EFFECTS OF HUMAN SUPEROXIDE DISMUTASE ON ISCHEMIC AND REPERFUSED MYOCARDIUM IN ISOLATED PERFUSED RAT HEART

SHIN-ICHI MAIE, M.D., KAZUHIRO NAKAMURA, M.D., KAZUO ICHIHARA, Ph.D.
AND YASUSHI ABIKO, M.D.

The effect of human superoxide dismutase (h-SOD) on the ischemic heart was studied in the isolated perfused working rat heart. Myocardial mechanical function expressed as pressure-rate product decreased and completely stopped within 5 min after the onset of global ischemia, and never recovered after reperfusion following 20 min of ischemia. In the ischemic myocardium, the levels of ATP, ADP, and creatine phosphate decreased, and those of AMP and lactate increased. Reperfusion of the ischemic heart did not restore the level of ATP completely. When the heart was treated with h-SOD, the perfusion medium was switched from the buffer containing no h-SOD to that containing h-SOD at either 100, 300 or 1,000 units/ml 5 min before the onset of ischemia. The pressure-rate products of the heart treated with 100, 300, and 1,000 units/ml of h-SOD were restored by reperfusion to 22%, 59%, and 51% of the preischemic level, respectively. The levels of ATP and creatine phosphate in the reperfused heart with 300 and 1,000 units/ml of h-SOD were significantly higher than those without h-SOD. However, a dose-response relationship was not observed when h-SOD was used in concentrations greater than 300 units/ml. These results indicate that a certain amount of h-SOD has some beneficial effects on the ischemic myocardium.

FREE radicals, such as the superoxide anion (O_2^-), the hydroxy radical ($\cdot OH$) and hydrogen peroxide (H_2O_2), have been implicated as important factors in tissue injury in various pathophysiological conditions! Reperfusion injury that follows a certain period of ischemia is also due partly to oxygen free radical generation^{2,3}. Because h-SOD alters O_2^- to H_2O_2 , and because catalase changes H_2O_2 to water, these enzymes scavenge the oxygen free radicals from the tissue. The free radical scavengers, therefore, can protect the heart from ischemia-

and reperfusion-induced injury. In fact, the combined administration of h-SOD and catalase reduces myocardial injury caused by ischemia and reperfusion; it decreases myocardial infarct size⁴ and improves myocardial segment function in dogs⁵. It also prevents mitochondrial dysfunction in rabbit⁶. In addition, h-SOD alone protects myocardium from ischemic and/or reperfusion injury. Administration of h-SOD alone reduces myocardial infarct size in dogs⁷, and improves recovery of mechanical function and tissue ATP and creatine phosphate during reperfusion after ischemia in isolated rat hearts⁸ and rabbit hearts⁹. Doses of h-SOD used in these experiments were up to 100 units/ml⁸⁻¹⁰. Larger amounts of h-SOD are now available for use in experimental anim-

Key words:

h-SOD
Perfused working rat heart
Ischemia
Energy charge potential

(Received January 6, 1990; accepted July 18, 1990)

Department of Pharmacology, Asahikawa Medical College, Asahikawa, Japan

Mailing address: Kazuo Ichihara, Ph.D., Department of Pharmacology, Asahikawa Medical College, 4-5 Nishikagura, Asahikawa 078, Japan

Fig.1. Effect of h-SOD on the pressure-rate products of "reperfusion" groups. Hearts were perfused as Langendorff preparation, and then perfusion was switched to the working heart preparation at 0 min (Work begun). After 10 min of the preliminary working perfusion, the perfusion was continued for another 5 min with either normal perfusate (0 units/ml; ●) or the perfusate containing 100 units/ml (◆), 300 units/ml (▲), or 1,000 units/ml (■) of h-SOD (SOD containing buffer). Ischemia was initiated at 15 min by lowering the afterload pressure to 0 mmHg (Ischemia). Twenty minutes after the initiation of ischemia, the afterload pressure was raised to 60 mmHg, so that the heart could be reperfused (Reperfusion). Values are means $\pm$ S.E. of 5–7 observations.

