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Protective Effects of the SOD-mimetic SC-52608 Against Ischemia/Reperfusion Damage in the Rabbit Isolated Heart

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K. S. Kilgore, G. S. Friedrichs, C. R. Johnson, C. S. Schasteen, D. P. Riley, R. H. Weiss, U. Ryan and B. R. Lucchesi. Protective Effects of the SOD-mimetic SC-52608 Against Ischemia/Reperfusion Damage in the Rabbit Isolated Heart. *Journal of Molecular and Cellular Cardiology* (1994) **26**, 995–1006. An experimental model of myocardial ischemia/reperfusion injury was used to assess the cardioprotective effects of SC-52608, a low molecular weight superoxide dismutase mimetic. Langendorff perfused rabbit isolated hearts were subjected to 30 min of global ischemia followed by 45 min of reperfusion. Hearts perfused in the presence of 20 μM SC-52608 exhibited a decrease in the release of creatine kinase and intracellular potassium compared to hearts receiving vehicle (control). A progressive increase in left ventricular end-diastolic pressure developed upon reperfusion in all hearts, but was significantly greater in control hearts when compared to hearts treated with SC-52608 ($P < 0.05$). In addition, results obtained with a radiolabeled monoclonal antibody to the intracellular protein myosin, indicate an increased degree of irreversible damage in vehicle-treated hearts. Myocardial protection was not significant in an additional group of hearts treated with 10 μM SC-52608. The hemodynamic, biochemical, morphological, as well as the antimyosin binding data, demonstrate that pretreatment with SC-52608 protects the myocardium from damage associated with global ischemia and reperfusion. The mechanism by which SC-52608 mediates the observed protective effect is most likely related to its ability to scavenge superoxide.

KEY WORDS: Ischemia-reperfusion; Oxygen radicals; Rabbit isolated heart; SC-52608; Superoxide dismutase.

Introduction

A number of factors have been implicated in the development of myocardial reperfusion injury. Calcium overload (Shen and Jennings, 1972), mediators of inflammatory response (Lucchesi *et al.*, 1989; Hill and Ward, 1971; Mathey *et al.*, 1986), and oxygen-derived free radicals (Guarnieri *et al.*, 1980; Hess and Manson, 1984; McCord, 1985) have been investigated for their role in reperfusion injury. Although the source of oxygen-derived free radicals has yet to be determined, mitochondria, endothelial cells, and neutrophils are potential sources for the generation of these reactive oxygen species (Babior, 1978).

Both direct and indirect experimental evidence suggests a role for free radicals in reperfusion injury. Direct evidence is derived from the use of electron-resonance spectroscopy and spin-trapping agents to detect free radicals in hearts subjected to ischemia and reperfusion (Kramer *et al.*, 1987; Zweier, 1988). Additional evidence includes the ability of free radical scavenger to limit infarct size in experimental models (Jolly *et al.*, 1984; Werns *et al.*, 1988). The present studies utilized the free radical scavenger superoxide dismutase (SOD) and the peroxide degrading enzyme, catalase, to protect the myocardium from reperfusion-induced injury. The ability of free radical scavengers to protect the myocardium from ischemia/reperfusion-induced

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damage has been documented. The combination of SOD and catalase has been utilized to protect the canine myocardium against ischemia/reperfusion-induced injury (Jolly *et al.*, 1984). Subsequently, SOD alone has been demonstrated to provide myocardial protection (Werns *et al.*, 1988). While an intracellular source of antioxidants exists in the myocardium, the endogenous cytoprotective mechanisms may be limited and subsequently overwhelmed by the excessive generation of free radicals upon reperfusion. Exogenous administration of antioxidants before reperfusion, especially those capable of entering the interstitial space, may serve to decrease the ability of the free radicals to induce cellular damage (Omar and McCord, 1991).

Conjugation of SOD with polyethylene glycol (PEG) increases the plasma half-life of the enzyme and enhances endothelial uptake, culminating in reduced infarct size in the canine heart (Tamura *et al.*, 1988). Other studies, however, do not support this conclusion (Tanaka *et al.*, 1989; Ooiwa *et al.*, 1989). It has been suggested (Omar and McCord, 1991) that PEG-SOD may in fact be too large of a molecule to readily pass across the endothelial cell, and therefore unable to scavenge the superoxide anion derived from intracellular sources. The rate of equilibration between the vascular compartment and the interstitial space will be determined by both the charge and size of the scavenger molecule, thus underscoring the need to develop low molecular weight SOD mimetics. In the present study, we demonstrate that the low molecular weight Monsanto SOD-mimetic, SC-52608 (Fig. 1), protects the rabbit isolated heart from myocardial ischemia/reperfusion damage.

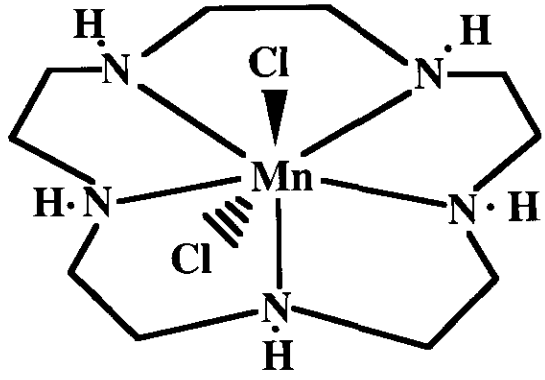

Figure 1 Chemical structure of SC-52608. MW 341.2.

