka, Fukushima 960-12, Japan.

Presented at the 65th Scientific Sessions of the American Heart Association Meeting November 18, 1992.

Received for publication May 27, 1996.

Accepted July 1, 1996.

blood flow may salvage one population of previously ischemic myocytes, yet have deleterious effect on other viable, previously ischemic cells. This phenomenon, so-called "reperfusion injury", is thought to be a consequence of oxygen-derived free radicals.^{1,2)}

The measurements of oxygen-derived free radical generation during ischemia and reperfusion have been carried out in isolated saline-perfused heart models³⁻¹⁰⁾ and *in vivo* heart models.^{11,12)} The major difference between isolated saline-perfused heart models and *in vivo* heart models is the presence of blood components including neutrophils, a potential source of superoxide. According to a study,¹³⁾ using an isolated blood or crystalloid-perfused rabbit heart with polyethylene glycol (PEG) -SOD, PEG-SOD protected the blood-perfused rabbit heart from the injury mediated by free radicals during ischemia and reperfusion. This protective action was observed during late reperfusion (> 10 mins). However, no protection was observed in the rabbit heart perfused with crystalloid. Blood-borne elements such as neutrophils are strong candidates for causing reperfusion injury mediated by free radicals.

In this way, the recruitment and activation of neutrophils in the reperfused myocardium may depend on the duration of ischemia. It may require an ischemic insult of greater severity over a long time period of ischemia and thereafter reperfusion than that which produces reversible dysfunction.¹⁴⁻¹⁶⁾ Neutrophils may not play an important role in the genesis of postischemic dysfunction after the occlusion of the coronary artery for 15 mins in the canine myocardium.¹⁷⁾ Therefore, in the isolated saline-perfused heart models and Bolli's stunned myocardium, i.e., reversible injury, of intact dogs,¹²⁾ it is difficult to consider that the neutrophils may have participated in the generation of the oxygen-derived free radicals which peaked within the first 30 secs to 5 mins of reperfusion.

In contrast, in an *in vivo* model which produced irreversible myocardial injury during ischemia and reperfusion,¹¹⁾ the oxygen-derived free radical signals were detected strongly during the late phase (1-3 hours) of reperfusion. However, the time course of oxygen-derived free radical generation during ischemia and reperfusion was not clarified in that study.

Our objective was to clarify the time course of the oxygen-derived free radical generation during ischemia and reperfusion in an *in vivo* irreversible myocardial injury rabbit heart model. We subjected the rabbit heart to 30 mins of coronary occlusion followed by 90 mins of reperfusion. In contrast to the stunned myocardium,¹²⁾ there may be enough ischemic injury for the rabbit myocardium with few native collaterals to activate the chemotactic mechanisms for neutrophils.¹⁴⁻¹⁶⁾ In addition, to detect continuously these oxygen-derived free radicals, we used a method of enhanced chemiluminescence with the agent 2-methyl-6-

[*p*-methoxyphenyl]-3, 7-dihydroimidazo [1, 2- α] pyrazin-3-one (MCLA) as a specific probe for detecting superoxide radicals.^{18,19)}

MATERIALS AND METHODS

Surgical preparation: Male New Zealand white rabbits (1.7~2.5 kg) were anesthetized with intravenous sodium pentobarbital (30 mg/kg). Anesthesia was maintained with the continuous infusion of pentobarbital (25 mg/h). Each animal was intubated through a tracheostomy and mechanically ventilated with room air at a rate of 25 strokes/min and a tidal volume of 30 ml. Catheters were inserted into both of the jugular veins for MCLA and drug, and into the left ventricle through the carotid artery for measurement of regional myocardial blood flow using the colored microsphere method,²⁰⁾ and into the femoral artery to monitor arterial pressure and to collect blood samples. A left lateral thoracotomy was made through the fifth intercostal space and the pericardium was excised, allowing us to visualize the lateral surface of the left ventricle. A 5-0 suture was passed around a branch of the left circumflex coronary artery with a tapered needle and then brought through a long rigid polyethylene tube. The latter served as a reversible snare occluder that was introduced into the light-proof box from the outside. The rabbit was placed in the box as close as possible to the window of the photomultiplier tube to measure the light emitted from the surface of the heart. The area that interrupted the counting of photons in the target area was masked with a black cloth. The animal's body temperature was monitored rectally and maintained at about 39°C by means of a jacket containing warm water (Figure 1).

