MnSOD in mouse heart: acute responses to ischemic preconditioning and ischemia-reperfusion injury

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MnSOD in mouse heart: acute responses to ischemic preconditioning and ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 288: H2986–H2994, 2005. First published February 4, 2005; doi:10.1152/ajpheart.01144.2004.—Manganese superoxide dismutase (MnSOD) is one of the main antioxidant enzymes that protects the heart against ischemia-reperfusion (I/R) injury. Ischemic preconditioning (IPC) is a short period of ischemia-reperfusion that reduces subsequent prolonged I/R injury. Although MnSOD localizes in mitochondria, the immediate subcellular distribution of MnSOD in heart after IPC and I/R has not been studied. In a Langendorff mouse heart model, IPC significantly improved cardiac function and reduced the infarction size induced by I/R. Immunoblotting and double immunostaining in fresh preparations revealed that I/R resulted in an increase in cytosolic MnSOD content accompanied by the release of cytochrome c. In contrast, IPC increased mitochondrial MnSOD and reduced cytosolic MnSOD and cytochrome c release induced by I/R. We found that compared with freshly prepared fractions, the freeze-thaw approach results in mitochondrial integrity disruption and release of large amounts of MnSOD into the cytosol along with mitochondrial markers even in the absence of I/R. In contrast, fresh preparations exhibit early MnSOD release into the cytosol after I/R that is prevented by IPC and cyclosporin A administration.

Manganese superoxide dismutase; reactive oxygen species; myocardial infarction; mitochondria; cytosol; permeability transition pore

**ISCHEMIC PRECONDITIONING (IPC) is defined as a short period of myocardial ischemia-reperfusion (I/R) that decreases subsequent lethal I/R injury (27).** Reactive oxygen species (ROS) are recognized as important intracellular signals that regulate cardiomyocyte viability during IPC (9) and I/R injury (8). ROS act as triggers of IPC (3, 30, 32), and excessive ROS production is implicated as a key mediator of myocardial I/R injury (10, 24). Mitochondria are the main source of ROS. Superoxide is the initial oxidant generated from NAD(P)H or other oxidases such as cytochrome P-450 and is also generated by the mitochondrial electron transport chain (13).

Mitochondrial manganese superoxide dismutase (MnSOD) is a key antioxidant enzyme that catalyzes the conversion of superoxide to hydrogen peroxide and molecular oxygen. MnSOD is a prototypical mitochondrial protein. It is encoded in the nuclear chromatin, synthesized as a precursor in the cytoplasm, and imported posttranslationally into the mitochondrial matrix in its mature form subsequent to the removal of its 23-amino acid NH2-terminal leader sequence by specific proteases (35). MnSOD is inducible (36), but the effects of IPC on mitochondrial MnSOD protein and enzyme activity in cardiac tissue have previously been described only in specimens that were frozen and then thawed for analysis (7, 16, 17, 25, 37–40). Data from freshly prepared cardiac tissue samples have not been previously reported. We therefore hypothesized that mitochondrial disruption and swelling, which are known to occur early after myocardial injury (18), should result in rapid release of MnSOD.

Accordingly, our goal was to determine the activity and content of MnSOD in cardiac mitochondrial and cytosolic fractions in a mouse model of IPC and acute I/R injury. We compared fresh and freeze-thawed preparations. Our findings indicate that it is essential to use fresh preparations of adult mouse hearts, because the freeze-thaw approach results in artifactual increases in extramitochondrial MnSOD. Early after I/R injury but not after a brief episode of IPC, cytosolic MnSOD activity and content are increased in fresh preparations, presumably due to mitochondrial disruption and release of MnSOD into the cytosol resulting from ischemia and subsequent reperfusion. IPC increases mitochondrial MnSOD content and reduces cytosolic MnSOD release induced by I/R injury.

**MATERIALS AND METHODS**

This study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (Washington DC: National Academnic Press, 1996). All procedures were approved by the Animal Care Subcommittee of the San Francisco Department of Veterans Affairs Medical Center.