Methods

Guidelines for animal research

The procedures used in this study were in accordance with the guidelines of the University of Michigan Committee on the Use and Care of Animals. Veterinary care was provided by the University of Michigan Unit for the Laboratory Animal Medicine. The University of Michigan is accredited by the American Association of Accreditation of Laboratory Animal Health Care, and the animal care use program conforms to the standards in "The Guide for the Care and Use of Laboratory Animals," DHEW Publ. No. (NIH) 86-23.

Langendorff perfused heart

Male New Zealand White rabbits (1.8–2.2 kg) were rendered unconscious by cervical dislocation. The hearts were excised quickly and the aorta attached to a cannula for perfusion with a modified Krebs-Henseleit (KH) buffer (pH 7.44, 37°C) delivered at a constant flow rate of 22–28 ml/min. The composition of the perfusion buffer, expressed in mM, was as follows: NaCl 117, KCl 4.0, $MgCl_2$ $6H_2O$ 1.2, KH_2PO_4 1.1, $NaHCO_3$ 25.0, $CaCl_2$ $2H_2O$ 2.6, glucose 5.0, L-glutamate 5.0 and pyruvic acid 5.0. The perfusate was recirculated throughout the experimental protocol. The KH buffer was passed through a membrane "lung" (Hamilton *et al.*, 1974) composed of 6 m of Silastic™ Medical Grade Tubing (0.058 inches i.d., 0.077 inches o.d., Dow Corning, Midland, MI, USA). The membrane "lung" was gased continuously with a mixture of 95% O_2/5% CO_2 to achieve an oxygen partial pressure of 500 mmHg. An in-line oxygen electrode and digital meter (Instech Laboratories, Plymouth Meeting, PA, USA) continuously monitored the oxygen tension in the KH buffer. The hearts were paced through the right atrium with electrodes attached to a laboratory stimulator (3 Hz, 2 ms duration, 4 V; Grass stimulator Model SD-5, Quincy, MA, USA).

The pulmonary artery was cannulated to facilitate collection of fluid from the coronary venous circulation. The pulmonary veins and the superior and inferior vena cava were ligated. A left ventricular drain, thermistor probe and a latex balloon were inserted via the left atrium and secured with a purse string suture at the atrial appendage. Isovolumetric left ventricular end-diastolic and systolic pressures were measured with the left ventricular fluid-filled latex balloon. The volume of the balloon was adjusted to achieve a left ventricular end-diastolic pressure of 5 mmHg. Physiologic parameters were monitored continuously and included: coronary perfusion pressure, left ventricular systo-

lic and end-diastolic pressures. Parameters were recorded using a direct-writing polygraph apparatus (Grass polygraph Model 79D, Quincy, MA, USA).

Experimental protocol

Four treatment groups were studied: (1) 10 μM SC-52608 ($n=6$); (2) 20 μM SC-52608 ($n=8$); (3) vehicle (physiologic saline, $n=8$); (4) native superoxide dismutase (nSOD, 16.5 μg/ml; 4450 U/mg protein; $n=4$). Hearts were allowed to equilibrate for 15–20 min under normoxic conditions. Five min. before the induction of ischemia, either SC-52608 nSOD, or vehicle was added to the reservoir containing the perfusion buffer. Thereafter, the hearts were subjected to normothermic, global ischemia by stopping delivery of the perfusion medium. Throughout the ischemic period, the hearts were exposed continuously to the drug or vehicle trapped within the coronary vascular bed. After 30 min of global ischemia, coronary artery perfusion was reinitiated by restoring the flow to the pre-ischemic flow rate. Functional parameters were recorded before the onset of ischemia and every 10 min thereafter throughout the reperfusion period. A constant temperature of 37°C was maintained throughout the protocol.

Preparation of SC-52608 and SOD

SC-52608 was a gift from the Monsanto Company (St. Louis, MO, USA). SC-52608 was prepared by the reaction of 1,4,7,10,13-pentaazacyclopentadecane with manganese(II) chloride in methyl alcohol (Riley DP and Weiss RH, 1994). The compound was soluble when dissolved in sterile, 0.9% sodium chloride solution or when a stock solution was prepared in the KH buffer. SC-52608 was prepared immediately before use in a stock solution from which aliquots were taken and added to the perfusion buffer to achieve the desired final concentrations of 10 or 20 μM. In order to relate the protective abilities of SC-52608 to that of nSOD (DDI Pharmaceuticals Inc., Mountain View, CA) nSOD was added to the KH buffer 5 min before induction of ischemia. Native SOD was present in the KH buffer until the end of the protocol. The final concentration of nSOD was 8.0 μg/ml (4450 U/mg protein). This concentration is equal to that of the 20 μM SC-52608.

Determination of SOD activity of SC-52608

The ability of SC-52608 to catalyse the dismutation of superoxide was determined by the direct technique of stopped-flow kinetic analysis (Riley *et al.*, 1991), which involves monitoring the spectrophotometric decay of superoxide in the presence and absence of SC-52608 in an aqueous buffer. The catalytic rate constant for SC-52608 at physiological pH was measured to be 4.13×10^7 M^{-1} s^{-1} (Riley DP and Weiss RH, 1994). Stopped-flow kinetic analysis is preferred over indirect methods of measuring SOD activity, such as the cytochrome *c* assay, as indirect methods can give false positive or negative results (Weiss *et al.*, 1993).