Measurement of regional myocardial blood flow: We used the colored

Figure 1. Diagram of the chemiluminescence of single-photon-counting apparatus and additional systems for measuring the light emission from the surface of the heart.

microsphere method²⁰⁾ in some experiments ($n = 4$) to measure regional myocardial blood flow in order to confirm the presence of myocardial ischemia and reperfusion while counting the light emission.

Colored microspheres (15 μm in diameter) were obtained from E-Z Trac (Los Angeles, CA). An amount of $2 \times 10^6/1.0 \text{ ml}$ of each color solution (red, blue, green, and yellow) was injected into the left ventricle via a long cannula from outside of the box. The reason for using these four colored microspheres is described later in the experimental protocol. The cannula was then flushed with 1.0 ml of heparinized saline. A reference blood sample was obtained from the femoral artery at the rate of 3.6 ml/min starting at 5 secs before the injection and continued for 60 secs after the injection (total collection time of about 90 secs). After completing the flow measurement, we injected 2000 units of heparin intravenously. The animal was then sacrificed by an overdose of pentobarbital. The heart was removed and the circumflex coronary artery was again occluded. The aorta was perfused in a retrograde manner with 0.25% Evans blue dye. The area at risk was identified by a lack of staining with this dye. The samples of ischemic and nonischemic tissue were then weighed and placed in individual test tubes to count the microspheres.

Photon counting system: The system for counting photons used in the present study (Figure 1) was essentially the same as that reported by Takahashi et al.²¹⁾ In brief, experiments were carried out in a light-proof box with an R1332 photomultiplier tube (Hamamatsu Photonics Inc., Hamamatsu, Japan), that was responsive in the range of 350–650 nm. We placed a dry air jacket in front of a shutter and a window of the photomultiplier tube to prevent condensation. After a dark current (a signal produced with the shutter closed) was recorded for 10 secs, the shutter was opened for 20 secs. This procedure was repeated throughout the experiment.

Chemicals: MCLA (Tokyo Kasei Co., Tokyo, Japan) was dissolved in saline and the concentration was calculated using the value of $\epsilon_{430 \text{ nm}} = 9600 \text{ M}^{-1} \text{ cm}^{-1}$. The solution was divided into 10 ml aliquots, stored at -80°C and then diluted to 100 μM with saline just before use. Recombinant human superoxide dismutase (rh-SOD), a gift from Ube Kosan (Tokyo) had an enzyme activity of 5340 U/mg.

Experimental protocol:

Group I (I/R-group, $n = 7$). In an ischemia-reperfusion group, we observed the natural light emission for 5–10 mins and then rapidly injected MCLA (100 μM , 1.0 ml/kg) followed by its continuous infusion at a dose of 1.0 ml/kg/h until the end of the experiment. After observing 45 mins of MCLA-dependent chemiluminescence as the control, we subjected the I/R-group to 30 mins of coronary occlusion followed by 90 mins of reperfusion. Light emission was continuously

observed. The MCLA-enhanced chemiluminescence, expressed in terms of light intensity (counts/10 secs), was calculated by subtracting the mean value of the natural light intensity for the initial 5–10 mins before infusion of MCLA. The steady-state value before ischemia was obtained as the mean value of the MCLA-enhanced chemiluminescence for the last 10 mins of the control period. Similarly, the value of increase in chemiluminescence during reperfusion was obtained as the mean value of the MCLA-enhanced chemiluminescence for the 90 mins of the reperfusion period. The beginning time of the increase in chemiluminescence during reperfusion was regarded as the time which showed the increase in chemiluminescence over the preischemic steady-state value. The time to peak value of the increase in chemiluminescence during reperfusion was regarded as the time which showed the end of the increase in chemiluminescence during reperfusion.

