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Cardioprotective actions of human superoxide dismutase in two reperfusion models of myocardial ischaemia in the rat

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1 In rats under ether anaesthesia, the left coronary artery was ligated and reperfused after 10 min of ischaemia. Forty-eight hours later the myocardium was analyzed for creatine kinase (CK) activity.

2 Human superoxide dismutase (h-SOD) given 1 min after occlusion and again 6 h later significantly improved survival and retarded the loss of myocardial CK.

3 In rat isolated hearts perfused at 15% of normal flow for 30 min followed by re-establishment of normal flow for 20 min, perfusion pressure increased by 72% and myocardial CK decreased by 44%. No significant changes occurred in wet-to-dry heart weight ratio.

4 Administration of h-SOD at 2.5 or 5.0 mg, significantly attenuated the elevated post-ischaemic perfusion pressure and the loss of myocardial CK activity in rat perfused hearts.

5 h-SOD appears to be an effective anti-ischaemic agent in the intact animal as well as the isolated perfused heart of the rat subjected to low flow followed by reperfusion at normal flow. The mechanism of this cardioprotective effect is not totally dependent upon the formed elements of the blood, but may be partially due to a direct cytoprotective effect.

Introduction

Early reperfusion remains one of the most effective means of reducing myocardial damage in acute myocardial infarction. However, even under these conditions, there is often significant cardiac injury. The mechanism of this myocardial cell injury is not fully understood. A variety of humoral mediators have been proposed including thromboxane A_2 (Lefer & Darius, 1987), platelet activating factor (Bracquet *et al.*, 1987), leukotrienes (Feuerstein, 1984) and oxygen derived free radicals (Stewart *et al.*, 1982; McCord, 1985; Mitsos *et al.*, 1986; Granger *et al.*, 1986).

There is a rapidly growing body of evidence indicating that oxygen derived free radicals play an important role in producing cellular damage associated with reperfusion of ischaemic organs (Feuerstein, 1984; McCord, 1985; Mitsos *et al.*, 1986; Granger *et al.*, 1986; Lefer & Darius, 1987; Bracquet *et al.*, 1987). It has recently been reported that administration of the oxygen derived free radical scavenger, superoxide dismutase, a scavenger of superoxide anions, and catalase, a scavenger of hydrogen peroxide, results in a significant decrease in infarct size in dogs (Gardner *et al.*, 1983; Jolly *et al.*, 1984; Myers *et al.*, 1985b). On the other hand, there are other reports that these free radical scavengers fail to alter the extent of myocardial infarction (Werns *et al.*, 1985; Myers *et al.*, 1985a; Gallagher *et al.*, 1986; Uraizee *et al.*, 1987). The purposes of this study were (a) to investigate the myocardial protective effects of human superoxide dismutase (h-SOD) in a reperfusion model of myocardial ischaemia, and (b) to determine if h-SOD can protect the isolated heart of the rat subjected to a severe global ischaemia perfused in the absence of blood cells.

Methods

Rat acute myocardial infarction in vivo

Myocardial infarction was produced by ligating the left anterior descending coronary artery (LAD). The surgical technique utilized for LAD ligation has been

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described previously (Hock et al., 1986). Male Sprague-Dawley rats (240–280 g) were lightly anaesthetized with ether prior to surgery. A 3 cm skin incision was made over the left thorax and the pectoral muscles were retracted to expose the ribs. A silk ligature was placed loosely through the skin and underlying muscle in a modified purse-string suture to facilitate rapid closure of the chest wall. A thoracotomy was performed at the level of the fifth intercostal space. Myocardial ischaemia (MI) was produced by briefly exteriorizing the heart from the thoracic cavity, locating the LAD and securing a 4-0 silk suture around the artery approximately 2 to 3 mm from its origin. The heart was repositioned in the thoracic cavity within 15–20 s. Air was then evacuated from the thorax, and the chest wall muscles and skin were rapidly closed by means of the previously placed purse-string suture.