Release of creatine kinase and electrolytes

Samples of the coronary venous return (pulmonary artery effluent) were collected before ischemia and every 10 min during reperfusion for assessment of creatine kinase and electrolyte release. Creatine kinase activity was determined using a commercially available kit (procedure 47-UV; Sigma Chemical Co., St. Louis, MO, USA). The assay procedure is based on a modified method described previously (Szasz *et al.*, 1976). Briefly, the assay measures the increase in absorbance at 340 nm produced by the reduction of NAD to NADH. The rate of change is proportional to the creatine kinase activity. One unit is defined as the amount of enzyme that produces one micromole of NADH per minute.

The determination of electrolyte concentrations (Na^+, K^+, and Ca^{2+}) in the perfusion medium before (arterial) and after traversing the coronary vascular bed (venous) was performed using a NOVA 6 electrolyte analyser (NOVA Biomedical Instruments, Waltham, MA, USA).

Preparation of antimyosin antibody

Murine monoclonal antimyosin antibody [Mifarmonab F(ab')™] was provided by Centocor, Inc. (Malvern, PA, USA). Radioiodination of the antibody was performed by the chloramine-T method (Sakahara *et al.*, 1987). After incubation with Na ^{125}I and chloramine-T, the free ^{125}I was removed by Sephadex G-50 column chromatography. The specific activity of the radiolabeled molecule was 85.4 μCi/mg protein.

Determination of ^{125}I-antimyosin uptake

Uptake of labeled antimyosin antibody was determined by perfusing 1.0 μCi of antibody through the isolated heart for 10 min before termination of the protocol. After administration of the antimyosin antibody, the hearts were washed for an additional 5 min with buffer to remove antibody not specifically bound to myosin. The hearts were dried overnight, weighed and the uptake of antimyosin determined by a well-type auto-gamma counter (Minaxi Auto-Gamma, Packard Instrument Co.; Downers Grove, IL, USA). The amount of antimyosin antibody retained by the myocardium was expressed as a percent of the perfused dose bound per gram dry weight of the tissue.

Electron microscopy

Upon completion of the designated protocol, hearts were perfused for 3 min with 2.5% glutaraldehyde and 1% $LaCl_3$ *in 0.1* M *sodium cacodylate buffer* (pH 7.44). The electron dense $LaCl_3$ served as an indicator of arterial capillary endothelial permeability (Huttner *et al.*, 1973; Jokelainen *et al.*, 1976). Tissue samples from the left ventricular myocardium were removed and cut into sections measuring approximately 1 mm on a side. The samples were fixed for an additional 2 h at 4°C in 2.5% glutaraldehyde in buffer. After washing with 0.1 M sodium cacodylate buffer, the samples were dehydrated in an ethanol series and embedded in EMbed-812 (Electron Microscopy Sciences, Ft. Washington, PA, USA). Tissue blocks were sectioned with a Reichert ultramicrotome and placed on formvar coated copper grids then stained with 3% uranyl acetate and 0.3% lead citrate. Sections were observed with a Philips CM-10 electron microscope.

Statistical analysis

Results are expressed as mean values ± S.E.M. An ANOVA (repeated measures) was performed for comparisons of parameters measured over time. A P value of < 0.05 was regarded as significant and is denoted by an asterisk. Statistical analyses were performed on a Macintosh computer using Statview SE+Graphics (Abacus Concepts, Berkeley, CA, USA).

Results

Left ventricular systolic and end-diastolic pressures

The contractile parameters of vehicle treated hearts [Fig. 2(a)] show increased left ventricular systolic and end-diastolic pressures after 30 min global ischemia and 45 min of reperfusion. The left intraventricular balloon was inflated to achieve a left ventricular end-diastolic pressure of 5 mmHg that resulted in a systolic pressure that was between 80 and 90 mmHg before the induction of global ischemia. During the 30-min period of global myocardial ischemia, systolic pressure decreased rapidly to 5 mmHg. At the onset of reperfusion, the systolic

