Free Rad. Res., Vol. 27, pp. 13–21
Reprints available directly from the publisher
Photocopying permitted by license only

© 1997 OPA (Overseas Publishers Association)
Amsterdam B.V. Published by
Harwood Academic Publishers
Printed in Malaysia

Superoxide Dismutase Does Protect the Cultured Rat Cardiac Myocytes Against Hypoxia/Reoxygenation Injury

ZHONG MING QIAN*, MEI FENG XU and PAK LAI TANG

Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong

Accepted by Prof. J. V. Bannister

(Received 16 August 1996; In revised form 18 January 1997)

The effect of superoxide dismutase (SOD) on membrane integrity and fluidity of the cultured neonatal rat cardiac myocytes *in vitro* was investigated under the condition of hypoxia and hypoxia/reoxygenation. Lactate dehydrogenase (LDH) concentration was used as the biochemical indicator for the loss of cell membrane integrity. Fluorescence polarization (*FP*), average microviscosity ($\bar{\eta}$) and anisotropy (*Ast*), which are inversely proportional to the fluidity of cell membrane, were assayed. Cells were respectively exposed to hypoxia or hypoxia/reoxygenation for different periods of time in the absence or presence of SOD at various concentrations. Hypoxia alone or hypoxia/reoxygenation brought injury to the cultured myocytes. This was demonstrated by changes in LDH and membrane fluidity. In the former LDH concentration gradually increased in a time-dependent manner and the values of *FP*, $\bar{\eta}$ and *Ast* were significantly increased. The changes in membrane integrity and fluidity induced by hypoxia or hypoxia/reoxygenation could be prevented by adding SOD to the culture medium. The results provide a direct evidence that SOD (740 $u.ml^{-1}$, the effective dose) was effective in protecting cultured myocytes against the injury as well as an indirect evidence of free radical generation. Based on the results obtained from this study and the establishment of concept of optimally effective dose by Bernier and Omar et al, it was suggested that some previous reports, in which no evidence was found both in protective effect of SOD and in free radical generation by using only one dose in hypoxia/reoxygenation model, should be interpreted with caution.

Keywords: Superoxide dismutase, Cultured rat cardiac myocyte, Hypoxia/reoxygenation, Membrane integrity and fluidity, Optimally effective dose (OPD)

INTRODUCTION

A number of studies have suggested that reperfusion-induced cellular injury of ischemic myocardium and reoxygenation injury of hypoxic myocardium are in part due to the production of oxygen-derived free radicals. In isolated hearts, direct measurement of oxygen radical generation by electron paramagnetic resonance spectroscopy has been documented showing a burst of oxygen radical generation occurs in the early moments of reperfusion and much less during the ischemic period itself.[1] Recently we reported[2] that superoxide dismutase (SOD), the most widely studied free radical

*Correspondening author. Tel.: 852-2766 6694. Fax: 852-2364 9932.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/16/11
For personal use only.

RIGHTSLINK

scavenger, improved significantly the contractile function, aortic output and coronary flow in isolated working heart and the protective effect on the cultured rat cardiac myocytes, providing indirect evidence of oxygen radical generation in hypoxia/reoxygenation model, the latter being similar to that obtained from the cultured chick ventricular myocytes by Murphy et al.[3]

However, Timerman et al.,[4] in contrast, provided evidence against oxygen radical generation by isolated mammalian cardiac myocytes. The study of Ziegelstein et al.[5] demonstrated that the hydroxyl radical scavenger protected isolated rat myocytes from hypoxic injury by inhibition of Na^+–Ca^{2+} exchange and not by its antioxidant effects, but it did not show any evidence of free radical generation by isolated mammalian cardiac myocytes. In addition, not all investigators have confirmed the protective effect of SOD in hypoxia/reoxygenation injury of cultured myocytes which was found in our previous study[2] and in hypoxic/reoxygenation-induced neuron injury reported by Cazevieille et al.[6] Several investigators have pointed out that SOD is ineffective in influencing cardiac myocytes survival in hypoxic/reoxygenation model,[5] and in preventing hydrogen peroxide cytotoxicity[7] as well as polymorphonuclear leukocytes-induced injury in hypoxic cardiac myocytes.[8] Similarly, SOD could not protect the cultured cardiac cell against the attack of extracellular oxygen radicals.[9] Obviously, it is still unclear and merits further investigation whether there are free radical generation and protective effect of SOD in cultured cardiac myocytes exposed to hypoxia/reoxygenation.