Sham-operated control rats (Sham-MI) underwent all the same surgical procedures except that the suture was passed under the LAD but was not tied. h-SOD (5 or 10 mg kg^{-1}) or its vehicle (i.e.. 0.9% NaCl) was given intravenously 1 min after ligation of the LAD. A total of 34 rats were subjected to occlusion of the LAD for 10 min followed by reperfusion for 48 h. They received the same dose of h-SOD or vehicle 6 h after the reperfusion. After 48 h, the rats were anaesthetized with pentobarbitone (35 mg kg^{-1}, i.p.) and their hearts were excised and placed in ice cold 0.9% NaCl. This has been shown to be the time of maximal depletion of myocardial creatine kinase with this method (Hock et al., 1985). The left ventricular free wall (LVFW) and septum were dissected free and homogenized in cold 0.25 M sucrose (1:10, w:v) containing 1 mM EDTA and 0.1 mM mercaptoethanol with a Polytron (PCU-2) homogenizer. Homogenates were centrifuged at 36,000 *g* at 4°C for 30 min. The supernatants were decanted and assayed spectrophotometrically for myocardial creatine kinase (CK) activities and free amino-nitrogen concentrations. The tissue activity of CK was measured by the Rosalki method (1967).

Rat isolated perfused hearts

Male Sprague-Dawley rats weighing between 200 and 270 g were given 1000 iu kg^{-1} of heparin 15 min before induction of anaesthesia with pentobarbitone sodium (35 mg kg^{-1}, i.p.). After a midsternal thoractomy, the hearts were rapidly removed and placed in ice-cold oxygenated (95% O_2 + 5% CO_2) Krebs-Henseleit (K-H) solution. Within 30 s, the hearts were transferred to a perfusion apparatus and perfused retrogradely via the aorta with K-H solution at pH 7.3 and 37°C according to the Langendorff technique. The hearts were adjusted to perfusion conditions at a constant pressure of 55 mmHg for 5 min and then changed to constant flow perfusion (i.e., 10 to 12 ml min^{-1}) at a perfusion pressure of 50 mmHg. The coronary perfusion pressure (CPP) was continuously monitored over the remainder of the experiment.

Hearts were subjected to a 30 min period of low flow by reducing the flow to 15% of control followed by 20 min of reperfusion at control flows. The treatment groups received h-SOD (2.5 or 5 mg per heart) which was added to the aortic inflow line perfusing the hearts. Drugs were infused over a period from 1 min after initiation of low flow until 1 min after restoration of normal flow. The vehicle groups received only 0.9% NaCl. CK activity was analyzed by the same methods as in the intact animal experiments.

For the purpose of confirming the free radical scavenging activity of h-SOD as a free radical scavenger, we compared it with bovine SOD (b-SOD) in a synthetic xanthine-xanthine oxidase superoxide generating system modified from McCord & Fridovich (1969) as previously described (Brezinski et al., 1988). Two types of superoxide dismutase, h-SOD and b-SOD, were used at concentrations of 50 ng ml^{-1}. In the b-SOD group, the difference of absorbance per min was 0.063 ± 0.002, and in the h-SOD group it was 0.066 ± 0.005. These values are significantly lower than those in the group receiving vehicle (0.156 ± 0.007, $P < 0.001$). As a result of this experiment, h-SOD was observed to be as effective as b-SOD as a free radical scavenger.

The h-SOD obtained from Grunenthal GmbH, Aachen, F.R. Germany had an activity of 3,100 SOD units mg^{-1}. The b-SOD obtained from Sigma Chemical Co., St. Louis, MO, USA, had an activity of 3,000 SOD units mg^{-1}.

Statistical methods

All values in the text are means ± s.e.mean. Differences among multigroup means were compared by analysis of variance (ANOVA). Tukey's pairwise comparison was used to determine the significance between specific pairs of data means. Analysis of survival was done by the Chi-square test.

Results

The effect of h-SOD on the degree of ischaemic damage during myocardial infarction and reperfusion in rats is summarized in Table 1. The difference in creatine kinase (CK) activity between the septum (non-ischaemic area) and the left ventricular free wall (LVFW) (ischaemic area) is shown. Loss of CK from the LVFW at 48 h was significantly ($P < 0.01$) attenuated in rats receiving h-SOD (10 mg kg^{-1}) compared to rats receiving only vehicle. This effect

Table 1 Effect of myocardial infarction (MI) plus reperfusion on the extent of ischaemic damage in the rat myocardium 48 h after ischaemia

Group	*CK activity* ($iu\,g^{-1}$) *Septum*	*LVFW*	*(Septum-LVFW)*	*Survival rate* (%)
Sham + h-SOD ($10\,mg\,kg^{-1} \times 2$) (8)	4.19 ± 0.27	4.42 ± 0.25	−0.23 ± 0.23	100
MI + vehicle (0.9% NaCl) (9)	4.27 ± 0.39	3.00 ± 0.29	1.27 ± 0.23*	64[a]
MI + h-SOD ($5\,mg\,kg^{-1} \times 2$) (8)	4.83 ± 0.21	4.41 ± 0.27	0.41 ± 0.25**	100
MI + h-SOD ($10\,mg\,kg^{-1} \times 2$) (9)	4.26 ± 0.34	4.11 ± 0.33	0.15 ± 0.20***	100