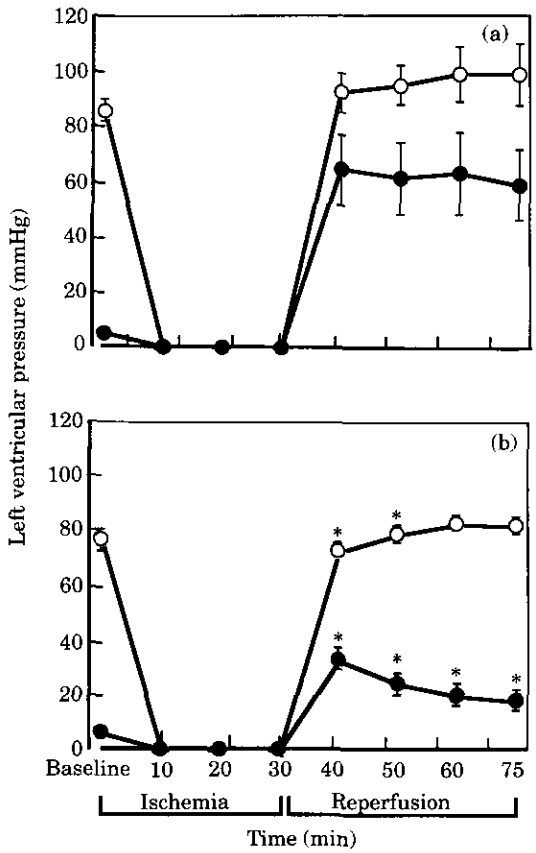

Figure 2 Diastolic and systolic pressures recorded from rabbit isolated hearts rendered globally ischemic (30 min) followed by reperfusion (45 min). (a) Hearts treated with vehicle prior to the induction of ischemia [(○) control LVPps, (●) control LVPed, $n = 7$]. The end-diastolic and systolic pressures increase and the developed pressure decreased upon reperfusion. (b) Data from hearts treated with 20 μM SC-52608 5 min prior to ischemia [(○) SC-52608 LVPps, (●) SC-52608 LVPed, $n = 7$]. *$P < 0.05$ *v* vehicle treated hearts at the same time point.

pressure increased to 90 mmHg within the first 10 min and continued to increase steadily throughout the remainder of the reperfusion period. The onset of reperfusion in control hearts was associated with an increase in left ventricular end-diastolic pressure, reaching a maximum of 60 mmHg by 10 min reperfusion. Developed left ventricular pressure (difference between left ventricular systolic pressure and left ventricular end-diastolic pressure) at the corresponding time point was approximately 50% of the pre-ischemic baseline value.

In hearts pretreated with SC-52608 [Fig. 2(b)], the systolic pressure was significantly lower than the corresponding time points in control hearts during the initial 20 min of reperfusion. The end-diastolic pressure reached a maximum value of 30 mmHg (T = 10 min reperfusion) and decreased steadily throughout the remainder of the reperfusion period. The end-diastolic pressure was significantly less than control hearts at all time points throughout reperfusion ($P < 0.05$). Both left ventricular systolic and end-diastolic pressures in hearts treated with 10 μM SC-52608 were not significantly different when compared to values from control hearts (Table 1).

Functional parameters of hearts receiving nSOD also were examined (Table 1). The left ventricular systolic pressure was not significantly different in hearts treated with 10 μM, 20 μM SC-52608 or with nSOD when compared to the corresponding vehicle value after 45 min reperfusion. Although the left ventricular end diastolic pressure in hearts treated with 10 μM SC-52608 and nSOD was lower than that seen in vehicle hearts, the difference was not found to be significant. However, the diastolic pressure in 20 μM SC-52608 treated hearts was significantly different when compared to vehicle at the same time point. Only hearts treated with 20 μM SC-52608 displayed a significant difference in the left ventricular developed pressure 45 min after the onset of reperfusion.

Coronary perfusion pressure

Coronary perfusion pressure (CPP) was used as an assessment of vasomotor tone. At the conclusion of the reperfusion period, CPP was greater in control hearts than in hearts treated with SC-52608 (Fig. 3). After the onset of reperfusion, the CPP in control hearts increased abruptly during the first 10 min of reperfusion and continued to increase throughout the reperfusion period. The CPP during reperfusion in hearts treated with 20 μM SC-52608 was not different from pre-ischemic baseline values. Moreover, CPP was significantly lower during this period compared to control hearts ($p < 0.05$). The CPP in hearts treated with 10 μM SC-52608, although greater than in hearts treated with 20 μM SC-52608, was not significantly less than the value in control hearts.

Release of myocardial creatine kinase

The increase in creatine kinase (CK) content in the

Table 1 Selected hemodynamic variables after 30 min of global ischemia in rabbit isolated hearts pre-treated with vehicle, SC-52608, or n-SOD

	Vehicle ($n=8$)	SC-52608 10 μM ($n=6$)	SC-52608 20 μM ($n=8$)	n-SOD 16.5 U/ml ($n=4$)
LVPps				
Baseline	86 ± 4	78 ± 4	77 ± 4	85 ± 3
Reperfusion (20 min)	96 ± 7	82 ± 6	79 ± 3	73 ± 11
Reperfusion (45 min)	100 ± 11*	83 ± 6	82 ± 3	72 ± 12
LVPed				
Baseline	5 ± 1	5 ± 1	6 ± 1	3 ± 1
Reperfusion (20 min)	62 ± 13*	45 ± 8*	24 ± 4*†	33 ± 10*
Reperfusion (45 min)	60 ± 13*	35 ± 8*	18 ± 4*†	29 ± 9
LVPdev				
Baseline	81 ± 4	73 ± 3	71 ± 4	82 ± 3
Reperfusion (20 min)	34 ± 8*	37 ± 6*	55 ± 5*	40 ± 10*
Reperfusion (45 min)	41 ± 8*	48 ± 3*	65 ± 6†	43 ± 10*

Data are mean ± S.E.M. LVPps, peak systolic left ventricular pressure; LVPed, end diastolic left ventricular pressure; LVPdev; left ventricular developed pressure.

* = $P < 0.05$ relative to corresponding baseline value, † = $P < 0.05$ v corresponding vehicle value.