In some experiments ($n = 4$) of the I/R-group, regional myocardial blood flow was measured before ischemia, during ischemia, and again after 5 mins and 90 mins of reperfusion.

Group II (S-group, $n = 4$). The same procedure, with the exception of coronary occlusion, was also performed in the sham-operated group.

Group III (SOD group, $n = 4$). Another ischemia-reperfusion group underwent the same procedure as the I/R-group (except for 20–30 mins of ischemia), and then received a bolus intravenous injection of rh-SOD (20 mg/kg) during reperfusion to observe the chemiluminescence response. All groups received an intramuscular injection of procainamide (100 mg/kg) to prevent arrhythmia before we counted the light emission.

Statistical analysis: Values are expressed as mean $\pm$ SEM. Statistical analysis employed analysis of variance (ANOVA), followed by paired Student's *t*-test. A level of $p < 0.05$ was considered statistically significant.

RESULTS

Chemiluminescence response: Results from a representative case of the sham-operated group are shown in Figure 2. After observing the natural light emission for 10 mins, MCLA was then injected. A strong burst of chemiluminescence over the natural light emission was detected from the surface of the heart. Nonspecific light emission that was not inhibited by rh-SOD gradually decreased and remained essentially stable for about 3 hours during the continuous infusion of MCLA. In the ischemia-reperfusion group (Figure 3), the enhanced chemiluminescence decreased during ischemia. However, some experiments showed a transient increase in chemiluminescence during ischemia (Figure 4). Following reperfusion, the chemiluminescence returned to the preischemic level. The in-

Figure 2. Photon counts in representative case in sham-operated group.

Figure 3. Photon counts in representative case of ischemia-reperfusion group. Ischemia (ISC) began when the nonspecific chemiluminescence was observed to be essentially constant. The chemiluminescence decreased during ischemia. With reperfusion (REP), the chemiluminescence gradually returned to the preischemic level. The increase in chemiluminescence began at 14 mins and peaked at 56 mins of reperfusion.

crease in chemiluminescence began at 13 ± 2 mins and peaked at 52 ± 12 mins of reperfusion.

In the I/R group, the mean value of the MCLA-enhanced chemiluminescence was 818 ± 350 counts/10 secs during the steady-state period before ischemia. During the 90 mins of reperfusion, the enhanced chemiluminescence significantly increased to a mean of 1077 ± 401 counts/10 secs (818 ± 350 vs 1077 ± 401 ; $p < 0.01$). In contrast, the mean value in the sham-operated group was 650 ± 141 counts/10 secs during the period consistent with the preischemic

Figure 4. Photon counts (upper panel) and regional myocardial blood flow (lower panel) in a case showing a transient increase in chemiluminescence during ischemia. Regional myocardial blood flow was simultaneously measured before ischemia, during ischemia, and again after 5 mins and 90 mins of reperfusion. Open circles = non-ischemic area; Closed circles = ischemic area. CON = preischemic control period; ISC = ischemic period; REP5' = after 5 mins of reperfusion; REP90' = after 90 mins of reperfusion.

steady-state period in the I/R group. During the period consistent with the 90 minute period of reperfusion in the I/R group, the mean value was 649 ± 155 counts/10 secs and not significantly changed in the sham-operated group (650 ± 141 vs 649 ± 155 : NS).

Regional myocardial blood flow: The mean regional myocardial blood flows (RMBF) are shown in Figure 5. In the ischemic region, RMBF decreased during the period of ischemia and recovered at 5 mins of reperfusion. The value at 90 mins of reperfusion tended to be lower than that before ischemia. RMBF was not significantly changed in the non-ischemic region.

