Targeted Disruption of the Mouse Sod I Gene Makes the Hearts Vulnerable to Ischemic Reperfusion Injury

Tetsuya Yoshida, Nilanjana Maulik, Richard M. Engelman, Ye-Shih Ho, Dipak K. Das

Abstract—The role of Cu/Zn–superoxide dismutase (SOD) in myocardial ischemic reperfusion injury was studied by using a mouse model with targeted disruption of the mouse Sod I gene. Inactivation of the functional mouse Sod I gene in hearts by gene targeting (Sod I−/−) resulted in a 50% reduction of Cu/Zn-SOD mRNA and significant reduction of Cu/Zn-SOD enzyme activity compared with that of wild-type Sod I+/+ mice. Cu/Zn-SOD mRNA could not be detected in Sod I−/− heart. The isolated buffer-perfused hearts from the knockout mice devoid of any functional copy of the Sod I (Sod I−/−) and matched nontransgenic control mice were subjected to 30 minutes of global ischemia followed by 2 hours of reperfusion. For both groups of mice, the postsischemic functional recovery for the hearts was lower than the baseline, but the recovery for the Sod I−/− was less compared with the wild-type mice. Thus, the postsischemic recovery of the developed force and the maximum first derivative of the developed force were consistently lower for the Sod I−/− mouse hearts compared with wild-type control hearts. The coronary flow was lower compared with the baseline levels for both groups of hearts, but there was no significant difference between the groups. The myocardial infarction determined from the ratio of infarct size/area of risk was higher for the Sod I−/− mice compared with the control mice. The amount of creatine kinase release from the wild-type mouse hearts was less compared with the Sod I−/− mouse hearts. These results documented that Sod I−/− mouse hearts were more susceptible to ischemic reperfusion injury compared with corresponding wild-type mouse hearts, suggesting that the Sod I gene constitutes an important defense element for the hearts. (Circ Res. 2000;86:264-269.)

Key Words: transgenic □ knockout □ ischemia/reperfusion □ free radicals □ oxidative stress

Reactive oxygen species, including superoxide and hydroxyl radicals, as well as oxidants such as hydrogen peroxide, have been implicated in the pathogenesis of myocardial ischemic reperfusion injury.1–3 In biological tissue, peroxide, have been implicated in the pathogenesis of myo-

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From the Cardiovascular Research Center (T.Y., N.M., R.M.E., D.K.D.), Department of Surgery, University of Connecticut School of Medicine, Farmington, Conn, and Institute of Chemical Toxicology (Y.-S.H.), Wayne State University, Detroit, Mich.
Correspondence to Dipak K. Das, University of Connecticut, School of Medicine, Cardiovascular Research Center, Department of Surgery, Farmington, CT 06030-1110. E-mail ddas@neuron.uche.edu
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Materials and Methods

Targeted Disruption of Mouse SOD1 Gene

Mouse Sod1 genomic clones were isolated from a 129/SVJ genomic library (Stratagene) by screening with a rat SOD1 cDNA probe. An ~7.2-kb SacI genomic fragment, which contains the entire mouse SOD1 gene, was isolated from clone 30 and used as the external to the genomic sequence presented in the targeting vector (Figure 1). To inactivate the mouse SOD1 gene, the Smal and HindIII restriction sites flanking the Smal-HindIII fragment, which contains sequences from introns 1 to 4, were converted into XhoI sites by linker ligation and then inserted into the XhoI site in plasmid vector pPNT13 (generously provided by Dr Richard Mulligan, Massachusetts Institute of Technology) (Figure 1). Similarly, linker ligation was also used to clone the EcoRI-Sall fragment containing the 3’-flanking sequence of the gene into the BamHI site in the pPNT vector.