* $p < 0.05$, compared with "0 units/ml" at the respective period of reperfusion.

als because of the development of recombinant h-SOD.

The present study was undertaken to examine the effect of high doses of h-SOD on both mechanical and metabolic changes induced by ischemia and by reperfusion following ischemia.

MATERIALS AND METHODS

Heart perfusion

Hearts were removed from fed male Sprague-Dawley rats weighing 200–250g, and perfused by the Langendorff and working heart techniques.¹¹ Krebs-Henseleit bicarbonate buffer containing 11 mM glucose equilibrated with a gas mixture of 95% O₂–5% CO₂ was used as perfusion medium (normal perfusate). The hearts were first perfused by the Langendorff technique with the normal perfusate. Ten minutes after the Langendorff perfusion, the hearts were perfused in turn by the working heart technique

using the normal perfusate for 10 min, with a left atrial filling pressure of 9 mmHg and a hydrostatic aortic afterload pressure of 60 mmHg. The perfusion was continued for another 5 min with either the normal perfusate or the buffer containing h-SOD, in which h-SOD (specific activity 4,667 units/mg protein) was added to the normal buffer at a concentration of 100 units/mg, 300 units/ml, 1,000 units/ml. Then ischemia (global ischemia) was induced by lowering the hydrostatic aortic afterload pressure to 0 mmHg, resulting in practically no coronary flow.¹² After 20 min of ischemia, the hearts were reperfused for 20 min as a working heart preparation by raising the hydrostatic afterload pressure to 60 mmHg again. In the present study, the cardiac mechanical function was defined as the product of peak aortic pressure and heart rate (pressure-rate product), because the pressure-rate product is closely related to oxygen consumption.¹¹

In control (non-ischemia) and ischemic ex-

Fig.2. Effect of h-SOD on energy charge potential. Hearts were removed 15 min after the working perfusion, 20 min after the initiation of ischemia, and 20 min after reperfusion. Perfusate was switched from the normal buffer to the buffer containing either 0 units/ml ($\square$), 100 units/ml ($\square$), 300 units/ml ($\square$), or 1,000 units/ml ($\square$) of h-SOD. Values are means $\pm$ S.E. of 5–7 observations.

* $p < 0.05$, compared with "0 units/ml" in each "Ischemia" and "Reperfusion" group.

$p < 0.05$, compared with "Non-ischemia" in the respective group.

periments, 5 rats were used in each of the 0, 100, 300, and 1,000 units/ml of h-SOD-treated groups. In the reperfusion experiment, 6 rats were used in each 0, 300, and 1,000 units/ml of h-SOD-treated group and 7 rats in 100 units/ml of h-SOD-treated group.

Biochemical assays

At the end of perfusion experiments, the hearts were frozen with freezing tongs chilled in liquid nitrogen. The frozen heart sample was pulverized in a mortar cooled with liquid nitrogen. A part of the pulverized tissue powder was weighed and put into an oven (90°C) for 24h to measure the tissue water content and dry weight of the tissue. The remainder of the tissue powder was used for determination of the tissue levels of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), creatine phosphate and lactate. These were measured by standard enzymatic procedures¹³ To estimate the myocardial energy state, we calculated energy charge potential according to the following equation¹⁴:

$$\frac{[(\text{ATP}) + 0.5(\text{ADP})]}{[(\text{ATP}) + (\text{ADP}) + (\text{AMP})]}$$

(AMP)]

Statistical analysis

All values are expressed as means $\pm$ SE. Results were evaluated by the one-way analysis of variance followed by Dunnett's t-test. a p value of 0.05 or less was considered significant.

