Postischemic Recovery of Contractile Function is Impaired in SOD2\textsuperscript{+/-} but Not SOD1\textsuperscript{+/-} Mouse Hearts

Gregory K. Asimakis, PhD; Scott Lick, MD; Cam Patterson, MD

Background—Reactive oxygen species (ROS) contribute to myocardial stunning. Superoxide dismutase (SOD) is a major defense mechanism against ROS. The purpose of this study was to evaluate the contributions of cytosolic (SOD1) and mitochondrial (SOD2) isoforms to protect against myocardial stunning.

Methods and Results—Isolated hearts from wild-type, heterozygous (+/−) SOD1 and SOD2 knockout mice received 30 minutes of ischemia followed by 60 minutes of reperfusion. After 60 minutes of reperfusion, the heart rate multiplied by the developed pressure (HR×DP) in the wild-type and SOD1\textsuperscript{+/-} hearts recovered to 92±9 and 85±7 of preischemic baseline values, respectively (P=NS). In contrast, the HR×DP was significantly lower (63±7%) in the SOD2\textsuperscript{+/-} hearts compared with the wild-type hearts. Western blot analysis and enzymatic activity of tissue lysates confirmed reduction of activities of specific SOD isoforms without compensatory increase in the other isoform in the knockout animals studied.

Conclusions—Postischemic functional recovery is more sensitive to a partial deficiency of SOD2 than a partial deficiency of SOD1. Therefore, modulation of the mitochondrial SOD isoform is a critical determinant in the tolerance of the heart to oxidative stress. (Circulation. 2002;105:981-986.)

Key Words: free radicals ■ ischemia ■ reperfusion ■ stunning, myocardial

Although coronary artery angioplasty and thrombolytic therapy are now routinely used to establish myocardial revascularization, persistent postischemic contractile dysfunction often hinders the clinical outcome of revascularization.\textsuperscript{1} This may be attributable in part to the generation of ROS, which can contribute to postischemic cell injury,\textsuperscript{2–5} thereby severely limiting the beneficial effects of reperfusion.

Unchecked generation of ROS may cause damage to nuclear and mitochondrial DNA, proteins, and membrane lipids. For this reason, cardiac cells contain specific ROS-scavenging enzymes, such as superoxide dismutase (SOD). Superoxide anion is formed in the extracellular space and intracellularly in the cytosolic and mitochondrial compartments. The importance of protecting against oxidative injury is indicated by the presence of unique SOD isoforms in these compartments. However, these endogenous protective mechanisms can be overwhelmed, as indicated by studies that show cardioprotection from ischemia/reperfusion injury when ROS-scavenging enzymes are overexpressed or added exogenously.\textsuperscript{6,7}

Although several studies have shown that augmentation with extracellular SOD, nuclear or cytosolic CuZnSOD (SOD1), or mitochondrial MnSOD (SOD2) provides significant cardioprotection from ischemia/reperfusion injury,\textsuperscript{7–9} the site of the most significant oxidative injury under these conditions remains controversial. Wang et al\textsuperscript{7} reported that overexpression of CuZnSOD almost totally quenched superoxide generation and attenuated post-ischemic injury, suggesting that the superoxide anions formed in the cytosolic compartment are most prevalent and injurious compared with other compartments. However, CuZnSOD overexpression itself alters both MnSOD and peroxidase activity,\textsuperscript{10} which complicates the interpretation of these and similar experiments. Moreover, overexpression of the various isoforms can be detrimental, potentially masking the protective effects of the native isoform.\textsuperscript{11–13} Therefore, studies using overexpression of SOD do not adequately address the significance or relative contribution of native SOD isoforms in protecting the heart from oxidative injury.

The purpose of the present study was to evaluate the contributions of endogenous cytosolic and mitochondrial SOD isoforms in protection against myocardial stunning in hearts from SOD knockout mice. Heterozygous SOD1 and SOD2 knockout mice were used. Postischemic mechanical function, lipid peroxidation, and enzyme washout were evaluated in hearts from the wild-type (WT) and knockout mice.
Methods

SOD Knockout Mice

Genetic engineering of mice to delete the SOD1 and SOD2 genes has been previously described.\(^1\)\(^,\)\(^2\) SOD1\(^{-/-}\) and SOD2\(^{-/-}\) are phenotypically indistinguishable from the WT mouse, yet have intermediate levels of SOD1 and SOD2 expression, respectively. Genotypes of mice were confirmed using polymerase chain reaction–based techniques from genomic DNA derived from tail preparations using appropriate primer pairs.