The Sod1 targeting vector, in which exons 5 was deleted, was linearized by HindIII digestion and transfected into R1 ES cells14 (a generous gift from Dr Andras Nagy, Mount Sinai Hospital at Toronto, Ontario, Canada). Clones resistant to G418 and ganciclovir (a gift from Syntex Inc, Palo Alto, CA) were screened by Southern linearized by dI/dt were recorded. The hearts of all mice were subjected to 30 minutes of thoracotomy, which in turn was attached to a force transducer. A round-bodied needle was passed through the apex of the heart and the perfusate to assess cellular injury. At the end of reperfusion, a 10% creatine kinase (CK) release from the heart was estimated in the perfusate to assess cellular injury. At the end of reperfusion, a 10% (w/vol) solution of triphenyl tetrazolium in phosphate buffer was infused into a side arm of the aortic cannula until the myocardium stained deep red.17 The hearts were excised, weighed, and stored at −70°C for subsequent determination of infarct size as described previously.16,17

Determination of Reactive Oxygen Species in the Heart

To estimate oxygen free radicals, an additional group of wild-type, Sod1lacZ+/+, and Sod1lacZ−/− hearts were perfused for 15 minutes in the presence of 2 mmol/L salicylate before ischemia as described previously.18 At the end of the 2 hours of reperfusion, hearts were frozen at −70°C for subsequent analysis of hydroxylated benzoic acids by HPLC using an electrochemical detector.18 Additionally, malonaldehyde was assayed19 to monitor the development of oxidative stress during ischemia/reperfusion.

Results

Generation and Characterization of Sod1 Knockout Mice

As shown in Figure 1, exons 5 of the mouse Sod1 gene (which encodes the C terminus of the protein from amino acid residues 120 to 154, which constitute both the structure and function of the active site channel) and some of the flanking intron sequences were replaced with the neomycin resistance cassette (neo). This also creates a new Psfl restriction site, resulting in a shorter Psfl genomic fragment from the targeted allele (12.5 kb) than that from the wild-type allele (16.5 kb). Mice heterozygous (Sod1lacZ+/−) for the targeted allele were interbred to generate homozygous knockout (Sod1lacZ−/−) mice. Male and female Sod1lacZ−/− mice grew normally and were apparently healthy under routine animal husbandry.

Inactivation of the functional mouse Sod1 gene in mouse hearts by gene targeting was initially determined by RNA blot analysis. An ~50% reduction of Cu/Zn-SOD mRNA was found in the heart of Sod1lacZ−/− mice compared with that of wild-type (Sod1lacZ+/+) mice (Figure 2A). Furthermore, no Cu/Zn-SOD mRNA could be detected in Sod1lacZ−/− heart, indicating that the truncated Cu/Zn-SOD or Cu/Zn-SOD-neo fusion mRNA is degraded rapidly in the heart. Reduction of heart Cu/Zn-SOD enzyme activity in Sod1lacZ−/− and Sod1lacZ−/− mice was also confirmed by SOD activity staining on a native polyacrylamide gel.21 Figure 2B shows that the Cu/Zn-SOD activities in the hearts of Sod1lacZ−/−, Sod1lacZ+/−, and Sod1lacZ−/− mice are proportional to the mRNA levels in these mice, as shown in Figure 2A. A decrease in Cu/Zn-SOD activity apparently
had no effects on the activity of other heart antioxidant enzymes, such as manganese SOD (Mn-SOD) (Figure 2B), catalase; glutathione peroxidase; and enzymes involved in the recycling of oxidized glutathione, including glutathione reductase and glucose-6-phosphate dehydrogenase (data not shown). A more detailed characterization of the Cu/Zn-SOD–deficient mice has been reported elsewhere.22 Male and female Sod1^+/+ and Sod1^−/− mice at 10 to 12 weeks of age were used for myocardial ischemia/reperfusion study.