The experiments in this paper were designed to investigate the degree of cellular injury and the effect of SOD concentrations on membrane integrity and fluidity of the cultured neonatal cardiac myocytes under the condition of hypoxia and hypoxia/reoxygenation. Lactate dehydrogenase (LDH) concentration in culture medium was measured and fluorescence polarization (*FP*), the average microviscosity ($\bar{\eta}$) and anisotropy (*Ast*) were assayed. The myocytes were exposed to hypoxia or hypoxia/reoxygenation for different periods of time respectively in a blinded and random fashion in the absence or presence of various concentrations of SOD. Based on the experimental results, the possible role of free radical generation involved in hypoxia/ reoxygenation-induced cellular injury and the effects of various doses of SOD were analysized and discussed.

MATERIALS AND METHODS

Materials

Superoxide dismutase (SOD) was purchased from Sigma Chemicals, St. Louis, MO, USA. Eagle's minimum essential medium (MEM) and 1, 6-Diphenyl-1,3,5-hexatriene (DPH) were obtained from Fluke Co. Japan. Trypsin (type III) was bought from Sigma and the fetal serum from Allgreen Biological. Huangzhou, PRC. The solution of DPH was prepared by dissolving the compound in tetrahydroforum to the concentration of 2 $mmol.L^{-1}$ and stored at 4°C. Before use, the solution was diluted to 2 $umol.L^{-1}$ with phosphate buffered saline (PBS, pH 7.4). Petri dish (Falcon 3001, 35 mm diameter) was obtained from Falcon Labware, Oxnard, Calif. LDH activity in cultured medium was measured by Spectrophotometer, Hitachi U2000, Japan. Polarization (*FP*) was determined by fluorescence spectrophotometer, (Perkin Elmer L550B).

Cardiac Myocyte Culture

Monolayer cultures of spontaneously contracting ventricular cells of neonatal rats were prepared by a slight modification of the method previously described.[2] Briefly, fragments of ventricles were aseptically removed from the 2–4 days old Wister rats and then transferred to Hank's solution. The cells were isolated with 0.1% trypsin. The cell suspension was diluted to $3 \times 10^6.ml^{-1}$ and placed in 35 mm culture dishes. The culture medium consisted of 15% heat-inactivated fetal bovine serum,

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/16/11
For personal use only.

RIGHTSLINK

85% MEM medium. The myocytes were kept in a humidified tissue culture incubator with 5% CO_2 and 95 % air supply at 37°C. Three days after plating, a confluent monolayer was formed and the cells beat spontaneously and synchronously. The culture was used for experiment.

Hypoxia/Reoxygenation Exposure

Myocytes of the control group were cultured in normal MEM medium without serum. Hypoxia was instituted by changing the normal MEM medium with the glucose and O_2-free (saturated with N_2) medium. After the hypoxic incubation, in certain experiments, reoxygenation was performed by replacing the hypoxic medium with normal MEM medium. Myocytes were exposed to hypoxia only or hypoxia/reoxygenation in a blinded and random fashion. To determine the degree of cellular injury, 120 dishes were divided into 15 groups as follows:

Groups	n (dishes)	Culture (hours)	Treatment
C1 (Control 1)	8	2	Normal MEM Medium
C2 (Control 2)	8	4	Normal MEM Medium
C3 (Control 3)	8	6	Normal MEM Medium
1 (H2)	8	2	Hypoxic Medium (Hypoxia)
2 (H4)	8	4	Hypoxic Medium (Hypoxia)
3 (H6)	8	6	Hypoxic Medium (Hypoxia)
4 (H2RO1)	8	3	Hypoxia 2 hours + Reoxygenation 1 hours
5 (H2RO2)	8	4	Hypoxia 2 hours + Reoxygenation 2 hours
6 (H2RO3)	8	5	Hypoxia 2 hours + Reoxygenation 3 hours
7 (H4RO1)	8	5	Hypoxia 4 hours + Reoxygenation 1 hours
8 (H4RO2)	8	6	Hypoxia 4 hours + Reoxygenation 2 hours
9 (H4RO3)	8	7	Hypoxia 4 hours + Reoxygenation 3 hours
10 (H6RO1)	8	7	Hypoxia 6 hours + Reoxygenation 1 hours
11 (H6RO2)	8	8	Hypoxia 6 hours + Reoxygenation 2 hours
12 (H6RO3)	8	9	Hypoxia 6 hours + Reoxygenation 3 hours