Effects of superoxide dismutase: Two representative cases from the group given rh-SOD are shown in Figure 6. When this SOD was injected as a bolus at

Figure 5. Mean regional myocardial blood flows (RMBF) in ischemic and non-ischemic region of ischemia-reperfusion group ($n = 4$). In non-ischemic region, RMBF did not differ significantly during the observation periods. Values are mean $\pm$ SEM. Cont. = pre-ischemic control period; Isc. = ischemic period; Rep. 5' = after 5 mins of reperfusion; Rep. 90' = after 90 mins of reperfusion. $p < 0.05$ compared between preischemic control period and ischemic period, and between ischemic period and after 5 mins of reperfusion.

38 $\pm$ 9 mins of reperfusion, the increase from the steady-state value of preischemic chemiluminescence before giving rh-SOD was 725 $\pm$ 48 counts/10 secs. The value of post-SOD decreased to 551 $\pm$ 66 counts/10 secs (Figure 7). The administration of rh-SOD significantly attenuated the increase in chemiluminescence by 24.1 $\pm$ 6.8% (725 $\pm$ 48 vs 551 $\pm$ 66; $p < 0.01$) for a period of 24.3 $\pm$ 6.8 mins. However, the increase in chemiluminescence after reperfusion was not completely inhibited by the amount of the administered rh-SOD to the preischemic steady-state value of chemiluminescence.

Hemodynamics: Aortic pressure and heart rate were almost constant in the sham-operated group during the experiment. The mean aortic pressure at the beginning of the experiment, after 60 mins, and after 120 mins was 93 $\pm$ 12, 92 $\pm$ 13, and 92 $\pm$ 16 mmHg, respectively, and each corresponding heart rate was 246 $\pm$ 22, 247 $\pm$ 30, and 259 $\pm$ 31 beats/min, respectively. The mean aortic pressure in the I/R-group averaged 93 $\pm$ 26 mmHg before ischemia. It fell to 81 $\pm$ 25 mmHg during ischemia and was at 82 $\pm$ 30 mmHg during reperfusion, which is not significantly different from each corresponding preischemic value. The heart rate in the I/R-group remained essentially unchanged throughout the experiment at 262 $\pm$ 16, 266 $\pm$ 21, and 270 $\pm$ 18 beats/min, respectively.

Figure 6. Photon counts in two representative cases of SOD-treated group. In case 1 (upper panel), superoxide dismutase (SOD) was injected as an iv bolus after 30 mins of reperfusion. It attenuated the increase in chemiluminescence by 31.8% for a period of 19.3 mins. Case 2 (lower panel) showed a transient increase in chemiluminescence during ischemia. SOD was administered as an iv bolus after 43 mins of reperfusion and attenuated the increase in chemiluminescence by 15.2% for a period of 21.0 mins. ISC = ischemia started; REP = reperfusion started; SOD = superoxide dismutase (20 mg/kg) was administered in a bolus form during reperfusion.

DISCUSSION

We continuously measured superoxide generation *in situ* during ischemia and reperfusion in the rabbit heart. The time to peak generation of free radicals during reperfusion (52 mins in the present study) was not as short as previously reported in isolated heart models.³⁻¹⁰ The generation of superoxide *in situ* in the heart appeared to increase gradually and to continue for a time during reperfusion.

Methodological considerations: Oxygen-derived free radicals have been measured in models of myocardial ischemia-reperfusion *in vivo* and *in vitro* by

Figure 7. Photon counts showing attenuating effect of superoxide dismutase (SOD) on an increased chemiluminescence during reperfusion ($n = 4$). Values are mean $\pm$ SEM. $p < 0.01$ before vs after administration of SOD.

ESR.^{3-8,11,12,22)} However, tissue ESR and the freeze-clamping of tissue,³⁾ can produce artificial radicals.^{8,22)} When using the spin-trapping technique, one must be cautious in conducting spectrum analysis because of the complex characteristics of the spin-trap agent itself.²³⁾ Moreover, ESR measurement cannot be conducted continuously because of the need to collect tissue samples from the myocardium or the coronary perfusate.