Myocardial Performance

All hearts recovered their beats spontaneously after 30 minutes of global ischemia followed by reperfusion. However, the heart rates remained lowered compared with baseline (Figure 3, top). No significant difference was found in heart rate between groups throughout the experiment. Coronary flow was significantly reduced after 30 minutes of reperfusion compared with baseline for all groups of mouse hearts (Figure 3, bottom). Although coronary flow was progressively reduced up to 2 hours of reperfusion, there was no difference between the 3 groups of hearts. The baseline values of dF (0.63 ± 0.07 g for wild type, 0.61 ± 0.05 g for Sod1^+/−, and 0.57 ± 0.08 g for Sod1^−/− mice) and maximal first derivative of dF (dF/dt; 265 ± 33 g/s for wild-type, 251 ± 25 g/s for Sod1^+/−, and 233 ± 27 g/s for Sod1^−/− mice) did not vary significantly among the groups. The dF during reperfusion was lower for the Sod1^−/− knockout mouse hearts than for control wild-type hearts throughout the reperfusion (Figure 4, top). In wild-type and Sod1^+/− hearts, dF recovered to the level of baseline after 30 minutes of reperfusion, and this level was maintained higher than the baseline through the end of the experiment. On the other hand, dF did not recover beyond the 90% level of baseline for the Sod1^−/− hearts. At all points, dF showed significantly lower recovery for Sod1^−/− hearts compared with nontransgenic control and Sod1^+/− hearts after 15 minutes of reperfusion (P<0.05). A similar trend was seen in dF/dt max (Figure 4, bottom). A significantly lower recovery of dF/dt max occurred in Sod1^−/− hearts after 15 minutes of reperfusion.

![Figure 2](image2.png)

**Figure 2.** Expression analysis of Cu/Zn-SOD gene in mouse hearts. A, RNA blot analysis of total cellular RNA isolated from the hearts of Sod1^+/+, Sod1^+/−, and Sod1^−/− mice (as indicated by +/+ , +/−, and −/−, respectively). Total heart RNA (25 μg) was separated on agarose gel for blot analysis. RNA blot filter was hybridized with a rat Cu/Zn-SOD cDNA probe. B, A native polyacrylamide gel showing activity staining for SOD in the hearts of Sod1^+/+, Sod1^+/−, and Sod1^−/− mice. Sod1 genotypes are shown at the top of the gel.

![Figure 3](image3.png)

**Figure 3.** Heart rate and coronary flow in Sod1^+/+, Sod1^+/− mice, and wild-type mice during ischemia/reperfusion. Sequential changes of heart rate (top) and coronary flow (bottom) during postischemic reperfusion in wild-type (□), Sod1^+/− (■), and Sod1^−/− (levator) mouse hearts. Results are expressed as mean±SEM (n=12) for each group. *P<0.05 compared with control. BL indicates baseline.

![Figure 4](image4.png)

**Figure 4.** dF and dF/dt max in Sod1^+/+, Sod1^+/−, and wild-type mice during ischemia/reperfusion. Shown are sequential changes of dF (top) and dF/dt max (bottom) during postischemic reperfusion in wild-type (□), Sod1^+/− (■), and Sod1^−/− (levator) mouse hearts. Results are expressed as mean±SEM for n=12 for each group. *P<0.05 compared with control. BL indicates baseline.
CK Release From Heart

Total CK release from the heart (Figure 5), which reflects cellular injury or tissue necrosis and membrane permeability, was only \(5 \pm 0.05\) U/mL for all of the groups before ischemia. After ischemia, CK release was increased in all 3 groups, but the amount of release was much higher for the \(Sod \, I^{-/-}\) knockout mouse hearts. For example, at 30 minutes of reperfusion, CK release was \(122 \pm 8.7\) IU/mL for \(Sod \, I^{-/-}\) mice as compared with \(70 \pm 6.8\) IU/mL \((P<0.05)\) for nontransgenic wild-type controls. The amount of CK release was progressively increased as the duration of reperfusion increased. At 60 minutes of reperfusion, CK release was \(151 \pm 7.7\) IU/mL for \(Sod \, I^{-/-}\) mouse heart compared with \(98 \pm 6.4\) IU/mL \((P<0.05)\) for nontransgenic controls. Similarly, after 120 minutes of reperfusion, CK release from the \(Sod \, I^{-/-}\) mouse hearts amounted to \(163 \pm 8.1\) IU/mL compared with that from nontransgenic controls, which was \(118 \pm 7.4\) IU/mL. Amount of CK release from the hearts of \(Sod \, I^{-/-}\) mice was not significantly different from that from the wild-type mouse hearts at any point.