RESULTS

Mechanical function

The effects of h-SOD on the pressure-rate product of "reperfusion" groups are shown in Fig. 1. When the heart was perfused by the Langendorff technique, the pressure-rate products of the "reperfusion" groups treated with 0, 100, 300, and 1,000 units/ml of h-SOD were $(17.8 \pm 0.6) \times 10^3 \text{ mmHg} \cdot \text{min}^{-1}$, $(18.2 \pm 0.6) \times 10^3 \text{ mmHg} \cdot \text{min}^{-1}$, $(18.0 \pm 0.9) \times 10^3 \text{ mmHg} \cdot \text{min}^{-1}$, and $(18.5 \pm 1.0) \times 10^3 \text{ mmHg} \cdot \text{min}^{-1}$ respectively. There were no significant differences among them. These pressure-rate products were increased to over $30 \times 10^3 \text{ mmHg} \cdot \text{min}^{-1}$ by switching the perfusion to the working heart mode (peak aortic pressure was about 100 mmHg and

TABLE I EFFECTS OF h-SOD ON TISSUE METABOLITES

	ATP	ADP	AMP	TAN	Creatine phosphate	Lactate
<i>Control (Non-ischemia)</i>						
0 units/ml	19.3±0.9	7.4±1.0	3.5±0.8	30.2±2.3	18.7±2.5	4.9±3.0
100 units/ml	19.4±1.5	4.8±0.4*	1.7±0.2	25.9±1.7	19.5±1.7	2.8±1.5
300 units/ml	18.1±0.7	6.2±0.6	2.8±0.5	27.1±1.0	19.9±1.8	0.8±0.6
1,000 units/ml	17.7±0.8	5.0±0.6	2.6±0.4	25.3±0.9*	20.1±1.2	6.5±2.0
<i>Ischemia</i>						
0 units/ml	4.4±0.1#	4.7±1.0	11.0±0.9#	20.0±1.1#	3.7±0.4	73.5±9.2#
100 units/ml	4.5±0.6#	4.4±0.3	8.4±0.8#	17.3±0.5#	4.7±0.3#	70.8±6.2#
300 units/ml	4.9±0.9#	4.5±0.5	9.7±0.7#	19.1±1.4#	4.7±0.4#	28.9±5.6#
1,000 units/ml	4.9±0.8#	6.3±1.5	7.8±1.2*#	19.0±2.0#	4.7±0.6#	24.4±4.9*#
<i>Ischemia + reperfusion</i>						
0 units/ml	8.5±0.8#	3.0±0.1#	4.0±0.4	15.5±0.6#	20.1±2.3	4.4±2.3
100 units/ml	10.0±0.6#	3.4±0.3#	3.0±0.6	16.5±0.8#	24.7±1.2#	6.8±1.4
300 units/ml	12.8±1.2*#	3.9±0.6#	2.6±0.4	19.4±1.4*#	30.9±1.9*#	1.8±1.1
1,000 units/ml	12.4±1.0*#	4.3±0.4	3.5±1.1	20.5±1.3*#	27.0±1.6*#	5.6±1.7

Heart samples were frozen 15 min after control working perfusion (Non-ischemia), 20 min after the onset of ischemia (Ischemia), or 20 min after reperfusion following 20 min of ischemia (Ischemia+reperfusion). TAN=total adenine nucleotide (ATP+ADP+AMP). Values are expressed as mean±S.E. (μ moles/g dry weight) of 5–7 observations.

* $p<0.05$, compared with "0units/ml" in each "Control", "Ischemia" and "Ischemia+reperfusion" group.

$p<0.05$, compared with the respective values of "Control" group.

heart rate was over 300 beats/min). The pressure-rate product was sustained for 10 min after the work was begun. An addition of h-SOD did not modify the pressure-rate product until ischemia was induced. When ischemia was initiated by lowering the after-load pressure, the pressure-rate product became near 0 mmHg·min⁻¹ immediately. After 20 min of ischemia, the heart was reperfused with the same buffer used during ischemia for 20 min by the working heart technique again. Although the pressure-rate product of the heart perfused without h-SOD did not recover at all during the reperfusion period, that of the heart perfused with either 100, 300, or 1,000 units/ml of h-SOD increased gradually after reperfusion. The difference in the pressure-rate product between 0 units/ml and 300 or 1,000 units/ml of h-SOD became significant 10 min after the onset of reperfusion. However, the pressure-rate product of 100 units/ml of h-SOD-treated heart did not recover significantly, although it increased after reperfusion. After 20 min of reperfusion, the recovery of the pressure-rate product was 22% at 100 units/ml of hSOD (not significant compared with 0 units/ml of h-SOD), 59% at 300

units/ml of h-SOD ($p<0.05$, compared with 0 units/ml of h-SOD), and 51% at 1,000 units/ml of h-SOD ($p<0.05$, compared with 0 units/ml of h-SOD).