To investigate the effect of SOD, 32 other dishes were assigned to one of the following 4 groups:

Groups	n (dishes)	Culture (hours)	SOD* ($u.ml^{-1}$)	Treatment
1 (Control G1)	8	5	No	Normal MEM Medium**
2 (Control G2)	8	5	No	Hypoxia 3 hours + Reoxygenation 2 hours
3 (SOD G1)	8	5	370	Hypoxia 3 hours + Reoxygenation 2 hours
4 (SOD G2)	8	5	740	Hypoxia 3 hours + Reoxygenation 2 hours

*SOD was added to hypoxic (glucose and oxygen-free) medium as well as reoxygenated (normal MEM) medium. ** After the first 3 hours of incubation, the culture medium was changed with new normal MEM medium and the cells were cultured for another 2 hours.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/16/11
For personal use only.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/16/11
For personal use only.

Lactate Dehydrogenase Measurement and Membrane Fluidity Assay

Cellular injury induced by hypoxia or hypoxia/reoxygenation was quantitated by the release of lactate dehydrogenase (LDH) in the cell culture media which correlated with the loss of cell membrane integrity.[7,10] LDH activity in cultured media of all groups was measured spectrophotometrically.[11] For assaying membrane fluidity, the cultured myocytes were washed with PBS from the cultured chamber and a single-stage hemolysis method in hypotonic solution was used for the preparation of myocyte membranes. Two ml of DPH (2.0 umol. L^{-1}) was added to 2.0 ml of membrane preparation and incubated at 25°C for 30 min, and then centrifuged at 2000 × g for 10 min. The precipitate was washed with PBS (0.01 mol.L^{-1}) and resuspended in 4 ml of PBS. The polarization (*FP*) of cultured myocyte membrane fluorescence labelled with DPH for each group was determined by RF 510 fluorescence spectrophotometer (λex = 363 nm, λem = 432 nm),[12] The degree of polarization was calculated by the following equation:

$$FP = (I_{VV} - GI_{VH})/(I_W + GI_{VH})$$

$$G = I_{VH} / I_{HH}$$

where I_{VV} and I_{VH} stood for the intensity of fluorescence recorded at the analysing orientations. *G* denoted a correction factor which could be used to correct the relative transmission of the emission monochrometer for the 2 polarization directions. I_{HH} was the intensity of emitted light when the 2 analysing polarizer orientations were both at horizontal directions. I_{HV} was the intensity of emission light when the emission polarizer at horizontal and the excitative polarizer at vertical orientations.[12,13] The polarization (*FP*) was the smaller, indicating that lipid membrane fluidity was the larger. The average microviscosity ($\overline{\eta}$) and anisotropy (*Ast*) were calculated from the following formula:

$$\overline{\eta} = 2FP/(0.46 - FP)$$

$$Ast = 2FP/(3 - FP)$$

Polarization *(FP)*, microviscosity ($\overline{\eta}$) and anisotropy (*Ast*) were inversely related to the fluidity of myocyte membranes.[12–15] When the effect of SOD on membrane fluidity was studied, various concentrations of SOD were added to hypoxia/reoxygenation culture media.

Statistical Analysis

All values were presented as mean ± SEM. Student's *t*-test was used to determine differences between means. $P < 0.05$ was taken to reflect a significant difference.

RESULTS

Relationship Between LDH Release and Period of Hypoxia and Hypoxia/Reoxygenation

The cultured neonatal rat myocytes of Groups 1–3 were exposed only to hypoxia for 2 (H2), 4 (H4), or 6 hours (H6) respectively. The concentration of LDH measured in culture medium of all three treatments was significantly higher than that obtained from control groups (C1 – C3) in which myocytes were incubated in normal medium for the same periods of time, *P* values being smaller than 0.05 (H2), 0.001 (H4) and 0.001 (H6). A progressively higher LDH concentration in the medium was found with increasing hypoxia time. The relationship between time and concentration (LDH) was determined by plotting the values of LDH measurements (u.ml^{-1}) and different hypoxic time (hours), and a very close correlation was observed (Fig. 1, Correlation coefficient (γ) = 0.9230).