With the chemiluminescence method, however, one can measure the generation of oxygen-derived free radicals both nondestructively and continuously in biological systems.^{9,10,24-30)} When measuring non-enhanced chemiluminescence in myocardial tissue,²⁷⁻³⁰⁾ it is difficult to identify the molecular source of photoemission because of the small concentration and low rate of production of the excited species. However, the use of luminescent compounds that selectively enhance the light emission associated with definite free radical reactions may make the chemiluminescence method more specific for investigating free radical reactions in biological systems. Luminol is widely used to enhance luminescence. Unfortunately, its chemiluminescence is brought about by oxidants other than the superoxide anion. While lucigenin is suggested to be more specific for the superoxide anion than luminol, *in vivo* proof is lacking particularly since this agent also enhances the chemiluminescence induced by hydrogen peroxide.^{25,26)}

Nakano et al. recently found that the Cypridina luciferin analog 2-methyl-

6-[p-methoxyphenyl]-3, 7-dihydroimidazo [1, 2-a] pyrazin-3-one (MCLA) is a highly sensitive and specific chemiluminescence probe for identifying the superoxide generated by activated leukocytes and monocytes.^{18,19,21)} We used MCLA to continuously observe the MCLA-dependent chemiluminescence from the surface of the rabbit hearts during ischemia and reperfusion. Limitations of this study method are: 1) MCLA has a nonspecific weak luminescence that is probably autoxidized by light emission at 465 nm,³¹⁾ which is not affected by superoxide dismutase³²⁾; 2) it is likely that the chemiluminescence from the subepicardium is the predominant source of the light detected in the tissue as dense as the myocardium, although it is uncertain which layer of the myocardium actually generates the chemiluminescence; and 3) the areas in which the chemiluminescence is detected from the surface of the heart may include not only the ischemic region, but also non-ischemic regions of various sizes because of the regional myocardial ischemia produced by selective coronary occlusion.

Time course of the generation of oxygen-derived free radicals in reperfused myocardium: Several investigators have reported the time course of the generation of oxygen-derived free radicals in the reperfused myocardium. Zweier et al., using ESR with freeze-clamping³⁾ and spin-trapping⁴⁾ techniques in isolated rabbit hearts, reported that a burst of free radical generation was observed within 30 secs of reperfusion. Kramer et al.,⁷⁾ using ESR with a spin-trapping technique in isolated buffer-perfused rat hearts, also demonstrated maximal spin-adduct detection after 3 mins of reperfusion, with a decrease observed in 7–10 mins. Garlick et al.,⁵⁾ using ESR, found that free radicals peaked after 4 mins of reperfusion as carbon-centered or alkoxyl radicals in the isolated ischemia-reperfused rat heart. Similarly, Henry et al.⁹⁾ investigated isolated buffer-perfused rat hearts using an enhanced chemiluminescence method with lucigenin as the luminescent probe. They showed that the lucigenin-induced luminescence was markedly increased, with the peak concentration observed within the first 3–5 mins of reperfusion. Okuda et al.,¹⁰⁾ using the luminol-enhanced chemiluminescence method with the same model, also demonstrated that the chemiluminescence increased further during the first 5 mins and then decreased slowly. Thus, findings in the isolated buffer-perfused heart model showed that the generation of free radicals peaked within a few minutes of reperfusion.

Bolli et al.,¹²⁾ using intact dogs *in vivo* subjected to only 15 mins of coronary occlusion, reported that the generation of oxygen-derived free radicals such as alkoxy and alkyl radicals, which could have been formed by a reaction between the primary oxygen radicals and membrane lipids, increased significantly within the first few minutes of reperfusion. Thus, it seems likely that the generation of oxygen-derived free radicals in the reperfused heart *in vivo* peaks within a few minutes of reperfusion when the period of ischemia is short.