CK = creatine kinase; h-SOD = human superoxide dismutase.
All values are means ± s.e.mean. Injections of drug or vehicle were made just before reperfusion and again 6 h following reperfusion. * $P < 0.01$ from sham; ** $P < 0.05$ from vehicle; *** $P < 0.01$ from vehicle.
[a] Represents 9 rats surviving of 14 subjected to MI; $P < 0.02$ from other groups. Numbers in parentheses are the number of rats studied in each group.

appeared to be dose-related as h-SOD ($5\,mg\,kg^{-1}$) slightly protected against myocardial CK loss, but to a lesser extent than a dose of $10\,mg\,kg^{-1}$. As would be expected, the survival rate of ischaemic rats treated with h-SOD was higher than ischaemic rats given only the vehicle (0.9% NaCl). Thus, all the evidence we obtained points to a cardioprotective effect of h-SOD in acute myocardial infarction in rats.

In an attempt to confirm this finding and to obtain additional information on the mechanism of this protective effect, we studied h-SOD in isolated perfused rat hearts subjected to low flow. The effects of h-SOD during low flow followed by reperfusion at normal flow in the isolated rat heart (*in vitro*) are shown in Tables 2 to 4.

Table 2 summarizes the ratio of the post-reperfusion perfusion pressure to that occurring in the initial control period in order to assess coronary vascular function. The control + vehicle group showed no change in perfusion pressure over time.

Table 2 Effect of human superoxide dismutase (h-SOD) on coronary perfusion pressure during low flow followed by reperfusion in the isolated constant flow perfused heart of the rat

Group	n	$\left[\frac{\text{Reperfusion pressure}}{\text{Pre-ischaemic pressure}}\right] \times 100$
Control + vehicle	15	97 ± 2
Control + h-SOD (5 mg per heart)	8	99 ± 2
Low flow + vehicle	18	171 ± 21*
Low flow + h-SOD (2.5 mg per heart)	9	129 ± 8**
Low flow + h-SOD (5 mg per heart)	12	112 ± 3**

All values are means ± s.e.mean.
* $P < 0.01$ from control; ** $P < 0.01$ from low flow + vehicle.
n = number of hearts studied in each group.
Pre-ischaemic pressure was taken 5 min before the onset of ischaemia.
Reperfusion pressure was taken 20 min following reperfusion.

However, the post-reperfusion to pre-low flow pressure ratio in the low flow + vehicle group significantly increased after reperfusion indicating an increase in post-reperfusion-induced coronary vascular resistance that could not be autoregulated. In contrast, both h-SOD treated groups exhibited a pressure ratio which was significantly lower than the untreated group and which was not significantly different from the control group not subjected to low flow.

Hearts receiving h-SOD (5 mg per heart) maintained significantly higher ($P < 0.01$) myocardial CK activities compared to those hearts given 0.9% NaCl. Furthermore, a significant cardioprotective effect was seen with 2.5 mg per heart h-SOD in another group of low flow hearts. Nevertheless, there was a moderate but significant difference in CK loss between control hearts given 0.9% NaCl and those hearts given 5 mg per heart. We checked for interference by h-SOD in the CK assay technique, but no significant effect of h-SOD on the CK assay was found. In addition, we measured the dry and wet weight ratio in the rat isolated perfused heart to determine the extent of cardiac oedema during the perfusion protocol and to determine whether h-SOD prevented it. There was no difference in dry to wet weight ratios among each group of perfused rat hearts indicating that there was no significant degree of cardiac oedema during this short perfusion period. These results are shown in Tables 3 and 4. Thus, h-SOD exerts a cardioprotective effect in perfused rat hearts independent of formed elements in the blood, and this protective effect does not appear to be related to an anti-oedema mechanism.

Table 3 Effect of human superoxide dismutase (h-SOD) on left ventricular (LV) creatine kinase (CK) activity during low flow followed by reperfusion in the isolated perfused heart of the rat

Group	n	*LV CK activity* ($iu\,g^{-1}$)
Non-perfused	8	1.62 ± 0.07*
Control + vehicle	11	1.29 ± 0.07
Control + h-SOD (5 mg)	8	1.51 ± 0.10*
Low flow + vehicle	14	0.74 ± 0.07*
Low flow + h-SOD (2.5 mg)	9	1.22 ± 0.04**
Low flow + h-SOD (5 mg)	12	1.28 ± 0.05**

All values are means ± s.e.mean.
* $P < 0.01$ from control + vehicle; ** $P < 0.01$ from low flow + vehicle.
n = number of rat hearts studied in each group.