Isolated Perfused Mouse Heart

Eight-week-old animals were used. Thirty minutes after an intraperitoneal injection of heparin, each animal was anesthetized with an intraperitoneal injection of pentobarbital. Each heart was quickly excised and placed in ice-cold saline. The aorta was quickly secured to a cannula, and the heart was perfused with Krebs-Henseleit buffer (KHB) using a Langendorff preparation. The concentrations (expressed as mmol/L) of constituents of the KHB were KCl 4.7, CaCl\(_2\) 2.5, MgCl\(_2\), 1.25, KH\(_2\)PO\(_4\) 1.25, NaHCO\(_3\) 25, NaCl 118, and glucose 11. The temperature of the buffer was kept constant at 38°C. Each group consisted of male and female animals. The WT group consisted of 3 male and 8 female animals. The SOD2 group consisted of 6 male and 3 female animals.

Because the groups had mixed male and female animals, we tested if there were differences in postischemic contractile function of hearts from male and female control C57BL/6 mice. Hearts from 9 male and 10 female mice were subjected to 30 minutes of ischemia and 60 minutes of reperfusion. Preischemic values for heart rate (HR), developed pressure (DP), HR and coronary flow rates were obtained as described for Western blotting. After centrifugation, protein concentration of the supernatant was determined. Supernatant was added to the cuvette. To this was added 16.7 mol/L acetylated cytochrome c, 25 mol/L Na\(_2\)HPO\(_4\), and 0.1 mmol/L EDTA in a cuvette. This was added 16.7 μL of solution B (0.2 μM xanthine oxidase in 0.1 mmol/L EDTA), and absorbance was read at 550 nm at 1-minute intervals for 10 minutes. Results were compared with a standard curve, and SOD activity was expressed as U/μg. To determine SOD2 activity, lysates were treated with 5 mmol/L KCN

<table>
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<th>TABLE 1. SOD Activities of Heart Lysates</th>
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<td>CuZnSOD Activity</td>
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</tr>
<tr>
<td>WT</td>
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<tr>
<td>SOD1(^{-/-})</td>
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<td>SOD2(^{-/-})</td>
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Activity is presented as units/mg protein. Results are mean±SD, \(n=4\) samples. *\(P<0.05\) compared with wild-type.
to inactivate SOD1 before performing assays. To determine SOD1 activity, lysates were treated with 10 mmol/L NaCN to inactivate SOD2 before performing assays.

### Lipid Peroxidation

Lipid peroxides (malondialdehyde plus 4-hydroxynonenal) were measured in homogenized tissue extracts by reaction with N-methyl-2-phenylindole using a commercially available assay (Oxish Research). Extracts were obtained from left ventricles of nonischemic hearts or of hearts after the ischemia-reperfusion protocol.

### Enzyme Release

Coronary effluent samples (1-minute collection time) were collected at selected times during the perfusion protocol. Lactate dehydrogenase (LDH) activity of each sample was determined using an LDH assay kit (Sigma Diagnostics). The absorbance was read at 340 nm.

### Statistical Analysis

Data are reported as mean±SEM. Group comparisons were made by ANOVA. If statistical differences were indicated by ANOVA, multigroup comparisons were made using the Bonferroni test. A value of *P*<0.05 was considered significant.

### Results

SOD1 protein levels were 42±12% of control in SOD1+/− mice, and SOD2 protein levels were 53±10% of control in SOD2+/− mice (Figure 1), confirming that SOD protein levels corresponded to the genotype of these mice. Moreover, Cu/ZnSOD and MnSOD enzymatic activity was reduced approximately 50% in the hearts from SOD1+/− and SOD2+/− mice, respectively (Table 1). Also, there was no evidence of dosage compensation of one isoform of SOD for the other at the level of protein expression in any of the genetic lines studied.