Rat cardiac myocytes of Groups 4–12 were first exposed to hypoxia for 2, 4, or 6 hours and then reoxygenated for 1, 2, or 3 hours respectively. The

RIGHTSLINK

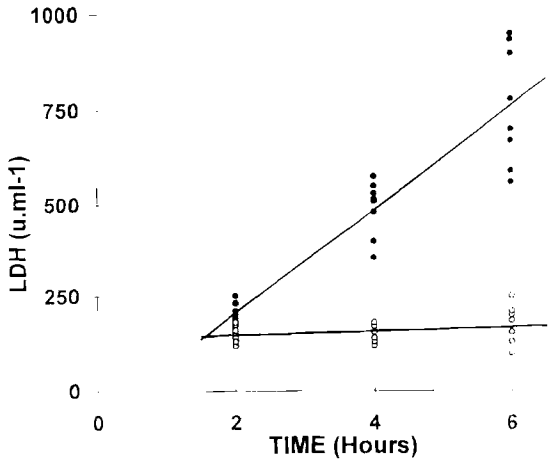

FIGURE 1 Effect of different hypoxic duration on LDH release. Neonatal rat myocytes were cultured in glucose and O_2-free medium (●, groups 1–3) or normal MEM medium (○, control groups C1–C3) for 2, 4, and 6 hours respectively. The measurements of LDH were made at the end of incubation and number of dishes in each group is 8. LDH concentration gradually increased with the increase of hypoxic time in time-dependent manner, correlation coefficient (γ) being 0.9230 and Y = 139.2X – 72. *$P < 0.001$ vs control.

results for LDH measurements of these groups were summarized in Table I. LDH released into the media from reoxygenated myocytes gradually increased in a time-dependent manner, correlation coefficient (γ) being 0.942, 0.906, and 0.886 in Groups 4–6, 7–9 and 10–12 respectively. No appreciable difference was found between LDH released from reoxygenated myocytes pre-cultured in hypoxic medium for 2 or 4 hours, suggesting no significant effect of hypoxic time on LDH release when the time was or less than 4 hours ($P > 0.05$). LDH concentration of Groups 10–12 (6 hours hypoxic pretreatment) was significantly lower than those of Groups 4–6 (H2) and 7–9 (H4) ($P < 0.05$), probably due to serious cell injury.

Effect of SOD on LDH Release

The value of LDH in SOD G2 was much lower than that in control G2 ($P < 0.05$), suggesting that a concentration of 740 $u.ml^{-1}$ of SOD could significantly decrese LDH concentration in the medium (Table II). However, the results from lower SOD dose group (SOD Gl) indicated that 370 $u.ml^{-1}$ of SOD had no significant effect on the increased LDH concentrations in hypoxic or reoxygenated medium induced by 3 h hypoxia and/or 2 h reoxygenation. No appreciable difference was obtained between the LDH concentrations of control G2 and SOD G1. The values of LDH in SOD G1 were still higher than those in control G1.

Changes in Polarization (FP), Average Microviscosity ($\bar{\eta}$) and Anisotropy (*Ast*) Induced by Hypoxia/reoxygenation and Effect of SOD on Membrane Fluidity of Cultured Myocytes

3 hours hypoxia/2 hours reoxygenation led to an increase in the values of fluorescence polarization (*FP*), the average microviscosity ($\bar{\eta}$) or

TABLE I Effect of hypoxia/reoxygenation on LDH release from myocytes

Groups (n = 8)	LDH ($u.L^{-1}$)	Groups (n = 8)	LDH ($u.L^{-1}$)	Groups (n = 8)	LDH ($u.l^{-1}$)
4(H2RO1†)	149 ± 16	7(H4RO1)	163 ± 30*	10(H6RO1)	129 ± 23
5(H2RO2)	187 ± 23*	8(H4RO2)	181 ± 24*	11(H6RO2)	130 ± 18
6(H2RO3)	232 ± 28*	9(H4RO3)	222 ± 24*	12(H6RO3)	167 ± 27
	(γ = 0.942)		(γ = 0.906)		(γ = 0.886)

Rat myocytes (3×10^6 ml^{-1}) were cultured in hypoxic (glucose and O_2-free) medium for 2 (groups 4–6), 4(groups 7–9), or 6 hours (groups 10–12), and then hypoxic medium was replaced by normal MEM medium in which hypoxic myocytes were reoxygenated for 1, 2, or 3 hours respectively. The measurements of LDH in the normal culture medium were made by the end of reoxygenation, method being described in the text. The results were expressed as Mean ± SEM. † H2RO1: Hypoxia 2 hours and Reoxygenation 1 hours. * $P < 0.05$ vs groups 10–12 respectively. γ: correlation coefficient.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/16/11
For personal use only.