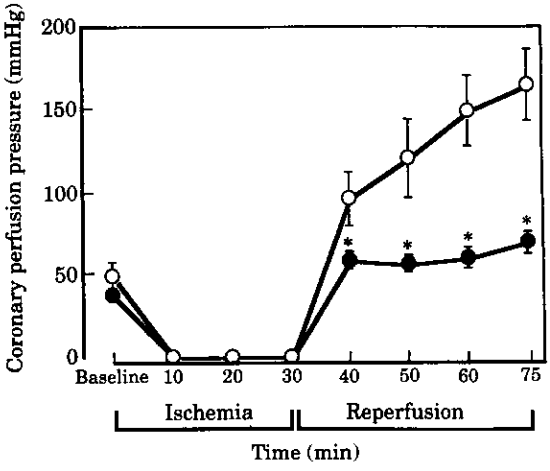

Figure 3 Coronary perfusion pressure (CPP) recorded from globally ischemic/reperfused isolated hearts [(○) control, (●) SC-52608]. Hearts pretreated with SC-52608 ($n=6$) show slightly elevated CPP upon reperfusion. After 10 min reperfusion, the CPP is stabilized for the remainder of the reperfusion period. The CPP in vehicle treated hearts continues to increase throughout the reperfusion period. *$P<0.05$ *v* control hearts at same time point.

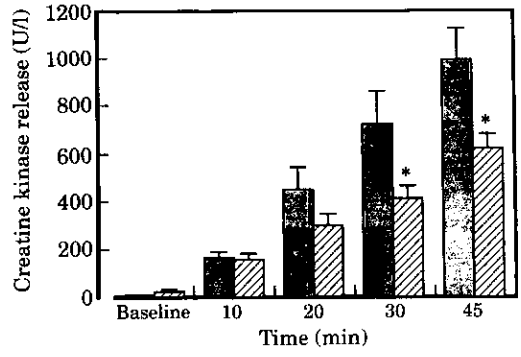

Figure 4 Release of cytosolic creatine kinase (CK) into the pulmonary effluent of isolated hearts. Control hearts (■) show increased release of CK after 10 min reperfusion as compared to hearts pretreated with SC-52608 (▨). At the 30 and 45 min time points the difference in CK release is significant. *$P<0.05$ *v* control hearts.

coronary venous drainage (pulmonary artery effluent) was used as an indicator of myocardial cell injury (Fig. 4). Baseline values of CK were similar for all groups. CK was detected in the coronary venous drainage throughout the reperfusion period in both control and SC-52608-treated hearts. However, after 20 min of reperfusion, the total CK release was less in hearts treated with SC-52608 when compared to that of control hearts. At the 30 and 45 min time points, CK loss was significantly greater from control hearts when compared to hearts pretreated with SC-52608 ($P<0.05$). CK release from vehicle treated hearts, 45 min after the start of reperfusion, was 997 ± 136 U/l as compared to 623 ± 57 U/l from SC-52608-treated hearts ($P<0.05$).

Release of intracellular electrolytes

Tissue injury was assessed further by determining the release of intracellular electrolytes into the coronary venous drainage collected from the pulmonary artery (Table 2). Baseline concentrations in each group were identical. For the first 20 min of reperfusion, the release of potassium was similar in all groups. However, at 30 and 45 min after the onset of reperfusion, the cellular loss of K^+ was significantly greater in control hearts ($P<0.05$). In

Table 2 Coronary sinus effluent K^+ (mM) in the presence and absence of SC-52608 after 30 min of global ischemia

		Reperfusion			
	Baseline	10 min	20 min	30 min	45 min
Control ($n=7$)	5.03±0.04	5.30±0.08	5.35±0.07	5.39±0.08	5.39±0.05
SC-52608 (10 μM, $n=6$)	5.13±0.02	5.34±0.05	5.30±0.03	5.31±0.03	5.32±0.04
SC-52608 (20 μM, $n=7$)	5.09±0.06	5.22±0.05	5.23±0.04	5.22±0.05*	5.22±0.05*

Data are mean±S.E.M.
*=$P<0.05$ relative to corresponding vehicle value.

control hearts K^+ efflux increased from a baseline value of 5.03 ± 0.04 mM to 5.39 ± 0.05 mM after 45 min of reperfusion. In contrast, K^+ efflux from hearts pretreated with 20 μM SC-52608 increased from 5.09 ± 0.06 mM to 5.22 ± 0.05 mM after reperfusion. Calcium and sodium ion concentrations in the pulmonary effluent also were determined. Release of these ions after reperfusion did not differ among groups.

Uptake of monoclonal antimyosin antibody

An ^{125}I-labeled monoclonal antibody to the intracellular protein myosin was used as an indicator of cellular damage in control and in hearts treated with 20 μM SC-52608. Binding of the antibody (Fig. 5) was 75% less in SC-52608 treated hearts as compared to control hearts ($P < 0.05$).

Morphological alterations/lanthanum chloride staining

Transmission electron microscopy provides morphological assessment in support of the functional changes and uptake of antimyosin antibody. Two different areas of left ventricular myocardium were examined from control and hearts treated with 20 μM SC-52608. The extravascular deposition of electron dense lanthanum chloride was used as an indicator of vessel damage. Control hearts [Fig. 6(a)] showed morphological alterations consistent with ischemia/reperfusion injury. Lanthanum chloride is present in the distended perivascular space with little deposition on the internal surface of the vessel. Endothelial cells lining the vessel appear disrupted with the appearance of numerous intracellular vesicles. Some lanthanum chloride can be seen between two endothelial cells (arrowhead). The mitochondria (M) were swollen with disrupted matrices and cristae. Large, amorphous densities (arrow), suggestive of irreversible injury, were noted within the damaged mitochondria. Extensive myofibrillar damage, as noted by blurring of the Z-bands and disruption of the myofibrils, are apparent.