However, Kuzuya et al.,¹¹⁾ using ESR in intact dogs subjected to 90 mins of coronary occlusion followed by 5 hours of reperfusion, detected the peak concentration of superoxide radicals at 60 mins of reperfusion. Similar to the findings of Kuzuya et al., we observed in the rabbit heart *in vivo* subjected to 30 mins of coronary occlusion followed by 90 mins of reperfusion that the peak concentration of superoxide was at 52 mins of reperfusion. While in previous studies it was generally uncertain when the generation of superoxide began during reperfusion of the heart *in vivo*, we clearly observed the beginning of superoxide generation from the surface of the heart at 13 mins, which then rose gradually following reperfusion.

Generation of oxygen-derived free radicals during ischemia: We observed a transient increase in MCLA-dependent luminescence during 30 mins of ischemia in 4 of the 11 rabbits, including the I/R group (7 cases) and the SOD-treated group (4 cases). While the mechanism of the generation of oxygen-derived free radicals during ischemia has been hypothesized,^{12,33,34)} it has not been confirmed.

Bolli et al.¹²⁾ showed a slight generation of oxygen-derived free radicals during 15 mins of occlusion in the canine coronary artery. Arroyo et al.⁶⁾ also detected oxygen-derived free radicals during ischemia using low-flow global ischemia (0.1 ml/min) with the isolated buffer-perfused rat heart. Zweier et al.³⁾ demonstrated that oxygen-derived free radicals increased during 10 mins of global ischemia (no coronary flow) in the isolated buffer-perfused rabbit heart. Other investigators^{9,10)} reported similar results.

In the present study, regional myocardial blood flow simultaneously observed in a case that showed a transient increase in chemiluminescence during ischemia apparently decreased in the center of the ischemic region (Figure 4). However, areas showing the MCLA-dependent luminescence from the surface of the heart may include not only the ischemic, but also non-ischemic regions of various sizes. Therefore, the increased chemiluminescence in this region during coronary occlusion could have been due to the presence of a partial ischemic area having a small blood supply at the periphery of the ischemic region.

Effects of superoxide dismutase: SOD administration significantly inhibited the increase in MCLA-dependent chemiluminescence. However, this SOD did not completely abolish the increase in chemiluminescence to the baseline level observed before induction of ischemia. Possible explanations for this finding are: 1) the administered dose of SOD was too low to abolish the generation of superoxide during reperfusion; and 2) SOD has only a brief half-life (6–10 mins). In this study, the time during which chemiluminescence was inhibited by the bolus injection of SOD was 24 mins. Thus, when superoxide was generated in the intravascular space, SOD would be able to catalyze immediately the dismutation

of superoxide. However, it is possible that SOD, a high molecular weight protein, failed to reach the interstitial space in which the superoxide could be generated, either fast enough or in adequate concentrations via the vascular endothelium; and 3) superoxide anions reportedly increase the vascular permeability of the skeletal muscle,³⁵⁾ brain,³⁶⁾ and lung.³⁷⁾ Thus, the superoxide generated in the reperfused myocardium may perhaps enhance the vascular leakage of plasma containing MCLA. Therefore some of the increased chemiluminescence during reperfusion would reflect not only the generation of superoxide, but also the nonspecific luminescence emitted by myocardial edema.

However, whatever the cause, it was apparent that the increase in chemiluminescence during reperfusion indicated, at least in part, the generation of superoxide since part of the increase in chemiluminescence during reperfusion was inhibited by the administration of SOD.

Conclusions: To clarify the time course of superoxide generation during ischemia and reperfusion in the irreversibly injured rabbit heart, we used a method involving enhanced chemiluminescence in which MCLA was used as a specific probe for detecting superoxide. We found that: 1) superoxide generation started at 13 mins and peaked at 52 mins of reperfusion; and 2) the administration of a bolus injection of SOD during reperfusion transiently and partially attenuated the generation of superoxide. These observations strongly suggest that superoxide generation in the ischemic irreversibly injured *in vivo* rabbit heart gradually increases and continues for a time during reperfusion.