*Isolation of mitochondria and cytosol-enriched fractions.* For these experiments, we used two different preparation methods. For *method 1* (freshly prepared samples), hearts were perfused, removed from the Langendorff apparatus, and minced in cold medium that contained 0.25 M sucrose, 20 mM Tri-HCl, and 5 mM EDTA, pH 7.4 and were then homogenized in a glass grinder. The homogenates were centrifuged at 800 g. The supernatant was centrifuged at 8,000 g for 10 min to generate the mitochondrial pellet and a soluble cytosol-enriched fraction. We then used 100,000 g for 30 min to prepare the cytosolic fraction.

For *method 2* (freeze-thawed samples), hearts were placed in liquid nitrogen and stored overnight at −80°C at the end of the experiment. The preparation was then thawed, and mitochondrial and cytosolic fractions were isolated using the steps described for *method 1*.

For both methods, all operations were performed at 4°C. The mitochondrial fraction used for Western blot analysis was treated with 1% Triton for 20 min at 4°C, whereas the mitochondrial fraction used for measuring MnSOD activity was sonicated using three 1-min bursts. Cytosolic and mitochondrial protein concentrations were determined using the Bradford method (Bio-Rad; Hercules, CA).

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For purposes of comparison, an identical approach was taken to isolate mitochondrial and cytosolic preparations from adult mouse liver. 

MnSOD activity measurement. MnSOD activity in mitochondria was determined by the modified nitro blue tetrazolium (NBT) method (29). Xanthine-xanthine oxidase was used to generate a superoxide flux. NBT reduction by superoxide anion to blue formazan was measured at 560 nm in a spectrophotometer (DU-65; Beckman; Fullerton, CA) at room temperature. Each 1-ml assay tube contained the final concentration of the following reagents: 0.05 mM bathocuproinedisulfonic acid (BCS) and 0.13 mg/ml defatted albumin from BSA, 1 mmol/l diethylenetriamine pentaacetic acid, 1 U catalase, 5.6 × 10⁻² mmol/l NBT, 0.1 mmol/l xanthine, and 80 μM xanthine oxidase. For measuring MnSOD activity, 5 mmol/l KCN was added to inhibit the activity of copper-zinc SOD. 

SDS-PAGE and Western blot analysis. For measurement of MnSOD protein in mitochondria or cytosol-enriched fractions, we used standard SDS-PAGE on a mini gel composed of 15% acrylamide continuous-resolving gel. Briefly, 20 μg of protein prepared from the mitochondria were electrophoresed on a 15% denaturing gel at 30 mA/lane for 1–2 h. Proteins were electrotransferred onto a nitrocellulose membrane (Bio-Rad) at 200 mA for 2 h. Adequate background blocking was accomplished by incubating the nitrocellulose membrane with 5% nonfat dry milk in phosphate-buffered solution (PBS, pH 7.4). Rabbit anti-MnSOD polyclonal antibody (Stressgen; Victoria, Canada) or mouse anti-cytochrome oxidase (COX) subunit I and IV monoclonal mitochondrial marker (Molecular Probes; Eugene, OR) was used to measure the expression of MnSOD or COX I or IV, respectively. Purified anti-cytochrome c antibody (BD Biosciences Pharmingen; San Diego, CA) was used to measure the expression of cytochrome c in both mitochondrial and cytosolic fractions. We also used an antibody to pyruvate dehydrogenase (PDH; Molecular Probes) to measure PDH expression. Western blots were performed with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG using enhanced chemiluminescence Western blotting detection reagents (Amersham International; Buckinghamham, UK) and were exposed to Kodak X-Omat AR film. The immunoreactive bands were exposed to Kodak X-Omat AR film. The immunoreactive bands were then subjected to 20 or 30 min of global ischemia and 30 min of reperfusion. Cyclosporin A (0.2 mg/kg, Sigma-Aldrich) was infused for 10 min before I/R injury. Infarct size was measured as previously described (20).

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RESULTS

MnSOD protein in freshly prepared mitochondrial and cytosolic fractions of mouse heart. Before we determined the relative concentrations of MnSOD in subcellular fractions during IPC and I/R, we used fresh preparations to probe the presence of MnSOD protein in the cytosol and mitochondria. Immunoprecipitation of proteins with anti-MnSOD antibody showed a single unique band with a calculated (4) molecular mass of 25 kDa (data not shown). This protein was predominantly present in the mitochondrial fraction, but a small proportion of the protein was also found in the cytosolic fraction (Fig. 1A).