Endothelial cells lining blood vessels in SC-52608-treated hearts [Fig. 6(b)] showed a continuous layer of lanthanum chloride on the luminal surface (LS). While lanthanum chloride was present in the interendothelial junction, very little was present in the perivascular space, indicative of a maintenance of vessel integrity. The morphological appearance of the tissue was essentially normal. Mitochondria (M) were densely packed with an intact matrix and showed little evidence of structural alteration in the cristae. No amorphous densities were noted. The myofibrils were intact with well aligned Z-bands and no evidence of intracellular edema.

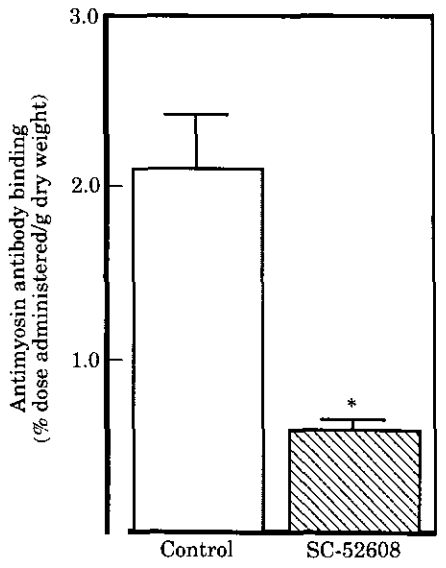

Figure 5 Uptake of ^{125}I-antimyosin antibody in control hearts ($n = 3$) and SC-52608-treated hearts ($n = 3$). Binding of antibody is increased significantly in vehicle hearts as compared to hearts treated with SC-52608 following 30 min ischemia and 45 min reperfusion. *$P < 0.05$ v vehicle treated hearts.

Discussion

In this study, the low molecular weight SOD-mimic SC-52608 was used to protect against ischemia/reperfusion damage in the rabbit isolated heart. The ability of SOD and other superoxide scavangers to protect the myocardium from ischemia/reperfusion-induced damage has been documented in a number of studies (Jolly *et al.*, 1984; Werns *et al.*, 1988). However, the protective effects of SOD in myocardial reperfusion injury are not accepted by all investigators (Jennings *et al.*, 1986). Species differences and the methods used to determine infarct size have been mentioned as possible explanations for the disagreement among different laboratories (Ooiwa *et al.*, 1989).

Scavenging superoxide may provide both direct and indirect protection to the reperfused myocardium. The direct action of agents such as SOD is to reduce the concentration of free radicals generated