Myocardial Infarction

In this study, global ischemia for 30 minutes was adopted; therefore, the whole ventricle was regarded as the area of risk. The area that was not stained by triphenyl tetrachloro was measured and calculated as the infarct area. Infarct size for each heart was defined as percentage of area at risk. Mean value of infarct size in the \(Sod \, I^{-/-}\) mouse heart group was significantly higher than that in the control group \((38.8 \pm 3.3\% \text{ versus } 22.5 \pm 1.8\% \text{ for the wild-type, } P<0.05)\) (Figure 6). Infarct size of the hearts from \(Sod \, I^{-/-}\) mouse hearts was similar to that of wild-type control. Our results indicated that \(Sod \, I^{-/-}\) mouse hearts had significantly higher myocardial necrosis.

Increased OH\(^{•}\) Formation in the \(Sod \, I^{-/-}\) Mouse Hearts

The levels of 2,3-dihydroxy benzoic acid (2,3-DHBA) in the hearts of wild-type, \(Sod \, I^{-/-}\), and \(Sod \, I^{-/-}\) mice are shown in Figure 7. The \(OH^{•}\) radical produces both 2,3-DHBA and 2,5-DHBA, but 2,5-DHBA may also be produced by the cytochrome P450 system.\(^{17}\) We therefore monitored 2,3-DHBA formation, which truly reflects the production of \(OH^{•}\) radical. Similar amounts of 2,3-DHBA were found in all 3 groups of hearts at baseline (before ischemia and reperfusion). The amount of 2,3-DHBA increased at the end of ischemia/reperfusion (after 2 hours of reperfusion) in all hearts, indicating an increased \(OH^{•}\) formation. A significantly higher amount of 2,3-DHBA was noticed in the hearts of \(Sod \, I^{-/-}\) mouse compared with \(Sod \, I^{-/-}\) mouse hearts and wild-type mouse hearts, demonstrating that significantly higher amounts of reactive oxygen species were formed in the \(Sod \, I^{-/-}\) mouse hearts on ischemia and reperfusion.

Malonaldehyde (MDA) Formation

MDA formation is considered a presumptive marker for oxidative stress. MDA was measured as MDA-DNPH derivative by HPLC. MDA formation was increased progressively and steadily during the postischemic reperfusion in all 3 groups of hearts (Figure 8). However, the amount of MDA production was significantly higher at all points in the \(Sod \, I^{-/-}\) mouse hearts compared with those for wild-type and \(Sod \, I^{-/-}\) mouse hearts, demonstrating that \(Sod \, I^{-/-}\) mouse hearts were subjected to an increased amount of oxidative stress during the postischemic reperfusion.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Discussion

The results of this study documented that \(Sod \, I^{-/-}\) mouse hearts are more susceptible to ischemic reperfusion injury.
compared with corresponding wild-type and Sod1<sup>+/−</sup> mouse hearts, suggesting that Sod1 gene constitutes an important defense element for the hearts. This gene encodes a Cu/Zn-dependent cytosolic enzyme of dimeric 32-kDa protein that dismutates O<sub>2</sub> into H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub>. Cu/Zn-SOD is considered primarily an antioxidant enzyme that can reduce oxidative stress. Our results also supported antioxidant action of Cu/Zn-SOD, because Sod1<sup>−/−</sup> knockout mouse hearts devoid of any Cu/Zn-SOD activities contained the highest amount of 2,3-DHBA, a marker for OH<sup>−</sup> formation and malonaldehyde, which is a presumptive marker for lipid peroxidation and oxidative stress. Postischemic left ventricular functional recovery of Sod1<sup>−/−</sup> mouse hearts was the lowest compared with those of wild-type controls and Sod1<sup>+/−</sup> mouse hearts, further supporting a cardioprotective role of Cu/Zn-SOD.