Comparison of freshly prepared vs. freeze-thawed preparations on MnSOD activity during IPC and I/R. We then directly compared the two preparative methods on MnSOD enzyme activity in hearts subjected to IPC and I/R. The data are summarized in Figs. 2 and 3. Figure 2 shows that cytosolic MnSOD enzyme activity was three- to sixfold higher in freeze-thawed samples compared with freshly prepared samples. MnSOD activity was somewhat increased from 8.2 ± 0.28 in freshly prepared mitochondria to 10.8 ± 0.48 U/mg protein in the freeze-thawed preparations (Fig. 2B). Although this differ-

Fig. 1. Manganese superoxide dismutase (MnSOD) Western blots relative to cytochrome c oxidase (COX) IV distribution in freshly prepared or freeze-thawed mouse hearts. A: MnSOD protein expression in cytosolic or mitochondrial fractions of freshly prepared normal mouse heart. α-Tubulin was used as a cytosolic-loading control. COX IV was used as a mitochondrial marker. B: MnSOD Western blots in cytosolic or mitochondrial fractions of freeze-thawed mouse heart. NC, normal controls; IR, ischemia-reperfusion. Data from eight separate experiments are shown.

Statistical analysis. Data are presented as means ± SE. The significance of the differences in mean values for the infarction size, MnSOD content, and activity between the treatment groups was evaluated by one-way ANOVA with subsequent post hoc testing (Newman-Keuls). P < 0.05 was considered statistically significant.
Fig. 2. MnSOD activity in cytosolic (A) and mitochondrial (B) fractions of freshly prepared and freeze-thawed isolated mouse heart. Note difference in scale between fresh and freeze-thawed preparations in A. NC, normal control; IPC, ischemic preconditioning; IR20, ischemia for 20 min and reperfusion for 30 min; IR30, ischemia for 30 min and reperfusion for 30 min. Data are means ± SE; *P < 0.05 vs. NC group; n = 6–8 hearts/group.

Fig. 3. MnSOD content in cytosolic (A) and mitochondrial (B) fractions of freshly prepared and freeze-thawed isolated mouse heart. Data were determined by Western blotting and subsequent NIH Image analysis and are expressed as ratios to control values. Data are means ± SE; *P < 0.05 vs. NC group; n = 6–8 hearts/group.
ence was not statistically significant, the preparative differences between freshly prepared and freeze-thawed samples could lead to variation in control results. Moreover, enzyme activity in the freeze-thawed preparations remained constant when control and I/R values were compared. In contrast, enzyme activity almost doubled during I/R vs. control in freshly prepared samples.

Figure 3 shows that relative cytosolic MnSOD content increased in parallel with enzyme activity in fresh preparations with I/R but was little changed in the freeze-thawed preparations. Quantitation of the relative changes shown in the bar graphs of fresh preparations reveals that the increase in MnSOD protein content determined by Western blotting after 20 min of ischemia and 30 min of reperfusion was 1.62 ± 0.24%, whereas mitochondrial MnSOD protein content declined to 0.90 ± 0.05% of control values. There was a relative increase in mitochondrial MnSOD content after IPC in freeze-thawed compared with control samples, and there was a reciprocal decrease in cytosolic MnSOD content. The decline in the much greater pool of mitochondrial MnSOD in large part accounts for the increase in the much smaller pool of cytosolic MnSOD observed after I/R.

In these experiments, infarct size was reduced after IPC (18 ± 4 vs. 42 ± 7% area at risk in the control group; \( P < 0.05 \)). It should be noted that a large infarct area resulted from a relatively short ischemic time of 20 min and reperfusion time of 30 min. These infarct-size results in the control group are virtually identical to those reported by Gray et al. (12) and by us in previous studies (20, 21) in which C57BL/6 mice, a strain that is highly sensitive to I/R injury, were also used.