Myocardial Metabolism

The tissue levels of ATP, ADP, AMP, creatine phosphate and lactate in the heart just before ischemia (control), after 20 min of ischemia (ischemia), and after 20 min of reperfusion (ischemia + reperfusion) were determined (Table I). In the control heart, an addition of h-SOD did not affect the levels of all metabolites, except that the total adenine nucleotide level (ATP + ADP + AMP) significantly decreased in the heart treated with 1,000 units/ml of h-SOD, and the ADP level significantly decreased in the heart treated with 100 units/ml of h-SOD. In the ischemic heart, the levels of ATP and creatine phosphate decreased, while those of AMP and lactate increased after 20 min of ischemia. h-SOD at either dose did not affect the changes in ATP, ADP, AMP, and creatine phosphate levels caused by ischemia, except that h-SOD at 1,000 units/ml prevented the increase in AMP level significantly ($p<0.05$). The increase in the level of

lactate, however, was significantly ($p < 0.05$) inhibited by the presence of 300 units/ml and 1,000 units/ml of h-SOD. In the reperfused heart, the levels of ATP, AMP, creatine phosphate and lactate that had been changed by ischemia returned toward their respective control values. The levels of ATP, creatine phosphate and calculated total adenine nucleotide in the heart treated with 300 units/ml and 1,000 units/ml of h-SOD were restored by reperfusion more than those in the heart without h-SOD. Those levels in the heart with 100 units/ml of h-SOD were not significantly restored by reperfusion. The energy charge potential calculated is illustrated in Fig. 2. The values of energy charge potential in the control heart, were 0.766 ± 0.018 at 0 units/ml of h-SOD, 0.839 ± 0.011 at 100 units/ml of h-SOD, 0.784 ± 0.006 at 300 units/ml of h-SOD, and 0.800 ± 0.018 at 1,000 units/ml of h-SOD. All these values significantly ($p < 0.05$) decreased 20 min after the onset of ischemia. The energy charge potential in the ischemic myocardium treated with h-SOD appeared to be higher than that without h-SOD, but the difference was not significant. Reperfusion restored the energy charge potential that had been decreased by ischemia. In the absence of h-SOD and in the presence of 100 units/ml of h-SOD, the energy charge potential of the reperfused heart was still significantly ($p < 0.05$) lower than that of the control heart. In the presence of 300 units/ml and 1,000 units/ml of h-SOD, however, there was no significant difference in the energy charge potential between the control and the reperfused heart. The energy charge potential of the reperfused heart treated with 300 units/ml and 1,000 units/ml of h-SOD was significantly higher ($p < 0.05$) than that without h-SOD.

DISCUSSION

Ischemia or reperfusion after ischemia causes myocardial injury. Oxygen free radicals may play an important role in these harmful events, because free radical scavengers prevent the myocardial injury induced by ischemia or reperfusion^{15,16}. In addition, oxygen free radicals produced by electrolysis of a physiological buffer directly cause damage to the normal isolated perfused rabbit heart.¹⁰ In fact, some investigators have

observed a release of free radicals from the heart during ischemia in humans¹⁷ and in the experimental animal model^{2,18,19}.