Table 4 Dry to wet weight ratio of isolated perfused hearts of the rat

Group	n	*Dry/wet weight ratio* × *100*
Non-perfused	4	20 ± 0.4
Control + vehicle	4	20 ± 0.3
Low flow + vehicle	4	20 ± 0.2
Low flow + h-SOD (5 mg per heart)	5	19 ± 0.7

All values are means ± s.e.mean. There is no significant difference among any of the groups.
n = number of rats.

Discussion

The results of this study suggest that h-SOD exerts a marked protective effect in the intact rat during ischaemia and reperfusion and in the isolated perfused rat heart subjected to low flow followed by reperfusion. Furthermore, this beneficial effect of h-SOD appears to be dose-related. In addition to retarding the extension of ischaemic myocardial damage, h-SOD significantly improved the survival rate in rats subjected to MI plus reperfusion. In the constant flow isolated perfused rat hearts, h-SOD significantly moderated the post-reperfusion increase in coronary vascular resistance and protected against loss of myocardial CK from low flow followed by reperfusion. These results suggest that one component of the cardiac damage sustained during ischaemia and reperfusion may involve direct injury caused by oxygen derived free radicals. This is consistent with the known finding that oxygen-derived free radicals inactivate the endothelium-derived relaxing factor (EDRF) (Rubanyi & Vanhoutte, 1986). This could help explain the increase in post-reperfusion coronary vascular pressure.

Engler *et al.* (1983) reported that neutrophils accumulate in the vascular space of the reperfused ischaemic myocardium, where they may adhere to the endothelium and release oxygen-derived free radicals. While this may occur *in vivo*, in the isolated heart perfused with Krebs-Henseleit solution, h-SOD protected the ischaemic reperfused rat myocardium without blood components (i.e., white blood cells and platelets) suggesting a cytoprotective effect upon either myocardial or endothelial cells or both.

Previous studies have shown that myocardial injury by oxygen-derived free radicals occurs primarily during the initial period of reperfusion (Jolly *et al.*, 1984). Although h-SOD was given shortly after the ischaemic period, cellular injury may already have occurred before addition of h-SOD. The mechanism of the protective effect of h-SOD may be

due to three distinct actions. First, h-SOD may improve control of the coronary circulation, particularly preventing an inappropriate increase in coronary vascular resistance. Secondly, h-SOD may exert a protective effect on the endothelium. Thirdly, h-SOD may protect myocardial cells via a direct cytoprotective effect, perhaps related to a myocardial membrane stabilization. From the point of view of the dry to wet weight ratio, h-SOD had no significant effect on fluid transport. This suggests that microvascular permeability may not be dramatically influenced by h-SOD. Since h-SOD scavenges superoxide anions but is not effective against other reactive oxygen compounds (i.e., hydrogen peroxide and hydroxyl radical), other free radical scavengers may be useful along with SOD in exerting a greater degree of cardiac protection during periods of ischaemia. Catalase and glutathione peroxide both catalyze reactions that reduce intracellular levels of hydrogen peroxide. However, there is no enzyme system that can scavenge excessive quantities of hydroxyl radical. The hydroxyl radical can be derived from activated neutrophils and from xanthine oxidase which is localized in the endothelial cells of the coronary vasculature (Werns *et al.*, 1986). It is not totally clear why others have failed to observe a protective effect of SOD in myocardial ischaemia (Myers *et al.*, 1985a; Werns *et al.*, 1985; Gallagher *et al.*, 1986; Uraizee *et al.*, 1987). Some of the differences between these studies and ours is that all were in dogs, none used human SOD, and the timing was different in all experiments. These differences in experimental design may account for the different results, but more work is needed to clarify these differences.

In summary, we have shown a protective effect of h-SOD in the ischaemic rat myocardium with and without the presence of blood components. These results suggest that oxygen-derived free radicals may play a significant role in the propagation of myocardial ischaemic injury following reperfusion in the rat, and that the blood cells are not the only source of the oxygen-derived free radicals, since h-SOD also protected in rat isolated hearts perfused with Krebs-Henseleit solution and subjected to low flow followed by reperfusion.

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