Comparison of freshly prepared vs. freeze-thawed preparations on determination of MnSOD protein content. In subsequent experiments, we used selected mitochondrial markers to compare protein content in freshly prepared mitochondrial and cytosolic fractions using the freeze-thaw technique, a method commonly reported by others in studying I/R injury (7, 16, 17, 37–40). As markers, we used antibodies to pyruvate dehydrogenase (PDH), an enzyme that is released upon mitochondrial disruption (1), and to subunit I or IV of COX, an enzyme that is tightly bound to the inner mitochondrial membrane (2).

Examples of differences in subcellular distribution of MnSOD in fresh cardiac tissue compared with samples that were frozen and then thawed are shown in Figs. 1 and 4. As can be seen in Fig. 4, considerable PDH was released by the freeze-thaw procedure relative to fresh preparations, whereas COX I and IV (see Fig. 1) were not, which indicates that the freeze-thaw approach causes substantial mitochondrial disruption. The freeze-thaw procedure also resulted in much larger amounts of MnSOD protein release into the cytosol. When mouse liver was compared with mouse heart, the results were similar (Fig. 4B). These data are summarized in Fig. 5.

Fig. 4. MnSOD Western blots relative to pyruvate dehydrogenase (PDH) or COX I distribution in freshly prepared or freeze-thawed mouse tissue (heart and liver). A: Western blots of MnSOD expression in freshly prepared (F) or freeze-thawed (F/T) mouse heart cytosol. PDH and COX I are mitochondrial markers. As can be seen, more PDH was released into the cytosol with the freeze-thaw technique, whereas COX I, which is tightly bound to the inner mitochondrial membrane, was not released. Data are from five separate experiments. B: comparison of MnSOD Western blots in cytosolic and mitochondrial fractions of mouse heart and liver tissue.

Fig. 5. Summary of MnSOD content in freshly prepared or freeze-thawed cytosolic (A) or mitochondrial fractions (B) of mouse heart and liver. Data are means ± SE; \( *P < 0.05 \) vs. Heart (F) group; \( \#P < 0.05 \) vs. Liver (F) group; \( n = 4–8 \) hearts/group.
It should be noted that in the fresh preparation, a small amount of PDH appeared in the cytosol (Fig. 4A). This observation is consistent with the presence of cytosolic MnSOD in fresh preparations detected by immunoblotting (see Figs. 1A and 4A) and suggests that the preparative procedure leads to some mitochondrial damage resulting in MnSOD release. In contrast, the identical preparative techniques led to virtually undetectable MnSOD levels in cytosolic preparations of rat liver (see Figs. 4B and 5), which suggests that heart mitochondria are more damaged than liver mitochondria by the preparative procedure.

Double immunostaining of MnSOD and COX I in mouse heart. We next asked whether MnSOD protein shifts during IPC and I/R could be detected by an alternative and less disruptive method, namely, double immunostaining. COX I served as the mitochondrial marker. As shown in Fig. 6A, a small amount of MnSOD could be detected in the cytosolic fraction from normal control hearts. The scanned data (Fig. 6A, right) show that MnSOD and COX I were almost completely colocalized, which indicates that the majority of MnSOD was in the mitochondria. There was no increase in MnSOD content in the mitochondria after IPC (Fig. 6B), which is consistent with the data shown in Fig. 3. After I/R injury, mitochondrial staining was reduced consistent with mitochondrial disruption and swelling, whereas MnSOD staining was increased in the cytosolic fraction (Fig. 6C). Because COX I is an integral mitochondrial inner-membrane protein, it is not released into the cytosol when mitochondria are rendered dysfunctional by I/R. As shown in Figs. 1 and 4, the extremely disruptive freeze-thaw technique releases the more soluble mitochondrial matrix protein PDH, whereas little or no membrane-bound COX I or IV can be found in the cytosol after differential centrifugation.