RIGHTSLINK

TABLE II Effect of SOD on LDH release from rat myocytes cultured under hypoxia(3 h)/reoxygenation(2 h)

Groups (N = 8)	LDH u.L^{-1} (Mean ± SEM)	
	Hypoxia or Normal Incubation (3 hours)	Reoxygenation or Normal Incubation (2 hours)
Control G1	190.0 ± 17.9	80.0 ± 22.9
Control G2	251.9 ± 15.4‡	147.5 ± 12.4‡
SOD G1	221.9 ± 23.4	136.3 ± 16.1
SOD G2	209.4 ± 19.6*	92.5 ± 13.1†

Control G1: Myocytes were cultured in normal MEM medium for 5 hours (medium was changed after 3 hours); Control G2: H3 + RO2 only; SOD G1: H3 + RO2 + 370 u.ml^{-1} of SOD; SOD G2: H3 + RO2 + 740 u.ml^{-1} of SOD. *$P < 0.05$, †$P < 0.01$ vs Control G2 respectively; ‡ $P < 0.01$ vs Control G1.

anisotropy (*Ast*) of myocyte membrane, the results of control G2 being significantly higher than those of control G1 ($P < 0.05$). It indicated that hypoxia/reoxygenation treatment could decrease the membrane fluidity of the cultured myocytes. Both doses of 370 and 740 u.ml^{-1} of SOD prevented the increase in values of *FP*, $\bar{\eta}$, and *Ast* (Table III) induced by hypoxia/reoxygenation. In certain experiments, the effect of larger doses of SOD on membrane fluidity of myocytes under normal culture condition was investigated. Rat myocytes were cultured in normal MEM medium without (control) and with (experimental) various doses of SOD (617, 1234 or 1850 u.ml^{-1}) for 3 hours at 37°C. No appreciable difference was found between the values of *FP*, $\bar{\eta}$, and *Ast* in control and 617 and 1234 u.ml^{-1} of SOD treated groups. Interestingly, the use of dose 1850 u.ml^{-1}, the highest dose of SOD used in the experiment, resulted in a decrease in all values which were significantly lower than those in the control group ($P < 0.05$). The phenomenon suggests that very high doses of SOD may promote oxidative damage and further investigation is needed. The results are presented in Table IV.

DISCUSSION

In the present paper, we have shown that the values of LDH released from myocytes exposed to hypoxia only or hypoxia/reoxygenation and those of membrane fluidity indicators (*FP*, $\bar{\eta}$, and *Ast*) of cells exposed to 3 hours hypoxia/2 hours reoxygenation are significantly higher than those in normoxic myocytes, demonstrating that both hypoxia alone and hypoxia/reoxygenation may lead to a time-dependent progressive injury of the cultured neonatal rat cardiac myocytes. SOD at 740 u.ml^{-1} (an effective dose in this experiment) is effective in reducing the increased LDH level as well as the values of membrane fluidity indicators induced by hypoxia alone or hypoxia/reoxygenation, confirming that SOD does protect the culture myocytes against hypoxia or hypoxia/reoxygenation induced injury. The study also provides an indirect evidence of free radical generation and free radical-mediated cell injury in hypoxia/reoxygenation model although the related measurements of free radical were not performed in this experiment.

SOD is one of the most widely studied and used of all antioxidants[16] and a vast literature

TABLE III Effect of SOD on membrane fluidity of rat myocytes cultured under hypoxia(3h)/reoxygenation(2h)

Groups (n = 8)	Polarization (*FP*)	Microviscosity ($\bar{\eta}$)	Anisotropy (*Ast*)
Control G1	0.1858 ± 0.0333	1.3976 ± 0.2029	0.1323 ± 0.0252
Control G2	0.2169 ± 0.0322*	1.8524 ± 0.2702*	0.1566 ± 0.0245*
SOD G1	0.1836 ± 0.0355†	1.3852 ± 0.2435†	0.1310 ± 0.0270†
SOD G2	0.1769 ± 0.0264†	1.2756 ± 0.1552†	0.1255 ± 0.1255†

Treatment of all groups was the same as that described in Table 2. The results were Mean ± SEM. *$P < 0.05$ vs Control G1; †$P < 0.05$ vs Control G2.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/16/11
For personal use only.