To test the role of SOD isoforms in protection against reperfusion injury, we subjected isolated hearts to ischemia for 30 minutes and measured posts ischemic function. Baseline hemodynamic and LDH release data for perfused hearts of the different groups are shown in Table 2. There were no significant differences between the groups with respect to the hemodynamic measurements or LDH release. The posts ischemic recovery of heart rate multiplied by developed pressure (HR×DP) is shown in Figure 2. After approximately 10 minutes of reperfusion, all hearts in all three groups developed reperfusion arrhythmias and ventricular tachycardia, with a concomitant reduction in pressure development. The dysrhythmias occurred for about 20 minutes, after which the hearts spontaneously converted to sinus rhythm. Although the SOD1+/− hearts seemed to be more susceptible to dysrhythmias between 10 and 30 minutes of reperfusion, these hearts recovered to near 100% of baseline values after 1 hour of reperfusion and were indistinguishable from hearts taken from WT mice. In contrast, the SOD2+/− hearts recovered only approximately 60% of baseline values. Reduced functional recovery in the SOD2 knockouts was observed after 45 minutes of reperfusion (Figure 2).

We also measured the EDP of each experimental group during the experimental protocol (Figure 3). During ischemia, all hearts developed ischemic contracture, as evidenced by increased ventricular pressure above baseline EDP. There were no significant differences between groups with respect to the extent of ischemic contracture. During early reperfusion, all hearts had EDPs 2 to 3 times higher than baseline values. After 60 minutes of reperfusion, the EDP values of the WT and SOD1+/− recovered to near baseline values (10 mm Hg). In contrast, the EDP of the SOD2+/− hearts remained elevated (approximately 30 mm Hg) throughout the 60-minute reperfusion period. These results are consistent with the values determined for HR×DP and indicate impair-
ment in myocardial function and hence worsened ischemic injury in SOD2+/− but not SOD1+/− hearts.

We also measured coronary flow to determine the extent to which impaired myocardial function reflected reduced flow after ischemia. The postischemic recovery (expressed as percent of baseline values) of the coronary flow among the groups, indicating that results in Figures 2 and 3 are not a function of decrements in postischemic myocardial perfusion.

Levels of lipid peroxidation products were measured as a marker for accumulation of oxidative injury in hearts before and after ischemia-reperfusion (Figure 5). After reperfusion, there was a 12-fold increase in lipid peroxidation products in the WT group, as expected. Consistent with a previous study by Yoshida et al., there was a trend toward greater accumulation of lipid peroxides in hearts of SOD1+/−/− mice after reperfusion, although this trend did not reach significance in our studies. In contrast, lipid peroxidation products were significantly greater in hearts from SOD2+/−/− mice after reperfusion compared with hearts from WT mice (481±58 versus 259±42 pmol/mg; P<0.05). (Figure 5.)

Figure 6 shows the release of LDH activity in the hearts. Although reperfusion resulted in elevated levels of LDH activity in the coronary effluents, there were no significant differences among the groups with respect to the rate of LDH release at any reperfusion time.

Discussion

A role for ROS in ischemia-reperfusion injury has been indicated by numerous studies showing increased levels of oxidative species in the heart during reperfusion. The consequences of ROS generation in this setting are diverse and include alterations in adrenergic signaling, impaired partitioning of calcium, and apoptotic cell death. Several pathways exist to protect against damage induced by ROS, with those best characterized in the heart being the superoxide dismutases and the glutathione/glutathione peroxidase system. However, the precise roles of these systems have been difficult to delineate. Studies that measure antioxidant levels have provided contradictory results regarding the role of antioxidant defenses in ischemia-reperfusion injury. Effects of exogenously administered SOD are variable and dose-dependent. Transgenic overexpression of either SOD1 or SOD2 is protective against ischemia-reperfusion injury in mice. However, the relevance of these experiments to endogenous systems is difficult to determine, insofar as overexpressed SOD isoforms may have effects that do not reflect their endogenous counterparts. In addition, overexpression of SOD isoforms may confound interpretation by altering the activity of other antioxidant systems or by producing excess free radicals.