Effects of IPC alone and I/R on MnSOD activity and content in cytosolic fraction in relation to cytochrome c release. We compared release of another mitochondrial marker, cytochrome c, with changes in MnSOD content and activity brought about by IPC alone and I/R. During I/R but not IPC, cytosolic cytochrome c content in fresh preparations increased (Fig. 7). This was accompanied by an increase in MnSOD content and activity (Figs. 2A, 3A, and 7), which indicates augmented mitochondrial release of MnSOD during I/R but not during IPC. In contrast with cytosolic changes, there were no detectable alterations in mitochondrial MnSOD activity or content (see Figs. 2 and 3) or in mitochondrial cytochrome c protein content (Fig. 8B).
IPC and cyclosporin A decreased MnSOD and cytochrome c release induced by I/R injury. We reasoned that if I/R caused MnSOD release from mitochondria, IPC should prevent this release. In these experiments, we again used cytochrome c release as a marker to document mitochondrial damage. As shown in Fig. 8A, IPC reduced both MnSOD and cytochrome c release into the cytosol induced by I/R and increased mitochondrial MnSOD content (Fig. 8B). Because cytochrome c is thought to be released via opening of the mitochondrial permeability transition pore (MPTP; Ref. 23), we wondered whether MnSOD might escape during mitochondrial damage by the same mechanism. Accordingly, we infused cyclosporin A, a known inhibitor of MPTP opening (15), before I/R. The data in Fig. 9 indeed show that cyclosporin A reduced MnSOD and cytochrome c release induced by I/R.

**DISCUSSION**

In this study, we investigated the subcellular distribution of MnSOD after IPC and I/R injury. We tested the hypothesis that mitochondrial disruption should lead to early MnSOD release, a response that has not previously been studied in myocardial tissue. There are two principal new findings of this study. First, the freeze-thaw methodology previously employed by others results in the release of spuriously large amounts of MnSOD into the cytosol along with the appearance of mitochondrial markers, which indicates that this method seriously disrupts mitochondrial integrity (see Figs. 2 and 3). A second important observation is that 20 min of ischemia followed by 30 min of reperfusion in isolated mouse heart results in substantial early increases in cytosolic MnSOD activity and content accompanied by the release of cytochrome c. IPC can prevent MnSOD release, which appears to occur via the MPTP as indicated by inhibition of MnSOD escape after pretreatment with cyclosporin A. These data are consistent with prior observations of mitochondrial dysfunction induced by ischemia (18), but to our knowledge, this is the first report to document the rapid release of MnSOD into the cytosol in myocardium. Of note, 2 min of IPC, which is sufficient to reduce myocardial infarction size in the Langendorff mouse model, does not by itself alter either mitochondrial or cytosolic MnSOD content or activity, perhaps because of its brief duration, which is insufficient to cause mitochondrial disruption.

Prior studies of the subcellular localization of MnSOD using the rat liver indicate that MnSOD is almost exclusively confined to the mitochondria of this organ (32). In rat hearts, a number of studies have been reported (7, 16, 17, 25, 37–40) regarding the response of MnSOD to various forms of preconditioning and I/R injury. These investigations have uniformly indicated that MnSOD content and activity increase after ischemic, hyperthermic, and pharmacological preconditioning and constitute an important defense mechanism particularly against I/R injury that occurs late (24–72 h) after the original insult. Overexpression of MnSOD, either in transgenic models or by adenoviral gene transfer, has provided direct evidence that MnSOD is a key component of cardioprotection during I/R injury (5, 22). Although such studies are important in establishing proof of principle, they rely on nonphysiological levels of the enzyme. Moreover, there is little information regarding early subcellular responses of MnSOD to IPC or I/R injury in wild-type animals of any species.

Most prior studies, save for those on transgenic mice, have been on the rat or dog and have used a method in which cardiac tissue is rapidly frozen at −80°C, stored, and subsequently thawed for analysis (7, 16, 17, 25, 37–40). These studies have analyzed only whole tissue responses and have not investigated the subcellular distribution of MnSOD content and activity either in the basal state or after IPC or I/R injury. Similarly, when isolated neonatal rat ventricular myocytes were employed for analysis of MnSOD during hypoxia-reoxygenation studies, no investigations of subcellular fractions were performed (39). No data have been reported in nongenetically altered mouse models, and in genetically altered mice, MnSOD in subcellular compartments was not studied (22).