RIGHTSLINK

TABLE IV Effect of SOD on membrane fluidity of normal cultured myocytes

SOD ($u.ml^{-1}$)	Polarization (*FP*)	Microviscosity ($\bar{\eta}$)	Anisotropy (*Ast*)
0.0†	0.1808 ± 0.0250	1.3186 ± 0.2980	0.1284 ± 0.0190
617.0	0.1647 ± 0.0341	1,1535 ± 0.3750	0.1165 ± 0.0255
1234.0	0.1771 ± 0.0248	1.2737 ± 0.2803	0.1257 ± 0.0186
1850.0	0.1506 ± 0.0240*	0.9894 ± 0.2381*	0.1059 ± 0.0178*

Myocytes were incubated with normal MEM medium in the absence (control) or the presence of 617, 1234 and 1850 $u.ml^{-1}$ of SOD for 5 hours at 37°C. The results were Mean ± SEM, Number of all groups is 8. *$P < 0.05$ vs control. †control group.

related to the free radical scavenger has been documented. In spite of numerous investigations, there is still no general agreement as to whether there is any protective effect of SOD in myocardial ischemic/reperfusion injury of the working heart[16,17] as well as hypoxia/reoxygenation-induced injury of the cultured heart cells.[2,5,7–9,18,19] Also it is not fully understood whether there is free radical generation in cultured myocytes exposed to hypoxia/reoxygenation.[4,5,19,20] The understanding of the latter question has been mainly based on the results of studies on whether there is the protective effects of SOD or other free radical scavengers.

Protective Effect of SOD. Is SOD effective in protecting the cultured neonatal rat cardiac myocytes against hypoxia/reoxygenation injury? The answer should be "yes" on the bases of our results. However, why is it that the protective effect of SOD is not found in some other studies? Several explanations are probably involved in the discrepancy among studies, the most important one may be the dose of SOD used. Recently, Bernier et al[21] described an anomalous bell-shaped dose-response curve for the prevention of reperfusion-induced arrhythmias in the isolated rat heart and found that protection increased with dose of SOD but declined dramatically when the concentration of SOD reached 120,000 $u.l^{-1}$. Omar and Nelson et al.[22–24] reported similar curves for the protection of reoxygenated rat and rabbit hearts by SOD. Also, it was documented[24] that high-dose SOD does indeed increase net lipid peroxidation in reperfusion injury model. Based on these findings, the concept has been established that there is an Optimally Protective Concentration or Dose (OPC or OPD) of SOD for any specific set of conditions,[24] including the experimental condition of cultured myocytes in hypoxia/reoxygenation model. Therefore it is very difficult to get correct information on whether there is any protective effect of SOD by using only one dose in some studies (1000 u/ml—Ziegelstein et al.,[5] 800 u/ml (6×10^5 cells/ml)—Byler et al.,[7] and 10 ug/ml (5×10^4 cell/dish)—Kuzuya et al.,[8]) because the dose used probably was not an OPD or protective dose of SOD under their experimental conditions. Our results show that 740 $u.ml^{-1}$ of SOD presents the protective effect but 370 $u.ml^{-1}$ does not. If only one dose of 370 $u.ml^{-1}$ was used, the conclusion might be totally different.

Free Radical Generation. The results obtained favour the suggestion that hypoxia/reoxygenation may lead to free radical generation and cell injury. It is mainly supported by (1) changes in membrane fluidity: all values of fluidity indicator were significantly increased when cells were exposed to hypoxia or hypoxia/reoxygenation; and (2) protective effect of SOD: the increased values of membrane fluidity indicator could be reduced by 740 $u.ml^{-1}$ of SOD. Various lines of evidences suggest that the decreased membrane fluidity can occur as a result of free radical reaction and lipid peroxidation.[14,25,26] SOD is the most extensively studied enzymatic scavenger of free radical, scavenging superoxide by dismutating it to the oxygen and hydrogen peroxide,[16,17]

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/16/11
For personal use only.