To address the unresolved issues regarding the importance of SOD isoforms in protection against ischemia-reperfusion injury, we isolated hearts from mice with genetically decreased levels of SOD isoforms and tested their postischemic hemodynamic functions. Although the oxidative defenses of the SOD1− and SOD2-deficient hearts are expected to be compromised, no significant functional decrements were noted under normoxic, preischemic conditions. Moreover, we found that SOD1−/− hearts were indistinguishable from WT hearts with respect to postischemic functional recovery. Conversely, we found that SOD2+/− mice, expressing the mitochondrial SOD activity at levels 50% of WT mice, had a potent deficit in postischemic myocardial function compared...
with WT hearts. These results are consistent with our findings that lipid peroxide formation produced by ischemia/reperfusion was significantly greater in the SOD2 deficient hearts than that observed in the WT and SOD1 hearts. We observed no differences in coronary flow or LDH release in the SOD2 knockout mice compared with controls, suggesting that the effects of SOD2 deficiency on cardiomyocyte metabolism alter recovery from ischemia independent of significant changes in myocyte viability. SOD2 deficient knockout mice die within weeks of birth. In contrast, SOD1 mice have normal lifespans, although they are less fertile and prone to toxicity induced by high concentrations of paraquat. These observations support the argument that mitochondrial SOD has a greater role than the cytosolic isofrom in protection against ROS-induced injury under physiological conditions. However, the relative importance of cytosolic and mitochondrial SOD during ischemia-induced oxidative stress is not clear. The present study is, to our knowledge, the first to compare directly WT, SOD1-deficient, and SOD2-deficient mice hearts with respect to their tolerance to oxidative stress induced by ischemia/reperfusion. Our observation that hearts are especially sensitive to deficiency of SOD2 is consistent with recent observations implicating mitochondrial reactive oxygen species in hypoxic preconditioning and reperfusion injury in cardiomyocytes. We can speculate that mitochondria may be particularly susceptible to ROS-induced damage in the setting of reperfusion, that the species of ROS differ in the mitochondrial and cytoplasmic compartments, or that ROS produced in mitochondria may have a specific ability to trigger signaling events that are deleterious to cardiomyocyte function. The lack of sensitivity of the SOD1 hearts to ischemia/reperfusion injury and postischemic dysfunction was not attributable to a compensatory increase in SOD2 activity (Figure 1 and Table 1). This is consistent with the previous observations that mice lacking the SOD1 enzyme showed no increased sensitivity to hyperoxia. In agreement with our results, Yoshida et al. reported that hearts from SOD1 mice were not more susceptible to ischemia/reperfusion injury compared with WT hearts. Nevertheless, we did observe that the SOD1 hearts tended to be more susceptible to tachycardia and arrhythmias between 10 and 30 minutes of reperfusion compared with WT and SOD2 hearts. Moreover, lipid peroxidation oxidation tended to be higher in the SOD1 hearts compared with WT hearts. Therefore, our results do not exclude a role for cytosolic SOD in protection against ischemia-reperfusion injury, particularly in its most severe forms. Indeed, Yoshida et al. have demonstrated increased indices of injury after ischemia-reperfusion in mouse hearts totally lacking SOD1.

The relative importance of the mitochondrial SOD is demonstrated by our observation that SOD2 mice hearts had significantly impaired postischemic function and greater oxidative stress compared with WT hearts, whereas the SOD1 hearts were more similar to the WT hearts. It is not clear if this is attributable to less redundancy of scavenging mechanisms in the mitochondrial compartment, if the ROS generated in the mitochondria differ qualitatively, or if there are relatively more critical targets of ROS in the mitochondria compared with the cytosolic compartment. Our data suggest that modulation of the mitochondrial SOD activity is a critical determinant in the tolerance of the heart to oxidative stress. In fact, alteration of mitochondrial SOD expression may be an endogenous cardioprotective mechanism elicited by exercise, suggesting that modulating ROS generation and antioxidant defense mechanisms within the mitochondrial compartment may be a relevant therapeutic goal. The model described in this study may be an ideal means to establish the functions and therapeutic impact of mitochondrial antioxidant defenses in myocardial ischemia-reperfusion injury in future studies.

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References
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