Because Mn-SOD is conventionally described as an enzyme with localization and activity that are confined to the mitochondrial matrix and associated with the inner mitochondrial membrane (26, 28, 33, 34), we were surprised to find low levels of both MnSOD enzyme activity and protein in the cytosolic fractions of adult mouse myocytes in the basal state. To confirm the absence of mitochondrial contamination in our preparations, we used COX IV, which is tightly bound to the inner mitochondrial membrane, as a mitochondrial marker in immunoblotting experiments. We found that COX IV was absent in cytosolic preparations (see Fig. 4A). However, another mitochondrial marker, PDH, which is released into the cytosol upon mitochondrial disruption, was detected by immunoblotting in fresh preparations (see Fig. 4A). Consistent with our findings, Chen et al. (5) reported cytoplasmic MnSOD
labeling by electron microscopy in wild-type mouse hearts as well as in hearts transgenic for MnSOD. Both cytoplasmic and mitochondrial labeling could be removed by antisera absorption on recombinant human MnSOD (5). These observations suggest that the small amount of cytosolic MnSOD we noted could be the result of one or both of the following: identification of MnSOD that is in transit to the mitochondria after nuclear synthesis and/or a small degree of mitochondrial disruption caused by the preparative techniques used. Of note is that an identical approach on liver revealed virtually no cytosolic MnSOD (see Figs. 4 and 5), which suggests greater damage to cardiac mitochondria. These explanations are supported by detection of a minor amount of cytosolic MnSOD in the control immunostaining experiments (see Fig. 6A).

After I/R, however, a large amount of MnSOD protein is released into the cytosol, and there is a concurrent increase in MnSOD activity (see Figs. 2 and 3). As shown Fig. 3, in freeze-thawed samples, there was a relative increase in mitochondrial MnSOD content after IPC compared with control, and there was a reciprocal decrease in cytosolic MnSOD content. This might represent a possible protective effect of IPC that is more pronounced in the freeze-thaw preparation. Although the degree of susceptibility of mouse mitochondria to I/R injury or to disruption in the preparative process remains to be determined, there is precedent for cytosolic localization of MnSOD after I/R. In a model of renal I/R injury in rat, Cruthirds et al. (6) reported that I/R but not ischemia alone resulted in MnSOD release into the cytosol. This was accompanied by the appearance of a small fraction of cytochrome c in the cytosol. These observations are consistent with our results (see Figs. 6–9).

Of note, when we performed additional experiments at baseline with subsequent IPC and I/R injury interventions and assayed MnSOD content and activity using the freeze-thaw method as performed by others (7, 16, 17, 25, 37–40), the results differed markedly from those obtained in freshly isolated mitochondria and cytosol (see Figs. 2 and 3). In contrast with the present studies, previous reports of studies that used the freeze-thaw method did not distinguish mitochondrial from cytosolic MnSOD content and activity (7, 16, 17, 25, 37–40). Because the freeze-thaw method likely results in substantial mitochondrial disruption, we believe that only fresh preparations should be used to assay subcellular fractions for mitochondrial enzyme content and activity and release of other mitochondrial enzymes and proteins such as PDH and cytochrome c. It should be noted that a wide range of values have been reported for MnSOD activity after I/R ranging from 10 to 300 U/mg protein at baseline (7, 16, 17, 25, 37–40). Our results for freeze-thawed preparations are in the lower range of values noted in previous reports (16, 25, 39), whereas our results for fresh preparations, which involve minimal mitochondrial disruption, are even lower (see Fig. 2).

To explore the mechanism of MnSOD exit from mitochondria, we performed experiments aimed at elucidating the role
of the MPTP, a nonspecific channel in the inner mitochondrial membrane (15). After ischemia, reperfusion of the heart is associated with opening of the MPTP, which causes mitochondrial uncoupling and hydrolysis of ATP that ultimately leads to myocardial cell necrosis (14). A marker of MPTP opening is the release of cytochrome c (23). Both the fungal metabolite cyclosporin A and IPC have been reported to inhibit MPTP opening (14, 19). We reasoned that if MnSOD is released via the MPTP opening during myocardial reperfusion, then IPC or pretreatment with cyclosporin A could serve as an additional marker of mitochondrial disruption. Moreover, serum MnSOD levels have been used as an index of myocardial damage in patients with acute myocardial infarction (11, 31). Whether this early release of MnSOD into the cytosol could represent a previously unrecognized acute homeostatic response aimed at reducing the intensity of I/R injury deserves additional investigation.

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