REFERENCES

1. McCord JM. Oxygen-derived free radicals in postischemic tissue injury. *N Engl J Med* 1985; 312: 159-63.
2. Przyklenk K, Kloner RA. Reperfusion injury by oxygen-derived free radicals? Effect of superoxide dismutase plus catalase, given at the time of reperfusion, on myocardial infarct size, contractile function, coronary microvasculature, and regional myocardial blood flow. *Circ Res* 1989; 64: 86-96.
3. Zweier JL, Flaherty JT, Weisfeldt ML. Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proc Natl Acad Sci USA* 1987; 84: 1404-7.
4. Zweier JL. Measurement of superoxide-derived free radicals in the reperfused heart. *J Biol Chem* 1988; 263: 1353-7.
5. Garlick PB, Davies MJ, Hearse DJ, Slater TF. Direct detection of free radicals in the reperfused rat heart using electron spin resonance spectroscopy. *Circ Res* 1987; 61: 757-60.
6. Arroyo CM, Kramer JH, Dickens BF, Weglicki WB. Identification of free radicals in myocardial ischemia/reperfusion by spin trapping with nitron DMPO. *FEBS Lett* 1987; 221: 101-4.
7. Kramer JH, Arroyo CM, Dickens BF, Weglicki WB. Spin-trapping evidence that graded myocardial ischemia alters post-ischemic superoxide production. *Free Radic Biol Med* 1987; 3: 153-9.
8. Baker JE, Felix CC, Olinger GN, Kalyanaraman B. Myocardial ischemia and reperfusion: direct evidence for free radical generation by electron spin resonance spectroscopy. *Proc Natl Acad Sci USA* 1988; 85: 2786-9.
9. Henry TD, Archer SL, Nelson D, Weir EK, From AHL. Enhanced chemiluminescence as a measure of oxygen-derived free radical generation during ischemia and reperfusion. *Circ Res* 1990; 67: 1453-61.