In the present study, we detected significant increases in cardiac mechanical function and in energy charge potential due to h-SOD administration during reperfusion following ischemia (Fig. 1 and Fig. 2). The mechanism by which h-SOD protects the myocardium from ischemic injury is still not clear. Most of the drugs that we have tested previously have had a cardiodepressant effect, and this effect may be responsible for their protective effect on the ischemic myocardium.¹² Propranolol and diltiazem reduce the ischemic damage of the heart decreasing the pressure-rate product of the perfused rat heart prior to the onset of ischemia.¹² These drugs can preserve the energy store during ischemia. However, h-SOD itself did not decrease the pressure-rate product before the onset of ischemia (Fig. 1). Pretreatment with h-SOD did not prevent a decrease in ATP and creatine phosphate caused by ischemia significantly (Table I). On the other hand, h-SOD (300 and 1,000 units/ml) significantly reduced the ischemia-induced increase in tissue lactate level (Table I). Ischemia converts the myocardial metabolism from aerobic to anaerobic, leading to some toxic metabolic end products in the ischemic myocardium, such as lactate, hydrogen ions, fatty acid esters. A reduction of the increase in lactate in the ischemic tissue may be responsible for the protection of the ischemic myocardium achieved by h-SOD. During not only reperfusion but also ischemia, oxygen free radicals may play an important role in causing damage to the myocardial tissue, because h-SOD prevented the lactate accumulation during ischemia.

A significant loss of free radical scavengers in the myocardial tissue is observed in the ischemic myocardium^{20,21}. If the increase in free radical generation in the ischemic and reperfused myocardium is due to the reduction of h-SOD activity in the tissue, exogenous h-SOD may contribute to a decrease in free radicals, resulting in protection of the myocardium from ischemic damage.

Treatment with a large amount of h-SOD alone as well as combined administration of h-SOD and catalase can protect the myocardial tissue against ischemic injury. Adminis-

tration of h-SOD alone reduces myocardial infarct size⁷, improves recovery of mechanical function and tissue ATP and creatine phosphate during reperfusion after ischemia^{8,9} and reduces reperfusion-induced arrhythmias⁸. Taking therapeutic use of h-SOD into account, we examined the effect of h-SOD alone on the ischemic heart. The dose of h-SOD used in the present study was higher than that used in other reports⁸⁻¹⁰. Because recombinant h-SOD is now available, we were able to use a large amount of h-SOD in the present experiment. Heuer et al⁸ infused SOD at a rate of 250 units/min into the aortic cannula of the perfused rat heart, and Ambrosio et al⁹ injected 60,000 units of h-SOD at the time of reflow, followed by infusion of 100 units/ml to the perfused rabbit heart. In their experiments, the final concentration of h-SOD was up to 100 units/ml. We dissolved h-SOD in perfusion medium, and used the medium containing h-SOD as the perfusate so that the precise dose of h-SOD could be obtained. However, more than 300 units/ml of h-SOD produced no further protection of the ischemic myocardium (Fig. 1 and Fig. 2).

In conclusion, administration of h-SOD alone at high doses improves recovery of mechanical function and ATP and creatine phosphate during reperfusion following ischemia in the isolated perfused working rat heart. A significant recovery of energy charge potential was also observed in the perfused heart with more than 300 units/ml of h-SOD, compared to that without h-SOD. These results suggest that h-SOD improves the recovery of cardiac function in clinical situations when it is used after open heart surgery and after recanalization of the coronary artery in the ischemic heart disease.

Acknowledgement

h-SOD was kindly supplied by Nippon Kayaku Co., Ltd., Tokyo, Japan.

REFERENCES

1. ROY RS, McCORD JM: Superoxide and ischemia: Conversion of xanthine dehydrogenase to xanthine oxidase. In *Oxy Radicals and Their Scavenger Systems. Cellular and Medical aspects*, ed by GREENWALD RA, COHEN G, Elsevier, New York, 1983, vol.11, p145
2. RAO PS, MUELLER HS: Lipid peroxidation and