The present study utilized both Sod1<sup>+/−</sup> and Sod1<sup>−/−</sup> mouse hearts. As mentioned earlier, Sod1<sup>+/−</sup> mouse hearts showed ≈50% reduction of Cu/Zn-SOD mRNA compared with that of wild-type Sod1<sup>+/+</sup> mouse hearts. No Cu/Zn-SOD mRNA was detected in the hearts of Sod1<sup>−/−</sup> mice. The activities of other key antioxidant enzymes, including Mn-SOD, catalase, glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase, remained unaltered in the Sod1<sup>+/−</sup> and Sod1<sup>−/−</sup> mouse hearts compared with wild-type controls. The results thus truly reflect the effects of Cu/Zn-SOD gene knockout in myocardial ischemic reperfusion injury.

SOD is present in both the cytosolic and the mitochondrial fractions of the mammalian heart. A significant number of studies exist in the literature to show the cardioprotective role of mitochondrial SOD or Mn-SOD. For example, overexpression of Mn-SOD has been found to protect myocardial ischemia/reperfusion injury in transgenic mice. Increased expression of Mn-SOD in the hearts transfected with Mn-SOD showed significant improvement in tolerance against ischemia/reperfusion injury. The activation of Mn-SOD by the free radicals produced during hyperthermia was found to be important for early-phase and late-phase cardioprotection against ischemia/reperfusion injury in rats. In another related study, the same investigators have found that heat shock–induced Mn-SOD enhanced the tolerance of cardiac myocytes to hypoxia-reoxygenation injury. Preconditioning of a heart by cyclic episodes of ischemia and reperfusion has been found to induce Mn-SOD, suggesting its importance in the myocardial defense mechanism. Mn-SOD could protect mitochondrial complex I against Adriamycin-induced cardiomyopathy in transgenic mice. Another recent study also showed the induction of Mn-SOD, supporting this previous observation.

In contrast, only a little information is available regarding the cardioprotective ability of cytosolic Cu/Zn-SOD. Initial studies have resulted in conflicting reports, showing Cu/Zn-SOD to be both protective and nonprotective. Efficacy of Cu/Zn-SOD in cardioprotection was believed to be limited by its inability to enter the cells, as well as by its nonspecific deleterious effects. However, our initial study has shown Cu/Zn-SOD to be cardioprotective against ischemia/reperfusion injury. A recent study has indicated that overexpression of human Cu/Zn-SOD prevents postischemic injury. The present study supports this previous report and further documents that Sod1<sup>−/−</sup> mouse hearts are subjected to an increased amount of oxidative stress during ischemia and reperfusion. The amount of 2,3-DHBA was the highest in the hearts of Sod1<sup>−/−</sup> mouse, indicating a significantly higher amount of OH<sup>−</sup> radical formation in these hearts compared with either wild-type or Sod1<sup>+/−</sup> mouse hearts. Sod1<sup>−/−</sup> mouse hearts contained ≈50% of the mRNA and enzyme activity compared with wild-type mouse hearts. Our results indicate that 50% of the total Sod1 activity provided a comparable degree of cardioprotection (as compared with wild-type hearts) against ischemia/reperfusion injury.

A large number of studies exist in literature demonstrating development of oxidative stress due to increased formation of reactive oxygen species and decreased antioxidant reserve in the ischemic reperfused myocardium. The reactive oxygen species include superoxide anions and hydroxyl and peroxyl radicals, as well as oxidants such as H<sub>2</sub>O<sub>2</sub>. Mammalian hearts are protected from the oxidant challenge by several defense systems, which include antioxidant enzymes such as SOD, catalase, and glutathione peroxidase, and antioxidants, which include glutathione, ascorbic acid, and α-tocopherol. Our results imply that Cu/Zn-SOD may also be a part of the antioxidant reserve, because Sod1<sup>+/−</sup> mouse hearts were subjected to increased oxidative stress in concert with increased ischemic/reperfusion injury.

Acknowledgments

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MATERIALS AND METHODS.