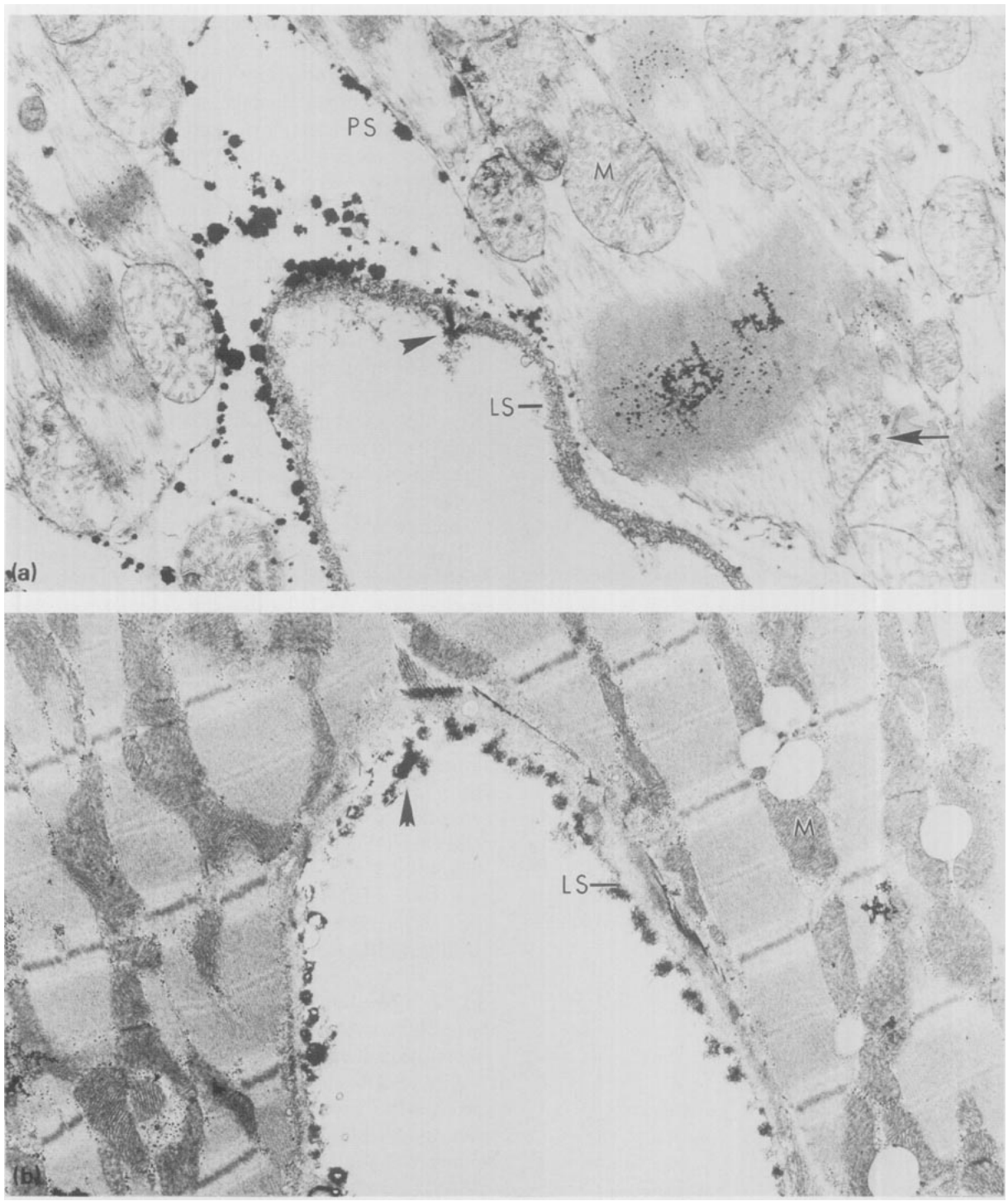

Figure 6 Electron microscopy of cardiac muscle following 30 min global ischemia and 45 min reperfusion. (a) Control hearts reveal numerous morphological changes indicative of ischemia/reperfusion injury. The mitochondria (M) are swollen and disrupted with large, amorphous densities, suggestive of irreversible injury (arrow). The amount of lanthanum staining surrounding the luminal surface (LS) is markedly reduced with increased lanthanum deposition in the perivascular space (PS). Lanthanum chloride may be seen between two endothelial cells. × 4420. (b) Morphology of hearts pretreated with SC-52608 is unremarkable with uniform lanthanum staining surrounding the luminal surface (LS) of the vessel. Cristae of the mitochondria (M) are compact and well organized. Myofibrillar structure appears normal. × 4420.

from superoxide at the time of reperfusion. It has been demonstrated (Johansson *et al.*, 1990) that the generation of free radicals is reduced when human recombinant SOD (hrSOD) and catalase are added to the perfusate of a rat isolated heart. Free radical scavengers also may indirectly protect the myocardium by mediating the inflammatory response associated with ischemia/reperfusion. Episodes of hypoxia/reoxygenation have been shown to promote adhesion of neutrophils to cultured endothelial cells, a phenomenon reversible by the addition of free radical scavengers (Palluy *et al.*, 1992; Yoshida *et al.*, 1992). Furthermore, it has been shown (Suzuki *et al.*, 1991) that the combination of SOD and catalase results in a decreased accumulation of leucocytes within the myocardium after ischemia/reperfusion of the rat heart. In addition to promoting leucocyte adherence, free radicals have been shown to activate the complement system by converting C5 to a functionally active C5b-like form (Vogt *et al.*, 1986, 1989). These observations are supported in a study (Shingu *et al.*, 1992) demonstrating that the generation of hydrogen peroxide by neutrophils will activate complement.

The lack of neutrophils and plasma-derived complement components in our isolated heart model suggest that free radicals, generated from intracellular sites, may be acting in a direct fashion to induce myocardial damage. Reperfusion of the rabbit isolated heart has been previously shown to result in the formation of superoxide-derived free radicals (Zweier, 1988). The reactive radicals damage both the cellular membrane lipids and membrane bound proteins (Kako, 1985). Multiple lines of evidence in our study demonstrate the ability of SC-52608 to protect the isolated heart from ischemia/reperfusion-induced injury. The decreased release of CK and intracellular potassium are indicative of protection of the myocytes against reperfusion damage. The release of CK is considered to be a manifestation of increased cellular permeability (Shell *et al.*, 1971), and the reduction in CK loss in the presence of SC-52608 is indicative of a cytoprotective effect. The ability of SC-52608 to protect against membrane damage is supported further by the decreased binding of labeled antimyosin antibody in the drug treated hearts. The antimyosin antibody has been shown to bind only to myocytes that have undergone an extensive degree of damage and exhibit a loss of membrane integrity (Khaw *et al.*, 1979; Haber *et al.*, 1982). A previous study from our laboratory demonstrated that reoxygenation of the hypoxic myocardium is associated with increased antimyosin binding (Kilgore and Lucchesi, 1993). The results from this study demonstrate that the use of labeled antimyosin is useful for quantitating the extent of myocardial injury due to ischemia/reperfusion. The decreased antimyosin binding in SC-52608-treated hearts implies that this compound affords protection to the reperfused myocardium, through protection of the membrane from attack by reactive oxygen metabolites. The protection of membrane integrity in drug treated hearts was further substantiated by the decreased release of cytosolic creatine kinase. Release of this and other intracellular enzymes from the isolated heart has been attributed to the "oxygen paradox" (Hearse, 1977). However, it should be emphasized that the release of CK after reperfusion is not characteristic of the CK release seen in the oxygen paradox, where there is a sudden release of CK upon reoxygenation. This may be indicative of continuing myocardial damage throughout the reperfusion period due to generation of free radicals or the presence of focal or regional ischemia. The increase in extracellular potassium in control hearts is an effect associated with the compromised myocardium. It has been suggested that this loss of potassium is a major reason for changes in the electrophysiological properties of the heart (Harris *et al.*, 1956). The presence of SC-52608 attenuated the efflux of potassium which may aid in reducing the occurrence of electrical disturbances (arrhythmias) associated with reperfusion.