- acute myocardial ischemia. *Adv Exp Med Biol* **161**: 347, 1983
3. WERNIS SW, SHEA MJ, LUCCHESI BR: Free radicals in ischemic myocardial injury. *J Free Radic Biol Med* **1**: 103, 1985
4. JOLLY SR, KANE WJ, BAILIE MB, ABRAMS GD, LUCCHESI BR: Canine myocardial reperfusion injury. Its reduction by the combined administration of superoxide dismutase and catalase. *Circ Res* **54**: 277, 1984
5. GROSS GJ, FARBER NE, HARDMAN HF, WARLTIER DC: Beneficial actions of superoxide dismutase and catalase in stunned myocardium of dogs. *Am J Physiol* **250**: H372, 1986
6. OTANI H, TANAKA H, INOUE T, UMEMOTO M, OMOTO K, TANAKA K, SATO T, OSAKO T, MASUDA A, NONOYAMA A, KAGAWA T: In vitro study on contribution of oxidative metabolism of isolated rabbit heart mitochondria to myocardial reperfusion injury. *Circ Res* **55**: 168, 1984
7. AMBROSIO G, BECKER LC, HUTCHINS GM, WEISMAN HF, WEISFELDT ML: Reduction in experimental infarct size by recombinant human superoxide dismutase: Insights into the pathophysiology of reperfusion injury. *Circulation* **74**: 1424, 1986
8. HEUER HJ, MULLER E, BERNAUER W: Differential effects of superoxide dismutase on high energy phosphates, creatine kinase release, and arrhythmias during post-ischemic reperfusion in isolated rat hearts. *Basic Res Cardiol* **83**: 149, 1988
9. AMBROSIO G, WEISFELDT ML, JACOBUS WE, FLAHERTY JT: Evidence for a reversible oxygen radical-mediated component of reperfusion injury: Reduction by recombinant human superoxide dismutase administered at the time of reflow. *Circulation* **75**: 282, 1987
10. JACKSON CV, MICKELSON JK, POPE TK, RAO PS, LUCCHESI BR: O₂ free radical-mediated myocardial and vascular dysfunction. *Am J Physiol* **251**: H1225, 1986
11. NEELY JR, LIEBERMEISTER H, BATTERSBY EJ, MORGAN HE: Effect of pressure development on oxygen consumption by isolated rat heart. *Am J Physiol* **212**: 804, 1967
12. ICHIHARA K, ABIKO Y: Effects of diltiazem and propranolol on irreversibility of ischemic cardiac function and metabolism in the isolated perfused rat heart. *J Cardiovasc Pharmacol* **5**: 745, 1983
14. ATKINSON DE, WALTON GM: Adenosine triphosphate conservation in metabolic regulation. Rat liver citrate cleavage enzyme. *J Biol Chem* **242**: 3239, 1967
15. BURTON KP: Superoxide dismutase enhances recovery following myocardial ischemia. *Am J Physiol* **248**: H637, 1985
16. TAMURA Y, CHI L, DRISCOLL EM Jr, HOFF PT, FREEMAN BA, GALLAGHER KP, LUCCHESI BR: Superoxide dismutase conjugated to polyethylene glycol provides sustained protection against myocardial ischemia/reperfusion injury in canine heart. *Circ Res* **63**: 944, 1988
17. YAMAMOTO H: Studies on clinical applications

- of electron spin resonance (ESR) spectrometry; Application to diagnosis of ischemic heart disease. *Jpn Circ J* **35**: 1257, 1971
18. AKIYAMA K: Studies on myocardial metabolism in the ischemic heart. Part II. Studies by the method of electron spin resonance. *Jpn Circ J* **33**: 146, 1969
 19. SUZUKI Y: Studies on the free radicals in myocardial mitochondria by electron spin resonance (ESR) spectrometry (Studies on experimental infarction dogs). *Jpn Circ J* **39**: 683, 1975
 20. MEERSON FZ, KAGAN VE, KOZLOV YP, BELKINA LM, ARKKHIPENKO YV: The role of lipid peroxidation in pathogenesis of ischemic damage and the antioxidant protection of the heart. *Basic Res Cardiol* **77**: 465, 1982
 21. RAO PS, MUELLER HS: Lipid peroxide production and glutathione peroxidase depletion in rat myocardium after acute infarction (abstr). *Clin Chem* **27**: 1027, 1981