Targeted Disruption of the Mouse SOD1 Gene:

Eleven mouse Sod1 genomic clones were isolated from a 129/SVJ genomic library purchased from Stratagene, La Jolla, CA by screening with a rat SOD1 cDNA probe. An approximately 7.2-kb SauI genomic fragment, which contains the entire mouse SOD1 gene, was isolated from clone 30 and used in construction of the gene targeting vector. To inactivate the mouse SOD1 gene, the Smal and HindIII restriction sites flanking the Smal-HindIII fragment, which contains sequences from intron 1 to intron 4, were converted into Xhol sites by linker ligation, and then inserted into the Xhol site in plasmid vector pPNT, generously provided by Dr. Richard Mulligan of Massachusetts Institute of Technology (Fig. 1). Similarly, linker ligation was also used to clone the EcoRI-Sall fragment containing the 3' flanking sequence of the gene into the BamHI site in the pPNT vector.

The Sod1 targeting vector, in which exon 5 was deleted, was linearized by HindIII digestion and transfected into R1 ES cells, a generous gift from Dr. Andras Nagy of Mount Sinai Hospital at Toronto, Canada. Clones resistant to G418 and ganciclovir (a gift from Syntex Inc., Palo Alto, CA) were screened by Southern blot analysis using a probe 5' external to the genomic sequence present in the targeting vector (Figure 1). Targeted clones were microinjected into C57BL/6 blastocysts following the standard procedure. Chimeric mice with near 100% chimerism were generated using Sod1 knockout clone 5, and showed 100% transmission of the 129/SvJ chromosomes.
Isolated Mouse Heart Preparation and Measurement of Contractile Function.

Thirty-two mice at 10 to 12 weeks of age were divided into three groups: Cu/Zn-SOD knockout homozygotes (Sod I-/-) (n = 12), heterozygotes (Sod I+/-) (n=12) and nontransgenic wild-type control (n = 12). They were anesthetized with an intraperitoneal injection of sodium pentobarbital (200 mg/kg) and heparin (sodium) (500 U/kg) administered at the same time to prevent intravascular coagulation of blood. The heart was excised immediately after thoracotomy, and placed in cold perfusion buffer. The aorta was cannulated, and the heart was perfused with Krebs-Henseleit bicarbonate (KHB) buffer by the retrograde Langendorff method. A small incision was made at the main trunk of the pulmonary artery to drain coronary effluent. The effluent was collected for one minute before ischemia, after ischemia and during reperfusion and stored at -20°C for subsequent measurement of creatine kinase (CK). A 4-0 silk suture on a round bodied needle was passed through the apex of the heart and attached to the apex which in turn was attached to a force transducer. The heart rate (HR), force developed by the heart (DF) and first derivative of developed force (dF/dt) were recorded. Data of myocardial contractile function were recorded and analyzed in real time using the Cordat II data acquisition, analysis, and presentation system (Data Integrated Scientific Systems, Pinckney, MI; Triton Technologies, Inc, San Diego, CA). After stabilization, preischemic baseline contractile function was measured. The hearts of all mice were subjected to 30 min of ischemia under normothermia by clamping the aortic cannula, followed by 2 hr of reperfusion. Coronary flow was calculated before ischemia and during reperfusion by quantifying the release of coronary effluent over one minute.

Estimation of CK Release.

CK release from the heart was estimated in the perfusate collected from the heart before ischemia, and during reperfusion using a CK assay kit obtained from Sigma Chemical Company (St. Louis, Mo). The enzyme activity was expressed as units/ml perfusate. The results are expressed as total CK release from the heart after the indicated time periods.
Determination of Reactive Oxygen Species in the Heart

In order to estimate the formation of oxygen derived free radicals during ischemia and reperfusion, an additional group of wild-type, Sod1+/− and Sod1−/− hearts were perfused for 15 min in the presence of 2 mM salicylate prior to ischemia/reperfusion protocol as described previously. Reperfusion was also performed with the KHB buffer containing 2 mM salicylate. At the end of the reperfusion, hearts were frozen at liquid nitrogen temperature for subsequent analysis of hydroxylated benzoic acids by High Performance Liquid Chromatography (HPLC). At a later date, hearts were homogenized in 1 ml Tris-sucrose buffer and 800 μl homogenate was treated with 40 μl of 3 M HCl. The precipitate was removed by centrifugation, and supernatant was filtered through a Rainin nylon-66 membrane filter (0.22 μm pore size). A 20-μl volume of the sample was injected onto an Altex Ultrasphere 3 mm ODS (75 mm x 4.6 mm) (Rainin) equipped in a Waters Assoc (Milford, MA) HPLC unit consisting of a Model 510 pump and a Model 460 electrochemical detector. 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-DHBA (hydroxylated products of salicylic acid after interaction with OH− radical) were eluted by buffer containing 0.03 M sodium acetate and 0.03 M citric acid (pH 3.6) at a flow-rate of 1 ml/min. The detection potential was maintained at 0.6 V, employing the Ag/AgCl reference electrode. Peaks were identified by authentic standards as well as by injecting the hydroxylated products of salicylic acid from a pure OH− generating system.