Immediately upon reperfusion in control hearts, the systolic and end-diastolic pressures increased concomitant with a decrease in developed pressure. After 20 min of reperfusion, the developed pressure in control hearts increased, reaching a maximum value 40 min after initiating reperfusion. In SC-52608-treated hearts, the end-diastolic pressure remained significantly lower throughout the reperfusion period. Unlike SC-52608 treated hearts, in vehicle treated hearts the late stages of ischemia and early stages of reperfusion were characterized by an excessive degree of contracture. The latter could result in impaired flow, leading to the formation of ischemic areas throughout the myocardium and a subsequent increase in tissue damage, an observation that may explain the prolonged release of CK observed in these studies. The protection seen during the ischemic period may be due to the ability of SC-52608 to preserve ATP and calcium concentrations, thus preventing the development of myocardial contracture. Another explanation for the protective effect of SC-52608 is the scavenging of oxygen-derived free radicals that may form throughout the ischemic period. Free radical formation occurs even under low oxygen tensions such as

those seen during a period of ischemia (Rao *et al.*, 1983). Thus, it is possible that SC-52608 exerts its protective effects not only during reperfusion, but also during the ischemic period.

Electron microscopy was performed using lanthanum chloride as a marker of vessel wall damage and increased cellular permeability (Hoffstein *et al.*, 1975; Haack *et al.*, 1981). Lanthanum chloride binds to an acidic mucopolysaccharide layer that lines the luminal surfaces of vessels and may play a role in maintaining vessel integrity (Luft, 1966). Disruption of the endothelial layer results in the loss of this mucopolysaccharide layer and a reduction in the amount of lanthanum chloride deposited on the luminal surface of the vessel (Luft, 1966). Hearts treated with SC-52608 showed a distinct deposition of lanthanum chloride on the luminal surface of blood vessels indicating that the coronary vascular bed had not been damaged substantially as a result of global myocardial ischemia and reperfusion. In contrast, vehicle treated hearts showed little or no lanthanum deposition, due to an extensive degree of cellular damage. Morphological changes seen in vehicle treated hearts were similar to those reported by others and known to be associated with irreversible injury resulting from global myocardial ischemia and reperfusion (Jennings *et al.*, 1985). The ultrastructural changes include, separation of the myofibrils and mitochondrial alterations manifested by disorganization of the cristae and the presence of large amorphous densities, that are indicative of irreversible injury. The presence of contracture bands were prominent in tissue from vehicle treated hearts. The latter ultrastructural change has been observed under conditions associated with the oxygen paradox (Schluter *et al.*, 1991). This finding has been substantiated by the observation that 2,3-butanedione monoxime (BDM), an inhibitor of contractile function, is able to prevent the formation of the myocardial contraction bands and changes associated with the oxygen-paradox (Armstrong and Ganote, 1991). It should be noted however, that the morphological changes seen in vehicle treated hearts also may be due to the oxygen-paradox as well as increased concentration of intracellular calcium. Thus, SC-52608 prevents contraction band formation after global ischemia and reperfusion by virtue of its ability to act as a scavenger of superoxide or by preventing excessive calcium overload.

The results of this study demonstrate that the low molecular weight SOD-mimic, SC-52608, has a salutary effect of reducing the extent of myocardial injury in the isolated perfused heart subjected to global ischemia and reperfusion. SC-52608 may offer advantages over other free radical scavengers such as native SOD, which due to its molecular weight, may be too large to gain access readily to the interstitial space and/or intracellular compartment, thereby limiting its ability to scavenge intracellular radicals. Theoretically, the relatively low molecular weight of SC-52608 would facilitate its entry to the interstitial and perhaps the intracellular compartment. Therefore, SC-52608 would have a distinct advantage over nSOD due to its ability to scavenge superoxide derived from intracellular sources. The observation that SC-52608 provides a greater degree of protection when compared to nSOD in these studies supports the concept that SC-52608 displays some apparent advantages in eliciting protective effects. However, additional studies are needed to substantiate the advantages of SC-52608 over those of the larger molecular weight nSOD. Previous studies using this rabbit isolated heart model have shown that SC-52608 rapidly equilibrates within the interstitial compartment (unpublished data), suggesting that the compound is able to gain access within the intracellular space. Thus, SC-52608 may protect intracellular membranes that form organelles such as the mitochondria and sarcoplasmic reticulum in addition to the plasmalemma. Others (Omar and McCord, 1991) have examined the cardioprotective activity of various forms of superoxide dismutase in the isolated heart subjected to global ischemia and reperfusion. The most effective cardioprotective agents were those that gained access to the interstitial space as evidenced for their presence in the lymphatic drainage (Omar and McCord, 1991).

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