RIGHTSLINK

and the widely used antioxidant in many areas including the effect on transferrin-free iron uptake by mammalian cells.[27,28] The protective effect of SOD has been demonstrated in this study. The result provides the indirect evidence of cell injury mediated by free radical generation. The true protective action of SOD has been questioned for a long time in view of its high molecular weight which makes its intracellular penetration unlikely,[29] however, it has been shown that free radicals could be present extracellularly.[30] Also it should be noted that SOD might be able to pass through the cultured cell membrane because of the increased membrane permeability resulted from severe hypoxia.

In summary, the study demonstrates that hypoxia and hypoxia/reoxygenation both lead to cultured myocytes injury which is effectively prevented by 740 $u.ml^{-1}$ of SOD under our experimental conditions. Our findings provide an indirect evidence of free radical generation and free radical-mediated cell injury. Based on the results obtained from the treatment with various doses of SOD and the establishment of the concept of optimally protective dose by Bernier and Omar et al.[21–24] it was suggested that some previous reports, in which no evidence was found both in the protective effect of SOD (or other free radical scavengers) and in free radical generation by using only one dose of SOD in hypoxia/reoxygenation model, should be interpreted with caution.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/16/11
For personal use only.

Acknowledgments

The study was supported in part by The Hong Kong Polytechnic University Research Grants (A/C 351/055, 350/480 and 350/314) and RGC grant (A/C 357/026).

References

[1] J. L. Zwier, J. T. Flaherty and M. L. Weisfeldt (1987). Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proceedings of the National Academy of Sciences USA*, **84**, 1404–1407.

[2] M. F. Xu, D. S. Wang and X. Z. Chen (1990). Effect of superoxide dismutase on ischemic reperfusion injury in isolated working heart and cultured myocardial cells on rats. *Acta Pharmacologia Sinica*, **11**, 324–328.

[3] J. G. Murphy, T. W. Smith and J. D. Marsh (1988). Mechanisms of reoxygenation-induced calcium overload in cultured chick embryo heart cells. *American Journal of Physiology*, **254**, H1133–1141.

[4] A. P. Timerman, R. A. Altschuld, C. M. Hohl, G. P. Brierley and A. J. Merola (1990). Cellular glutathione and the response of adult rat heart myocytes to oxidant stress. *Journal of Molecular and Cellular Cardiology*, **22**, 565–575.

[5] R. C. Ziegelstein, J. L. Zweier, E. D. Mellits, A. Younes, E. G. Lakatta, M. D. Stern and H. S. Silverman (1992). Dimethylthiourea, an oxygen radical scavenger, protects isolated cardiac myocytes from hypoxic injury by inhibition of Na^+–Ca^{2+} exchange and not by its antioxidant effects. *Circulation Research*, **70**, 804–811.

[6] C. Cazevieille, A. Muller, F. Meynier and C. Bonne (1993). Superoxide and nitric oxide cooperation in hypoxia/reoxygenation-induced neuron injury. *Free Radical Biology and Medicine*, **14**, 389–395.

[7] R. M. Byler, N. A. Sherman, J. S. Wallner and L. D. Horwitz (1994). Hydrogen peroxide cytotoxicity in cultured cardiac myocytes is iron dependent. *American Journal of Physiology*, **266**, H121–H127.

[8] T. Kuzuya, H. Fuji, S. Hoshida, M. Nishida, K. Goshima, M. Hori, T. Kamada and M. Tada (1994). Polymorphonuclear leukocytes-induced injury in hypoxic cardiac myocytes. *Free Radical Biology and Medicine*, **17**, 501–510.

[9] M. Mohsen, A. Pinson, R. Zhang and A. Samuni (1995). Do nitroxides protect cardio-myocytes from hydrogen peroxide or superoxide? *Molecular and Cellular Biochemistry*, **145**, 103–110.

[10] L. J. Eddy, D. V. Goeddel and G. H. W. Wong (1992). Tumor necrosis factor-a pretreatment is protective in a rat model of myocardial ischemia-reperfusion injury. *Biochemical and Biophysical and Research Communications*, **184**, 1056–1059.

[11] F. Wroblewski and J. S. LaDue (1955). Lactic dehydrogense activity in blood. *Proceedings of the Society for Experimental Biology*, **90**, 210–213.

[12] D. Li, Q. Han, X. Y. Yang, D. M. Rong and Z. J. Lin (1994). Effect of kappa-selenocarragenan on membrane fluitidy and ghost reseal ability of rat erythrocyte. *Acta Pharamcologia Sinica*, **15**, 211–214.