Estimation of Malonaldehyde (MDA) Content in the Perfusate

Malonaldehyde (MDA) was assayed as described previously to monitor the development of oxidative stress during ischemia reperfusion. Coronary perfusates were collected at the time of recording baseline functional parameters, and thereafter, at timepoints of 5 min, 30 min, 60 min, and 120 min into reperfusion for the measurement of MDA. The MDA in the collected coronary perfusate
samples was derivatised using 2,4-dinitrophenylhydrazine (DNPH). 2 ml of perfusate was added to 0.1 ml of DNPH reagent (310 mg DNPH in 100 ml 2N HCl, 1.56 mmol DNPH) in a 20 mL Teflon lined screw-capped test tube, contents were vortexed and 10 ml of pentane was added prior to intermittent rocking for 30 minutes. The aqueous phase was extracted 3 times with pentane, blown down with N₂ and reconstituted in 200 µL of acetonitrile. Aliquots of 25 µL in acetonitrile were injected onto a Beckman Ultrasphere C18 (3mm) column in a Waters HPLC (Waters Corp., Milford, MA). The products were eluted isocratically with a mobile phase containing acetonitrile-H₂O-CH₃COOH (34:66:0.1, v/v/v) and detected at three different wavelengths of 307 nm, 325 nm and 356 nm. The peak for malonaldehyde was identified by co-chromatography with a DNPH derivative of the authentic standard, peak addition, comparison of the UV patterns of absorption at the three wavelengths and by GC-MS. The amount of MDA was quantitated by performing peak area analysis using the Maxima software program (Waters) and expressed in pmol/ml.

**Measurement of Infarct Size.**

At the end of reperfusion, a 10% (w/v) solution of triphenyl tetrazolium in phosphate buffer (Na₂HPO₄ 88mM, NaH₂PO₄ 1.8mM) was infused into a side arm of the aortic cannula until the myocardium stained deep red¹⁶,¹⁷. The hearts were excised, weighed and stored at -70°C. Frozen hearts were sliced perpendicularly to the long axis from apex to base in 0.8 mm thick sections. Sections were then fixed in 2% paraformaldehyde. Thin mouse heart cross sections were placed between two cover slips and digitally imaged using an IBM-compatible PC and a Microtek ScanMaker 600z, a 600 dot per inch, flat-bed, full color scanner. The cross section was imaged at the maximum scaling and dot resolution that the scanner would allow. The digitized image was stored in Adobe TIFF file format by the software package. PhotoStyler, v.1.0.3, by U-Lead Systems, Inc. For analysis of infarct areas, some enhancement of the image was necessary at times to more
clearly visualize the areas of staining by Corel Photo-Paint 4.0 (Corel Inc.). Corel was also used to mark the stained areas. To quantitate the areas of interest in pixels, a NIH Image 5.1 (a public-domain software package) was used. The entire area of risk (transmural) was quantified in pixels using the computer software, and the measured infarct areas were compared to the entire area at risk in a blinded fashion.

**STATISTICAL ANALYSIS**

For statistical analysis, a two-way analysis of variance (ANOVA) followed by Scheffe's test was first carried out using Primer Computer Program (McGraw-Hill, 1988) to test for any differences between groups. If differences were established, the values were compared using Student's t-test for paired data. The values were expressed as mean ± SEM. The results were considered significant if p was less than 0.05.