10. Okuda M, Ikai I, Chance B, Kumar C. Superoxide dismutase, but not catalase or mannitol, decrease oxygen radical formation in perfused rat heart during ischemia-reperfusion as monitored by luminol-enhanced chemiluminescence. *J Appl Cardiol* 1991; 6: 113-21.
11. Kuzuya T, Hoshida S, Kim Y, et al. Detection of oxygen-derived free radical generation in the canine postischemic heart during late phase of reperfusion. *Circ Res* 1990; 66: 1160-5.
12. Bolli R, Patel BS, Jeroudi MO, Lai EK, McCay PB. Demonstration of free radical generation in "stunned" myocardium of intact dogs with the use of the spin trap *a*-phenyl *n*-tert-butyl nitron. *J Clin Invest* 1988; 82: 476-85.
13. Galinanes M, Qiu Y, Ezrin A, Hearse DJ. PEG-SOD and myocardial protection: studies in the blood- and crystalloid-perfused rabbit and rat hearts. *Circulation* 1992; 86: 672-82.
14. Go LO, Murry CE, Richard VJ, Weischedel GR, Jennings RB, Reimer KA. Myocardial neutrophil accumulation during reperfusion after reversible or irreversible ischemic injury. *Am J Physiol* 1988; 255: H1188-98.
15. Engler RL, Dahlgren MD, Morris D, Peterson MA, Schmid-Schonbein G. Role of leukocytes in response to acute myocardial ischemia and reflow in dogs. *Am J Physiol* 1986; 251: H314-22.
16. Lucchesi BR, Mullane KM. Leukocytes and ischemia-induced myocardial injury. *Ann Rev Pharmacol Toxicol* 1986; 26: 201-24.
17. Bolli R, Roberts R. Role of oxy-radicals in postischemic myocardial dysfunction, In: Singal PK, editor. *Oxygen Radicals in the Pathophysiology of Heart Disease*. Boston, Kluwer Academic Publishers, 1988; 203-25.
18. Nakano M. Determination of superoxide radical and singlet oxygen based on chemiluminescence of luciferin analogs. *Methods in Enzymology* 1990; 186: 585-91.
19. Nishida A, Kimura H, Nakano M, Goto T. A sensitive and specific chemiluminescence method for estimating the ability of human granulocytes and monocytes to generate O_2^- . *Clin Chem Acta* 1989; 179: 177-82.
20. Hale SL, Alker KJ, Kloner RA. Evaluation of nonradioactive, colored microspheres for measurement of regional myocardial blood flow in dogs. *Circulation* 1988; 78: 428-34.
21. Takahashi A, Nakano M, Mashiko S, Inaba H. The first observation of O_2^- generation in *in situ* lungs of rats treated with drugs to induce experimental acute respiratory distress syndrome. *FEBS Lett* 1990; 261: 369-72.
22. Nakazawa H, Ichimori K, Shinozaki Y, Okino H, Hori S. Is superoxide demonstration by electron-spin resonance spectroscopy really superoxide? *Am J Physiol* 1988; 255: 213-5.
23. Pou S, Hassett DJ, Britigan BE, Cohen MS, Rosen GM. Problems associated with spin trapping oxygen centered free radicals in biological systems. *Anal Biochem* 1989; 177: 1-6.
24. Boveris A, Cadenas E, Reiter R, Filipkowski M, Nakase Y, Chance B. Organ chemiluminescence: noninvasive assay for oxidative radical reactions. *Proc Natl Acad Sci USA* 1980; 77: 347-51.
25. Archer SL, Nelson DP, Weir EK. Simultaneous measurement of O_2 radicals and pulmonary vascular reactivity in the rat lung. *J Appl Physiol* 1989; 67: 1903-11.
26. Archer SL, Nelson DP, Weir EK. Detection of activated O_2 species *in vitro* and in rat lungs using chemiluminescence. *J Appl Physiol* 1989; 67: 1912-21.
27. Cadenas E, Boveris A, Chance B. Low-level chemiluminescence of bovine heart submitochondrial particles. *Biochem J* 1980; 186: 659-67.
28. Barsacchi R, Camici P, Bottigli U, et al. Correlation between hydroperoxide-induced chemiluminescence of the heart and its function. *Biochim Biophys Acta* 1983; 762: 241-7.
29. Barsacchi R, Pelosi G, Camici P, Bonaldo L, Maiorino M, Ursini F. Glutathione depletion increases chemiluminescence emission and lipid peroxidation in the heart. *Biochim Biophys Acta* 1984; 804: 356-60.
30. Barsacchi R, Coassin M, Maiorino M, Pelosi G, Simonelli C, Ursini F. Increased ultraweak chemiluminescence emission from rat heart at postischemic reoxygenation: protective role of vitamin E. *Free Radic Biol Med* 1989; 6: 573-9.
31. Nakano M. Assay for superoxide dismutase based on chemiluminescence of luciferin analog. *Methods in Enzymology* 1990; 186: 227-32.
32. Kimura H, Nakano M. Highly sensitive and reliable chemiluminescence method for the assay of

- superoxide dismutase in human erythrocytes. *FEBS Lett* 1988; 239: 347-50.
33. Thompson JA, Hess ML. The oxygen free radical system: a fundamental mechanism in the production of myocardial necrosis. *Prog Cardiovasc Dis* 1986; 28: 449-62.
 34. Ferrari R, Curello S, Cargnoni A, et al. Importance of free radicals generated by endothelial and myocardial cells in ischemia and reperfusion, In: Piper HM, editor: *Pathophysiology of Severe Ischemic Myocardial Injury*. Dordrecht, Kluwer Academic Publishers, 1990; 221-38.
 35. Korthuis RJ, Smith JK, Carden DL. Hypoxic reperfusion attenuates post-ischemic microvascular injury. *Am J Physiol* 1989; 256: H315-9.
 36. Kontos HA, Wei EP, Ellis EF, et al. Appearance of superoxide anion radical in cerebral extracellular space during increased prostaglandin synthesis in cats. *Circ Res* 1985; 57: 142-51.
 37. Risberg B, Smith L, Stenberg B. Prevention of edema formation in the perfused lung preparation by oxygen radical scavengers. *Eur Surg Res* 1985; 17: 230-6.