[13] T. Azumi and S. P. Mcglynn (1962). Polarization of the luminescence of phenanthrene. *Journal of Chemical Physico*, **37**, 2413–2420.

[14] J. J. Chen and B. P. Yu (1994). Alterations in mitochondrial membrane fluidity by lipid peroxidation products. *Free Radical Biology and Medicine*, **17**, 411–418.

[15] H. Wiseman and P. Quinn (1994). The antioxidant action of synthetic oestrogens involves decreased membrane fluidity: relevance to their potential use as anticancer and cardioprotective agents compared to tamoxifen? *Free Radical Research*, **21**, 187–194.

[16] J. M. Downey, B. Omar, H. Ooiwa and J. McCord (1991). Superoxide dismutase therapy for myocardial ischemia. *Free Radical Research Communications*, **12–13**, 703–720.

[17] P. Menasche and A. Piwnica (1989). Free radicals and myocardial protection: a surgical viewpoint. *Annals of Thorac Surgery*, **47**, 939–945.

RIGHTSLINK

[18] W. F. Tzeng, T. J. Chiou, C. P. Wang, J. L. Lee and Y. H. Chen (1994). Cellular thiols as a determinant of responsiveness to menadione in cardiomyocytes. *Journal of Molecular and Cellular Cardiology*, **26**, 889–897.

[19] M. Kaneko, H. Hayashi, A. Kobayashi, N. Yamazaki and N. S. Dballa (1991). Stunned myocardium and oxygen radicals—sarcolemmal membrane damage due to the oxygen free radical. *Japanese Circulation Journal*, **55**, 885–892.

[20] C. M. Hohl and R. A. Altschuld (1991). Response of isolated adult canine cardiac myocytes to prolonged hypoxia and reoxygenation. *American Journal of Physiology*, **260**, C383–C391.

[21] M. Bernier, A. S. Manning and D. J. Hearse (1989). Reperfusion arrhythmias: Dose-related protection by anti-free radical interventions. *American Journal of Physiology*, **256**, H1344–H1352.

[22] B. A. Omar, N. M. Gad, M. C. Jordan, S. P. Striplin, W. J. Russell, J. M. Downey and J. M. McCord (1990). Cardioprotection by Cu.Zn.Superoxide dismatuse is lost at high doses in the postischemic isolated rabbit heart. *Free Radical Biology and Medicine*, **9**, 465–471.

[23] B. A. Omar and J. M. McCord (1990). The cardioprotective effect of Mn-superoxide dismutase is lost at high doses in the postischemic isolated rabbit heart. *Free Radical Biology and Medicine*, **9**, 473–478.

[24] S. K. Nelson, S. K. Bose and J. M. McCord (1994). The toxicity of high dose superoxide dismatuse suggests that superoxide can both initiate and terminate lipid peroxidation in the reperfused heart. *Free Radical Biology and Medicine*, **16**, 195–200.

[25] M. T. Curtis, D. Gilfor and J. L. Farber (1984). Lipid peroxidation increases the molecular order of microsomal membrane. *Archives ???of Biochemistry and Biophysics*, **235**, 644–649.

[26] D. L. Schmucker, R. K. Wang, D. A. Vessey, J. L. James and A. Maloney (1984). Aged-dependent alterations in the physicochemical properties of rat liver microsomes. *Mechanisms of Ageing and Development*, **27**, 207–217.

[27] Z. M. Qian and P. L. Tang (1995). Mechanisms of iron uptake by mammalian cells. *Biochimica et Biophysica Acta*, **1269**, 205–214.

[28] Z. M. Qian, P. L. Tang and E. H. Morgan (1996). Effect of lipid peroxidation on transferrin-free iron uptake by rabbit reticulocytes. *Biochimica et Biophysica Acta*, **1310**, 293–302.

[29] H. S. Silverman, L. A. Siddoway, Z. Yavin, A. Gonenne, M. L. Weisfeldt and L. C. Becker (1990). Pharmacokinetics of recombinant human superoxide dismutase. *Journal of Cardiovascular Pharmacology*, **16**, 107–111.

[30] H. A. Kontos, E. P. Wei and E. F. Ellis (1985). Appearance of superoxide anion radical in cerebral extracellular space during increased prostaglandin synthesis in cats. *Circulation Research*, **57**, 142–151.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/16/11
For personal use only.

RIGHTSLINK